Quantitative Fluorescence Nanospectroscopy of Nucleotide Excision Repair

— From Single Molecules to Cells

Dianwen Zhang

**QUANTITATIVE FLUORESCENCE NANOSPECTROSCOPY OF
NUCLEOTIDE EXCISION REPAIR**

— FROM SINGLE MOLECULES TO CELLS

Dianwen Zhang

The research presented in this thesis was carried out in the Biophysical Engineering Group, Faculty of Science and Technology, University of Twente, the Netherlands and in collaboration with 1) the Genetics and Cell Biology Department of the Erasmus University Rotterdam, the Netherlands; 2) Department of Pharmacology, SUNY Stony Brook, Stony Brook, United States; 3) Laboratory of Bioorganic Chemistry of Enzymes, Institute of Chemical Biology and Fundamental Medicine, Novosibirsk State University, Russia.

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DISSERTATION

**to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
prof.dr. W.H.M. Zijm,
on account of the decision of the graduation committee,
to be publicly defended
on Thursday the 11th of December at 13.15**

by

Dianwen Zhang

born on the 29th of December 1976

in Jilin, P. R. China

Promotor: Prof. Dr. Vinod Subramaniam

Assistant Promotor: Dr. Cees Otto

Members of the Committee:

Prof. dr. G. van der Steenhoven	University of Twente	the Netherlands	Chairman
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Prof. dr. W. Kruijer	University of Twente	the Netherlands	

For my family

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
6-4PP	Pyrimidine (6-4) pyrimidone photoproduct
aa	Amino acid
bp	Base pair
cts	Counts
eGFP	Enhanced green fluorescent protein
pI	Isoelectric point
AAF	N-acetoxy-2-acetylaminofluorene
APD	Avalanche photo diode
ATP	Adenosine-5'-triphosphate
BER	Base excision repair
CPD	Cyclobutane pyrimidine dimer
Da	Dalton
DtA	Distance to average center
DDB	DNA binding protein
DNA	Deoxyribonucleic acid
DPB	DNA-protein binding
DPP	DNA-protein pair
ERCC1	Excision repair cross-complementation group 1
FA	Fluorescence anisotropy
FCS	Fluorescence correlation spectroscopy
FCFS	Fluorescence cross-correlation spectroscopy
FLSF	Full least squares fit
FOV	Field of view
FP	Fluorescent protein
FRET	Fluorescence resonance energy transfer
FWHM	Full width half maximum
GFP	Green fluorescent protein
GG-NER	Global genomic nucleotide excision repair
HPLC	High-performance liquid chromatography
(h)HR23B	(human) Homologue B of yeast Rad23 protein
IMA	Interfacial molecular aggregation
LSF	Least squares fit
NA	Numerical aperture
NER	Nucleotide excision repair
NP40	Nonidet P-40
NSC	Nanometer-precision single-molecule colocalization
NSL	Nanometer-precision single-molecule (or single-particle) localization

PAA	Polyacrylamide
PSF	Point spread function
QDot	Quantum dot
ROI	Region of interest
SD	Standard deviation
SFA	Steady-state fluorescence anisotropy
SLSF	Simplified least squares fit
SFCM	Single-molecule fluorescence confocal microscope
SNR	Signal to noise ratio
SROI	Square region of interest
TC-NER	Transcription-coupled nucleotide excision repair
TDt	Translational diffusion time
TFA	Time-resolved fluorescence anisotropy
TFIIH	Transcription factor II H
TIRFM	Total internal reflection fluorescence microscope
TX100	Triton X-100
UV	Ultraviolet
XP	Xeroderma pigmentosum
XPA (to -G)	Xeroderma pigmentosum complementation group A (to -G) protein

Table of Contents

Chapter 1 Introduction.....	1
1.1 Aim of this thesis.....	1
1.2 Fluorescence microscopy	2
1.2.1 Fluorescence	2
1.2.2 Single-molecule fluorescence microscopy	3
1.2.3 Confocal fluorescence microscopy	3
1.2.3.1 Fluorescence correlation spectroscopy.....	3
1.2.3.2 Fluorescence anisotropy	4
1.2.3.3 Advantages of FCS and FA	6
1.2.4 Total internal reflection fluorescence microscopy	6
1.3 DNA repair	7
1.3.1 Nucleotide excision repair.....	8
1.3.1.1 NER disorders	8
1.3.1.2 Global genomic NER.....	9
1.3.1.3 Transcription-coupled NER	10
1.4 GG-NER specific proteins in this thesis	10
1.4.1 XPC/HR23B	11
1.4.2 Rad4/Rad23.....	11
1.5 DNA-Protein binding	12
1.6 Outline of this thesis.....	12
Chapter 2 Development of a Fluorescence Confocal Microscope	15
2.1 Introduction	16
2.2 Single-molecule fluorescence confocal microscope	16
2.2.1 Microscope configuration	16
2.2.2 Microscope functions.....	17
2.3 Single-molecule intensity imaging and spectral imaging	18
2.3.1 Materials and methods	18
2.3.1.1 Materials	18
2.3.1.2 Coverslips.....	18
2.3.1.3 Confocal microscope measurements	18
2.3.1.4 Microscope configuration	18
2.3.2 Results.....	18
2.3.3 Discussion.....	19
2.3.4 Conclusions.....	20
2.4 Fluorescence correlation spectroscopy.....	20
2.4.1 Introduction.....	20
2.4.2 Correlation analysis.....	20
2.4.3 Validation of correlation analysis program for FCS	21
2.4.4 Available models of FCS	22
2.4.4.1 2D and 3D free diffusion models in FCS.....	22
2.4.4.2 Free diffusion of a single species with triplet blinking.....	23
2.4.4.3 Restricted diffusion.....	23
2.4.4.4 Diffusion in flow.....	24
2.5 Experimental aspects of FCS.....	24
2.5.1 Introduction.....	24

2.5.2 Confocal volume	24
2.5.3 Photophysical and photochemical dynamics of fluorophores	25
2.5.3.1 Optical saturation for some free fluorescent dyes	26
2.5.3.2 Fluorescence triplet blinking for some free fluorescent proteins	26
2.5.4 Interfacial effects	28
2.5.4.1 Materials	28
2.5.4.2 Coverslip surface passivation	29
2.5.4.3 Confocal microscopic measurements	29
2.5.4.4 Sample solutions	29
2.5.4.5 Results	29
2.5.4.6 Discussion	33
2.5.4.7 Conclusions	35
2.5.5 How to prevent artifacts in FCS?	35
2.5.5.1 On-line quantitative FCS	35
2.5.5.2 Coverslip surface preparation	35
2.5.5.3 Identical experimental conditions and internal FCS calibration	36
2.5.5.4 Water Raman band as internal excitation intensity marker	36
2.6 Steady-state fluorescence anisotropy	36
2.7 Conclusions	37
Chapter 3 Single-molecule Localization Accuracy: Theory	39
3.1 Introduction	40
3.2 Results	40
3.2.1 Analytical expressions for localization accuracy	40
3.2.2 Numerical simulations	41
3.2.3 Relationship between the Airy pattern and the Gaussian beam in LSF	43
3.3 Discussion	44
3.4 Conclusions	44
3.5 Appendix	44
3.5.1 2D Gaussian fitting with the least squares minimization	44
3.5.2 LSF of an Airy pattern and a Gaussian beam	45
3.5.3 Theoretical localization accuracy derivation	46
3.5.3.1 Simplified LSF – SLSF	46
3.5.3.2 Full LSF – FLSF	47
3.5.4 Comparison with the Thompson formula	48
3.5.5 Fundamental limitations of localization accuracy in LSF	49
3.5.6 Conditions for the localization accuracy formulas	49
3.5.7 Additional simulation results	49
Chapter 4 Nanometer-precision Single-molecule Localization: Experimental Evidence	53
4.1 Introduction	54
4.2 Materials	55
4.3 Methods	56
4.3.1 Biotin-PEG functionalized coverslips	56
4.3.2 TIRF microscope	57
4.3.3 Sample preparation for microscopic measurements	57
4.3.4 Image acquisition and processing	58
4.4 Results	59
4.4.1 Single quantum dots	59

4.4.2 Two quantum dots in a diffraction-limited spot	61
4.4.3 A comparison of the experimental and theoretical localization accuracy	63
4.4.4 The analysis of microscope resolution	64
4.5 Discussion	64
4.5.1 The selection of quantum dots	64
4.5.2 Identify multiple QDots in a diffraction-limited spot.....	64
4.5.3 Comparison between FLSF and SLSF	65
4.5.4 Microscope spherical aberration	65
4.5.5 Experimental and theoretical localization accuracy	65
4.6 Conclusions	66
4.7 Appendix.....	66
4.7.1 Distance determination for two QDots.....	66
4.7.2 Centroid coordinates of two-QDot system.....	66
4.7.3 Detailed localization results	67
Chapter 5 Protein Stability in Fluorescence Microscopy.....	69
5.1 Introduction	70
5.2 Materials	71
5.3 Methods	71
5.3.1 Single-molecule fluorescence confocal microscope	71
5.3.2 Coverslip surface passivation.....	72
5.3.3 On-line FCS analysis	72
5.3.4 Buffers	73
5.3.5 Purification of fluorescent proteins	73
5.3.6 Preparation of FP sample solutions.....	73
5.3.7 Preparation of double-stranded DNA.....	74
5.3.8 DNA restriction reaction	74
5.4 Results.....	74
5.4.1 Protein adsorption to surfaces	74
5.4.2 Loss of fluorescence of FPs in aqueous solutions	75
5.4.3 Activity of FPs in the presence of surfactant TX100	77
5.4.4 Activity of restriction enzymes in the presence of surfactant TX100	78
5.5 Discussion	80
5.5.1 Molecular crowding environment and protein functionality lifetime.....	80
5.5.2 Estimation of TDt of eGFP	82
5.6 Conclusions	82
5.7 Appendix.....	83
5.7.1 Additional figure	83
Chapter 6 Nucleotide Excision Repair Protein Complexes XPC/HR23B and Rad4/Rad23	
Binding to DNA – Investigation by Combined Fluorescence Correlation	
Spectroscopy and Steady-state Fluorescence Anisotropy	85
6.1 Introduction	86
6.2 Materials and methods.....	88
6.2.1 Chemical materials.....	88
6.2.2 Preparation of proteins	88
6.2.2.1 XPC/HR23B	88
6.2.2.2 Rad4/Rad23.....	89
6.2.2.3 XPC/Alexa488-HR23B	89

6.2.3 Preparation of DNA substrates.....	89
6.2.4 XPC/HR23B and DNA binding reactions for gel mobility shift assay	91
6.2.5 Protein titration binding reaction	91
6.2.6 Competition binding reaction for XPC/HR23B complex	91
6.2.7 Single-molecule fluorescence confocal microscope	91
6.2.8 Data analysis	93
6.2.8.1 <i>One-site binding model</i>	93
6.2.8.2 <i>Competition binding model</i>	93
6.2.8.3 <i>Binding model for FCS</i>	93
6.2.8.4 <i>Binding model for SFA</i>	94
6.2.8.5 <i>Non-linear least squares fitting for titration curves</i>	95
6.2.9 Combined FCS and SFA analyses	96
6.3 Results.....	96
6.3.1 Validation of SFA measurements	96
6.3.2 Gel mobility shift assay of XPC/HR23B and DNA binding reactions.....	96
6.3.3 Binding of XPC/HR23B to DNA substrates	97
6.3.4 Competition titration for XPC/HR23B binding specificity.....	100
6.3.5 Binding of Rad4/Rad23 to dsDNA and damaged bubble DNA	104
6.3.6 Binding of XPC/Alexa488-HR23B to UV-irradiated plasmid DNA	106
6.4 Discussion	107
6.4.1 Comparison of both FCS and SFA techniques.....	107
6.4.1.1 <i>Global translational motion and local rotational motions</i>	107
6.4.1.2 <i>Resolution</i>	108
6.4.1.3 <i>Sensitivity to significant fluorescent intensity bursts</i>	110
6.4.1.4 <i>Additivity of diffusion constants and SFAs</i>	111
6.4.2 Translational diffusion time of binding complex	111
6.4.3 Multiple binding sites on a single molecule.....	112
6.4.4 Mechanism of XPC/HR23B binding to DNA.....	113
6.5 Conclusions	114
Chapter 7 Multiparameter Fluorescence Imaging in Single Cells	115
7.1 Introduction	116
7.2 Materials and methods.....	117
7.2.1 Cell culture.....	117
7.2.2 UV irradiation	117
7.2.3 Multiparameter fluorescence imaging microscopy	118
7.2.4 Multiparameter fluorescence imaging measurements in cells.....	118
7.2.5 Image processing and data analysis	119
7.3 Results.....	119
7.3.1 Distribution of GFP-XPC in mammalian nuclei	119
7.3.2 Autofluorescence	120
7.3.3 SFA imaging in cells.....	121
7.3.4 Single point FCS and SFA in cells.....	125
7.4 Discussion and perspective	127
7.4.1 Rotational mobility of GFP-XPC in cells by SFA	128
7.4.2 Fluorescent label for SFA imaging in cells.....	128
7.4.3 Perspectives for fluorescent intensity time trace analysis	128
7.5 Conclusions	129

Chapter 8 Nanometer Single-molecule Colocalization of Nucleotide Excision Repair

Protein complexes XPC/Alexa488-HR23B Binding to Cy5 Labeled DNA.....	131
8.1 Introduction	132
8.2 Materials and methods.....	134
8.2.1 Materials	134
8.2.2 Preparation of XPC/Alexa488-HR23B	134
8.2.3 Preparation of DNA substrates.....	134
8.2.4 DNA-protein binding reaction	135
8.2.5 Streptavidin functionalized protein-resistant surface.....	135
8.2.6 TIRF microscope.....	137
8.2.7 Sample preparation for microscopic measurements.....	138
8.2.8 Image acquisition and processing	138
8.3 Results.....	140
8.3.1 Single-molecule TIRF imaging.....	140
8.3.2 Photostability of Cy5 and Alexa488 dyes for NSL.....	140
8.3.3 XPC/Alexa488-HR23B and Cy5-dsDNA	141
8.3.4 XPC/Alexa488-HR23B and Cy5-B5-DNA	143
8.4 Discussion	146
8.4.1 Photophysical properties of Cy5 and Alexa488 dyes.....	146
8.4.2 Theoretical localization in NSL	148
8.4.3 DNA length measurement.....	149
8.4.4 Binding model of protein XPC/Alexa488-XPC to DNA	149
8.4.5 Binding degree of XPC/HR23B complex to DNA substrates.....	150
8.5 Conclusions	150
Chapter 9 General Discussion and Prospects.....	153
9.1 Quantitative on-line FCS.....	154
9.2 Quantitative on-line FCCS	156
9.3 Two photon FCS.....	156
9.4 Fluorescence anisotropy.....	157
9.5 Total internal reflection fluorescence microscopy.....	157
9.6 DNA repair	158
Summary	161
Samenvatting	165
内容简介	169
References.....	173
Acknowledgements	187
List of publications	191
Curriculum Vitae	193

Chapter 1

Introduction

1.1 AIM OF THIS THESIS

The aim of this thesis is to study the architecture of nucleotide excision repair (NER) complexes using single-molecule spectroscopy methods. NER is one of the most important mammalian DNA repair processes to eliminate a variety of frequently occurring damages on genomic DNA [1]. Much information about the molecules participating in a successful repair event has come from biochemical research during the last few decades. However, mechanistic aspects are easily obscured by ensemble averaging. The NER process is commonly divided in distinct steps [1-3] including 1) damage recognition, 2) dual incision in DNA around the damage, 3) release of a 24-32 bps nucleotide, which contains the damage, 4) DNA synthesis and 5) ligation of the new oligonucleotide into the gap. In each of these steps, different NER related proteins are sequentially recruited to the damage site on DNA. Biochemical methods have been essential to recognize the participating proteins, the sequence of binding and releasing events and the interactions between proteins. However, detailed structural and functional insight in how the NER process evolves is difficult to acquire by existing bulk biochemical methods. This thesis is devoted to the development of alternative, single molecule approaches, which will further our understanding of how DNA and protein molecules interact in complex molecular events like NER. Here it will be shown how laser scanning single-molecule confocal fluorescence microscopy and total internal reflection fluorescence microscopy can be used to study interactions between proteins and DNA in the macromolecular assembly that are part of NER.

1.2 FLUORESCENCE MICROSCOPY

1.2.1 Fluorescence

Fluorescence is the ability of certain chemical substances to absorb light (energy) of a certain wavelength (color) and to emit light with a different wavelength which appears in different degrees of redshift compared to the absorbed light. Fluorescence spectroscopy and microscopy are now at the basis of many modern methods in the life sciences. The various energy levels involved in the absorption and the emission of fluorescent light by a fluorophore are classically illustrated by a Jablonski diagram [4] (Fig. 1-1). After absorption of a photon, a fluorophore is usually excited to higher vibrational energy levels of the first (S_1) or second (S_2) singlet energy state from the vibrational energy levels of the ground state (S_0) as shown in Fig. 1-1. This will be followed by one or more of several processes including internal conversion,

Fig. 1-1 Typical Jablonski diagram.

vibrational relaxation, fluorescence, phosphorescence, intersystem crossing, and non-radiative relaxation, all with varying probabilities and lifetimes of energy levels [4]. Internal conversion or vibrational relaxation generally occurs on a picoseconds time scale after the energy absorption. An excited fluorophore exists in the lowest excited singlet state for periods on the order of nanoseconds before it relaxes to the ground state with or without emission of a fluorescent photon. As shown in Fig. 1-1, the fluorescence emission process competes with several other relaxation pathways since the excited state energy can dissipate also by non-radiative relaxation, such as heat, fluorescence quenching for instance as a result of after collisions, or intersystem crossing transition to the lowest excited triplet state (T_1). Upon transition from an excited singlet state to the excited triplet state, fluorophores may undergo phosphorescence emission or delayed fluorescence by a transition back to the excited singlet state. Fluorophores can also interact with other molecules to produce

irreversible covalent modifications, which manifest themselves optically for instance as photobleaching events. Intersystem crossing, a transition between states with a different electron-spin configuration, may for instance result in “blinking”. Photobleaching may also occur from a triplet state because the long lifetime offers a higher probability for the fluorophore to undergo chemical reactions with other components in the environment. The average number of excitation and emission cycles that occur for a particular fluorophore before photobleaching is a stochastic variable and highly dependent upon the molecular structure and the local environment.

1.2.2 Single-molecule fluorescence microscopy

Fluorescence microscopy has become an essential tool in biological and biomedical sciences, as well as in materials science due to attributes that are not readily available in other contrast techniques in traditional optical microscopy. Fluorescence microspectroscopy is capable of retrieving information about physical parameters of fluorescent molecules with high spatial and temporal resolution. Among all these fluorescence microscopy techniques, single-molecule fluorescence microscopy may be contrasted with measurements on an ensemble or bulk collection of molecules, where the individual behavior cannot be distinguished and only average characteristics can be measured, and it is a powerful method to study the individual behavior of biological systems as opposed to the ensemble averaging in bulk experiments [3-16].

1.2.3 Confocal fluorescence microscopy

Unlike wide-field microscopy, in which both in- and out-of-focus light is collected, confocal laser scanning microscopy is commonly used to improve contrast and increase sensitivity in fluorescent intensity images through the use of a pinhole in the light path, which suppresses out-of-focus fluorescence light. In confocal microscopy, small spontaneous variations of the fluorescence intensity in a microscopic volume (typically ~ 1 femtoliter) can be detected and analyzed. The sensitivity and selectivity of fluorescence allows measurements at the single-molecule detection level, and thus it has been a promising method to study the behavior of biomolecules *in vitro* or in living cells at physiologically relevant concentrations. Among many fluorescence techniques, Fluorescence correlation spectroscopy (FCS) has been used to monitor physical parameters like local particle concentration, molecular brightness, translational diffusion behavior and internal dynamics of fluorescent molecules while fluorescence anisotropy (FA) has been used to study rotational diffusion of fluorescent molecules.

1.2.3.1 Fluorescence correlation spectroscopy

FCS was first introduced [3-5] as a method for measuring molecular diffusion and reaction kinetics, and in most modern applications it is based on confocal microscopy

Fig. 1-2 (a) Schematic configuration of a confocal FCS setup. A short wavelength laser beam is first expanded by a beam expander (L1 and L2), then focused by a high-NA objective lens (OBJ) on a fluorescent sample (FS). The fluorescence is collected by the same objective, reflected by a dichroic mirror DM1, filtered (F), focused by a tube lens (TL), and passed through a confocal aperture (P) onto two avalanche photodiode (APD) detectors. The dichroic mirror DM2 is used for selectively recording two different color fluorescent signals. (b) Magnified focal volume (blue) within which the sample particles (green spheres) are illuminated. The focal volume is the distribution of laser illumination at the focus of the objective. On the other hand, the observation volume, contained within the focal volume, is the region in space where fluorescent molecules are both excited and detected. (c) A typical fluorescence intensity time trace in a FCS measurement. (d) After auto-correlation analysis, measured auto-correlation curve $G(t)$ can be fitted using various analytic functions to extract information about molecular concentration, brightness, diffusion, and chemical kinetics for one or more diffusing fluorescent species.

[5]. In confocal FCS, as schematically shown in Fig. 1-2, light is focused in a volume of approximately one femtoliter, and intensity fluctuations in the fluorescence are recorded for correlation analysis. These fluctuations are caused by random diffusion of fluorophores in and out of the illuminated focal volume or by photophysical changes in their fluorescence emission. FCS is used to determine the molecular mass, density and rate of diffusion of ensembles or even individual biomolecules.

1.2.3.2 Fluorescence anisotropy

FA measures the depolarization (Fig. 1-3) of fluorophores, which can be caused by intrinsic sources, e.g., fluorescence excitation photoselection and fluorescence energy transfer, as well as external sources including rotational motion of fluorophores in solution, reactions, *etc.* FA was first described by Perrin (1926) [6]. The theory of fluorescence polarization is based on the observation that when a linearly polarized light beam of appropriate wavelength passes through a dilute aqueous solution of fluorophores, fluorophores with their absorption transition vectors aligned parallel to the polarization plane of the light are preferentially excited. The polarization plane of

Fig. 1-3 Theory of fluorescence anisotropy (FA). (a) Excitation photoselection of fluorophores. Illustrated by the linearly polarized excitation propagating along x-axis, the probability of excitation photon absorption for immobile fluorophores is proportional to $\cos^2\theta$, where θ is the angle between the absorption dipole and the incident polarization direction indicated by the blue arrows parallel to the z-axis. Upon absorption of an exciting photon a transition dipole is created in the fluorophore usually with different magnitude and direction from the ground state dipole. (b) As an example, we consider that a single immobile fluorophore (grey ellipsoid) has two excited dipoles corresponding to the $s_0 \rightarrow s_1$ (green) and the $s_0 \rightarrow s_2$ (blue) transitions and an emission dipole (red) for the $s_1 \rightarrow s_0$ transition (see the Jablonski diagram in Fig. 1-1). Any one of these dipoles usually has one average orientation as illustratively indicated in Fig. 1-3(b) and may be oriented with certain probability at an arbitrary angle close to the average orientation in each excitation and emission cycle. Hence, If an ensemble of randomly oriented immobile fluorophores (c1) are separately excited with two short wavelength excitation beams – green for $s_0 \rightarrow s_1$ (green) transitions (c2) and blue for the $s_0 \rightarrow s_2$ transitions (c4), due to photoselection a population of molecules which nominally have their excited dipoles lined up with the polarization direction of the excitation are excited as shown in Fig. 1-3(c2) and Fig. 1-3(c4). For the $s_0 \rightarrow s_2$ transitions (c4), rapid internal conversion (see Fig. 1-1) leaves the excited fluorophores (c5) in the s_1 state, but the orientation of the excited dipoles will thus have a different average orientation than the $s_0 \rightarrow s_2$ absorption dipoles originally photoselected by the exciting light, and consequently the average orientation (c6) of their emission dipoles are different from those (c3) from the fluorophores in Fig. 1-3(c2). Therefore, the depolarization from immobile fluorophores is dependent on the excitation wavelength and has intrinsic contribution to fluorescence anisotropy. In the case that the fluorophores can rotate during the fluorescence lifetime, the dipole rotation becomes an external source for the additional depolarization which can also contribute to the measured fluorescence anisotropy.

the fluorescence photon is determined by the actual orientation of the molecule at the moment of emission. The emitted fluorescent light is depolarized because molecules tumble rapidly in solution during their fluorescence lifetime and emit photons from a distribution of orientations. If the fluorescent tracer is bound by a larger molecule, its effective molecular volume is increased, and thus the tracer's rotation is slowed down so that the polarization plane of emitted light is close to the excitation polarization plane. The bound and free states of the tracer each have an intrinsic polarization value: a relatively high value for the bound state and a low value for the free state. The measured anisotropy is a weighted average of the two values, thus providing a direct measure of the fraction of tracer bound to a receptor. FA has been used to monitor molecular volume (or mass) increase due to molecular binding, e.g. the molecular

interactions of receptor-ligand [7], DNA-protein [8, 9], peptide-protein [10], as well as molecular volume (or mass) decrease due to dissociation or enzymatic degradation [11, 12].

1.2.3.3 Advantages of FCS and FA

Many microscopic studies on biomolecular interactions have been based on measurements of individual molecules immobilized onto dielectric surfaces (such as glass coverslips) in the absence of an aqueous environment. However, it is known that the surrounding environment affects the properties of molecules. Coatings and protection to avoid the interaction between molecules and surface help to minimize surface effect. Because most biological events occur in water, it is important to evaluate the behavior of freely diffusing molecules in solution. Both FCS and FA technologies provide a view of molecular binding events in solution and enable true equilibrium analysis in the low concentration range (nanomolar to picomolar). Both technologies do not affect samples, so they can be changed and reanalyzed in order to ascertain the effect on binding by changes as pH, temperature, and salt concentration. In addition, because both technologies enable real-time data acquisition, experiments are not limited to equilibrium binding studies, and are thus well-suited to kinetic analyses of association and dissociation reactions. For both techniques, the measurements are fast, relatively simple, and require small amounts of material, and are therefore well-suited to investigate binding conditions, which involves changes in buffers, detergents, DNA sequences and the addition of non-specific proteins or nucleic acids.

1.2.4 Total internal reflection fluorescence microscopy

When light travels from a medium of higher refractive index to a medium of lower refractive index, it is refracted away from the normal at the boundary (Fig. 1-4(a)). At higher angles of incidence a critical point is reached where the light will not transmit into the lower refractive index medium and will instead be totally reflected (Fig. 1-4(b)). This phenomenon is known as Total Internal Reflection. When the incident light is totally reflected at the glass-water interface, an evanescent wave is generated with an associated electromagnetic field, which decays exponentially from the interface. A typical maximal penetration depth is only some 200 nm into the sample medium (Fig. 1-4(c)). A total internal reflection fluorescence (TIRF) microscope uses evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface, and this selective visualization of fluorophores in TIRF microscopy renders high axial resolution. Two common technical approaches to TIRF microscope have been developed, namely objective-based (Fig. 1-4(d)) and prism-based (Fig. 1-4(e)).

Fig. 1-4 (a) A laser beam is reflected and refracted in an optical prism. (b) Total internal reflection. (c) Evanescent wave penetrates to ~ 200 nm into the sample medium (fictitious image). (d) Schematic configuration of objective-based TIRF microscope. (e) Schematic configuration of prism-based TIRF microscope. In Fig. 1-4(d) and Fig. 1-4(e), s, cs, Obj and p represent sample solution, coverslip, objective and optical prism, respectively; the colors green and red represent excitation beam and fluorescence light, respectively.

The objective-based TIRFM has the advantage that the sample is easily placed and is accessible for manipulation. In objective-based TIRFM, the laser excitation is introduced through the microscope objective. To achieve total internal reflection, an oil-immersion objective with a $NA > 1.4$ is required. This objective configuration can result in signal to background limitations because of a combination of autofluorescence from the immersion oil and/or stray light reflections from the excitation laser, which are total internally reflected back through the objective again.

The prism configuration has the disadvantage that the alignment of the laser excitation area and the objective observation region needs to be carefully done. The sample is located between the objective and the prism. As a consequence, more elaborate methods of sample placement and manipulation often have to be considered. Except for the Rayleigh scattering by dust on glass surfaces, almost all the excitation light with the prism configuration is reflected away from the objective detection area, and thus the background photon noise is very low.

1.3 DNA REPAIR

DNA repair is a collection of processes by which a cell recognizes and corrects damage to the DNA molecules that encode its genome. In human cells, both normal metabolic activities and environmental factors such as certain plant toxins, UV light and other radiation can cause DNA damage, resulting in as many as ~ 1 million individual molecular lesions per cell per day [13]. Though this constitutes a very small fraction of the human genome, which consists of approximately 3 billion base pairs, these lesions can alter or impede the cell's ability to exactly transcribe genes and for instance increase the likelihood of tumor formation, or affect the vitality of its daughter cells after it undergoes mitosis. Therefore, the DNA repair process must be constantly active so it can respond rapidly to any damage to the DNA molecule in the cell. DNA damage plays a major role in mutagenesis, carcinogenesis and ageing [14,

15]. Failure to correct DNA molecular lesions in cells can introduce mutations into the genomes of the offspring and thus influence the rate of evolution [16, 17].

Many DNA lesions result in structural changes in the DNA molecule. In eukaryotic cells, DNA is usually supercoiled or wound around histones; this does not protect DNA from harmful effects of chemicals or radiation. The vast majority of DNA damage directly affects the primary structure of the double helix by chemical modification of the bases by introducing non-native chemical bonds or bulky adducts that do not fit in the double helix structure [16, 17].

DNA repair has been commonly separated into the five pathways as follows [16, 17]: direct damage reversal, base excision repair, nucleotide excision repair (NER), mismatch repair, and double-strand break repair, each of which deals with specific types of DNA lesions. Among all these pathways, NER is considered as the most versatile DNA repair system.

1.3.1 Nucleotide excision repair

Nucleotide excision repair (NER) is a versatile repair pathway, involved in the removal of a variety of bulky DNA lesions such as UV induced cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4PP) [1]. A general mechanical model for NER has been proposed and can be dissected into the following steps [1, 16-18]: (1) DNA damage recognition; (2) unwinding of the DNA helix around the damage site; (3) double incision of the damaged strand on both sides of the lesion; (4) removal of the damaged oligonucleotide and resynthesis of the gap, and (5) ligation of the residual nick. Many details of NER as a versatile multi-protein and multi-step system are still unknown. NER in the eukaryotic cell nucleus can be accomplished through two distinctive pathways, known as global genomic NER (GG-NER, see Fig. 1-5) and transcription-coupled NER (TC-NER). As indicated by the two names, GG-NER activity works throughout the entire genome, whereas TC-NER action is performed within active transcriptional regions. Although the mechanical models of both GG-NER and TC-NER show significant overlap, the initial actions of both NER pathways are quite different.

1.3.1.1 NER disorders

Xeroderma pigmentosum (XP), cockayne syndrome (CS) and trichothiodystrophy (TTD) are rare hereditary disorders. Although all these patients exhibit defects in the NER system, their clinical symptoms are quite different. XP patients are extremely photosensitive and exhibit 1000-fold higher incidence in sunlight-induced skin cancers [18]. Eight complementation groups can be distinguished among XP patients, and each group has a distinct defective gene, *i.e.*, XP complementation groups A to G (XPA to XPG) and an XP variant group. The XPA to XPG genes are all directly involved in NER, but most of them have other functions in cellular activities as well. CS patients exhibit hardly any similarity to XP patients, and present a range of defects,

such as dwarfism, mental retardation, sun sensitivity and so on [19]. CS patients fall into two genetic complementation groups: CSA and CSB. However, a defect in NER does not seem to be sufficient to explain complex clinical phenotypes of CS patients, and CS is a clinically heterogeneous multi-system disorder [20]. TTD is another rare multi-system disorder [21], and its patients have brittle hair, photosensitivity, mental retardation, *etc.* TTD is thought to be caused by the defects of Transcription factor II H (TFIIH) [22].

1.3.1.2 Global genomic NER

GG-NER can resolve DNA damage in the genome and is thus responsible for the majority of lesion removal [21-23]. The efficiency is known to depend on the type of DNA damage. The repair kinetics of GG-NER can be faster for specific lesions that disrupt the DNA double helix. Therefore, the initial damage sensing process may be

Fig. 1-5 Multi-step model [1] for mechanism of global genome nucleotide excision repair (GG-NER). (a) Non-damaged DNA (top) and damaged DNA (bottom) with a bulky lesion indicated with three red bases. (b) The DNA lesion is initially recognized by XPC/HR23B complex. (c) Multi-subunit transcription factor TFIIH has two helicase subunits, XPA and RPA, and is recruited by the XPC/HR23B complex to the lesion. XPA and RPA verify the damage and then the TFIIH opens ~30 base pairs of DNA around the lesion. (d) The endonucleases, XPG and ERCC1/XPF, cleave 3' and 5' of the borders of the opened stretch only in the damaged strand, respectively. (e) The resynthesis machinery fills in the gap and DNA ligase I seals the nick. For more detailed about the last step, refer to Ref. [2].

the major determinant for the repair rate of GG-NER. Fig. 1-5 is the multi-step model [1, 16-18] for mechanism of GG-NER.

Considerable progress on the mechanism of damage recognition in GG-NER has been made over the recent years [23], but its precise elucidation remains a matter of discussion in this field. Different proteins including XPA [24], RPA [25], XPC/HR23B [26], yeast Rad4/Rad23 [27], and several combinations [28, 29] of these proteins were shown to have a higher binding affinity for damaged DNA than for undamaged DNA [25, 26, 29], and have been suggested to be responsible for initial damage recognition in GG-NER. It is now widely accepted that the DNA damage is recognized by human XPC/HR23B complex [26] or yeast Rad4/Rad23 [27]. GG-NER can be briefly summarized as follows: after recognition of the helical distortions, the presence of an actual lesion is verified [1, 29], and subsequently endonucleases, XPG and ERCC1-XPF (for excision repair cross complementation group 1 and XPF heterodimer), incise the damaged strand at the junctions between the damage site and double-stranded DNA. This leads to an open complex of 25-30 bps. After dual incisions, the gap is filled by polymerization which involves the presence of replication factor C, proliferating cell nuclear antigen and DNA polymerase δ and ϵ . Subsequently ligase I seals the residual nick [2, 3].

1.3.1.3 Transcription-coupled NER

The discovery of TC-NER came from the observation that active genes were repaired much faster than inactive genes [30, 31]. The faster repair rate observed in TC-NER seemed to be a consequence of enhanced lesion removal from the actively transcribed strand of a gene [32, 33]. TC-NER differs from GG-NER in the initial steps of the repair activity and does not require the GG-NER specific proteins XPC-HR23B [34, 35]. An adduct present in the template strand of a transcribed gene can block RNA Polymerase II (RNA Pol II) at the damage site. TC-NER is mediated by the TC-NER specific proteins CSA and CSB, as well as XPA-binding protein 2 [36]. Although the exact molecular mechanism of TC-NER has remained largely elusive, a possible mechanism of TC-NER is that stalling of the elongating RNA Pol II at the lesion site serves as the damage recognition element [36-38]. When the jammed polymerase is either removed or displaced, the core NER factors are recruited to the damage site to repair the DNA damage. The subsequent sequence of steps is thought to be the same for TC-NER as for GG-NER.

1.4 GG-NER SPECIFIC PROTEINS IN THIS THESIS

Eukaryotic NER appears to be very complex. There are approximately 30 gene products directly implicated in the repair reaction [1, 23]. However, the work in this thesis was focused on the GG-NER initial step – DNA damage recognition. In this step of human GG-NER, the specific protein complex XPC/HR23B was used to study its interaction properties with different DNA substrates. Recently, the yeast XPC

orthologue Rad4 has been studied [27] and found to have similar function and structure as XPC in human cells. In addition to high affinity and specificity observed from both human XPC and yeast Rad4 binding to damaged DNA, both proteins have substantial affinity for undamaged DNA [26, 27, 39-41].

1.4.1 XPC/HR23B

Xeroderma pigmentosum group C (XPC) protein (MW: 125 kDa) is an initiator of GG-NER and plays an essential role in the damage recognition step of GG-NER [3, 26, 42]. The XPC protein exists *in vivo* as a heterotrimeric complex with one of the two mammalian homologs of *S. cerevisiae* Rad23p (HR23A or HR23B, MW: 58 kDa) [43] and centrin 2, an 18 kDa centrosome protein [44, 45]. However, centrin 2 is not essential for GG-NER at least *in vitro*. Most XPC is bound to HR23B rather than to HR23A [45]. In the case of UV damage, XPC-HR23B binds to 6-PPs *in vitro* without the requirement of XPA and RPA [26, 39, 40, 46], and in ultraviolet-irradiated cells XPC localizes to DNA lesions before other NER factors [42]. Both observations indicate a central role for XPC-HR23B in damage recognition of GG-NER. However, recognition of CPDs damage by the XPC/HR23B complex is found to be inefficient, and the binding of XPE to CPDs damage improves the localization of XPC to these lesions [47, 48]. XPC-HR23B complex has a high affinity for single-stranded DNA [43, 49], and exhibits a higher affinity for damaged DNA than for undamaged templates [26, 39, 40]. *In vitro*, GG-NER assays [26, 50] have shown that XPC-HR23B is required when several base pairs around a lesion are artificially unpaired. This seems to indicate that impaired Watson-Crick base pairing in double-stranded DNA results in the specificity of XPC-HR23B the specificity of binding to DNA damage site. A feasible mechanism for XPC-HR23B function is that it binds to and recognizes DNA damages by detecting the local helix distortion including improper base pairing which can be induced by adducts. XPC/HR23B complex binding to DNA damage locally melts the DNA duplex and potentially promotes recruitment of other NER proteins [51].

The function of HR23B in NER is not clear, but it has been proposed that HR23B protects XPC from ubiquitin-mediated degradation [52]. XPC protein itself is easily destabilized *in vivo* as well as *in vitro*, and the HR23B protein is essential for efficient GG-NER [53]. It is an experimental problem that both XPC proteins and XPC/HR23B complexes tend to aggregate *in vitro* [39], and special care has to be taken in *in vitro* experiments.

1.4.2 Rad4/Rad23

Rad4 protein is the *Saccharomyces cerevisiae* orthologue of XPC and appears *in vivo* to be always associated with yeast protein Rad23. The structural domains of the yeast protein Rad4 and human XPC share a large amount of sequence identity [27]. The crystal structure [27] of the protein Rad4 bound to DNA, which contains a CPD lesion,

shows that Rad4 inserts a β -hairpin through the DNA duplex and alters the Watson-Crick double helix by flipping out two damaged bases. This potentially promotes recruitment of other NER proteins. Similar to the human XPC/HR23B protein complex, yeast Rad23 has been proposed to protect Rad4 protein from ubiquitin-mediated degradation [54].

1.5 DNA-PROTEIN BINDING

Protein binding to DNA plays a fundamental role in the regulation of cellular and viral functions. The mechanisms by which proteins and DNA interact to control many cellular activities, including transcription and replication, are slowly being elucidated. DNA-protein interactions are studied using a variety of methods such as gel-shift assays, footprinting, single-molecule imaging, fluorescence resonance energy transfer (FRET), FCS and FA. Each of these methods may contribute distinct information about the formation or conformation of binding complexes. As illustrated in this thesis, among these methods, FCS and FA provide a rapid, non-radioactive method for measuring DNA-protein binding directly in aqueous solution. In a basic DNA-protein binding experiment, both proteins and DNA have fluorescent labels, or either proteins or DNA are separately labeled with fluorophores. After the binding reactions are allowed to reach equilibrium, the fluorescent parameters (e.g. molecular translation diffusion time in FCS or FA) of each sample are measured and the data are used to construct an equilibrium binding curve. The analysis of protein-DNA binding is of importance to understand many processes in the field of molecular biology. The simplest system involves the binding of a protein to a single site on a DNA molecule.

1.6 OUTLINE OF THIS THESIS

The work in this thesis describes the development of state-of-the-art fluorescence microscopy methods in the project titled “In vitro and in vivo studies of the architecture of nucleotide excision repair complexes” granted by the Human Frontiers in Science Program (HFSP) RGP0007/2004-C. Chapter 2 presents our developments on a single-molecule fluorescence confocal microscope (SFCM) and a few applications. With the SFCM, it was possible to perform fluorescent intensity imaging, steady-state FA imaging, fluorescent spectral imaging, single point on-line quantitative FCS (or FCCS) and steady-state FA. It will be shown in Chapter 2 that FCS and other techniques are of great importance for many biological applications since most biomolecular interactions actually take place in solution. It will be shown that the presence of a glass-solution interface may give rise to undesirable aspecific adsorption but may also lead to interesting interface effects, related to the “like charge attraction” [55-63] or the entropic “excluded-volume” effects [64-66]. Chapter 3 is a theoretical study on single-molecule localization accuracy in wide field fluorescence microscopy. Exact analytical expressions for the localization accuracy are derived and they are in precise agreement with the simulations and the experimental results

presented in Chapter 4. In Chapter 4, water soluble quantum dots were used to demonstrate nanometer localization accuracy of single-molecules in total internal reflection fluorescence. Chapter 5 is a study on soluble protein stability in aqueous solution by on-line quantitative FCS technique. In this chapter, we reported the observations of a 1000-fold increase in lifetime of protein functionality by quantitative fluorescence microscopy. These observations suggest that soluble biomolecules can extend an influence over much larger distances than suggested by their actual volume. This study actually provides information to help the buffer preparation for the binding reactions between NER proteins and DNA substrates in Chapter 6. In Chapter 6, the binding properties of NER proteins to various DNA substrates were studied by using combined FCS and FA techniques to determine the binding affinities and specificity of the human XPC/HR23B complex and the yeast Rad4/Rad23 complex to various DNA substrates in combination with titration binding experiments and competition titration experiments. Chapter 7 presents the multiparameter fluorescence imaging technique, including fluorescent intensity imaging, fluorescent spectral imaging and steady-state FA imaging, for single living XP4PA cells to study the interaction of the DNA repair protein XPC tagged with green fluorescent protein and DNA. The limitations of FCS applications in cells are presented and the potential use of other mathematical data processing methods for fluorescent time trace analysis is suggested. In Chapter 8, single-molecule imaging and nanometer-precision localization technique were used to study the binding properties of the human GFP-XPC/HR23B complex to damaged and undamaged DNA substrates in TIRF microscopy. Chapter 9, finally, is a prospect that addresses some major remaining questions on both microscopic technique improvement and the biological NER system.

Chapter 2

Development of a Fluorescence Confocal Microscope

ABSTRACT

A state-of-the-art laser scanning fluorescence confocal microscope is described in this chapter. The instrument allows performing fluorescent intensity imaging, steady-state fluorescent anisotropy (SFA) imaging, fluorescent spectral imaging, and single point on-line quantitative fluorescence correlation spectroscopy (FCS) or fluorescence cross-correlation spectroscopy (FCCS) and SFA. Latest developments allow on-line measurement and processing of almost all microscopic functions, which provides unique fidelity for measured parameters in the single-molecule fluorescence confocal microscope, and are the groundwork for advanced applications presented in the following chapters.

On-line quantitative FCS and SFA are the two most frequently used techniques in this thesis. However, they are known to be susceptible to give artifacts since they are very sensitive to many factors, for example, microscope configuration and alignment. For both techniques, the artifact sources are carefully studied in this chapter and various strategies are proposed and applied to avoid artificial results in actual measurements.

It was found in the FCS measurements that many investigated molecules exhibited significantly different dynamic behaviors and conformations at the glass-solution interface than in solution. Therefore, a useful lesson learnt from this study is that the single-molecule surface imaging may not exactly reflect the dynamic behavior and conformation of the investigated molecules in solution and perhaps only represents results created at the glass-solution interface by the interfacial effect. Therefore, FCS or other techniques that can detect molecules in solution are of great importance for many biological applications since most biomolecular interactions actually take place in solution, and are potentially distorted at the glass-solution interface.

2.1 INTRODUCTION

For the project titled “In vitro and in vivo studies of the architecture of nucleotide excision repair complexes” granted by the Human Frontiers in Science Program (HFSP) RGP0007/2004-C, several experiments have been performed in a single-molecule fluorescence confocal microscope (SFCM) in Biophysical Engineering Group, Faculty of Science and Technology, University of Twente, the Netherlands. Nevertheless, it should be noted that significant groundwork had been done in building the microscope [67, 68] prior to the start of this thesis. A fluorescent spectrometer with single-molecule sensitivity, on-line quantitative fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS), and steady-state fluorescence anisotropy (SFA) as well as the inverted configuration of the microscope are most recent developments.

2.2 SINGLE-MOLECULE FLUORESCENCE CONFOCAL MICROSCOPE

2.2.1 Microscope configuration

The home-built SFCM with imaging capability consisted of an Ar^+/Kr^+ mixed gas laser (Coherent, Innova 70 spectrum, Santa Clara, CA) and an inverted optical microscope and has been described elsewhere [67, 68]. It was redesigned to accommodate on-line quantitative FCS and steady-state fluorescence anisotropy measurements as well as single-molecule spectral measurements. In the SFCM, a water immersion objective (Zeiss C-Apochromat 63 $\times$, NA=1.20, working distance = 0.24 mm) is used. Linearly polarized laser lines including the commonly used 448 nm, 514 nm, 568 nm, and 647 nm lines are separated by a prism-based beam splitter [67] and used to excite fluorescent molecules. In the microscope, after color selection and separation, excitation light passes a beam expander, a high-reflection/low-transmission (HRLTBS, reflection 5%, transmission 95%) beam splitter and the objective. Emitted and reflected light then re-enters the objective. The interface between the measured solution and the coverslip surface (glass-solution interface) can be readily identified by observing the brightness of back-scattered laser light peaks on a video camera. Fluorescence emission is collected by the same objective and 95% of fluorescent light passes through the HRLTBS beam splitter towards the detectors via one or more notch filters (NF). A folding mirror is used to direct fluorescent light to two APDs in a sealed enclosure or to a single-prism spectrometer equipped with a liquid nitrogen cooled charged-coupled device (CCD) camera (SPEC-10 System, Princeton Instruments, Trenton, NJ) for single-molecule sensitivity spectral measurements. Spectral data were acquired frame by frame using WINSPEC software (Roper Scientific, Duluth, GA). In the sealed APD enclosure, a dichroic beam splitter or a polarizing cube can be installed in between both APDs for FCCS and fluorescence anisotropy, respectively.

A color schematic diagram of the SFCM is presented in Fig. 2-1.

Fig. 2-1 The diagram of single-molecule fluorescence confocal microscope (SFCM).

2.2.2 Microscope functions

A custom computer program was written in Labview (National Instruments, Austin, TX) to control the fluorescence microscope piezo nano-positioning stage and to record fluorescent amplitude time traces or spectra. The single-molecule fluorescence confocal microscope could be operated in five modes:

- 1) Fluorescent intensity imaging;
- 2) Fluorescent anisotropy imaging;
- 3) Fluorescent spectral imaging;
- 4) Intensity time trace for single point FCS and fluorescence anisotropy;

- 5) Spectral time trace for single point spectral correlation spectroscopy and single fluorescent molecule identification.

The on-line quantitative FCS [69] and fluorescence anisotropy were recently developed and integrated in the microscope. See Chapter 6 (section 6.2.7) for the details of the development and applications of the on-line quantitative FCS and SFA.

2.3 SINGLE-MOLECULE INTENSITY IMAGING AND SPECTRAL IMAGING

A number of techniques have been developed and applied to detect and manipulate single biological molecules, since optical imaging and analysis of single-molecules continue to be powerful approaches to study the individual behavior of biological systems in contrast to ensemble averaging [70-83]. To demonstrate the ability of the SFCM for single-molecule intensity imaging and spectral imaging, an example of imaging enhanced green fluorescent proteins (eGFP) immobilized on glass surfaces is given in the following.

2.3.1 Materials and methods

2.3.1.1 Materials

The recombinant proteins eGFP (Molecular mass: 27 kDa, selling concentration: 1 mg/ml) were purchased from Clontech (Palo Alto, CA). Triton X-100 (TX100, product No. T8787) was purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) and used for all buffer preparations and dilution steps.

2.3.1.2 Coverslips

The coverslips were first cleaned in 65 % HNO_3 , washed with milli-Q water, and then dried using N_2 gas. The surface of the cleaned coverslips presents many silanol groups for protein adsorption. See Chapter 5 for the details.

2.3.1.3 Confocal microscope measurements

For single-molecule microscopic measurements, eGFP molecules were diluted to concentrations of 1×10^{-10} M in 0.23 mM TX100 to prevent protein degradation in aqueous solution (See Chapter 5 for the details). 5 μl of diluted eGFP solution was applied on a cleaned coverslip and all eGFP molecules were completely immobilized onto the coverslip surface in a few minutes.

2.3.1.4 Microscope configuration

The microscope configuration for imaging the single eGFP molecules was the same as described in Chapter 5. A linearly polarized 488 nm laser was used for excitation of eGFP. The laser power was 3 μW (or approximately 2.4 kW/cm^2) at the sample.

2.3.2 Results

Fig. 2-2 (a) Scanning confocal image of eGFP molecules immobilized on a cleaned glass coverslip. Image size is $6.2 \times 6.2 \mu\text{m}^2$, 128×128 pixels, dwell time 0.5 ms/pixel, excitation 2.4 kW/cm^2 at 488 nm. The indicated positions 1 and 2 (arrows) are molecules on which spectral intensity (b) and fluorescence intensity time trace (c) were respectively measured. (b) Data acquisition time is 200 ms. (c) Data bin time in the time trace is 0.5 ms.

Fig. 2-2 shows the experimental results of fluorescently imaging the single eGFP molecules. Fig. 2-2(a) is a representative confocal fluorescent intensity image of single eGFPs. Two molecules labeled 1 and 2 in Fig. 2-2(a) were separately used for a single-point spectral measurement (Fig. 2-2(b)) and a single-point intensity time trace measurement (Fig. 2-2(c)). Single fluorophores have the natural signature of almost constant emission intensity terminating with abrupt photobleaching, so Molecule 2 was confirmed to be a single molecule from the intensity time trace in Fig. 2-2(c) while Molecule 1 in Fig. 2-2(a) was characterized as eGFP via its spectrum in Fig. 2-2(b). The light spots in Fig. 2-2(a) have inhomogeneous distribution of fluorescent intensity, which results from the well-known triplet blinking phenomenon [84] of the eGFP fluorescence emission. This is clearer from the intensity time trace in Fig. 2-2(c) where eGFP molecule 2 was observed to blink, indicating that it underwent transitions between fluorescent “on” and “off” states from time to time.

2.3.3 Discussion

Characterization of single fluorophores like in Fig. 2-2(b) using the single point spectral measurements in the SFCM is often very important since in single-molecule experiments one always has to work with extremely low concentration sample

solutions (from nanomolar to picomolar) where fluorescent contaminations may come from anywhere including air, buffer, container walls, glass surface, *etc.* and often easily have an amount comparable with that of the sample molecules. Single point spectral measurements on the images of single eGFP molecules immobilized in agarose gel revealed many fluorescent spots on the images which are like single-molecules and often can show fluorescent emission for a longer time but actually are not eGFP molecules. Therefore, spectral measurement with single-molecule sensitivity is a very useful function in the SFCM.

2.3.4 Conclusions

As shown in Fig. 2-2, this experiment manifests the capacity of the SFCM for fluorescent intensity imaging and fluorescent spectral imaging with single-molecule sensitivity, which is the groundwork for all other functions of the SFCM (see section 2.2.2).

2.4 FLUORESCENCE CORRELATION SPECTROSCOPY

2.4.1 Introduction

Since its early introduction in the 1970s [85-87], fluorescence correlation spectroscopy (FCS) has been extensively used in many applications of biophysical investigation. For many biological applications, FCS has a key advantage that it can be performed in aqueous solutions. Many single-molecule studies have been based on the measurements of individual fluorescent or fluorescently labeled molecules immobilized onto dielectric surfaces (such as glass coverslips) in the absence of an aqueous environment. However, it is known that the surrounding and supporting environments can affect the photophysical properties of fluorescent molecules as well as the biological function of many biological molecules. It is thus very important to evaluate the behavior of freely diffusing molecules in aqueous solution. A number of recent reviews [88-105] attest to the popularity of the FCS technique in the fields of biophysics and biology.

2.4.2 Correlation analysis

In general, the correlation curve is computed by

$$g(\tau) = \frac{\langle \delta F(0) \cdot \delta F(\tau) \rangle}{\langle F \rangle^2} \quad (2-1)$$

where F denotes fluorescence intensity signal, and $\delta F(\tau) = F(\tau) - \langle F \rangle$. However, Eq. (2-1) has an equivalent form,

$$g(\tau) = \frac{\langle F(0) \cdot F(\tau) \rangle}{\langle F \rangle^2} - 1 \quad (2-2)$$

Although both Eqs. (2-1) and (2-2) can give the same correlation curves, the computation for Eq. (2-2) is more efficient than that for Eq. (2-1). This is because the arrays, $\delta F(0)$ and $\delta F(\tau)$, in Eq. (2-1) have to be stored in the data type of float (4 bytes) or double (8 bytes) in computer memory, but raw intensity data arrays $\delta F(0)$ and $\delta F(\tau)$ can be stored in one byte (Here it is assumed that more than 2^7 counts / $12.5 \mu\text{s} = 10.24$ million counts per second will not occur in practical situation). For a long data array, multiplication operations in two one-byte data array stored in computer memory are much faster than those for multiple-byte data. For example, using our custom correlation analysis program based on C++ language and integrated with the Labview control software for the SFCM, the calculation of a 186-point auto-correlation function (ACF) for 10-second 80 kHz fluorescent intensity trace took 1.5 seconds for a computer with 3.2 GHz CPU and 1 GB RAM, which is approximately 100-fold faster than that by a commercial time-correlated single photon counting (TCSPC) card [106].

2.4.3 Validation of correlation analysis program for FCS

In the single-molecule fluorescence confocal microscope, control software for FCS continuously recorded a fluorescence intensity time trace and calculated the ACF for a presettable time. Correlation analysis was coded in C++ language and integrated with the Labview control software for fast calculations of the ACF or the cross-correlation function (CCF). The correlation analysis program can be calibrated by simple use of a room light lamp source which is an incandescent bulb supplied with room electrical power at voltage of 220 V and nominal frequency of 50 Hz. Laser source was stopped in the microscope and the room light was switched on. Due to light scattering, reflection and diffraction, part of lamp light travelled through a pinhole to an APD sealed in a light tight casket. The APD signal was transferred to a computer. The correlation program calculated the ACF by using Eq. (2-2) and the experimental result is shown in circles in Fig. 2-3. As the lamp light intensity is proportional to the applied electrical power (or applied current times applied voltage), we can generalize the lamp light intensity to be

$$I(t) = \Xi + A^2 \cdot \sin^2(2\pi\omega t) \quad (2-3)$$

where Ξ is DC level, A is the amplitude related to the applied voltage and ω is frequency. Obviously, Eq. (2-2) should be simplified to a large extent, since all parameters in Eq. (2-2) are constant for the system. Therefore, Eq. (2-2) can be rewritten as

$$g(\tau) = \lim_{L \rightarrow \infty} \left(\frac{\frac{1}{L} \int_0^L I(t) I(t+\tau) dt}{\left(\frac{1}{L} \int_0^L I(t) dt \right)^2} \right) - 1 \quad (2-4)$$

With Eq. (2-3), the analytical ACF for lamp light can be expressed as

Fig. 2-3 Experimental ACF (circles) and its curve fitting (lines) to Eq. (2-5) for the room light of an incandescent bulb supplied with room electrical power at voltage of 220 V and nominal frequency of 50 Hz. Linear x-scale used in Fig. 2-3(b) is for clarity.

$$g(\tau) = \frac{A^4}{4(A^2 + 2\Xi)} \cos(4\pi\omega\tau) + \frac{A^2}{2} + \Xi - 1 \quad (2-5)$$

Using non-linear least squares fitting, the experimental ACF (circles in Fig. 2-3) can be fitted to Eq. (2-5). The line in Fig. 2-3 is the fitted ACF with $A = 0.4288$, $\Xi = 0.9081$, and $\omega = 49.994$ Hz. These data demonstrate that the correlation analysis program is correct.

2.4.4 Available models of FCS

So far a number of mathematical models [107] have been designed in FCS and used for relevant biochemical or biophysical investigations.

2.4.4.1 2D and 3D free diffusion models in FCS

If it is assumed that the fluorescent brightness of a molecule is not changed during the movement through the observation volume, and the spatial profile of the observation volume can be approximated by a 3D Gaussian, the ACFs for a single species result in a 3D freely diffusing model:

$$G(\tau) = \left[\langle N \rangle \cdot \left(1 + \frac{\tau}{\tau_D} \right) \left(\sqrt{1 + \left(\frac{\omega_r}{\omega_z} \right)^2 \frac{\tau}{\tau_D}} \right) \right]^{-1} \quad (2-6)$$

where the parameters N , τ_D , ω_r and ω_z represent the average number of molecules in the confocal observation volume, the molecular translational diffusion time (TDT), the lateral and axial widths of the focal volume, respectively. Here the τ_D represents the time in which fluorophores stay in the confocal volume, which is related to the translational diffusion coefficient D by

$$\tau_D = \frac{\omega_r^2}{4D} \quad (2-7)$$

and related to the size and shape of the fluorescent molecules. In case molecules can be described as approximate compact spheres in a viscous medium [108], the diffusion coefficient can be expressed by the Stokes-Einstein relation:

$$D = kT/6\pi\eta R \quad (2-8)$$

where k is the Boltzmann constant, T is absolute temperature, η is the solvent viscosity and R is the hydrodynamic radius. Therefore, when the investigated molecules have a globular shape, the molecular mass (m) can be estimated from FCS measurements by

$$\tau_D \propto \frac{6\pi\eta}{kT} \sqrt[3]{m}. \quad (2-9)$$

In the derivation [104] of the 3D free diffusion model, each of the directions of translational motion results in a term like $(1+t/\tau)^{-1/2}$ of Eq. (2-6). The 2D diffusion model in the x - y plane becomes:

$$G(\tau) = \frac{1}{\langle N \rangle} \cdot \frac{1}{1 + \frac{\tau}{\tau_D}} \quad (2-10)$$

In practice, Eq. (2-10) is usually a good approximation to the 3D model, Eq. (2-6), when the confocal volume fulfils the condition $-\omega_r/\omega_z < 1$.

2.4.4.2 Free diffusion of a single species with triplet blinking

For 2D- and 3D free diffusion models the fluorescent brightness of a molecule should not change in the observation volume. However, an additional process commonly observed in FCS is intersystem crossing, a reversible transition of the fluorophores from an excited singlet state to a relative long-lived excited triplet state. The molecular chromophore takes a comparably long time to relax back to the ground state. This results in a common phenomenon of triplet blinking – a series of dark intervals interrupt the otherwise constant fluorescence emission of the molecule diffusing through the excitation volume. If the intra- or intermolecular reactions give rise to fluorescence fluctuations and the correlation curves change on time-scales much faster than those caused by the diffusional motion of the investigated molecules, then the 3D free diffusion in FCS has to be described by Eq. (2-6) multiplied by an additional simple exponential decay for the triplet blinking:

$$G(\tau) = \left[\langle N \rangle \cdot \left(1 + \frac{\tau}{\tau_D} \right) \left(\sqrt{1 + \left(\frac{\omega_r}{\omega_z} \right)^2 \frac{\tau}{\tau_D}} \right) \right]^{-1} \left[1 + \frac{T}{1-T} \exp\left(-\frac{\tau}{\tau_T}\right) \right]. \quad (2-11)$$

where T is average equilibrium fraction of molecules in triplet state, and τ_T is the triplet correlation time related to rate constants for intersystem crossing and the triplet decay.

2.4.4.3 Restricted diffusion

Restricted diffusion is also known as anomalous diffusion and is generally caused by interactions between the investigated fluorescent molecules and fixed or mobile structures. Based on the 3D free diffusion model (Eq. (2-6)), a generalized model for the restricted diffusion in FCS can be expressed as follows:

$$G(\tau) = \left[\langle N \rangle \cdot \left(1 + \left(\frac{\tau}{\tau_D} \right)^\zeta \right) \left(\sqrt{1 + \left(\frac{\omega_r}{\omega_z} \right)^2 \left(\frac{\tau}{\tau_D} \right)^\zeta} \right) \right]^{-1} \quad (2-12)$$

where ζ is the restriction coefficient. For restricted diffusion, $\zeta < 1$, and for free diffusion $\zeta = 1$.

The restricted diffusion model has been used in confocal FCS to probe GFPs diffusion *in vivo* [109] and DNA diffusion *in vitro* [110-112].

2.4.4.4 Diffusion in flow

FCS has been used to map three-dimensional fluid velocities [113-116] for the characterization of flow in microfluidic devices. In this application, the FCS technique is applied to fluorescent particles pumped through a microchannel. The FCS model for the diffusion in flow is formalized [116] as follows:

$$G(\tau) = \left[\langle N \rangle \cdot \left(1 + \frac{\tau}{\tau_D} \right) \left(\sqrt{1 + \left(\frac{\omega_r}{\omega_z} \right)^2 \frac{\tau}{\tau_D}} \right) \right]^{-1} \exp \left[- \left(\frac{v\tau}{\omega_r} \right)^2 \frac{\tau_D}{\tau_D + \tau} \right], \quad (2-13)$$

where v is transport velocity in flow.

2.5 EXPERIMENTAL ASPECTS OF FCS

2.5.1 Introduction

Although FCS is now a well established technique that is widely used in a variety of biological, biophysical and biochemical applications, it has been found that FCS is susceptible to generating misleading results (artifacts) [105]. This is mostly due to the extremely high sensitivity of the FCS technique to many factors. A number of research publications focusing on understanding and avoiding experimental artifacts in FCS have been published [117-126].

2.5.2 Confocal volume

A major physical source of FCS artifacts is due to the difficulty to precisely determine the confocal volume in which the fluorescent molecules are excited and detected when they diffuse. Numerical modeling and simulations [123] have revealed that the standard assumption of a 3D Gaussian profile that has been used for the 3D free diffusion model (Eq. (2-6)) is not a valid approximation for the confocal volume under many common measurement conditions. As revealed by another detailed theoretical study [119], the shape and size of the confocal volume could be affected by at least three optical effects:

1. Refractive index of sample solution. This is a particularly significant factor for intracellular FCS measurements [127, 128] (see Chapter 7). As in Fig. (3) in Ref. [119], an increase of the refractive index from 1.33 to 1.38 results in ~10% increase of the measured TDT in FCS.

2. Confocal pinhole position. This is particularly important for one-photon excitation confocal microscopy, and therefore an important issue for most FCS measurements. The responsible and constant alignments of the detection optics and the position of the pinhole along the optical axis in the fluorescence detection path are cumbersome.
3. Coverslip thickness. Compared with the above two factors, coverslip thickness deviation has only little contribution to the profile of the confocal volume as demonstrated in Fig. (6) of Ref. [119]. The factor actually can be omitted for many practical FCS experimental conditions and especially for this thesis since coverslips with a thickness of 170 μm were used for all the FCS work.

2.5.3 Photophysical and photochemical dynamics of fluorophores

Besides the change of the confocal volume by the optical effects mentioned above, rotational motion of the fluorescent molecule or other fast photophysical and photochemical processes such as fluorescent triplet state dynamics and photoisomerization [129] can also influence the FCS ACF profile over a complete time-scale of tens of nanoseconds to seconds and particularly strongly in the time-scale range from tens of nanoseconds to a few microseconds [4]. However, our SFCM instrument can only work on the time-scale slower than 12.5 μs and is limited by the data acquisition card for acquiring fluorescent intensity signals, so it is difficult to judge the effects of the photophysical and photochemical dynamics of fluorophores in the FCS ACF calculated from an intensity time trace data.

Fortunately, two major photophysical effects, fluorescent triplet blinking and saturation, in FCS measurements have been theoretically studied and it is known how they can affect the determination of TDt from FCS [119, 126]. Optical saturation can lead to a flattening top on the FCS ACF profile and thus results in a slower decay of the FCS ACF curve at larger lag times [119]. Therefore, the TDt of fluorescent molecules measured from the FCS measurements under optical saturation condition can be found by measuring the excitation power dependence. In general, increasing the excitation power results in a larger TDt due to optical saturation. On the contrary, if fluorescent molecules transit into a dark state and disappear in the confocal volume due to fluorescence triplet blinking, the TDts of the molecules in the focal volume are shortened. As a result, the measured TDt by FCS is smaller and also dependent on the excitation power. Normally, the increase of the excitation power causes a decrease of the measured TDt due to fluorescence triplet blinking.

To address the effects of optical saturation and fluorescence triplet blinking, we will show the different experimental observations for the excitation power dependence of the FCS measured TDt for three fluorescent dyes and three fluorescent proteins used in Chapter 5.

Fig. 2-4 FCS measurements of Atto 488, Alexa 488 and Alexa 568 dyes as a function of the power. τ_D and N are the TDt and the average number of molecules in focal volume determined by FCS, respectively, and $\langle I \rangle$ represents average fluorescent intensity rate (counts per second, cps). In each of FCS measurements, 50 10-second fluorescent intensity time traces were measured in sample solution and separately fitted to the 3D free diffusion model for statistical analysis.

2.5.3.1 Optical saturation for some free fluorescent dyes

The dyes Alexa Fluor 488 (Alexa488) and Alexa Fluor 568 (Alexa568) were purchased from Invitrogen (Carlsbad, CA) and the Atto 488 (Atto488) dye was purchased from ATTO-TEC (Siegen-Weidenau, Germany), and directly diluted into HPLC grade water at ~ 2 nM concentration. The FCS measurements were performed using the SFCM and the microscope configuration for imaging the single eGFP molecules was the same as described in Chapter 5. Linearly polarized 488 nm laser light was used for excitation of Atto488 and Alexa488 dyes and separately the 568 nm laser line was used to excite Alexa568 dye. The resulting ACF curves were simply fitted to the 3D free diffusion model (Eq. (2-6)). Fig. 2-4 shows all the FCS measurement results on these three dyes. Apparently in Fig. 2-4, the TDts τ_D and the average numbers N of the dye molecules in focal volume measured by FCS for all three dyes increased with the excitation power, which can be explained by optical saturation effect as mentioned before.

2.5.3.2 Fluorescence triplet blinking for some free fluorescent proteins

FCS has been used to study the dynamics of fluorescence fluctuations in green fluorescent protein (FP) [130-132], yellow FPs [133], and other FPs DsRed and Citrine [134-136]. FPs often exhibit fluorescence triplet blinking which is dependent

Fig. 2-5 FCS measurements of fluorescent proteins, mStrawberry, mCherry and eGFP as a function of the power. τ_D and N are the TDts and the average number of molecules in focal volume determined by FCS, respectively, and $\langle I \rangle$ represents average fluorescent intensity rate (counts per second, cps). In each of FCS measurements, 50 10-second fluorescent intensity time traces were measured in sample solution and separately fitted to the 3D free diffusion for statistical analysis.

on excitation power and pH in buffers [130, 133, 134]. Here we show FCS measurements on three FPs, *i.e.*, mStrawberry, mCherry and eGFP. FPs were the same as those used in Chapter 5 in 0.23 mM Triton X-100 (TX100, Sigma-Aldrich, St. Louis, MO) solutions. The FCS measurements were performed using the SFCM and the microscope configuration was the same as described in Chapter 5. Linearly polarized 488 nm laser light was used for excitation of eGFP and 568 nm laser line was used to excite mStrawberry and mCherry. Although there has been a free diffusion model (Eq. (2-11)) for a single species with triplet blinking in FCS, the resulting ACF curves were simply fitted to the 3D free diffusion model (Eq. (2-6)) because the minimal intensity data acquisition time of 12.5 μ s in the SFCM cannot give a full ACF curve in the time-scale range from nanoseconds to seconds for the application of Eq. (2-11). Fig. 2-5 shows all the FCS measurement results on these three FPs. As shown in Fig. 2-5, for eGFP and mCherry, the TDts τ_D measured by FCS decreased when increasing the excitation power, which can be attributed to fluorescence triplet blinking, while the average number N of the FP molecules in the focal volume exhibited a slight increase with the excitation power for both FPs, which can be caused by fluorescence triplet blinking or simultaneously optical saturation effect. In contrast, the mStrawberry showed nearly constant TDts τ_D and average

number N of the molecules in the focal volume measured by FCS at different excitation power. The protein fluorescence is not dependent on the excitation power in the range from 20 μW to 400 μW . However, to the best of our knowledge, no FCS measurements have been reported photophysics of the FP mStrawberry over a time scale from nanoseconds to seconds. The photodynamic properties of mStrawberry seem to be significantly different from other FPs like eGFP and mCherry.

In conclusion, as shown in Fig. 2-5, the photophysics of fluorescent proteins are rather complicated. These photophysical dynamic processes are too fast to be characterized by the current configuration of the SFCM.

2.5.4 Interfacial effects

For many biomolecular interaction applications, the interfacial effect is another source of FCS artifact and it can lead to the observation of more complicated diffusion of the investigated molecules measured at the glass-solution interface than in solution. The interactions between the molecules in solution and the coverslip surface can completely change the dynamic properties of diffusing molecules. High NA objectives widely used in FCS setup often have very small working distances (240 μm in the SFCM), so the confocal volume may be very close to the coverslip (170 μm thickness) surfaces where strong interfacial effects may take place and be measured. As a result, the experimental results in FCS are disturbed. In practice, one common interfacial effect is the surface adsorption, which is usually caused by the sticky glass coverslip surfaces. For instance, plenty of silanol groups on coverslip surface are sticky to many biomolecules and easily immobilize the fluorescent molecules on the surface. As a result, a photobleaching intensity time trace can be observed, and the resulting TDT from the FCS measurement must be incorrect. Besides the surface adsorption, other interfacial phenomena can also take place and result in that the fluorescent molecules near the glass-solution interface behave significantly different from those freely diffusing in solution. To illustrate these phenomena, in the following we show a few experimental FCS results on Cy5 dye and Cy5 labeled 55-bp double-stranded DNA (Cy5-55-bp-dsDNA) in the absence and presence of cations in sample solutions using the SFCM.

2.5.4.1 Materials

A cyanine derivative, Cy5 mono reactive dye (Cy5, product No. PA25001), was purchased from Amersham Biosciences (Piscataway, NJ). The original DNA sample was 5 μM Cy5 labeled 55-bp double-strand DNA (Cy5-55-bp-dsDNA), and it was made by annealing equimolar amounts of Cy5 labeled 55-mer oligonucleotide and its complementary strand (DNA Technology A/S, Risskov, Denmark) in the DNA annealing buffer (50 mM Tris-HCl, pH = 7.4, 5 mM EDTA, 0.5 M NaCl). The sequences of the oligonucleotides are the same as the Alexa488-55-bp-dsDNA in Fig. 5-7 of Chapter 5. The quality of the resulting DNA products was checked using

polyacrylamide gel electrophoresis (data not shown). Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TFOS, product No. 448931) and Triton X-100 (TX100, product No. T8787) were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) and used for all buffer preparations and dilution steps.

2.5.4.2 Coverslip surface passivation

Coverslips (diameter = 25mm) were first cleaned in 65% HNO₃, washed with milli-Q water, and dried using N₂ gas. To avoid biomolecule adsorption, silanol groups on the coverslip outer surface were removed by TFOS treatments. The TFOS was applied by placing cleaned coverslips into a glass desiccator containing 1 ml of TFOS under vacuum for 5 minutes. The vacuum was then removed quickly to produce a uniform dispersion of gaseous TFOS, and afterwards vacuum was created again and kept for more than three hours. TFOS-treated coverslips were washed with 100% ethanol and dried using N₂ gas before use.

2.5.4.3 Confocal microscopic measurements

FCS measurements were performed within the SFCM using an on-line quantitative FCS technique (see Chapter 5 for details) specially developed for this purpose. The objective used is a water immersion C-Apochromat 63× (Zeiss, numerical aperture 1.20) with a working distance of 0.24 mm, which allows the focal volume to reach 70 μm deep in solutions. The interfaces between sample solutions and the coverslip surfaces (glass-solution interface) can be readily identified because the brightness of the back scattered laser light peaks when the interface is in focus. For all the measurements in the sample solutions, the focal volume was positioned in the solution as far as possible away from the glass-solution interface unless otherwise noted. The FCS measurements at the interface between coverslip surface and the sample solutions were performed when the back scattered laser light was observed to form a sharpest spot on the video camera (see section 2.2.1). 20 μW of linearly polarized 647nm laser light was used to excite Cy5 dye in this work.

All sample solutions were prepared at 5 nM concentration and 10 μl of each was applied to TFOS-treated coverslips for microscopic measurements.

2.5.4.4 Sample solutions

Four sample solutions were used in the experimental measurements, and they were 1) Cy5 in water; 2) Cy5 in 0.23 mM TX100 solution; 3) Cy5-55-bp-dsDNA in water 4) Cy5-55-bp-dsDNA in 10 mM NaCl solution. To obtain the same chemical conditions for both Cy5 dye and Cy5-55-bp-dsDNA, 5 μM original Cy5 solution was first prepared in the DNA annealing buffer.

2.5.4.5 Results

Fig. 2-6 The histograms of the TDts τ_D for Cy5 measured in solution (a) and at the glass-solution interface (b-c). For Fig. 2-6(c), 0.23 mM TX100 was added to the Cy5 solution.

For on-line FCS measurements, Cy5 dyes were directly diluted in HPLC grade water for Fig. 2-6(a) and Fig. 2-6(b) while for Fig. 2-6(c) the Cy5 solution contained also 0.23 mM TX100. In Fig. 2-6, each histogram was acquired from auto-correlation analyses of 70 5-second fluorescent intensity time traces. All ACFs were fitted to the 3D freely diffusion model (Eq. (2-6)). The histograms of the TDts τ_D for Cy5 dyes in water measured in solution and at the glass-solution interface were shown in Fig. 2-6(a) and Fig. 2-6(b), respectively. As shown in Fig. 2-6(b), the TDts measured at the interface were much higher than those in solution, so the diffusion of Cy5 dyes was strongly reduced at the interface. However, as shown in Fig. 2-6(c), the use of the neutral surfactant TX100 in the solution eliminated the interfacial effect in Fig. 2-6(b), and resulted in the same TDts as measured in solution. Strikingly, the Cy5 dye is not impeded by the addition of TX100 and therefore does not seem to bind to TX100.

A straightforward interpretation for the abnormal diffusion (Fig. 2-6(b)) of the Cy5 dye at the glass-solution interface is to assume surface adsorption for the dye. Fig. 2-7 gives two representative fluorescent intensity time traces measured at the glass-solution interface (Fig. 2-7(a)) and in solution (Fig. 2-7(c)) and their histograms for the sample solution in which Cy5 dyes were directly dissolved in HPLC grade water. Photobleaching would be expected in Fig. 2-7(a) if the Cy5 molecules were immobilized at the coverslip surface. However, the use of superhydrophobic TFOS-treated surface coverslips minimized the surface adsorption of Cy5 dye molecules. As shown in Fig. 2-7(a), Cy5 molecules were still mobile at the glass-solution interface. A comparison of the measured TDts (Fig. 2-6(b)), the intensity time trace (Fig. 2-7(a)) and its histogram (Fig. 2-7(b)) at the glass-solution interface to those (Fig. 2-6(a) for TDts, Fig. 2-7(c) and Fig. 2-7(d) for intensity time traces and its histogram) in solution suggests very specific behavior of the Cy5 molecules in the interface. The higher intensity bursts in Fig. 2-7(a) than that in Fig. 2-7(c) (or the higher intensity rate in Fig. 2-7(b) than that in Fig. 2-7(d)) indicates that Cy5 molecules aggregated

Fig. 2-7 Representative fluorescence intensity time traces measured at glass-solution interface (a) and in solution (c) and their histograms (Fig. 2-7(b) for Fig. 2-7(a) and Fig. 2-7(d) for Fig. 2-7(c)). Cy5 is dissolved in HPLC grade water.

only at the glass-solution interface, so we call this phenomenon interfacial molecular aggregation (IMA). However, the IMA does not seem to be able to explain the very large TDts (Fig. 2-6(b)) measured in the interface, and this suggests that some other interfacial effects also played roles near the glass surface and is discussed in section 2.5.4.6.

In the following experiment, Cy5-55-bp-dsDNA was directly dissolved in HPLC grade water and measured by using on-line quantitative FCS separately at glass-solution interface and in solution. Fig. 2-8 shows the histograms of the measured TDts, each of which was conducted by 50 10-second fluorescent intensity time traces. As shown in Fig. 2-8, both measurements at glass-solution interface and in solution produced the same experimental results, and thus no interfacial effects for the Cy5-55-bp-dsDNA molecules was observed.

Fig. 2-8 The histograms of the TDts τ_D for Cy5-55-bp-dsDNA measured at the glass-solution interface (a) and in solution (b). The measured TDts are in good agreement with those for Alexa488 dye labeled dsDNA (Alexa488-55-bp-dsDNA) which has the same sequence as Cy5-55-bp-dsDNA and was dissolved in 0.23 mM TX100 solution as shown in Fig. 5-7 in Chapter 5.

Fig. 2-9 The histograms of the TDts τ_D measured by FCS for 4 nM Cy5-55-bp-dsDNA solution in the presence of 10mM NaCl at different distances Z between the focal volume and the glass-solution interface, as indicated in the figure ($z = 0 \mu\text{m}$ is the glass-solution interface). Larger values and broader distribution ranges of the TDts indicates strong interfacial effects near the glass-solution interface.

Fig. 2-9 presents FCS results for the Cy5-55-bp-dsDNA solution in the presence of 10mM NaCl. In Fig. 2-9, the histograms of the TDts τ_D were shown as a function of the distance Z between the focal volume and the glass-solution interface. The distance Z was set manually by a translation stage installed on the objective in the SFCM and $Z = 0 \mu\text{m}$ means that the FCS measurement was performed at the glass-solution interface and the sharpest reflected laser spot was observed in the video camera. As shown in Fig. 2-9, when the focal volume is deeper in the solution with $Z > 20 \mu\text{m}$, FCS reported the same TDts as those in Fig. 2-8 for the Cy5-55-bp-dsDNA molecules in water. In contrast, the larger values and wider distributions of the measured TDts at the positions close to the glass-solution interface in Fig. 2-9 were observed and displayed interfacial effects for the Cy5-55-bp-dsDNA molecules in 10 mM NaCl solution. Increasing the cation concentration in solution resulted in larger TDts and more Cy5-55-bp-dsDNA molecules near the glass surfaces (data not shown). However, it was also found that the addition of 0.23 mM TX100 in solution can

effectively eliminate the interfacial effects for the Cy5-55-bp-dsDNA molecules in the presence of the cations, NaCl and MgCl₂ (data not shown), which concentrations were up to 500 mM and 10 mM, respectively.

2.5.4.6 Discussion

Multiple Cy5 molecules aggregate at glass surface, which has been reported by single-molecule surface imaging technique [137, 138], and our observations (Fig. 2-7) indicated that the Cy5 molecules are only possible to form the aggregations at the glass-solution interface, and in solution they still behave monomers. In addition, in Fig. 2-9 we show that the experimental TDts for Cy5-55-bp-dsDNA in 10 mM NaCl solution is a function of the distance between the focal volume and the glass-solution interface. Both examples (Fig. 2-7 and Fig. 2-9) provide evidence that single-molecule surface imaging may only reveal surface specific dynamic behavior which is quite distinct from that of molecules in solution.

The IMAs of Cy5 in water and Cy5-55-bp-dsDNA in 10 mM NaCl solution are similar to the mysterious phenomenon called “like charge attraction” [55-63] that has been theoretically studied by a number of proposed theory [139-147] and numerical simulations [148, 149]. Two like-charged colloidal spheres were observed that they seemed to attract each other near a container wall, and the theory of “like charge attraction” was then proposed to explain the phenomenon [55, 57]. Eventually the similar phenomena have been found in many biological systems containing DNA or proteins [150-152]. Interestingly, similar to our observations (Fig. 2-8 and Fig. 2-9) with Cy5-55bp-dsDNA, this phenomenon occurs only near a wall and is mediated by the concentration of multivalent counterions in solution [55, 57]. However, the existence of like-charge attraction has been challenged [139-147] and in particular the theory itself hardly interprets the intermediary role of counterions in solution. In our experiments, Cy5 dye is negatively charged [153], so if it is true that like-charges attracts one another at the glass-solution interface, it can be expected that the Cy5 molecules can still aggregate under the same experimental conditions when they are bound to other negatively charged molecules, for instance, DNA. However, as shown in Fig. 2-8, no evidence for like-charge attraction was found. Todd M. Squires and Michael P. Brenner [142] demonstrated analytical results and Brownian dynamics simulations and suggested that attractive interaction measured between like-charged colloidal spheres near a wall can be accounted for by a nonequilibrium hydrodynamic effect, but their theory cannot explain the intermediary role of counterions as well.

Another persuasive theory is entropic excluded-volume effects [64-66], as larger molecules can make more space for little ones by clustering together or moving to the sides of a container resulting in a maximization of the entropy of the system as a whole. On the basis of this theory, amphiphiles tend to form micelles (intermolecular aggregates) and analogous intramolecular interactions in an aqueous medium. This is

known as hydrophobic effect [154] and is mainly due to the entropic gain of the water structure by not being in contact with the hydrophobic part. At the molecular level, the hydrophobic effect is an important driving force for many biological structures and often responsible for protein folding, protein-protein interactions, formation of lipid bilayer membranes, nucleic acid structures, and protein-small molecule interactions [154]. The formation of micelles requires special molecular constitution of the amphiphile, for instance, surfactants have a hydrophilic head group and a long hydrophobic tail and the overall structure can be described as being of cylindrical or conical shape [154], so many molecules (for instance, the Cy5 dye) have hydrophobic and hydrophilic groups, but most of them cannot form micelles in aqueous solution, because. However, near a hydrophobic glass surface, water may directly interact with the hydrophilic groups of the molecules, and according to the entropic excluded-volume effects, water may also tend to pack a number of hydrophobic groups of the molecules together to the hydrophobic glass surface platform. As a result, the molecules show IMA and also potentially become rolling, tumbling or moving slowly along the hydrophobic surface as found in Fig. 2-6 and Fig. 2-7 for Cy5 dye in water. Dissolution of molecules in water is mostly attributed to their interactions with water molecules [155]. Apparently for highly charged molecules (for instance, DNA) in water, the entropic excluded-volume effect should take place on the premise of weakened attractive interaction between the molecules and water molecules as well as simultaneously weakened electrostatic repulsion between the molecules. This can be done by screening the charges on the molecules to a certain extent with a certain concentration of counterions in solution. In Fig. 2-8, the elimination of the interfacial effects is perhaps because the Cy5 binding with DNA improves the attractive interaction between Cy5-55-bp-dsDNA molecules and water molecules. The net charge of DNA molecules may be reduced by cations in solution, because the cations at a certain concentration can effectively screen the negative charges of the DNA molecules [156]. Consequently, direct interactions between water and Cy5-55-bp-dsDNA and between the Cy5-55-bp-dsDNA molecules may be decreased, and thus the entropic excluded-volume effect can start to play important roles in the system. As shown in Fig. 2-9, the interfacial effects reemerged for the Cy5-55-bp-dsDNA molecules when the sample solutions containing 10 mM NaCl.

In conclusion, the interfacial effects can arise from various sources and is thus generally very complicated. It is important to avoid FCS artifacts and distinguish interfacial effects. Glass surface adsorption is a common interface effect and can simply to be avoided. Here the use of superhydrophobic TFOS-treated surface coverslips has largely prevented surface adsorption. Alternatively surface adsorption can be avoided by pre-illumination in order to photobleach the fluorescent molecules immobilized on the surface, or simply by removing the photobleaching time trace data from the total intensity time trace. However, other interfacial effects, for instance, the

entropic excluded-volume effect, are more complicated and cannot be directly avoided by implementing the methods for surface adsorption. In section 2.5.4.5, it was shown that TFOS and also some non-ionic surfactants are very useful to prevent interfacial effects, and the use of surfactants in FCS experiments were also proposed to overcome interfacial effects in Ref. [126], but it is important to first study the interactions between the investigated molecules and the surfactants used in the buffer. In addition, in Chapter 5 the use of non-ionic surfactants significantly extends the functionality lifetime of many soluble proteins and will be hypothesized that the surfactant molecules form micelles and change the hydrogen-bond network in aqueous solution.

2.5.4.7 Conclusions

FCS measurements are often affected by the interfacial effects. To avoid these effects, special care must be paid to coverslip surface preparation and buffers. The fluorescent molecules, Cy5 and Cy5-55-bp-dsDNA, displayed different dynamic behavior at glass-solution interface from those in solution, even after the exclusion of the surface adsorption by using superhydrophobic surface coverslips. Intriguingly, our experimental results may be understood entropic excluded-volume effect, and thus potentially provides evidence for quantitative guidance for the study of the hydrophobic driving force. Superhydrophobic glass surface and also some non-ionic surfactants are very useful to prevent interfacial effects if the surfactants do not interact with the fluorescent molecules under study.

2.5.5 How to prevent artifacts in FCS?

Here procedures will be presented that were developed in order to overcome various FCS artifacts.

2.5.5.1 On-line quantitative FCS

In the SFCM we have developed an on-line quantitative FCS mode that allows the determination of TDt τ_D and average number N of molecules in the focal volume typically within ~ 12 seconds for a frame of 10-second fluorescent intensity time trace. Therefore, in about 10 minutes, 50 experimental results of τ_D and N can be obtained and presented on the monitor. Two apparent advantages of the on-line quantitative FCS technique for the avoidance of FCS artifacts are as follows:

- 1) On-line direct information about experimental parameters is useful to acquire stable results in long-term experiments (see the examples in Chapter 5).
- 2) Direct observation of evaporation effects, interfacial effects, fluorescent biomolecule degradation, *etc.* As a result, critical events can be measured and recognized either to establish effects or to investigate possibilities to avoid these effects.

2.5.5.2 Coverslip surface preparation

The surfaces of ordinary coverslips have many silanol groups and are therefore very sticky for most biomolecules, especially for proteins. Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TFOS) treatment of the surface was found very useful for the elimination of the surface adsorption for many biomolecules, for instance, soluble proteins.

2.5.5.3 Identical experimental conditions and internal FCS calibration

Using the inverted configuration of the SFCM and the spherical structure of the sample solution on the hydrophobic coverslip surface, one 24×50 mm TFOS-treated hydrophobic coverslip can be applied for 25 droplets of sample solutions of 8 µl each for FCS measurements. After a measurement in one droplet of sample solution, the coverslip was shifted to a new position where another new droplet of sample solution was put onto the focal volume, without changing any of optics (including the objective for a measurement in solution). This provided an identical focal volume and thus comparable experimental results from one sample to another for an entire experiment

For long-term FCS experiments, a stably fluorescing fluorescent sample has been used as an internal marker for calibration, and it can be any of fluorescent molecules with well-known parameters of fluorescent properties and translational diffusion. The TDT of the internal marker was first determined. Optimizations were carried out to generate consistent results. This process was facilitated by on-line data analysis.

2.5.5.4 Water Raman band as internal excitation intensity marker

The concentration of fluorophores in the nano- to picomolar range is difficult to determine directly, particularly due to the existence of photo-physical and photo-chemical dynamics, such as optical saturation, blinking and photobleaching. These effects may easily affect the concentration over time. In fluorescence spectroscopy the intense band of the Raman scattering of water can be easily observed and it is red-shifted 3380 cm⁻¹ relative to the laser line. This band is a robust intensity marker from a known and constant concentration of sample solution. The fluorescence intensity has been directly related to this band which thus provides a convenient standard for the assessment and control of (sub-) nanomolar concentrations of fluorescent components. A reliable intensity standard can be acquired from the spectral measurement. (see Chapter 5 for detailed applications).

2.6 STEADY-STATE FLUORESCENCE ANISOTROPY

Fluorescence anisotropy (FA) can provide information on molecular size and shape, and local viscosity of the probe molecules' environment (see section 1.2.3.2). By measuring the parallel and perpendicular polarized fluorescent emission components,

$I_{\parallel}(t)$ and $I_{\perp}(t)$, relative to the polarization vector of the exciting beam, the SFA $r(t)$ can be determined by:

$$r(t) = \frac{I_{\parallel}(t) - G \cdot I_{\perp}(t)}{I_{\parallel}(t) + 2G \cdot I_{\perp}(t)}, \quad (2-14)$$

where the G -factor accounts for different transmission and detection efficiencies of the imaging system at parallel and perpendicular polarization [157] and usually can be determined by calibration experiments using fluorescent samples with well-known FA values. In contrast to time-resolved FA (TFA), which requires a pulsed light source and high speed intensity measurements, SFA is performed using constant illumination and observation. SFA is another recently developed fluorescence spectroscopic technique in SFCM. It can be performed in the SFCM by using a linear polarizer in the optical excitation path to control the excitation polarization and a polarization analyzer in between two right-angle APDs in the fluorescent detection path (see section 2.2.1).

Similar to the FCS technique, SFA based on confocal microscopy was found to be sensitive to the microscope configuration as well, especially for the use of high NA objective [158, 159] for single-molecule sensitivity. High NA objectives have a natural depolarization effect on the excitation light delivered to the sample, and it can decrease the measured SFA from both the excitation and observation focal volume [160]. Excitation light shining on the sample through high NA objectives contains polarization components that are not oriented strictly along the direction of that of linearly polarized incident light [161, 162]. These components of the electrical field of the excitation light tend to disappear for propagation vectors near the optical axis, but farther away they can become comparable in magnitude. Fluorescence detection is also susceptible to errors induced by high NA objectives that allow the collection of polarization components along all three Cartesian coordinates in object space [158]. These high NA objective effects may lead to significantly smaller (nearly 25% as Ref. [158]) measured SFA values than the actual value. However, it has been found that the high NA objective effects can be minimized by carefully controlling the microscope alignment and using under-filled-objective excitation light [163, 164]. To control the size and the shape of the excitation volume, a diaphragm was placed in front of the objective in the excitation path of the SFCM to provide a beam which underfilled the objective for SFA measurements (see Chapter 6 and Chapter 7). However, this results in a larger excitation volume and thus a larger intensity background, further accompanied by a larger number of observed fluorescent molecules and larger TDts of the sample molecules in FCS. The optimal diaphragm setting is a tradeoff between increasing SNR and minimizing the artifacts by underfilling the objective back aperture [123].

2.7 CONCLUSIONS

We have built a state-of-the-art laser scanning fluorescence confocal microscope, which allows fluorescent intensity imaging, steady-state fluorescent anisotropy imaging, fluorescent spectral imaging, single point on-line quantitative FCS (or FCCS) and steady-state fluorescence anisotropy. Almost all these microscopic functions were eventually implemented with on-line measurement and processing ability, which provide unique fidelity for measured parameters in the SFCM.

The FCS technique is very sensitive to many factors including microscope configuration and alignment, photophysical dynamics of sample fluorophores, and interfacial effects, so special care was taken to avoid errors in FCS measurements. Particularly, the FCS measurements on the molecules, Cy5 and Cy5-55-bp-dsDNA, disclose that molecular dynamic behavior at glass-solution interface can be significantly different from those in solution even after suppressing surface adsorption effect. This shows that the interfacial effects can be glass surface adsorption as well as the interfacial aggregations. The designed experiments (see section 2.5.4) show that the interfacial aggregation phenomenon can be better explained by the theory of entropic excluded-volume effect [64-66] than “like-charge attraction” [55-63]. SFA is another mature technique developed in the SFCM. Both FCS and SFA techniques are combined in the application for the study of the binding interaction between the nucleotide excision repair protein complexes XPC/HR23B and Rad4/Rad23 and DNA, and compared in detailed in Chapter 6.

Chapter 3

Single-molecule Localization Accuracy: Theory

CHAPTER

3

ABSTRACT

The single-molecule localization approach in far field fluorescence microscopy with nanometer precision has been widely applied. In this chapter we will present exact analytical expressions for the localization accuracy in both the simplified least squares approximation and the full least squares approximation. It will be shown that the analytical expressions are in precise agreement with earlier simulations [165] as well as our own simulations. The analytical formula enables an unequivocal interpretation of the experimental localization accuracy and resolves the discrepancy between simulated and experimentally observed data [165-167]. Furthermore, since two-dimensional Gaussian function is commonly used to describe the Airy pattern in far field microscopy, relationships will be presented between Gaussian parameters and Airy pattern parameters. We conclude that the analytical expressions derived here provide a transparent relationship between relevant microscope specifications and the experimentally achievable localization accuracy.

3.1 INTRODUCTION

Fluorescence microscopy provides ultimate sensitivity down to the single-molecule level in micro- to milliseconds time [83] and has been successfully applied to single-molecule localization and tracking in a large number of studies [166-172]. Both confocal fluorescence microscopy [173] and global illumination and imaging [168, 169] have been used in localization strategies. Particularly global illumination techniques, such as total internal reflection fluorescence (TIRF), have become a method of choice, due to the inherent speed of image acquisition and the relative insensitivity to perturbations. In TIRF microscopy the diffraction limited image of a single fluorescent molecule results in an Airy pattern as a result of fundamental optical principles [174-176].

In spite of the physical limitations to the resolution, nanometer-precision single-molecule localization (NSL) is a powerful strategy to study sparse systems with single-molecule fluorescent microscopy (SMFM). Since the proposition of Bobroff in 1986 [177] to extract position information beyond the diffraction limit from resolution-limited and noisy data, it has been of utmost importance to connect the relevant experimental parameters to a theoretical model of the localization accuracy. We will extend here the centroid localization strategy [165]. An alternative approach has been presented as well [178] with similar results. In a previous study by Thompson *et al.* [165] the relationship between the variance (or uncertainty) σ_i^2 in centroid localization and the detected photon counts N , the imaging detector pixel size a , the variance σ_{bk}^2 of the background noise, and the waist widths s_i of the point spread function (PSF) was formalized to:

$$\sigma_i^2 = \frac{s_i^2}{N} + \frac{a^2}{12N} + \frac{8\pi s_i^4 \sigma_{bk}^2}{a^2 N^2} \quad (3-1)$$

where i labels the x - and y -Cartesian coordinate, respectively. However, Eq. (3-1) is not completely accurate [165, 167, 171, 173], and also shows a difference between the simulations obtained in this chapter with the simplified least square fitting method (referred to as “Gaussian mask method” [165]).

In this chapter we will derive analytical expressions for the localization accuracies in the simplified least squares fitting (SLSF) approach and in the full least squares fitting (FLSF) approach, and compare the results with simulations. To facilitate a direct comparison with earlier results [165], simulations were performed for identical experimental parameters.

3.2 RESULTS

3.2.1 Analytical expressions for localization accuracy

As usual, we define the localization accuracy as the standard deviation (SD) of molecular centroid coordinates determined by centroid localization in SMFM

expressed in unit of pixel number instead of nanometer as in Eq. (3-1). Least square fitting (LSF) in two dimensions is implemented with the following equation in agreement with [177]:

$$\chi^2(a_1, a_2, \dots, a_l) = \sum_{j=1}^M \sum_{i=1}^N \left[s_{ij} - f(x_{ij}, y_{ij}, a_1, a_2, \dots, a_l) \right]^2 / \sigma_{i,j}^2 \quad (3-2)$$

where s_{ij} denotes the actual values measured at point (x_{ij}, y_{ij}) , and f is the fit function with l parameters a to be optimized, and $\sigma_{i,j}^2$ is the expected variance from a measurement. Eq. (3-2) can be used in two different forms in practical applications. The first method is the simplified LSF (SLSF) [179] in which $\sigma_{i,j}^2$ in Eq. (3-2) is taken to be 1 and any variation in the variance is neglected. The second method is the full LSF (FLSF) [177] in which $\sigma_{i,j}^2$ in Eq. (3-2) is supposed to be known. In single-molecule imaging, $\sigma_{i,j}^2$ in Eq. (3-2) is generally taken to be the fit function that the dominant noise-term is Poisson distributed photon noise [165]. For both methods, SLSF and FLSF, analytical expressions were derived for the theoretical localization accuracy, assuming a 2D-Gaussian model, as follows (in accordance with Ref. [165]):

$$G(x, y) = B + A \cdot \exp \left[-\frac{(x - x_0)^2}{2s_x^2} - \frac{(y - y_0)^2}{2s_y^2} \right] \quad (3-3)$$

A detailed derivation is available in the Appendix. The formula for the localization accuracy of x -Cartesian coordinate for SLSF becomes:

$$\langle \sigma_{x_0} \rangle = \left(\left(\frac{8}{9\pi A} + \frac{2\sigma_{bk}^2}{\pi A^2} \right) \cdot \frac{s_x}{s_y} \right)^{1/2} \quad (3-4)$$

and for FLSF is:

$$\langle \sigma_{x_0} \rangle = \left(2\pi \left(A + \sigma_{bk}^2 \times Li_2 \left(-\frac{A}{\sigma_{bk}^2} \right) \right) \cdot \frac{s_y}{s_x} \right)^{-1/2} \quad (3-5)$$

In Eqs. (3-4) and (3-5), σ_{bk} is the SD of background, and Li_2 is dilogarithm function. Exchanging the subscripts x and y in Eqs. (3-4) and (3-5) readily results in the localization accuracy formulas of y -Cartesian coordinate. The total number of photon counts N from an object can be obtained from the fitting result as follows:

$$N = 2\pi A s_x s_y \quad (3-6)$$

3.2.2 Numerical simulations

To test the analytical expressions of the theoretical localization accuracy, numerical simulations were done using custom programs in Matlab (The Mathworks, Natick, MA, USA). For ease of comparison, the same parameters were chosen as for Fig. 5 in Ref. [165], namely a Gaussian distributed background noise ($\sigma_{bk} = 0.7$) and a waist width $s_x = s_y = 1.6$ pixels. Simulated noised PSF images were created by using a “Gaussian object” in Eq. (3-3) as the image model and adding Poisson distributed noise with a Matlab function *poissrnd*. The LSF algorithms were implemented by

using the *fminunc* function in optimization toolbox in Matlab (see the manual of Matlab). For Fig. 3-1, 500 32×32 pixels PSF images were created and subsequently fitted one by one to the 2D Gaussian function (Eq. (3-3)). The simulation results were compared with the results of the analytical expressions Eqs. (3-4) and (3-5) in Fig. 3-1, where the localization accuracy is plotted versus the total number of collected photons from a fluorescent molecule. The simulation results were in exact agreement with those reported in Ref. [165]. The localization accuracies of the centroid coordinates x_0 were calculated and plotted in Fig. 3-1 with triangles for the FLSF and with circles for the SLSF. Identical statistical results were obtained for the y -coordinates. The simulations were compared with the results from the analytical expressions for the SLSF (Eq. (3-4)) and for the FLSF (Eq. (3-5)) in lines in Fig. 3-1. As expected, increasing the number of collected photons from the fluorescent molecule improves

Fig. 3-1 The localization accuracy plotted versus total detected photon counts N . The theoretical localization accuracy was calculated from Eq. (3-4) for the SLSF (dot line) and Eq. (3-5) for the FLSF (dash line). For comparison, the localization accuracy (Eq. (3-1)) is plotted as a solid line. The localization accuracies of centroid coordinate x_0 by the SLSF (open circles) and by the FLSF (open triangles) were obtained from numerical simulation. Identical results were obtained for y -coordinate. See the text for the parameters in the numerical simulation.

the localization accuracy. It is clear from Fig. 3-1 that the localization accuracies from the theoretical formulas, Eqs. (3-4) and (3-5) are in excellent agreement with those obtained by the simulation results. It should be noted that we have omitted the second term for the pixelation noise in Eq. (3-1). A typical value for this term in our experimental conditions, $a = 100$ nm, $N = 10^4$, was approximate 0.1 nm, which is much smaller than the rest terms in Eq. (3-1). Neglecting this term is valid in most realistic situations as it contributes minimally to the accuracy in relation to the other two terms.

Clearly, the FLSF method allowed higher localization accuracy than the SLSF method, as demonstrated both by simulations and by analytical expressions. However,

SLSF has been more common in practice since LSF programs are coded as SLSF instead of FLSF in most commercial mathematic softwares (e.g. Matlab).

3.2.3 Relationship between the Airy pattern and the Gaussian beam in LSF

Although classical diffraction theory predicts an Airy pattern for an object in single-molecule imaging, it is customary [168, 169] to use Gaussian formulas to economize mathematical calculations. We will here derive the relationship between a Gaussian- and an Airy function in the least squares sense. The PSF of a circular aperture will be described by the Airy function as follows [180]:

$$\text{PSF}(r) = A_A [2J_1(ar)/ar]^2 \quad (3-7)$$

where

$$a = 2\pi\text{NA}/\lambda \quad (3-8)$$

NA is the numerical aperture of the objective, λ is the wavelength of emission light, and J_1 is the Bessel function of the first kind. The limit of resolution of a microscope objective refers to its ability to distinguish between two closely spaced Airy patterns in the diffraction pattern, and is generally expressed to be the radius of the central bright spot of an Airy pattern as follows:

$$R = 0.61\lambda/\text{NA} \quad (3-9)$$

A Gaussian profile can be expressed in polar coordinates as

$$G(r) = A_G \exp(-r^2/2s^2) \quad (3-10)$$

In a straightforward approximation of Eq. (3-7), the relationships between the amplitudes and radii of both functions, Eqs. (3-7) and (3-10), are as follows:

$$\zeta = A_G/A_A \approx 1.0459 \quad (3-11)$$

and

$$s \approx R/2.9633 \quad (3-12)$$

Details for the derivation are shown in the Appendix,

Fig. 3-2 shows the cross sections through the curves of the Airy pattern profile and the Gaussian profile with A and s as given in Eqs. (3-11) and (3-12). Clearly, when

Fig. 3-2 The curves of the Airy pattern (circles, Eq.(3-7)), and the Gaussian function (line) in Eq. (3-10) with the parameters from Eqs. (3-11) and (3-12).

Eqs. (3-11) and (3-12) are satisfied, the Gaussian $G(r)$ is closest to the actual PSF. It is clear from these equations that the utilization of the Gaussian beam model (Eq. (3-3)) to express the PSF gives rise to a numerically smaller width of objects, *i.e.*, a higher microscope resolution. In agreement with [173], we conclude that this is merely a matter of definition.

3.3 DISCUSSION

In this chapter, we have presented analytical formulas for localization accuracy in SMFM. The formulas resolve a discrepancy between simulated and experimental data. In Ref. [165], a discrepancy of 30% is significant in the range of a few nanometers, the localization accuracy of interest as the example below will show. Assume a diffraction-limited single-molecule image in which the pixel size equals 100 nm, the background noise SD σ_{bk} equals 10 cts and the fitted Gaussian waist widths s_x equals s_y equals 1.5 pixels. In case that the integrated number of photons is 1,350, the centroid localization accuracy is already 10 nm, however, a total of 2×10^4 photons would be required to achieve an accuracy of 1.5 nm, but the accuracy predicted by Eq. (3-1) would be 1.2 nm. To achieve this improvement in accuracy, *i.e.* from 1.5 nm to 1.2 nm, a total of 3.05×10^4 photons, which corresponds to $\sim 50\%$ more photons, would be required from the fluorophore. Although this is in practice possible, it would reduce the total number of images from a single molecule at this localization accuracy.

3.4 CONCLUSIONS

In this chapter, we have presented analytical formulas, Eqs. (3-4) and (3-5), to predict the localization accuracy in the NSL for sparse systems of nano-objects. The analytical expressions and numerical simulations are in precise agreement with each other and our experimental results that are shown in Chapter 4.

3.5 APPENDIX

3.5.1 2D Gaussian fitting with the least squares minimization

Least squares fitting (LSF) is a common mathematical procedure which was proposed [179] to find the best-fitting curve to a given set of points by minimizing the sum of the squares of the offsets (or the residuals) of the points from the curve. Gaussian fitting is a nonlinear LSF, in which a number of unknown parameters are determined by least squares minimization.

LSF in two dimensions is implemented with Eq. (3-2) [177] by minimizing the sum of the squares of the deviations χ^2 of a set of $M \times N$ data points. The square deviations from each point are summed, and the resulting residual is then minimized to find optimized parameters a_k of the best fit curve f . The condition for the minimum χ^2 is

$$\frac{\partial(\chi^2)}{\partial a_k} = 0, \quad a_k = a_1, \dots, a_l \quad (3-13)$$

If the signal s is a 2D image, it can generally be regarded as a combination of noise n and an actual image f . The accuracy of each optimized parameter a_k in the LSF can be obtained by expanding Eq. (3-13) around the actual value a_{k0} of a_k , and assume that the error $\Delta a_k = a_k - a_{k0}$ is sufficiently small to disregard higher than second order terms in Δa_k . Then it is obtained:

$$\Delta a_k = \frac{\sum_{j=1}^M \sum_{i=1}^N \left\{ \left(n^2 / \sigma_{i,j}^2 \right)' \right\} \Big|_{a_{k0}}}{\sum_{j=1}^M \sum_{i=1}^N \left\{ \left(n^2 / \sigma_{i,j}^2 \right)'' \right\} \Big|_{a_{k0}}} \quad (3-14)$$

In Eq. (3-14), n replaces the term $s(x_{i,j}, y_{i,j}) - f(x_{i,j}, y_{i,j})$ in Eq. (3-2) and $(n_{i,j}^2 / \sigma_{i,j}^2)' \Big|_{a_{k0}}$ and $(n_{i,j}^2 / \sigma_{i,j}^2)'' \Big|_{a_{k0}}$ are the first and the second derivatives of $n_{i,j}^2 / \sigma_{i,j}^2$ at $a_k = a_{k0}$. In the SLSF procedure, Eq. (3-14) can be rewritten to:

$$\Delta a_k = \frac{\sum_{j=1}^M \sum_{i=1}^N \left\{ n \cdot f'(a_{k0}) \right\}}{\sum_{j=1}^M \sum_{i=1}^N \left\{ \left(f'^2(a_{k0}) - n \cdot f''(a_{k0}) \right) \right\} \Big|_{a_{k0}}} \quad (3-15)$$

and the variance can be obtained as error propagation [179] by, as follows:

$$\sigma_{a_k}^2 = \frac{\sum_{j=1}^M \sum_{i=1}^N \left\{ \sigma_n^2 f'^2(a_{k0}) \right\}}{\left(\sum_{j=1}^M \sum_{i=1}^N \left\{ f'^2(a_{k0}) - \langle n \rangle f''(a_{k0}) \right\} \right)^2} \quad (3-16)$$

In the treatment for FLSF, the variance $\sigma_{i,j}^2$ in Eq. (3-2) is assumed to be the same as the fit function. The variance of the error Δa_k can be determined [165] by:

$$\sigma_{a_k}^2 = 1 / \sum_{j=1}^M \sum_{i=1}^N \left\{ f'^2(a_{k0}) / \sigma_n^2 \right\} \quad (3-17)$$

Here σ_n^2 is the variance of noise. Eqs. (3-14) - (3-17) are general formulas to evaluate the average error and the variance for each parameter in LSF under the condition that Δa_k is sufficiently small.

3.5.2 LSF of an Airy pattern and a Gaussian beam

As discussed previously, a 2D Gaussian function is usually selected to describe the Airy disk function in LSF, because the Gaussian function is mathematically more tractable. In addition, computations with Gaussian functions are generally more efficient. The relationship between the parameters of the fitted Gaussian function (Eq. (3-10)) and the Airy PSF (Eq. (3-7)), can be obtained in a least squares minimization:

$$\chi^2(\zeta, s) = \int_0^\infty \left(A_A \left(\frac{2J_1(ar)}{ar} \right)^2 - A_G \exp \left(-\frac{r^2}{2s^2} \right) \right)^2 r dr \quad (3-18)$$

For generalization, we define $\zeta = A_G / A_A$. Solving Eq. (3-18), we have

$$\chi^2(\zeta, s) = \frac{12\pi^2 - 64 + 3\pi^2 \zeta (\zeta a^2 s^2 - 8) + 24\zeta \pi^2 e^{-a^2 s^2} [I_0(a^2 s^2) + I_1(a^2 s^2)]}{6a^2 \pi^2} A_A^2, \quad (3-19)$$

where I_0 and I_1 are modified Bessel functions of the first kind with order 0 and 1, respectively. The condition for the minimum $\chi^2(\zeta, \sigma)$ in Eq. (3-18) is:

$$\frac{\partial(\chi^2)}{\partial\zeta} = 0, \quad \frac{\partial(\chi^2)}{\partial\sigma} = 0 \quad (3-20)$$

Numerically solving both equations, the solutions in Eqs. (3-11) and (3-12) are determined.

3.5.3 Theoretical localization accuracy derivation

The general form for the Gaussian function G (Eq. (3-3)) has been used in the NSL with the LSF algorithm [168-170]. A different form for the 2D Gaussian has also been proposed [181, 182], which lacks a term for the background level B [181, 182]. The resulting parameters A , s_x and s_y (or s) do depend on this choice and the present treatment is based on the Gaussian G function in the form of Eq. (3-3).

Substituting the Gaussian function G in Eqs. (3-15) - (3-17), and replacing the summations with infinite integrals gives the analytical expressions for the accuracies of the estimates of parameters. The expectation value of the mean error of x_0 and y_0 in Eq. (3-15) is zero in all cases, so both SLSF and FLSF are unbiased estimates (see also Eq. (10) in Ref. [165]).

Background noise and photon shot noise affect the accuracy in NSL [75]. The photon shot noise is Poisson distributed and its variance σ_p^2 can be expressed as collected photon counts in each point of image, *i.e.*,

$$\sigma_p^2 = G \quad (3-21)$$

A Gaussian distribution function is generally considered for the background noise. The localization accuracies for the SLSF and for the FLSF were separately calculated for three cases: a) for photon shot noise only, b) for pure background noise with a Gaussian distribution, and c) for a combination of photon shot noise and background noise.

3.5.3.1 Simplified LSF – SLSF

From Eq. (3-16), the variances of localization error in the SLSF are:

$$\sigma_{x_0}^2 = \frac{8}{9\pi A} \cdot \frac{s_x}{s_y}, \quad \sigma_{y_0}^2 = \frac{8}{9\pi A} \cdot \frac{s_y}{s_x} \quad (3-22)$$

for the case of photon shot noise, and

$$\sigma_{x_0}^2 = \frac{2\sigma_{bk}^2}{\pi A^2} \cdot \frac{s_x}{s_y}, \quad \sigma_{y_0}^2 = \frac{2\sigma_{bk}^2}{\pi A^2} \cdot \frac{s_y}{s_x} \quad (3-23)$$

for the case of pure background noise with a SD σ_{bk} . In both equations, the units for σ_{x_0} and σ_{y_0} are pixel number. The terms s_x/s_y and s_y/s_x take the potential asymmetry (e.g. as a result of spherical aberration) of the PSF into account. Eq. (3-23), for the case of the pure background noise, predicts that the localization accuracy is

proportional to the image signal-to-noise ratio (SNR) A/σ_{bk} , and is independent of other parameters in SLSF. For the case of the combination of the photon shot noise and the background noise the localization accuracy of the SLSF can be determined directly by the summation of Eqs. (3-22) and (3-23) because both noise sources can in general be considered to be independent.

3.5.3.2 Full LSF – FLSF

In the FLSF, the variances of the localization error for the case of photon shot noise only are:

$$\sigma_{x_0}^2 = \frac{1}{2\pi A} \cdot \frac{s_x}{s_y}, \sigma_{y_0}^2 = \frac{1}{2\pi A} \cdot \frac{s_y}{s_x} \quad (3-24)$$

and for the case of only background noise the same equation as Eq. (3-23) is obtained. However, from Eq. (3-17) it follows that the variances of the different noise terms are not additive in the FLSF method. Instead Eq. (3-17) should be calculated by substitution of $\sigma_n^2 = \sigma_{bk}^2 + \sigma_p^2$ replacing summations with infinite integrals. Eq. (3-17) for x_0 (the same for y_0) has to be solved in the following form:

$$\sigma_{x_0}^2 = \left[\int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \frac{\left(\frac{Ax}{s_x^2} \exp\left(-\frac{x^2}{2s_x^2} - \frac{y^2}{2s_y^2}\right) \right)^2}{\sigma_{bk}^2 + \exp\left(-\frac{x^2}{2s_x^2} - \frac{y^2}{2s_y^2}\right)} dx dy \right]^{-1} \quad (3-25)$$

To solve the above equation, we first transfer it into the polar coordinates:

$$\sigma_{x_0}^2 = \left[\frac{A^2}{s_x^4} \int_0^{2\pi} \cos^2(\theta) \int_0^{\infty} \frac{r^2 \text{Exp}[-r^2\omega]}{\sigma_{bk}^2 + A \text{Exp}[-r^2\omega/2]} r dr d\theta \right]^{-1} \quad (3-26)$$

with

$$\omega = \frac{\cos^2(\theta)}{s_x^2} + \frac{\sin^2(\theta)}{s_y^2} \quad (3-27)$$

We now define: $F = \text{Exp}(-r^2\omega/2)$, i.e.,

$$\text{Log}(F) = -r^2\omega/2 \quad (3-28)$$

and obtain:

$$dr = \frac{1}{-r\omega F} dF \quad (3-29)$$

Using the identities Eq. (3-28) and Eq. (3-29) in Eq. (3-26), we obtain:

$$\begin{aligned} \int_0^{\infty} \frac{r^2 \text{Exp}[-r^2\omega]}{\sigma_{bk}^2 + A \text{Exp}[-r^2\omega/2]} r dr &= \frac{2}{\omega^2} \int_0^1 \frac{F \log(F)}{\sigma_{bk}^2 + AF} dF \\ &= \frac{2}{\omega^2 A^2} \left(A + \sigma_{bk}^2 \cdot \text{Li}_2\left(-\frac{A}{\sigma_{bk}^2}\right) \right) \end{aligned} \quad (3-30)$$

and by using the following identity [183]:

$$\int_1^0 \frac{t \log(t)}{b^2 + At} dt = \frac{1}{A} + \frac{b^2}{A^2} Li_2\left(-\frac{A}{b^2}\right), \quad (3-31)$$

where Li_2 is dilogarithm function (or second-order polylogarithm). Eq. (3-26) can now be simplified to:

$$\begin{aligned} \sigma_{x_0}^2 &= \left[\frac{2}{s_x^4} \left(A + \sigma_{bk}^2 \cdot Li_2\left(-\frac{A}{\sigma_{bk}^2}\right) \right) \int_0^{2\pi} \frac{\cos^2(\theta)}{\omega^2} d\theta \right]^{-1} \\ &= \left[2\pi \left(A + \sigma_{bk}^2 \times Li_2\left(-\frac{A}{\sigma_{bk}^2}\right) \right) \frac{s_x}{s_y} \right]^{-1} \end{aligned} \quad (3-32)$$

by using [184]

$$\int \frac{\cos^2(\theta)}{\omega^2} d\theta = \frac{1}{2} s_x s_y \left(\arctan\left(\frac{s_x \tan(\theta)}{s_y}\right) + \frac{s_x s_y \sin(2\theta)}{s_x^2 + s_y^2 + (s_y^2 - s_x^2) \cos(2\theta)} \right) \quad (3-33)$$

The same derivation of the variance of localization error holds for the y -Cartesian coordinate and we obtain:

$$\sigma_{y_0}^2 = \left[2\pi \left(A + \sigma_{bk}^2 \times Li_2\left(-\frac{A}{\sigma_{bk}^2}\right) \right) \frac{s_y}{s_x} \right]^{-1} \quad (3-34)$$

Both Eqs. (3-32) and (3-34) are the theoretical variances of the localization error in number of pixels for the case of the combination of photon shot noise and background noise. The terms s_x/s_y and s_y/s_x in Eqs. (3-32) and (3-34) are taking into account a possible asymmetry in the x - and y -coordinate, e.g., possibly in response to spherical aberration.

3.5.4 Comparison with the Thompson formula

Eqs. (3-22) - (3-23) can be easily rewritten in terms of the number N of total collected photons by taking $A = N/2\pi s^2$ [165]. For the pure background noise case, by replacing the Gaussian maximum amplitude A with $N/2\pi s^2$ and inserting $s_x = s_y = s$, the variance for the case of pure background noise in Eq. (3-23) can be rewritten as:

$$\sigma_{x_0}^2 = \sigma_{y_0}^2 = \frac{8\pi\sigma_{bk}^2 s^4}{N^2} \quad (3-35)$$

This expression has the same form as presented by Thompson *et al.* [165]. The units in Eq. (3-35) of σ_{x_0} , σ_{y_0} and s are in number of pixels. Repeating the same analysis for the FLSF, Eq. (3-24) becomes

$$\sigma_{x_0}^2 = \sigma_{y_0}^2 = \frac{1}{2\pi A} = \frac{s^2}{N} \quad (3-36)$$

which is given the same in Ref. [165]. However, for the case of pure photon shot noise in the SLSF, the variance of localization error from Eq. (3-22) is

$$\sigma_{x_0}^2 = \sigma_{y_0}^2 = \frac{16s^2}{9N} \quad (3-37)$$

in contrast to Eq. (3-36) in the FLSF. From Eqs. (3-36) and (3-37) it is obvious that the FLSF gives a 25% higher localization accuracy than the SLSF does. The result for the FLSF is in agreement with our simulations (Fig. 3-1) and it resolves the discrepancy between the theoretical prediction and the simulation result for the SLSF in Ref. [165].

3.5.5 Fundamental limitations of localization accuracy in LSF

The relationship between the Airy radius R and the Gaussian waist width s in Eq. (3-12) offers the following limit to the accuracy in NSL from Eqs. (3-36) and (3-37) as follows:

$$\sigma_{x_0} = \sigma_{y_0} \approx \frac{0.45R}{\sqrt{N}} \quad (3-38)$$

and

$$\sigma_{x_0} = \sigma_{y_0} \approx \frac{0.34R}{\sqrt{N}} \quad (3-39)$$

for the SLSF and the FLSF, respectively, in the absence of background noise and ignoring the possible aberration of the microscope system.

Eqs. (3-38) and (3-39) may be compared with an expression recently presented by Ober *et al.* [178], namely,

$$\sigma_{x_0}^2 = \sigma_{y_0}^2 \approx \frac{0.26R}{\sqrt{N}} \quad (3-40)$$

This result was obtained without taking the photon shot noise into consideration.

3.5.6 Conditions for the localization accuracy formulas

It should be noted that the selected image area, over which the fit will extend, should have boundaries sufficiently distant from the object. If the boundaries are too close to the center, the approximation to replace summations in Eqs. (3-15) - (3-17) with infinite integrals is not valid any more. For a typical Gaussian waist width of 1.5-3.0 pixels, the window size was tested and we conclude that a window of 30 pixels in x - and y -direction is sufficient for this approximation to hold.

3.5.7 Additional simulation results

The numerical method of the image generation and localization program has been described previously in the section 3.2.2. The initialization for each parameter to be fitted in the fit function is pivotal to carry out the fitting program in the LSF. In these simulations, we directly took the exact value of each parameter for the initialization. Practically a few fast centroid localization algorithms (typically, the centroid coordinates calculation in Ref. [181]) can be used to automatically give rough initializations for the centroid coordinates in this application, but they generally are less accurate than those obtained from the 2D Gaussian fitting, and especially for low SNR image these algorithms can often create the initializations far away from the

Fig. 3-3 The localization accuracy plotted versus the background SD σ_{bk} . The theoretical localization accuracy was calculated from Eq. (3-4) for the SLSF (dot line) and Eq. (3-5) for the FLSF (dash line). In the simulations, 500 single-molecule images were artificially generated from an image model, Gaussian G with $s_x = s_y = 1.6$ pixels and the maximum amplitude $A = 30$ and fitted by the SLSF and the FLSF, separately. The localization accuracies for centroid coordinate x_0 by the SLSF (open circles) and by the FLSF (open triangles) were obtained from the simulation. Identical results were obtained for the other centroid coordinate y_0 .

exact values, and thus become the bottleneck of improving the fitting precision in localization program.

Here we show three simulations to demonstrate the factors that influence the location accuracy in NSL applications. In these simulations, 500 32×32 pixels single-molecule images were first created using the method described previously in the section 3.2.2, and then the images were fitted to the 2D Gaussian G by the SLSF and by the FLSF, separately. The background noise was assumed to be Gaussian distributed. In Fig. 3-3 to Fig. 3-4, we took $s_x = s_y = s$ in the Gaussian, and $s_x/s_y = 0.8$ in Fig. 3-5 to investigate the spherical aberration effect on the localization accuracy.

Fig. 3-3 shows the theoretical localization accuracy (lines) and the simulation results (open circles and triangles) with respect to the background noise SD σ_{bk} . In the simulations, the 2D Gaussian maximum amplitude A was taken to be 30 (or total counts N was 483) and other parameters are taken the same as those used for Fig. 3-1. In Fig. 3-3, the localization accuracy can be better by lowering the SD of the noise as

Fig. 3-4 The localization accuracy varies with the waist width $s_x = s_y = s$. The theoretical localization accuracy was calculated from Eq. (3-4) for the SLSF (dot line) and Eq. (3-5) for the FLSF (dash line). In the simulations, 500 32×32 pixels single-molecule images were artificially generated from the same image model G (but total counts $N = 10000$) as in Fig. 3-1, and then fitted in the SLSF and the FLSF, separately. The localization accuracies of centroid coordinate x_0 by the SLSF (open circles) and by the FLSF (open triangles) were obtained from the simulation. Identical results were obtained for the other centroid coordinate y_0 .

revealed in Eqs. (3-4) and (3-5).

Fig. 3-4 demonstrates the influence on localization accuracy by the waist width s of 2D Gaussian. In the simulation, $\sigma_{bk} = 10$ and $N = 10000$. In Fig. 3-4, the theoretical localization accuracies were found in good agreement with those obtained from the simulation until the waist width is down to around half of pixel size, where the simulation results show excess error because of low sampling frequency. In this case, the pixelation noise [165] will become significant.

Fig. 3-5 shows that localization accuracy can also be affected by spherical aberration of the optical system of the microscope. In the simulation, single-molecule images were artificially generated using the Gaussian G with $s_x = 1.6$ pixels and $s_y = 2.0$ pixels, and $\sigma_{bk} = 0.7$, where $s_x/s_y = 0.8$ denotes spherical aberration. As shown in both figures, theoretical and experimental localization accuracies are influenced by the spherical aberration of the microscope.

Fig. 3-5 The influence of the spherical aberration of the microscopy system on localization accuracy. The localization accuracy are plotted versus Gaussian maximum amplitude A (and total counts N) for the FLSF in Fig. 3-5(a) and the SLSF in Fig. 3-5(b). Theoretical localization accuracies (dot lines for centroid coordinate x_0 and dash lines for y_0) were calculated using the analytical expressions of Eqs. (3-4) and (3-5). In the simulation, the image model G has $s_x = 1.6$ pixels and $s_y = 2.0$ pixels. Here $s_x/s_y = 0.8$ denotes the spherical aberration. The background noise has a SD $\sigma_{bk} = 0.7$. All images were then fitted by using the SLSF and the FLSF, separately. The SDs of the fitted centroid coordinates x_0 (open circles) and y_0 (filled circles) in the simulations were calculated and plotted in the figures.

Chapter 4

Nanometer-precision Single-molecule Localization: Experimental Evidence

ABSTRACT

CHAPTER

4

Water soluble quantum dots (QDots) were used to demonstrate nanometer localization accuracy of single nanoparticles in total internal reflection fluorescence (TIRF) microscopy. Approximately 100 images were acquired of individual QDots. A localization accuracy of ~ 2 nm could be obtained. Analysis of the dynamic emission patterns of two QDots in close proximity revealed an inter-particle distance of 14.5 ± 4.8 nm. This value is approximately 25 times better than the actual resolution in far field microscopy. The analyses were performed with adapted formulas for localization accuracy in Chapter 3.

4.1 INTRODUCTION

Our understanding of molecular processes in biological systems depends on our ability to actually visualize events on a nanometer scale. Far field fluorescence microscopy offers an extremely good signal-to-noise ratio, which makes it possible to track individual molecules in movies. Subsequent analysis of the individual frames in the movies gives accurate information on dynamic aspects of molecular motion. The motion of Myosin V moving on actin was investigated in detail with localization accuracies of the order of 2 nm [168, 169]. In biological complexes the distance between individual partners is often of importance. One typical example in this thesis is the process of global genomic nucleotide excision repair (GG-NER) in cells. In GG-NER, altogether, but not simultaneously, ~30 proteins participate [1]. The whole process can be divided in damage-recognition, damage excision, DNA synthesis and ligation in the gap and results in repaired double-stranded DNA. In the GG-NER study we have focused on the interaction between XPC, which is now generally accepted as the damage recognizing factor in GG-repair and DNA. Optical labels on XPC and DNA were used as markers to measure the distance in between.

Conventional light microscopy is limited to a resolution of ~200 nm according to the Rayleigh criteria [180]. Several techniques in fluorescent microscopy have been demonstrated to measure submicroscopic distance between biomolecules, such as stimulated emission depletion microscopy [185] and near field scanning optical microscopy [186]. A typical far field approach for nanometer scale distance measurements is fluorescent resonant energy transfer (FRET), which employs a Förster energy transfer process. Donor-acceptor intermolecular distances of < 10 nm [4] can be determined. Localization microscopy is based on nanometer-precision single-molecule (or single-particle) localization (NSL) technique and does not depend on point-spread function engineering, near field effects or energy transfer processes and seems to be a robust and versatile addition to nanometer scale metrology techniques. The interparticle distances can typically range from microscopic distances all the way through the mesoscopic range down to nanometers [169, 170, 172, 187-189]. Previous work Ref. [169, 170, 172, 187-189] is based on a theory for localization accuracy [165]. In chapter 3 a new approach was presented, which resolved a discrepancy between theoretical formulas and simulations.

As in Chapter 3, NSL can be performed on diffraction-limited image by fitting the image to two-dimensional Gaussian function in two mathematical methods: simplified least squares fitting (SLSF) and full least squares fitting (FLSF). The theoretical localization accuracy is defined to be the standard deviation (SD) of centroid coordinates in two-dimensional Gaussian fitting. Eq. (3-4) and Eq. (3-5) were derived in chapter 3 for the localization accuracy of the centroid coordinates in two-dimensional Gaussian fitting for SLSF and FLSF, respectively. It will be noticed that

the brightness of individual quantum dots (QDots) varies appreciably. This offers an opportunity to determine the localization accuracy for each particle in relation to the total emitted number of photons by this particle for all particles in the field of view (FOV). This result will be compared with the theoretical formulas, Eq. (3-4) and Eq. (3-5), acquired in Chapter 3. In order to obtain a localization accuracy of ~ 2 nm, a significantly large number of photons are required for microscopic images with a certain background SD. The high brightness and relative photostability of QDot-luminescence [190, 191] is very convenient. Recently Lagerholm *et al.* [166] have presented an analysis method for measuring submicroscopic distance between multiple QDots in the wide-field microscopic images which were generated by using software to overlap single-QDot images, but their images suffered from high background SD and less total photons collected from single QDots. To lower the image background SD for approaching the theoretical localization accuracy limitation, total internal reflection fluorescence (TIRF) microscopy was used in our work. Here we will present real experimental nanometric distance measurements between two QDots which were naturally aggregated, specifically immobilized on coverslip surfaces and thus imaged within the same diffraction limited light spot in TIRF microscopy. Another mathematic analysis method, independent component analysis (ICA) [192], was also reported to decompose the sequential images of multiple QDots residing within a diffraction-limited space into the separate images of each QDots, and then the distance between the QDots were resolved by using NSL in the images for each QDots. However, ICA assumes that the particles in a diffraction-limit light spot do not move over the time course of the experiment. This requirement gives this method a fundamental limitation in many biological applications. In chapter 8, it will be shown however that also the organic dyes Alexa488 and Cy5 have favorable properties to achieve high localization accuracy clearly below 10 nm. Here quantum dot was used. We will also show that distances between QDots can be accurately determined, even when the quantum dots are within the same diffraction limited laser focus, as a consequence of individual dynamic emission patterns.

4.2 MATERIALS

QDot 655 Streptavidin Conjugates (QDot655; product No. Q10151MP, 1 μ M) with an emission maximum at 655 nm was purchased from Invitrogen (Carlsbad, CA). Biotinylated polyethylene glycol amine (biotin-PEG-NH₂; product No. 12141-0895), CH₃O-PEG-NH₂ (product No.12-750-2) and (3-aminopropyl)dimethylethoxysilane (APDMES; product No. SIA0603.0) were purchased from PolyPure (Oslo, Norway), Rapp Polymere (Tübingen, Germany) and Gelest (Morrisville, PA), respectively. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride salt (EDC; product No. W001) and Morpholinoethanesulfonic acid, monohydrate (MES; product No. GB12) were purchased from Dojindo Laboratories (Kumamoto, Japan). Poly(acrylic acid)

sodium salts (PAA; average molecular mass, 15 000; product No. 416037) were purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin (product No. S888) was purchased from Invitrogen. Milli-Q water was characterized as having a resistance of 18 M Ω and was further purified by filtration over a 0.2 μ m filter. High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) for all buffer preparations and sample solution dilutions. Microscope immersion oil (product No.16212) used was obtained from Cargille Labs (Cedar Grove, NJ).

4.3 METHODS

4.3.1 Biotin-PEG functionalized coverslips

As schematically shown in Fig. 4-1, coverslip surfaces were coated with biotin following the method reported by Wazawa *et al.* [193] with a small modification.

Fig. 4-1 The schematic structure of APDMS/PAA/PEG-biotin coated coverslip surface for QDots immobilization.

Glass coverslips (24×60×0.17 mm³) were cleaned by dipping into 65% nitric acid for more than one day, washing with milli-Q water, after which they were dried with N₂ gas. Subsequently, coverslips were incubated in APDMS (2 % in ethanol) for more than 12 hours, APDMS has only one ethoxy group per molecule and can form a monolayer of propylamine on the glass surface. Coverslips were then rinsed with copious amounts of milli-Q water and treated with 0.01 mM PAA and 2 mM EDC in 50 mM MES-NaOH buffer (pH 6.0) for ~ 1 hour. After the reaction, the coverslips were rinsed with milli-Q water again and then functionalized with 0.01 mM biotin-PEG-NH₂ solution containing 1 mM CH₃O-PEG-NH₂, 1 mM EDC, and 50

mM MES-NaOH buffer (pH 6.0). This reaction was allowed to proceed for at least 3 hours. The resulting biotin-PEG functionalized coverslips were carefully rinsed with milli-Q water, dried with N₂ gas and stored in a cleaned desiccator until use.

4.3.2 TIRF microscope

A home-built prism-based total internal reflection fluorescence (TIRF) microscope as schematically shown in Fig. 4-2 was used to observe QDot655 in this work. The TIRF microscope was based on a Zeiss Axiovert 135 TV (Oberkochen, Germany) which was fixed on an optical table (Newport, Irvine, CA). A 100 $\times$ water immersion objective (numerical aperture 1.20, Leica, Wetzlar, Germany) was used for this work. The excitation source was 488 nm laser light from an Ar⁺/Kr⁺ mixed gas laser (Coherent, Innova 70 spectrum, Santa Clara, CA) working in single line mode. The light was guided to the prism through a single mode optical fiber (FFS-B88K61XX3-SFO953, SIFAM, UK) and passed through a beam collimator before it was focused by a lens into the prism at the back focal plane of the objective. The laser power in front of the prism was 8 mW, and the spot size was $\sim 50\ \mu\text{m} \times 100\ \mu\text{m}$. The fluorescent light was collected by the objective and passed through a tube lens, a band pass filter (FF01-695/75, Semrock, Rochester, NY) and detected by a back illuminated EMCCD camera (DU-897; 512 $\times$ 512 pixels; pixel size 16 $\times$ 16 μm^2 ; single photon sensitivity > 90% quantum efficiency; Andor, South Windsor, CT) cooled to -85 $^{\circ}\text{C}$. Andor Solis software was used for camera control and image acquisition. A baseline clamp ensured a stable background baseline value across a series of images. A shutter between the coherent laser and the fiber coupler was controlled by the software to automatically stop the laser illumination after data collection. The alignment of the excitation area with the objective focal plane was facilitated by a red light from above the prism and a rotatable mirror to guide the image to a video camera before actual measurements.

4.3.3 Sample preparation for microscopic measurements

The QDot655 stock solution was first centrifuged at 5000 $\times$ g for 3 minutes to remove most aggregates of particles. Subsequently 1 μl of the supernatant was diluted $10^4\times$ in 10 mM Tris-HCl buffer (pH 7.4) and 200 μl of the resulting solution was then applied

Fig. 4-2 The schematic diagram of TIRF microscope: (1) Ar⁺/Kr⁺ Laser, (2) shutter, (3) attenuator, (4) fiber coupler, (5) single-mode optical fiber, (6) beam collimator, (7) lens, (8) BK7 glass prism, (9) microscope translation stage, (10) objective, (11) tube lens, (12) tunable mirror, (13) band pass filter FF01-695/75, (14) Andor EMCCD DU-897, (15) laser stop, (16) beam collimator for (17) Epi-illumination, (18) top coverslip – biotin-PEG functionalized coverslip, and (19) bottom coverslip – a cleaned coverslip.

to a biotin-PEG functionalized coverslip for ~1 hour. After the incubation, the coverslip was carefully rinsed with HPLC grade water. Subsequently ~80 μl of Tris-HCl buffer (pH 7.4) was deposited on the coverslip before it was covered with a second clean coverslip. Finally the sample was placed on the microscope translation stage as shown in Fig. 4-2. A small drop of microscope immersion oil was applied in between the prism and the top coverslip, at bottom of which the evanescent wave excited the QDot655 immobilized on the biotin-PEG functionalized surface.

CHAPTER

4

4.3.4 Image acquisition and processing

TIRF images were acquired with an Andor EMCCD camera. The projected pixel size on the camera was determined to be $100\text{ nm} \times 100\text{ nm}$ by imaging a standard optical grating with known feature size. The image acquisition time was 0.1 seconds and the time between images was ~30 milliseconds. A single TIRF movie consisted of 300 images from a FOV and was acquired in 39 seconds. TIRF movies were saved to files.

A custom Matlab (The Mathworks, Natick, MA) program was used to read and display the TIRF movies, identify single QDot and localize the centroid of an individual QDot. The fluorescent emission of QDots is known to undergo blinking as a result of a transient state in which no fluorescence can be observed. However, all QDots in the FOV could be detected in an average image created from all images in the movie. Next a small square region of interest (SROI), typically 30×30 pixels, which contains a single bright spot close to the center, is chosen for all frames. The total number of counts in a chosen SROI for each image in the movie was acquired by integrating over all pixels and plotted as a function of time (or frame number). In spite of our measure to prevent aggregation, multiple single QDots may reside within a diffraction-limited spot. In that case, the position of each QDot is not resolvable when they are optically identical. However, differences in optical properties offer an opportunity to separately determine the position of individual QDot in an aggregate. Here we will use the unsynchronized behavior in the dynamics of the emission of QDots to distinguish individual particles. The occurrence of time intervals during which only one of the QDots is contributing to the emission can be used to localize with precision and even the distance between QDots [166] can be determined. In this work we will first identify and select individual QDots as follows: a histogram of the total counts J in the SROI over all frames was acquired, which was then fitted to a sum of q one-dimensional Gaussian functions, defined as:

$$C(J) = b + \sum_{i=0}^q A_i \exp\left[-\frac{(J - J_i)^2}{2\sigma_i^2}\right] \quad (4-1)$$

where σ_i is the waist width of each Gaussian and C means the occurrence value as the function of total SROI intensity J and b describes a baseline. In Eq. (4-1), $q = 0$ and $q = 1$ denote pure background and a single fluorescent QDot, respectively, but $q > 1$ denotes multiple-QDot in the SROI. Initially SROI with multiple QDots ($q > 1$) were

excluded from NSL analysis. Next, for an identified single QDot, the maximum pixel intensity in the SROI of each frame of a TIRF movie was plotted as a function of time and a threshold was set at 1/3 of the maximum pixel intensity to exclude those images in the movie during which the QDot was in an off-state. Finally, in all selected images NSL is performed by fitting the spot to a two-dimensional Gaussian, using the same method (section 3.2.2) as described in Chapter 3. The SD of the centroid coordinates is then calculated and compared with the theoretical localization accuracy formulas shown in Eq. (3-4) for the SLSF and Eq. (3-5) for the FLSF.

4.4 RESULTS

Fig. 4-3 shows a frame-averaged image of one representative TIRF movie, in which QDots were immobilized on a biotin-PEG functionalized coverslip as described in

Fig. 4-3 Frame-averaged image of one representative TIRF movie (totally 300 frames for 39 seconds) of QDots immobilized on a biotin-PEG functionalized coverslip. The excitation intensity is $\sim 1 \text{ kW/cm}^2$. Image size is $50 \times 50 \text{ } \mu\text{m}^2$, 512×512 pixels, image acquisition time 100 ms/frame.

Materials and Methods. Although the QDots were observed to undergo transitions between fluorescent on- and off-state from time to time, the frame-averaged image was able to show all QDots in the TIRF movie. As shown in Fig. 4-3, the light spots (QDot images) of interest were labeled with a number. For each of the QDots 1-16, an identical SROI was selected through all image frames for data analysis.

4.4.1 Single quantum dots

Fig. 4-4 demonstrates how a single QDot (No. 12) was identified. In Fig. 4-4(a), the time trace of the intensity shows clear on- and off-states. A histogram of the total photon counts J per frame in the SROI of 30×30 pixels was presented in Fig. 4-4(b). Since a transition from the “on” to the “off” state may occur during the image

acquisition time, also amplitudes were observed in between the background baseline and the maximum magnitude as shown in Fig. 4-4(a) and Fig. 4-4(b). The histogram in Fig. 4-4(b) shows two values of J with high reproducibility over time, which is indicative of a single QDot in the chosen SROI. The histogram was fit to Eq. (4-1) with $q = 1$ by use of nonlinear least squares fitting in Matlab, and the best fit of the histogram is shown as a line in Fig. 4-4(b). The fitted value of J_0 is 89,080 cts which corresponds to an average background in the TIRF image of 99 cts/pixel. The other fitted parameter J_1 was 108,100 cts and it follows that the maximum counts from the QDot in a single frame can be expected to be $J_1 - J_0 = 19,020$ cts if the QDot stayed in fluorescent on-state in the entire acquisition time for the frame. Similar analyses were also carried out for all other QDot images labeled in Fig. 4-3. The intensity time traces

Fig. 4-4 Analysis on single QDot 12 in the TIRF movie for Fig. 4-3. (a) The total counts J in a 30×30 SROI around QDot image 12. (b) Histogram (columns) of J in Fig. 4-4(a) over 300 frames of images and its fitting (line) by use Eq. (4-1). Fitted parameters: $q = 1$, $b = 0.44$, $A_0 = 20$, $A_1 = 20.7$, $\sigma_0 = 624.2$, $\sigma_1 = 708.6$, $J_0 = 89,080$ and $J_1 = 108,100$. (c) Spatial mapping of single QDot 12 by fitted centroid coordinates in 166 frames out of 300 sequential images. The colormap represents the time (second) for each image. (d) The histogram of the distance (DtA) from each QDot position to the average center.

show that all QDot images 1-15 are images from single QDots.

Subsequently QDots 1-15 images in 30×30 pixels SROI were fitted to a two-dimensional Gaussian function using the same approach (see section 3.2.2) as described in Chapter 3. Here FLSF and SLSF were separately used as the fitting method. As mentioned in Methods, a threshold I_T was used and the frames with maximum pixel intensity $I_M < I_T$ were excluded from the NSL analyses. In the Appendix (section 4.7.3), Table 4-2 shows all the parameters for QDot images 1-15 as obtained with the FLSF method and Table 4-3 contains the results for the SLSF method. The theoretically predicted localization accuracies were determined using Eq. (3-4) for the SLSF and Eq. (3-5) for the FLSF. The SD of the background in the

images was ~ 10 cts and the average amplitude of background per pixel is ~ 99 cts (see Table 4-2 and Table 4-3 in section 4.7.3).

The results for QDot 12, as an example, show an average total counts of 18375 ± 2895 cts/frame by FLSF and 18404 ± 2896 cts/frame by SLSF. The localization accuracy was determined in 166 images which were selected from all 300 images to be above the intensity threshold level. The FLSF method gives an expected better experimental localization accuracy of 2.0 nm (x -axis) and 1.7 nm (y -axis) versus respectively 2.1 nm and 1.9 nm for the SLSF method. The centroids from 166 frames were presented in Fig. 4-4(c) for the FLSF method and are nearly circular-symmetrically distributed in a ~ 10 -nm diameter circular area. In Fig. 4-4(c), the origin of coordinates is the average of all QDot positions. The distance from each QDot position to the center was determined and the histogram is presented in Fig. 4-4(d).

4.4.2 Two quantum dots in a diffraction-limited spot

In contrast with QDot images 1-15, QDot image 16 has a higher brightness in Fig. 4-3 and was found to be a typical TIRF image of two QDots in close proximity. Fig. 4-5(a) and Fig. 4-5(b) show the time trace of the total photon counts J per frame in the 30×30 pixels SROI around QDot image 16 against time of frames and its histogram,

Fig. 4-5 Analysis on QDot image 16 where two QDots resided together in the TIRF movie for Fig. 4-3. (a) The total counts J in a 30×30 SROI around QDot image 16. (b) Histogram of J in Fig. 4-5(a) over 300 frames of images and its fitting (line) by use Eq. (4-1). Fitted parameters: $q = 3$, $b = 0.69$, $A_0 = 12.6$, $A_1 = 16.2$, $A_2 = 13.2$, $A_3 = 14.4$, $\sigma_0 = 635.8$, $\sigma_1 = 665.8$, $\sigma_2 = 807.3$, $\sigma_3 = 816.1$, $J_0 = 89,540$, $J_1 = 103,500$, $J_2 = 109,400$ and $J_3 = 123,400$. (c) Spatial mapping of fitted centroid coordinates from three groups of images: group 1 (blue triangles) $J = J_1 \pm 2\sigma_1$, group 2 (red squares) $J = J_2 \pm 2\sigma_2$ and group 3 (green circles) $J = J_3 \pm 2\sigma_3$.

respectively. From the histogram, four values of J exhibit apparently high occurrence over time. However, the two QDots, Q1 and Q2, close together within diffraction-limited spot should have four states: $i = 0$ denotes background state, in which both QDots are in a dark state; $i = 1, 2$ represents the situation where one of them is in the fluorescent on-state and the other is in the off-state; $i = 3$ denotes that both of them are in the on-state, where i is the index in Eq. (4-1). The histogram in Fig. 4-5(b) was fitted to Eq. (4-1) with $q = 3$. The fitted values of J_i are $J_0 = 89,540$, $J_1 = 103,500$, $J_2 = 109,400$, $J_3 = 123,400$. Therefore, as indicated in Fig. 4-5(b), the maximal total counts from both QDots in one frame can be expected to be $J_1 - J_0 = 13,960$ cts and $J_2 - J_0 = 19,860$ cts, respectively, if one of them is individually fluorescing and the other is in dark state during the entire acquisition time for the frame. $J_3 - J_0 = 33,860$ cts is the expected maximal total counts when both QDots are simultaneously in the on-state. This is in good agreement with the sum of maximal total counts from the individual QDots.

As shown in Fig. 4-5(b), the maximal total counts from both QDots in QDot image 16 in one frame was expected to be 13,960 cts and 19,860 cts. Similar to the

Table 4-1 Detailed fitted results in NSL of three groups (see Fig. 4-5(c)) of images for QDot image 16 in Fig. 4-3 using two-dimensional fitting with FLSF method.

No.	Background		QDots images			Localization accuracy (nm)				<i>M</i>
	Mean (cts)	SD (cts)	<i>N</i> (cts)	<i>s_x</i> (nm)	<i>s_y</i> (nm)	Experimental		Theoretical		
						<i>x</i> ₀	<i>y</i> ₀	<i>x</i> ₀	<i>y</i> ₀	
1	100.0	11.0	13,792	152.1	155.5	3.1	3.4	2.0	2.0	65
2	99.9	11.2	19,870	150.8	155.2	3.2	3.7	1.6	1.6	65
3	99.7	11.9	34,071	152.0	155.8	1.8	1.8	1.2	1.2	69

No. – Group number, **SD** – standard deviation, **N** – total photon counts from QDot, **M** – total number of frames in the group, **(x_0, y_0)** – centroid coordinates.

NSL for QDot 12, corresponding localization accuracy can be expected for both QDots on their respective individual positions in the frames where only one of both is fluorescing. Using the fitted peak positions J_i and waist widths σ_i of each Gaussian in Eq. (4-1), all frames of QDot image 16 images in the SROI were divided into four groups: group 1 has all frames of total photon counts $J_1 \pm 2\sigma_1$, group 2 has all frames of total photon counts $J_2 \pm 2\sigma_2$ and group 3 has all frames of total photon counts $J_3 \pm 2\sigma_3$. Group 4 contains other frames, which were excluded from NSL analyses. NSL was performed on groups 1-3 by FLSF and the fitted values are listed out in Table 4-1. As expected, the average values of total counts for each group in Table 4-1 are in agreement with those determined in Fig. 4-5(b). The acquired centroid coordinates of each of the three groups were then mapped in Fig. 4-5(c). Triangles and squares in Fig. 4-5(c) indicate the tracks of centroids of both QDots. The positions for both QDots were calculated by separately averaging their fitted centroid coordinates and shown in Fig. 4-5(c) with the black symbols “+”. Finally the distance between both QDots was determined to be 14.5 ± 4.8 nm (see section 4.7.1 for the method). The size of a QDot655 is ~ 15 -20 nm [194]. Therefore, the two QDots in QDot image 16 are in

close proximity next to each other on the surface. Interestingly, we found that the mean spot centroid coordinates $(-0.91, 0.86)$ in group 2 are actually in good agreement with the centroid coordinates $(-0.34, 0.50)$ of both QDots weighted by their brightness (see section 4.7.2 in the Appendix).

4.4.3 A comparison of the experimental and theoretical localization accuracy

A comparison of the results of the experimental and the theoretical localization accuracies of single QDots 1-15 were plotted in Fig. 4-6. Although experimental localization accuracy is very close to the theoretical prediction (Eq. (3-4) for the SLSF and Eq. (3-5) for FLSF) in Chapter 3, all experimental SDs of QDot centroid coordinates are larger than theoretical expectation as shown in Fig. 4-6. An explanation for this is the potential influence of the nanometer-scale architecture of

Fig. 4-6 Experimental (solid) and theoretical (open) localization accuracy against total counts from the single QDots in the TIRF movie for Fig. 4-3. There are no any two single QDots which give the same total counts. x- (circles) and y- (triangles) coordinates of the centroids of single QDots 1-15 are separately determined by the FLSF (a) and by the SLSF (b). All detailed fitted parameters are available in the Appendix.

the biotin-PEG functionalized coverslips. QDot nanocrystals have been coupled to streptavidin through an active ester coupling reaction and typically 5-10 streptavidins are covalently attached on the QDot surface [194]. As shown in Fig. 4-1, depending on local density of biotin on the surface, one QDot can bind to Biotin-PEG=NH₂ through a single streptavidin conjugate. In this case, it may not be absolutely immobilized but instead diffuse in a confined small area. The centroid localization accuracy would reflect also the increased mobility. A lower mobility, as a result of the formation of multiple Biotin-PEG=NH₂ groups with multiple streptavidin molecules on the surface of the QDot would result in a more tightly fixed QDot and an experimental localization accuracy in even closer agreement with theoretical prediction.

4.4.4 The analysis of microscope resolution

The average waist widths of the Gaussian functions to which QDots 1-15 were fitted were $s_x = 147.5 \pm 3.3$ nm and $s_y = 151.0 \pm 3.5$ nm in SLSF and $s_x = 148.1 \pm 3.2$ nm and $s_y = 151.6 \pm 3.4$ nm in SLSF over 15 QDots (see Table 4-2 and Table 4-3 in the Appendix), consistent with those values reported in Refs. [166, 167, 169, 172]. Generally, microscopy resolution is well defined according to Abbe diffraction theory and Rayleigh resolution criterion [180] and it is the radius R of the centric bright spot of Airy disk. The relationship between Gaussian waist width s and microscope resolution R is $s \approx R/2.9633$ determined in Chapter 3. The fluorescent emission peak of QDot655 is at 655 nm, so according to Rayleigh criterion $R = 0.61\lambda/\text{NA}$, the limit of s is 112 nm. Therefore, using an identical theory, the size of the point spread function (PSF) of the TIRF microscope for NSL is not smaller than the theoretical prediction. However, as commented by Hänninen [174], Gaussian function used as fit function in NSL is indeed better than Rayleigh criterion since it has a smaller spatial span and thus give a higher spatial resolution.

4.5 DISCUSSION

4.5.1 The selection of quantum dots

We have demonstrated experimental evidence of localization accuracy in NSL for a single QDot. This method takes advantage of long fluorescent lifespan of QDots to get good statistics analysis. As shown in Fig. 4-6, from a few single QDots we experimentally obtained ~ 2 nm localization accuracy (SD in more than 100 pairs of fitted centroid coordinates), which is much better than that of ~ 10 nm reported by Lagerholm *et al.* [166]. However this reasonably verifies our theory in Chapter 3, because Lagerholm *et al.* used QDot605 with fluorescent emission peaks at 605 nm and with lower efficiency (or brightness) than QDot655 used in our work [194] potentially. Maybe also limited by the camera's sensitivity, only $\sim 2,500$ photons per frame were obtained as reported by Lagerholm *et al.* in Ref. [166], and background noise must be taken into account, so the reported ~ 10 nm localization accuracy from single QDots is actually very close to the theoretical prediction for SLSF (see section 3.3 in Chapter 3).

4.5.2 Identify multiple QDots in a diffraction-limited spot

The approach of using the integrated intensities of frames to identify single QDots or overlapped multiple-QDot was first proposed by Lagerholm *et al.* [166]. Due to 3-minute centrifugation of QDot655 stock solution before use, most QDots in the TIRF movies were found to be single QDots, and many of the rest were multiple-QDot aggregations. Fortunately, we found a few two QDots aggregations in TIRF movies, and Fig. 4-5 is a representative example in this chapter. Two QDots very close together and concealed in diffraction-limited spot have been studied. In principle, this

method can be used for multiple QDots aggregation in which more than two QDots reside. Their individual centroid positions can be resolved by NSL when only one of them is fluorescing due to blinking or nonradiant dark state of QDots. Only the problem will be more complicated to find out all the states from the histogram of integrated intensity of frames perhaps by longer observation. If a number n of QDots aggregates, the maximum value of q in Eq. (4-1) should be 2^n . Lagerholm *et al.* [166] assumed that two aggregated QDots emit similar number of photons, therefore they seemed to be able to use q in Eq. (4-1) as the total number of QDots in one aggregation. However, in our experiment, we found that the total number of photons from the QDots often randomly varies from each other even if two of them are very close in the image. Within a diffraction-limited light spot, the fluorescence emission intensity of each individual QDots may exhibit variability because of differences in the local environment and stochastic photodynamic property of each QDot. In addition, due to the Gaussian profile of the laser beam at the total internal reflection interface, the excitation intensity is higher in the center of the area than in the periphery. Thus, the fluorescent emission intensity should depend on the location of the QDot.

4.5.3 Comparison between FLSF and SLSF

In Chapter 3, it was found that FLSF shows better localization accuracy than SLSF does. Similar observation was found in the experiment as well. Only one exception was found from QDot 5, whose y -coordinates localization accuracy in SLSF is slightly better (0.03 nm) than in FLSF.

4.5.4 Microscope spherical aberration

In Chapter 3, we concluded that spherical aberration (or the difference between the waist widths of the Gaussian functions) can affect the localization accuracy. However, as pointed out previously, the difference between average values of s_x and s_y from both SLSF and FLSF is < 4 nm, therefore, it cannot produce apparent influence on localization accuracy as suggested by theory (Eqs. (3-4) and (3-5) in Chapter 3) and the spherical aberration in our microscope system is reduced to an acceptable level.

4.5.5 Experimental and theoretical localization accuracy

As mentioned above, we found that surface chemistry on coverslip can be important to achieve nanometer localization accuracy. Perhaps due to local non-uniform density of biotin-PEG on the surface, two impact factors – drift and confined diffusion of molecules might be involved and consequently larger difference between experimental and theoretical localization accuracy were observed in some TIRF movies. When experimental localization accuracy was much larger than theory, the fitted centroid coordinates were then plotted to map the position diagram in Fig. 4-4(c) and Fig. 4-5(c). Because theoretical localization accuracy is from 2 nm to 10 nm as

shown in Chapter 3, molecule centroid drift of > 10 nm can be readily recognized from the map of the centroid coordinates. However, it is difficult to find the impact of molecule confined diffusion that molecule is diffusing around its linker to the coverslip surface.

4.6 CONCLUSIONS

In this chapter, we demonstrate experimental evidence for NSL on QDot 655 in TIRF microscopy. The experiment localization accuracies are very close to the predictions from the theoretical formulas (Eq. (3-4) for the SLSF and Eq. (3-5) for FLSF) adapted in Chapter 3. The experimental localization accuracy was generally slightly larger than theoretical predictions, but Thompson's formula [165] predicts a smaller theoretical localization accuracy value for SLSF than Eq. (3-4), and therefore this proves that our adapted formula Eq. (3-4) is more accurate. Nanometer resolution of multiple QDots in a diffraction-limited spot can be achieved by using NSL on a single QDot when other QDots are in the nonradiant dark states. The empirically determined single-molecule localization accuracy is from 2 nm to 10 nm.

With an identical function, either the Gaussian in NSL or the Airy disk in Rayleigh criterion, the microscope resolution was never better than the theoretical prediction, but the localization accuracy in NSL can be approximately 100 times better than the actual microscope resolution.

4.7 APPENDIX

4.7.1 Distance determination for two QDots

The distance between two QDots residing in QDot image 16 was calculated from the from their centroid coordinates as

$$d = \left[(x_{01} - x_{02})^2 + (y_{01} - y_{02})^2 \right]^{1/2} \quad (4-2)$$

and the SD in the distance determination was calculated as error propagation principle from the variance of the centroid coordinates of each of the two QDots as

$$\langle \sigma_d^2 \rangle = \frac{1}{d^2} \left((x_{01} - x_{02})^2 (\sigma_{x_{01}}^2 + \sigma_{x_{02}}^2) + (y_{01} - y_{02})^2 (\sigma_{y_{01}}^2 + \sigma_{y_{02}}^2) \right) \quad (4-3)$$

where x_{0i} and y_{0i} are centroid coordinates of QDot number i , ($i = 1, 2$), and $\sigma_{x_{0i}}^2$ and $\sigma_{y_{0i}}^2$ are variance of the centroid coordinates.

4.7.2 Centroid coordinates of two-QDot system

The centroid coordinates of both QDots weighted by their brightness was calculated by

$$x = \frac{N_1 x_{01} + N_2 x_{02}}{N_1 + N_2}, y = \frac{N_1 y_{01} + N_2 y_{02}}{N_1 + N_2} \quad (4-4)$$

where N_i is total counts obtained from the QDot number i , x_{0i} and y_{0i} are centroid coordinates.

4.7.3 Detailed localization results

All detailed fitted parameters for single QDots 1-15 in Fig. 4-3 are listed out in Table 4-2 and Table 4-3.

Table 4-2 All the results of localization accuracy analysis in NSL using 2D fitting with FLSF method

No.	Image background		QDots images			Localization accuracy (nm)				M
	Mean (cts)	SD (cts)	N (cts)	s _x (nm)	s _y (nm)	Experimental		Theoretical		
						x ₀	y ₀	x ₀	y ₀	
1	102.2	10.7	7,629	144.2	147.6	4.0	4.5	2.7	2.8	154
2	99.4	10.6	6,341	144.2	146.4	3.5	3.5	3.0	3.0	104
3	97.0	10.1	19,743	151.0	153.3	1.7	1.8	1.6	1.7	128
4	100.2	10.9	16,108	145.3	149.5	2.0	1.9	1.8	1.8	185
5	99.9	11.1	14,139	151.0	158.4	2.2	2.2	2.0	2.1	184
6	97.9	11.0	16,885	152.0	155.8	2.4	2.6	1.8	1.8	178
7	98.3	10.2	12,491	147.9	151.7	2.4	2.5	2.0	2.1	190
8	97.8	9.9	13,252	152.2	153.2	2.2	2.3	2.0	2.0	169
9	100.1	10.3	6,108	146.7	150.8	3.3	3.2	3.1	3.2	106
10	98.1	10.0	12,500	147.5	152.8	2.8	2.6	2.0	2.1	158
11	100.0	10.3	12,271	147.7	151.1	2.7	2.8	2.1	2.1	188
12	99.2	10.7	18,375	150.9	152.3	2.0	1.7	1.7	1.7	166
13	101.2	10.9	8,313	143.5	149.0	4.2	4.0	2.6	2.7	168
14	100.2	10.9	5,848	143.2	145.7	4.6	4.9	3.1	3.2	148
15	98.1	10.4	9,266	144.5	147.3	2.9	3.1	2.4	2.4	194

No. – QDot image number, SD – standard deviation, N – total photon counts from a QDot, M – total number of frames in analysis, (x_0 , y_0) – centroid coordinates.

Table 4-3 All the results of localization accuracy analysis in NSL using 2D fitting with SLSF method

No.	Image background		QDots images			Localization accuracy (nm)				<i>M</i>
	Mean (cts)	SD (cts)	<i>N</i> (cts)	<i>s_x</i> (nm)	<i>s_y</i> (nm)	Experimental		Theoretical		
						<i>x</i> ₀	<i>y</i> ₀	<i>x</i> ₀	<i>y</i> ₀	
1	101.7	10.7	7,649	145.0	148.2	4.1	4.5	2.7	2.8	154
2	98.8	10.6	6,363	145.0	147.4	3.7	3.6	3.0	3.0	104
3	96.5	10.1	19,776	151.5	153.8	1.8	2.0	1.6	1.7	128
4	99.6	10.9	16,142	145.8	149.9	2.1	2.0	1.8	1.9	185
5	99.4	11.1	14,164	151.5	158.9	2.3	2.2	2.0	2.1	184
6	97.4	10.9	16,916	152.6	156.5	2.5	2.6	1.8	1.8	178
7	97.8	10.2	12,512	148.4	152.4	2.4	2.7	2.0	2.1	190
8	97.4	9.9	13,277	152.3	153.4	2.4	2.5	2.0	2.0	169
9	99.6	10.3	6,125	147.5	151.6	3.5	3.3	3.1	3.2	106
10	97.7	10.0	12,532	148.3	153.5	3.0	2.6	2.0	2.1	158
11	99.6	10.3	12,297	148.3	151.6	2.8	3.0	2.1	2.1	188
12	98.6	10.6	18,404	151.3	152.8	2.1	1.9	1.7	1.7	166
13	100.5	10.9	8,340	144.2	149.8	4.3	4.2	2.6	2.7	168
14	99.6	10.9	5,868	144.0	146.6	4.8	5.0	3.2	3.2	148
15	97.6	10.4	9,290	145.2	148.0	3.0	3.2	2.4	2.4	194

No. – QDot image number, SD – standard deviation, N – total photon counts from a QDot, M – total number of frames in analysis, (x_0 , y_0) – centroid coordinates.

Chapter 5

Protein Stability in Fluorescence Microscopy*

ABSTRACT

We have investigated dilute protein solutions with fluorescence correlation spectroscopy (FCS) and have observed that a rapid loss of proteins occurs from solution. It is commonly assumed that such a loss is the result of protein adsorption to interfaces. A protocol was developed in which this mode of protein loss can be prevented. However, FCS on fluorescent protein (enhanced green fluorescent protein, mCherry and mStrawberry) solutions enclosed by adsorption protected interfaces still reveals a decrease of the fluorescent protein concentration, while the diffusion time is stable over long periods of time. We interpret this decay as a loss of protein functionality, probably caused by denaturation of the fluorescent proteins. We show that the typical lifetime of protein functionality in highly dilute, approximately single-molecule per femtoliter solutions can be extended more than thousand-fold (typically from a few hours to >40 days) by adding compounds with surfactant behavior. No direct interactions between the surfactant and the fluorescent proteins were observed from the diffusion time measured by FCS. A critical surfactant concentration of more than 23 μM was required to achieve the desired protein stabilization for Triton X-100 (TX100). The surfactant does not interfere with DNA-protein binding, because similar observations were made using DNA-cutting restriction enzymes. We associate the occurrence of denaturation of proteins with the activity of water at the water-protein interface which was recently proposed in terms of the “water attack model”. Our observations suggest that soluble bio-molecules can extend an influence over much larger distances than suggested by their actual volume.

* Adapted from Biophys. J. **95**:3439-3446 (2008).

5.1 INTRODUCTION

Fluorescence Correlation spectroscopy (FCS) has developed [85-87, 195] into a powerful method to analyze, real-time diffusional properties of molecules, molecular binding and affinity and molecular dynamic processes. The fluorescence amplitude fluctuations can be directly acquired and the subsequent conversion to an autocorrelation trace (or cross-correlation) is easily performed. A large number of dynamic models have been theoretically analyzed and experimentally verified [95, 101, 104, 196, 197] and highlight the strength of FCS. Molecular diffusion is just one of the dynamic processes that can be successfully studied with this technique and theoretical models have been proposed to describe molecular diffusion in different regimes [85-87]. A model for free, three-dimensional diffusion is well-established [198]. The translational diffusion time (TDt, τ_D) and the average number of molecules in the measurement volume can be directly obtained from FCS measurements. It turned out, however, that reproducible measurements of the concentration of proteins in dilute, single-molecule concentrations is not easy to achieve, due to a gradual loss of protein and/or denaturation of the protein. The average TDt in a measurement volume can be accurately and reproducibly determined.

The inaccuracy in the number determination of proteins at dilute, nanomolar concentrations is commonly interpreted as interfacial protein loss and significantly interferes with the ability to measure quantitative properties of for instance protein binding parameters and protein aggregation constants. This, in practical terms, limits the consistency of FCS results. We concluded that in order to correctly assess both concentration and TDts in dilute, nanomolar protein solutions under ambient condition (room temperature 19-23 °C) special care should be taken to prevent such interfacial protein loss. We have used hydrophobic coverslips to prevent protein adsorption to surfaces and tested this approach down to the single-molecule regime. It was noticed that in spite of the measures taken to prevent protein adsorption a gradual loss of fluorescence occurred for dilute fluorescent protein solutions. Since no fluorescent molecules could be detected at the surfaces, even with single-molecule imaging capability, we concluded that the proteins were losing their fluorescence functionality, possibly as a result of denaturation. Usually, the stability of proteins has been investigated at relatively high concentrations (e.g. from millimolar to micromolar) protein solutions, because common techniques, such as circular dichroism, calorimetry, and nuclear magnetic resonance, are not sufficiently sensitive for nanomolar protein solutions. At such high concentrations, however, proteins may tend to stabilize each other and a minor loss of functional protein may be unnoticed.

In this work, we used three mutant FPs, one red-shifted variant of wild type green fluorescent protein (GFP) [199], known as enhanced GFP (eGFP) and two monomeric, red fluorescent derivatives of DsRed, known as mCherry and mStrawberry [200] to

study cause and characteristics of protein loss in nanomolar solutions. FCS has been successfully applied before [130, 133, 134] to study the kinetic stability and biological function of fluorescent proteins (FPs) in aqueous solution. The stability of FP's is directly related to their fluorescence output, which is in turn related to the integrity of the protein surrounding the fluorophore. We will demonstrate (vide infra) a gradual loss in the amplitude of the fluorescence emission in conjunction with an absence of interfacial protein loss and interpret this phenomenon as the result of a denaturation process. Subsequently we will show that the protein functionality lifetime in nanomolar protein solutions is increased >1000-fold in non-ionic surfactant solution at room temperature without any other specific conditions, while the diffusion properties of the fluorescent proteins are unaltered by the presence of the surfactant. The latter observation suggests that the protein functionality-lifetime is increased even without direct contact between protein and surfactant molecules. Subsequently we show that a certain minimal surfactant concentration is necessary to be effective. In order to test whether this observation extends also to other protein functionalities than "fluorescence", we show that the presence of surfactant also stabilizes the function of restriction enzymes (related to the "functionalities" DNA recognition, binding and cutting) over a period of 6 weeks.

5.2 MATERIALS

Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TFOS, product No. 448931), Rhodamine 6G (R6G, product No. R-4127; molecular mass, 479 g/mole) and Triton X-100 (TX100, product No. T8787) were purchased from Sigma-Aldrich (St. Louis, MO). The other surfactant Nonidet P-40 (NP40, product No.11754599001) was purchased from Roche Applied Science (Indianapolis, IN). Purified bovine serum albumin (BSA, product No. B9001S) used to stabilize FPs, restriction enzymes *FokI* (product No. R0109S, 4 units/ μ l) and *AvaI* (product No. R0152S, 10 units/ μ l) were purchased from New England Biolabs (Hitchin, UK). 5' Alexa-488 55-mer oligonucleotide and its complementary strand were purchased from DNA Technology A/S (Risskov, Denmark). Milli-Q water was characterized as having a resistance of 18 M Ω and was further purified by filtration over a 0.2 μ m filter. High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) and used for all buffer preparations and dilution steps.

5.3 METHODS

5.3.1 Single-molecule fluorescence confocal microscope

A single-molecule fluorescence confocal microscope (SFCM) with imaging capability was redesigned to accommodate FCS measurements. The SFCM was described elsewhere [67, 68]. A detailed description of the set up is presented in Chapter 2 (section 2.2). An Ar⁺/Kr⁺ mixed gas laser (Coherent, Innova 70 spectrum, Santa Clara,

CA) was used for excitation of eGFP and Alexa488 ($\lambda_{\text{exc}}=488$ nm.) and mCherry and Strawberry ($\lambda_{\text{exc}}=568$ nm.). The laser power was 20 μW in sample solution.

5.3.2 Coverslip surface passivation

The coverslips were first cleaned in 65 % HNO_3 , washed with milli-Q water, and then dried using N_2 gas. Protein adsorption at surfaces was prevented by passivating all coverslips with TFOS, which removes silanol groups at the coverslip surface (see section 2.5.4.2 in Chapter 2 for more details).

TFOS renders the surface highly hydrophobic resulting in water contact angles above 120° , ensuring that 8 μl of aqueous sample solution, which was used for FCS measurements, forms a spherical droplet. TFOS-treated coverslips were washed with 100% ethanol and dried using N_2 gas before use.

5.3.3 On-line FCS analysis

A custom computer program was written in Labview (National Instruments, Austin, TX) to control the fluorescence microscope piezo nano-positioning stage and to record fluorescent amplitude time traces or spectra. The SFCM could be operated in four modes: i) fluorescent intensity imaging, ii) fluorescent spectral imaging, iii) intensity time trace for FCS and iv) spectral time trace for sFCS (spectral FCS). Control software for FCS continuously recorded a fluorescence intensity time trace and calculated the autocorrelation trace for an adjustable preset time, which was 10 seconds. The autocorrelation trace was subsequently on-line fitted, with a non-linear least squares fitting procedure. Correlation analysis was coded in C++ language and integrated with the Labview control software for fast calculations of the autocorrelation function (ACF) or the cross-correlation function (CCF). Molecular TDts and average numbers of molecules in the focal volume could be rapidly determined by fitting each ACF to the generalized 3D diffusion model [198] in less than two seconds for a ten-second intensity time trace and a 128-point auto-correlation curve. Typical measurements consisted of 50 times 10-second time traces within 10 minutes. Temporal evolution of molecular TDts and average numbers of molecules in focal volume was directly monitored during measurements in a separate panel in the software display.

On-line FCS could directly be used to monitor molecular numbers and diffusion at different locations in the sample. Intensity time traces of surface adsorbed proteins gave rise to high amplitude when started from a fresh position, which was followed by rapid intensity decay due to photobleaching of (immobilized) fluorescent molecules. In some experiments, the piezo scanner was used to start each FCS time trace at a fresh position, which was randomly distributed within an area of $50 \times 50 \mu\text{m}^2$ to take into account a non-uniformed surface coverage. All FCS measurements were repeated 50 times to acquire accurate statistics on all observations. Typically, sample drops were placed on a single TFOS treated coverslip while the laser focus was moved from

one drop to another. All other components of the SFCM were fixed, avoiding any variation in the excitation and observation volume [123].

All intensity time traces were saved to file and analyzed off-line at all available delay times, typically 8×10^5 time points. Concentrations of sample molecules were estimated from the background-corrected amplitudes of the FCS curves [197] and compared with the expected concentrations during sample preparation. Sample solutions were hermetically sealed in between two cover glasses using a silicone spacer to avoid evaporation. Volumes of measured solutions were always much smaller than the space in the sealed well so that droplets of sample solution were isolated from the silicone spacer material.

5.3.4 Buffers

PGI buffer was used to store FPs and contained 20 mM Na_3PO_4 , (pH = 7.8), 300 mM NaCl, 10% glycerol and 60 mM imidazole (used for purification). Other buffers used were: a) 40 mM HEPES-NaOH buffer (pH 7.4), b) 25 mM Tris-HCl buffer (pH 7.4), c) phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 18.9 mM Na_2HPO_3 , 1.8 mM KH_2PO_3 , pH 7.5) and d) CP buffer [130, 133, 134] (10 mM citric acid, 100 mM K_3PO_4 , pH 7.0). TEK buffer, which was used to store restriction enzymes and consisted of 10 mM Tris-HCl (pH = 7.6), 0.1 mM EDTA and 50 mM KCl. TEKT buffer was similar to TEK buffer with the addition of 0.23 mM TX100. NEBuffer 4 (New England Biolabs) was used for all DNA restriction reactions and consisted of 50 mM CH_3COOK , 20 mM Tris-acetate, 10 mM $(\text{CH}_3\text{COO})_2\text{Mg}$, and 1 mM DL-Dithiothreitol (pH = 7.9). HPLC grade water was used in all sample preparations.

5.3.5 Purification of fluorescent proteins

The eGFP gene was fused to N-terminal (His)9- and HA-tags and inserted into the pET17B vector (Novagen, San Diego, CA), removing the pET17B T7-tag. Plasmids pRSET-B-mCherry and pRSET-B-mStrawberry, containing (His)6-Xpress tagged mCherry and mStrawberry, respectively, were kind gifts of Roger Y. Tsien [200], department of pharmacology and department of chemistry & biochemistry, university of California, San Diego, La Jolla, CA, USA. Recombinant proteins were produced by over expression in *E. coli* strain BL21(DE3) and subsequently purified using Ni-NTA chromatography (Qiagen, Valencia, CA) in PGI buffer. Protein concentrations were measured using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

5.3.6 Preparation of FP sample solutions

Preparation of fluorescent protein samples was as follows: 2 μl of stock solution of 2.5 μM eGFP in PGI buffer was first 10 $\times$ diluted in 23 μM (i.e., 0.002% (v/v)) TX100 solution. Next, this stock solution was 25 $\times$ diluted using HPLC grade water or

other buffers, producing 10 nM eGFP solutions. Five 100 μ l solutions (labeled FS1-5) of 34 days old and 5 freshly prepared solutions (labeled FFS1-5) were prepared containing 2.5 nM eGFP and different concentrations of TX100. TX100 concentrations in sample (F)FS1 to sample (F)FS5 were 2.3 μ M, 23 μ M, 230 μ M, 2.3mM and 23mM, respectively. To avoid any proton-induced effect on the optical properties [130, 133, 134, 199], pH=7.5 was used in all solutions.

5.3.7 Preparation of double-stranded DNA

Alexa-488 dye labeled 55 bps dsDNA (Alexa488-55bps-dsDNA) was made by annealing equimolar amounts of commercial 5' Alexa-488 55-mer oligonucleotide to its complementary strand. The quality of the resulting DNA products was checked using polyacrylamide gel electrophoresis (data not shown).

5.3.8 DNA restriction reaction

Restriction reactions were executed in 20 μ l reaction solutions (labeled RS1-4) respectively, containing NEBuffer 4, 0.23 mM TX100, 4.2 nM Alexa488-55bps-dsDNA and 2 μ l of the corresponding enzyme solution ES1-4. As control DNA solution RS5 was used, containing all components except enzyme. Restriction reactions were performed at 37 °C for one hour.

5.4 RESULTS

5.4.1 Protein adsorption to surfaces

Without special measures, proteins in low concentrations readily adhere to surfaces, since an excess of surface binding sites is available. As a consequence, protein concentrations in solution are at best approximate and experimentally derived parameters, such as affinity constants, are not reliable. To prevent protein adsorption, we tested treatment of glass surfaces by using TFOS to exposed silanol groups. In Fig. 5-1, eGFP adsorption of untreated (Fig. 5-1(c)) and treated surfaces (Fig. 5-1(b)) is shown. For ease of reference, Fig. 5-1(a) shows a representative image of a standard cleaned and TFOS passivated surface in the presence of HPLC water only. Color scales of Fig. 5-1(a) and Fig. 5-1(b) are identical (counts per 250 μ s). As can be clearly discerned, the adsorbed number-density of proteins at untreated glass is approximately 100 times higher than at treated glass. The data of Fig. 5-1(b) is represented at a ten times expanded color scale and only few adsorption events in an area of $9.3 \times 9.3 \mu\text{m}^2$ were observed. The maximum number of proteins per μm^2 in an 8 μ L drop of 1 nM protein solution is approximately 800. Thus, the occupied surface area would in that case be less than 1%. Moreover, the (relative) immobility of surface adsorbed molecules results in rapid photobleaching of the fluorescence amplitude in a time trace (Fig. 5-1(d)) as compared to that of molecules in solution (Fig. 5-1(e)). Thus, we conclude that using TFOS treated glass proteins do not stick to the surface.

Fig. 5-1 Protein adherence to surfaces is largely prevented by treating the surface with TFOS. Image size in (a), (b) and (c) is $9.3 \times 9.3 \mu\text{m}^2$, 256×256 pixels, dwell time 0.25 ms/pixel, excitation $7.9 \text{ kW}\cdot\text{cm}^{-2}$ at 488nm. (a) A representative image of a standard cleaned and TFOS passivated surface in the presence of HPLC grade water only is shown. (b) A representative image of a TFOS passivated surface after incubation with 10 nM eGFP in HPLC grade water is shown. (c) A representative image of a standard cleaned surface not passivated using TFOS after incubation of 10 nM eGFP in HPLC grade water is shown. Bar in Fig. 5-1(a) denotes 2 μm. Color scales in Fig. 5-1(a) and Fig. 5-1(b) are identical. In Fig. 5-1(d), a representative intensity time trace of the glass-solution interface as in Fig. 5-1(c) and Fig. 5-1(e) in solution is shown.

All following experiments were performed using TFOS treated glass surfaces.

5.4.2 Loss of fluorescence of FPs in aqueous solutions

In spite of the virtual absence of protein adsorption, gradual loss of fluorescent proteins from solution was still observed in our FCS experiments. In these experiments, fluorescence bursts were recorded during at least 500 seconds, resulting in a sequence of 50×10 second traces, which were subsequently calculated into an autocorrelation trace and fitted to a 3D-diffusion model [198] to extract molecular TDts and eGFP concentrations estimated from background-corrected amplitudes of the FCS curves. As shown in Fig. 5-2(a), eGFP fluorescent intensity in solution gradually decreased in spite of the virtual absence of protein adsorption. Molecular TDts are plotted in Fig. 5-2(b) and Fig. 5-2(c). From these measurements, it follows that the average TDt of eGFP is $149 \pm 7 \mu\text{s}$, which is in good agreement with Schenk et al. [136] and with the τ_D of R6G (see section 5.5.2). Similar results have been obtained using mStrawberry and mCherry (data not shown). In spectral time traces, spectral properties of diffusing species can be monitored. A typical spectral time trace of an eGFP solution (25000 spectra with a total recording time of 5000 s.) is presented in Fig. 5-3, which shows the decay in amplitude from spectral time traces and average spectra from subsequent time intervals. In order to test the potential influence of

Fig. 5-2 (a) The decrease of the fluorescence amplitude of a solution of 5 nM eGFP (open circles) in HPLC grade water and a stable fluorescence amplitude of a solution of 2.5 nM eGFP (solid circles) in 0.02% TX100. The excitation power was 16 $\text{kW}\cdot\text{cm}^{-2}$. Excitation wavelength was 488 nm. **(b)** Simultaneously with the time trace in (a) the TDT of eGFP in the focal volume, τ_D , was determined 360 times for 10 second intervals during one hour. **(c)** The histogram of Fig. 5-2(b). Average value and standard deviation of $\langle\tau_D\rangle = 149 \pm 7 \mu\text{s}$.

buffers used, similar experiments were conducted using eGFP dissolved in Tris-HCl buffer, HEPES-NaOH buffer, PBS buffer and CP buffer. Fluorescence amplitude decreased over time in all instances (Fig. 5-4(a)), while molecular TDTs remained constant (Fig. 5-4(b)). In contrast, 2.5 nM eGFP solution in 0.23 mM TX100 stably fluoresced until the experiment was stopped in one hour. This clearly shows photobleaching is not the source of fluorescence loss of eGFP in the solution. Experiments for mStrawberry and mCherry instead of eGFP gave similar results (data not shown).

Fig. 5-3 Spectral measurements of ~ 10 nM eGFP solutions in HPLC grade water. (a) Consecutive series of spectra each obtained from an average over 3600 spectra acquired during 12 minutes (time increases from top to bottom). **(b)** Spectral intensity time trace at an emission wavelength of 509 nm from 25200 spectra recorded during a total of ~ 84 minutes. Each spectrum was acquired for 200 ms. The excitation intensity was 16 $\text{kW}\cdot\text{cm}^{-2}$. The water Raman band (indicated with an arrow) was used as an internal intensity standard. The spectrum of the fluorescence emission decreases from top to bottom and reflects gradual loss of fluorescence of eGFP.

Fig. 5-4 (a) Decrease in fluorescence amplitude of eGFP in FCS time traces in Tris-HCl buffer (open circles), HEPES-NaOH buffer (solid circles), PBS buffer (open triangles), and CP buffer (solid triangles). (b) The average and standard deviation of TDts, τ_D , of eGFP as obtained from the time traces in Fig. 5-4(a).

To test the potential influence of protein concentration in the stock solution, the concentration of the stock solution was increased to 10 nM. FCS measurements from fresh samples over a two day time period revealed a similar loss of fluorescent protein signal in a few hours.

We conclude that in spite of the surface treatment using TFOS, a gradual decrease of fluorescence results under a wide range of conditions.

5.4.3 Activity of FPs in the presence of surfactant TX100

In order to develop a procedure to prevent loss of active fluorescent protein in solution, protein solutions were prepared containing varying concentrations of TX100, a well known surfactant, ranging over 5 orders of magnitude from 0.23 μM to 23 mM. Five 100 μl 2.5 nM eGFP solutions were prepared (labeled FS1-5, see section 5.3.6), which were all stored in a dark polyurethane box under ambient condition. Aperiodically, within a period of 34 days, 8 μl of each solution was used to monitor protein activity using FCS and spectral measurement. Apart from solution FS1, containing 2.3 μM of TX100, which completely lost fluorescence activity within a few hours, all solutions preserved fluorescence activity for more than one month (34 days). Fig. 5-5(a) shows the spectra of FPs in FS2-5 (from the top down) on day 34 compared with that of a freshly prepared sample in 23 mM TX100 (bottom). The Raman band of water was used as internal intensity marker. A constant ratio of fluorescent emission to this band was observed even after 34 days. A pair-wise comparison of molecular TDts (Fig. 5-5(b)) in the presence of 4 different TX100 concentrations showed near-identical behavior of fresh samples and samples stored for 34 days. In Fig 4(b) it is shown that the highest concentration of TX100 (samples FFS5 and FS5 at 23 mM) results in a slight increase in molecular TDt, which could be the result of increased viscosity of the solution.

Fig. 5-5 (a) Fluorescence emission spectra of 2.5 nM eGFP solutions FS2-5 (after 34 days under ambient condition) and FFS5 (freshly prepared) from top to bottom, by averaging 100 frames of spectra with a 200 ms acquisition time for each frame. Excitation used was $16 \text{ kW}\cdot\text{cm}^{-2}$ 488 nm laser. The water Raman band, indicated with arrows, served as internal intensity standard. (b) The TDts τ_D of eGFP as obtained from spectral time traces of eight different eGFP solutions FS2-5 (grey) and FFS2-5 (white). Solutions FFS2-5 were freshly prepared. Histograms of Fig. 5-5(b) are shown in Fig. 5-8 (see the Appendix).

Identical experiments were conducted using mStrawberry and mCherry or using NP40 as surfactant instead of TX100, which all led to similar results (Fig. 5-6).

The influence of high concentrations of other proteins was also tested. Nanomolar concentrations of eGFP were dissolved in buffer containing 0.1 mg/ml ($1.47 \mu\text{M}$) Bovine Serum Albumin (BSA). FCS measurements indicated that fluorescence activity of eGFP could be observed up to 72 hours following sample preparation (data now shown), suggesting that BSA does indeed stabilize functionality but not as efficiently as TX100 or NP40.

5.4.4 Activity of restriction enzymes in the presence of surfactant TX100

The extraordinary long functionality lifetime of fluorescent proteins in dilute solutions (vide supra) in the presence of TX100 raises the question whether these observations also extend to other protein functionalities, such as the activity of restriction enzymes [201]. To explore this, an Alexa-488 dye labeled 55 base pairs dsDNA (Alexa488-55bps-dsDNA) containing a recognition site for *FokI* was chosen (Fig. 5-7(a)), which was positioned such that in the presence of active *FokI* a short Alexa-488 labeled double-stranded DNA fragment would result. The expected rapid diffusion of the small DNA fragment as measured by FCS was used as reporter of the activity of the

Fig. 5-6 The TDts τ_D of eGFP in the focal volume was acquired using 2.5 nM eGFP solutions in the presence of NP40 at different concentrations (indicated in the figure) after a storage period of one month in a dark polyurethane box under ambient condition.

Fig. 5-7 (a) 55bps-dsDNA was labeled with Alexa488. *FokI* recognition sites are 5 base pairs (double underlines) in length. Cleavage positions on both strands are indicated by arrows. The restriction fragment containing the Alexa-488 dye is singly underlined. **(b)** The average TDts τ_D of Alexa488-DNA molecules from FCS measurements acquired at the indicated times during 48 days. Excitation for FCS measurements was $40\text{ kW}\cdot\text{cm}^{-2}$ 488 nm laser. Reaction solutions RS1-4 contained 4.2 nM Alexa488-55bps-dsDNA, 0.8 units *FokI* for RS1&2, and 0.08 units *FokI* for RS3&4, respectively. The *FokI* in the reaction solutions RS1-4 was obtained from the enzyme storage solutions ES1-4, respectively, which were stored at room temperature for the indicated times. ES2 and ES4 solutions contained 0.23 mM TX100. Solution RS5 contained similar components but no enzyme.

restriction enzyme *FokI*.

Four 40 μl enzyme solutions, labeled ES1-4, were prepared containing 0.4 units/ μl *FokI* in buffer TEK without TX100 (ES1), or in buffer TEKT containing 0.23 mM TX100 (ES2) or containing 0.04 unit/ μl *FokI* in buffer TEK without TX100 (ES3) or buffer TEKT containing 0.23 mM TX100 (ES4). All *FokI* solutions were prepared on ice and 2 μl of each solution ES1-4 was immediately quantified with DNA restriction reactions (see section 5.3.8) by FCS ($t = 0$ hrs). The enzyme solutions ES1-4 were subsequently stored under ambient conditions and enzyme activity was monitored by DNA restriction reactions and FCS over a period of 48 days. Fig. 5-7(b) shows the average TDts of DNA molecules in all samples which were obtained from 50 independent 10 second time traces. As can be observed, solutions containing low concentrations of *FokI* (0.04 units/ μl) in the absence of TX100 (ES3), completely lost activity within 72 hours. However, in the presence of 0.23 mM TX100 (ES4) low concentrations of *FokI* retained activity during 48 days. High concentration (0.4 units/ μl) of *FokI* solution in the presence (ES2) and absence (ES1) of TX100 showed similar activity after 72 hours, corroborating the results.

The same experiment was conducted using the restriction enzyme *AvaI*, for which similar results were obtained (data not shown). In conclusion, these results demonstrate that the addition of surfactant to solutions containing low protein concentrations prevents loss of protein activity.

5.5 DISCUSSION

5.5.1 Molecular crowding environment and protein functionality lifetime

We have used quantitative FCS to monitor the activity of proteins at dilute (nanomolar) concentrations in the presence and absence of surfactant. It was observed that the protein functionality lifetime at very dilute solutions was more than 1000 fold extended in the presence of surfactant TX100 and NP40. The effect of TX100 was achieved at a concentration of 23 μM and higher while no effect was observed when a concentration of 2.3 μM was used. The average aggregation number of TX100 in a TX100 micelle is approximately 147 [202]. This implies that the average distance between TX100 micelles in a 23 μM solution is ~ 216 nm, while the average distance between proteins in a 1 nM solution is ~ 1.2 μm . These numbers show that the intermolecular distances are much larger than the molecular size of the surfactant (between 5 nm and 10 nm) and the protein (~ 4 nm for eGFP). The molecular TDT obtained from FCS directly shows that no long-lasting contact interaction takes place between the surfactant and the protein. An order of magnitude estimate of the result of the interaction of a single micelle (molecular mass: $147 \times 628 \approx 90$ kDa) with a single eGFP molecule (molecular mass: 30 kDa), would result in a molecular TDT of ~ 240 μs . This number is much different from the values we have obtained (Fig. 5-2(c), Fig. 5-4(b) and Fig. 5-6) and clearly beyond the measurement error. Therefore, we conclude that the very large, 1000-fold effect of the detergent on the functionality lifetime of dilute protein in solution is achieved by another mechanism than direct contact interaction.

Structure and function of proteins are related to sub-nanometer scale interactions between protein sidechains. These interactions are facilitated by a 3D arrangement of secondary structure motifs. The role of water in protein function and stability is well known [203]. Water enables fast conformational changes and clusters of water molecules have been found to form in or near hydrophobic cores in proteins [204]. In very dilute protein samples the environment is dominated by water, the behavior of which seems to be affected by the presence of surfactant (vide supra). Intramolecular H-bonds contribute greatly [204] to protein structure and stability and they are particularly effective in the absence of competing, free water molecules in the interior of the protein. On the other hand interior water molecules do play an essential role in protein stability and they have much longer mean TDTs than water molecules in bulk. Nevertheless water molecules may dissociate and escape to the bulk as a result of or as part of a structural collapse [205] of a protein. The presence of free water

molecules in the interior of proteins may lead to a so called “water attack” [206, 207] on the intramolecular protein hydrogen-bond network. It was shown by computer modeling and molecular dynamics simulations that such a water attack could lengthen the intramolecular H-bonds and rearrangements in the hydrophobic side-chains in order to shield them from a more polar environment [206, 207]. Clearly, for eGFP in water to maintain its fluorescence, contacts between protein sidechains and the Ser-Tyr-Gly chromophore should be shielded from hydration.

In bulk water H-bond networks are governed by extremely fast dynamics, typically within ~ 1 ps timescale [208, 209]. The dispersive forces of rapidly fluctuating constructive and destructive water H-bond networks may destabilize proteins by the repeated recurrence of destructive forces with an, in itself, small probability. A lower population of destructive H-bond network or a slower evolution of water H-bond network patterns may increase protein stability and in this way lengthen the functionality lifetime of a protein. We have provided ample evidence that TX100 (and other surfactants) increase the stability of protein functionality without directly interacting with the proteins. The question that arises is: how does TX100 (and other surfactants like e.g. NP40 and BSA) stabilize the functionality lifetime of soluble proteins?

The hydration shell of soluble protein was proposed very early [210] to be of major influence on the functionality of proteins. Recent theoretical [211, 212] as well as experimental [213] evidence indeed revealed that water molecules in the hydration shell exhibit anomalous behavior [211, 212], such as sub-diffusive translational diffusion and non-exponential orientation relaxation. The solvation dynamics of optical probes near the protein surface also show a slow decay over time [208, 209]. The solvation dynamics on surfactant micelles is known to be very slow as well and may even extend to a few nanoseconds [214]. The general structure of non-ionic micelles containing a dense hydrophobic core and hydrophilic polar head groups on the surface is accompanied by a large effect on the dynamic properties of water molecules in the hydration shell of the micelle. Tight hydrogen bonding networks of water with polar head groups may lead to solvation dynamics in the hydration shell of micelles that are slowed down by as much as 3 orders of magnitude.

Our observation that the protein functionality is “remotely” stabilized by the presence of non-ionic surfactant micelles at a concentration higher than ~ 23 μM could reflect the slow water dynamics as a result of stable networks in the hydration shell of surfactants. This property effectively decreases the rate of occurrence of destructive dispersive water activity that competes with intramolecular protein hydrogen bonding and fixed water in proteins. An approximate average distance between TX100 micelles at a concentration of 23 μM is of the order of 200 nm. It follows that an influence of micelles on water dynamics extending over 100 nm would already lead to an, on average, continuous effect on water dynamics in solution. The

size of FP's and restriction enzymes, geometrically typically some 4 nm plus their respective water shells, further contributes to the overlap of organized water structures in between micelles at a concentration of some tens of μM . At a 10 times lower TX100 concentration, coinciding with an approximate average distance of 450 nm, we have not observed an effect on the stabilization of protein functionality, suggesting that the influence of the micelle does not extend much further than approximately 100 nm into the solvent.

Since, using FCS, we have measured directly relatively slow molecular diffusion, it is interesting to note that no effect on protein diffusion rate was observed until approximately 23 mM of TX100. This concentration coincides with an approximate average distance of 22 nm between TX100 micelles. This agrees with our reasoning that particularly the fast dynamics of the solvent are affected at relatively low surfactant concentrations, while viscous effects of the surfactant influence the relatively slow molecular mobility at much higher concentration when the average distances between micelles become of "molecular size".

It follows that in the cells of organisms, where the concentration of proteins in the cytoplasm is between 100 and 250 mg/ml [215], protein functionality is stabilized in such a molecular crowded environment.

5.5.2 Estimation of TDt of eGFP

Under low light conditions, photophysical effects on FPs are minimal. An average TDt $\tau_D = (149 \pm 7) \mu\text{s}$ (mean $\pm$ standard deviation) for the eGFP was determined as in Fig. 5-1(b). A relation between the TDts of two fluorescent molecules (roughly defined as globular molecules), the eGFP and the internal marker R6G, can be found with the help of the Stokes-Einstein relation, resulting in:

$$\tau_D^{SP} / \tau_D^{R6G} = (m^{SP} / m^{R6G})^{\frac{1}{3}} \quad (5-1)$$

Here τ_D and m represent average TDt of molecules in focal volume and molecular mass, respectively (*SP* denotes "soluble protein"). Average TDt of R6G has been determined to be around $40 \pm 2 \mu\text{s}$ using our setup. Using Eq. (5-1) and $m^{\text{eGFP}} = 30 \text{ kDa}$, the estimated average TDt of eGFP is $159 \mu\text{s}$. Given the simplification using this approach, the reported average TDt of eGFP seems to be in reasonable agreement with that expected for eGFP and is consistent with the value reported by Schenk, *et al.* [136]

5.6 CONCLUSIONS

Quantitative fluorescence correlation spectroscopy of dilute, nanomolar solutions of fluorescent proteins and the restriction enzymes *FokI* and *AvaI* reveals that the lifetime of protein functionality is extended at least 1000-fold in the presence of a sufficient concentration of surfactant molecules. We have argued that this effect is a result of the influence that micelles, and to a somewhat lesser degree the proteins

themselves, have on the dynamic rearrangement of water hydrogen networks, limiting the occurrence of hydrogen bond networks in competition with the intramolecular protein networks that stabilize proteins. This model connects to ideas that have recently been proposed under the denominator “water attack” model [206] for protein destabilization. We feel that the reported effect at nanomolar dilutions of proteins contributes to a concept of “biological water” and could have a much broader range of applications. We also conclude that experiments in the single-molecule optical regime can be carried out with fidelity in the presence of surfactant and passivated surfaces.

5.7 APPENDIX

5.7.1 Additional figure

CHAPTER

5

Fig. 5-8 Histograms associated with the data shown in Fig. 5-5(b). TDts of eGFP in focal volume measured for eGFP solutions were $\langle \tau_D \rangle_{\text{FFS5}} = 149 \pm 7 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FS5}} = 150 \pm 7 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FFS4}} = 144 \pm 7 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FS4}} = 145 \pm 6 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FFS3}} = 146 \pm 6 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FS3}} = 144 \pm 5 \mu\text{s}$, $\langle \tau_D \rangle_{\text{FFS2}} = 144 \pm 7 \mu\text{s}$ and $\langle \tau_D \rangle_{\text{FS2}} = 145 \pm 7 \mu\text{s}$, where the subscripts of $\langle \tau_D \rangle$ label the corresponding eGFP solution.

Chapter 6

Nucleotide Excision Repair Protein Complexes XPC/HR23B and Rad4/Rad23 Binding to DNA – Investigation by Combined Fluorescence Correlation Spectroscopy and Steady-state Fluorescence Anisotropy

ABSTRACT

Disruption of DNA repair may eventually result in the loss of genetic integrity and cause various cancers. Failure in nucleotide excision repair (NER), one of important DNA repair pathways, in eukaryotes gives rise to xeroderma pigmentosum (XP) skin cancer predisposition syndrome. Recognition of DNA damage by the human XPC/HR23B and the yeast Rad4/Rad23 protein complexes plays a central role in initiating global-genome NER. We have performed a quantitative study on the bindings of XPC/HR23B and Rad4/Rad23 to damaged and undamaged DNA substrates by using combined fluorescence correlation spectroscopy (FCS) and steady-state fluorescence anisotropy (SFA) techniques. Human XPC/HR23B binds with higher affinity ($K_D \sim 0.8$ nM by FCS and 3.7 nM by SFA) to damaged fluorescent DNA substrate (Alexa488-B5-DNA) containing a 5 unpaired base pairs than the yeast Rad4/Rad32 ($K_D \sim 66$ nM by FCS and 69 nM by SFA). Competition titration experiments revealed that XPC/HR23B complex preferentially binds to damaged DNA substrates with a few improperly pairing bases. For the study of binding reaction of fluorescent molecules in solution, FCS and SFA are expected to measure the translational and rotational Brownian motions taken by overall binding complex, respectively. However, as presented in this chapter, SFA can be strongly influenced by the local rotational motion of the fluorescence label on the molecule under study and FCS was found more sensitive to the formation of binding complex than SFA. This observation suggests that FCS can offer more reliable information than SFA in the application of molecular interaction. The combined FCS and SFA techniques can provide complementary information for overall molecular mobility and show excellent ability and advantages for the study of DNA-protein binding. The experiments showed our techniques a promising future to elucidate the architectures of complicated biological systems.

CHAPTER

6

6.1 INTRODUCTION

For the integrity of the genome and cellular functions, living organisms naturally have multiple DNA repair systems that can treat with a wide variety of DNA lesions [16, 216]. Of all DNA repair pathways, nucleotide excision repair (NER) is a versatile repair pathway, involved in the removal of a variety of bulky DNA lesions such as UV induced cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4PP) [1, 217]. NER in the eukaryotic cell nucleus can be accomplished through two distinctive pathways, known as global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). As indicated from both two names, GG-NER activity works throughout the entire genome, whereas TC-NER action is performed within active transcriptional regions. Impaired NER activity has been associated with several human genetic disorders, e.g., xeroderma pigmentosum (XP). XP patient is characterized clinically by cutaneous hypersensitivity to UV exposure and increased susceptibility to skin cancer. Due to the importance of NER for human health, seven NER-deficient genetic XP groups, XPA to XPG, have been identified, and their responsible genes have been cloned. A multistep model for GG-NER mechanism has been proposed [2, 3], and it can be dissected into: (1) DNA damage recognition; (2) unwinding of the DNA helix around the damage site; (3) double incision of the damaged strand on both sides of the lesion; (4) removal of the damaged oligonucleotide and resynthesis of the gap, and (5) ligation of the residual nick. Many NER proteins are involved in the model and the ordered arrival and release of each factor has been found to repair the DNA damage [2, 3]. Among all these NER proteins, the XPC protein has been found to play a central role in initiating global-genome NER by recognizing the lesion and recruiting other NER proteins to start NER machine [218]. However, XPC proteins were found to tend to aggregate *in vitro* [39], and thus for quantitative measurements, careful protein preparation is required [40]. Recently the yeast XPC orthologue Rad4 protein [27] was found to have a similar structure as well as similar functions to bind to the DNA with lesions as the human XPC protein has and play a role in the DNA damage recognition.

Fluorescence correlation spectroscopy (FCS) was first proposed by Magde *et al.* [87] and it is based on the correlation analysis of fluorescence intensity fluctuations of fluorescent molecules randomly passing through a small volume due to Brownian motion. The auto-correlation function (ACF) profile of the fluorescence intensity fluctuation in focal volume represents the instantaneous average of the translational diffusion by all molecules in various conformational states, but is weighted solely by the number of molecules in each of those states. Other techniques like microscopic surface imaging were often used to study the molecules' conformation states as well, but can obscure the typical behavior of most molecules within the population. FCS provides information about the translational Brownian motion of fluorescent

molecules in solution. Since it was invented in the beginning of 1970s, booming applications has been proposed and developed in FCS [88, 89, 91, 219-221]. Recently, we reported an on-line quantitative FCS technique for the study of soluble protein stability [69].

Fluorescence anisotropy (FA) is a popular fluorescence spectroscopic technique and has been extensively used to study molecular interactions both in biology and in chemistry [222-225]. The experimental principle of FA is that a linearly polarized light of appropriate wavelength passes through a diluted aqueous solution of fluorophores, fluorophores with their absorption transition vectors aligns parallel to the polarization plane of the light are preferentially excited, and the emitted fluorescent light depolarized because molecules tumble rapidly in solution during its fluorescence lifetime. FA is to measure the fluorescence molecule depolarization that can reveal a wealth of information about the intrinsic properties of fluorescent molecules as well as the environment in which they reside. Due to the dependence on molecular size and environment, FA allows probing the change of molecular conformation in solution and thus suits to the DNA-protein binding (DPB) study.

FA measurements can be classified into two types of measurements: steady-state and time-resolved [4]. The steady-state FA (SFA) measurements are using constant illumination and observation, while for the time-resolved FA (TFA) measurements, the illumination is pulsed light source and the intensity observation requires high speed for measuring intensity decays or anisotropy decays. The relationship between the TFA and the SFA is

$$\langle r \rangle = \int_0^\infty I(t) r(t) dt / \int_0^\infty I(t) dt \quad (6-1)$$

where $\langle r \rangle$ and $r(t)$ are SFA and TFA, respectively, and $I(t)$ is fluorescence intensity decay function. For single-exponential photophysics, *i.e.*, $I(t) = I_0 e^{-t/\tau_F}$, the TFA for spherical molecules is a single-exponential $r(t) = r_0 e^{-t/\tau_R}$ [4], and thus according to Eq. (6-1), the corresponding SFA is $\langle r \rangle = r_0 / (1 + \tau_F/\tau_R)$. Here τ_F , r_0 and τ_R are fluorescence lifetime, fundamental anisotropy and rotational correlation time (RCt). Therefore, for a spherical fluorescent molecule, FA is only determined by one RCt. However, nonspherical macromolecules have multiple RCts that generally are difficult to resolve from FA data. For example, there are five possible RCts for an elliptic macromolecule [226, 227]. If a fluorophore label also experiences local depolarizing motions independent on the rotational motions taken by a macromolecule, the FA data analysis is compounded further because even more RCts must be resolved and interpreted [228-231]. As a result, FA can be dependent on the position of the fluorophore label on the macromolecule due to heterogeneous environment and complex depolarizing motions in many biomolecular system [231].

SFA technique has been used to investigate binding of XPC/HR23B complexes to damaged DNA [40]. However, upon XPC/HR23B binding to DNA, the structure of

the binding complex are unknown, and thus it remains a question whether the SFA data [40] were influenced by potential complex rotational motions of the fluorescent labels. Alternatively, FCS can also be used to study the protein-DNA binding by determining the translational diffusion time (TDt, τ_D) of fluorescent macromolecules. Compared with RCt in FA, TDt in FCS is generally insensitive to the position of the fluorophore label on the macromolecule. Therefore, we combined FCS and SFA techniques to study the binding properties of NER proteins, human XPC/HR23B and yeast Rad4/Rad23, to various DNA substrates. In the work, on-line quantitative FCS and SFA analyses are performed for the same fluorescent intensity time traces measured in sample solutions from titrations of NER proteins binding to four fluorescently labeled DNA substrates. Both combined FCS and SFA techniques can provide complementary information for overall molecular mobility and thus exact knowledge of binding interactions.

6.2 MATERIALS AND METHODS

6.2.1 Chemical materials

Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TFOS, product No. 448931), Rhodamine 6G (R6G, product No. R-4127, molecular mass: 479 g/mole) and Triton X-100 (TX100, product No. T8787) were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 dye (Alexa488, molecular mass: 643 Da, product No. A20000) was purchased from Invitrogen (Carlsbad, CA). Glutaraldehyde (product No. 16220) was purchased from Electron Microscopy Sciences (Hatfield, PA). The Milli-Q water was distilled and deionized from a Milli-Q water purification system (Millipore, Bedford, MA) and filtered through a 0.2 μm filter. Solutions and buffers were prepared in High-performance liquid chromatography (HPLC) grade water which is purchased from Merck (Whitehouse Station, NJ) for all buffer preparations and dilution steps. Except as otherwise noted, other used chemicals were purchased from Merck.

6.2.2 Preparation of proteins

6.2.2.1 XPC/HR23B

The human XPC protein has a molecular mass of 125 kDa and exists *in vivo* associated with the 58 kDa HR23B protein. The basal isoelectric points for both the human XPC protein and the human HR23B protein are 9.54 and 4.79, respectively [232]. XPC/HR23B protein complex was produced as recombinant FLAG-XPC/Rad23B-His and kindly provided by K. Sugawara, RIEN, Institute of Physical and Chemical Research, Saitama 351-0198, Japan. Briefly, Recombinant human HR23B protein, the carboxy-terminal of which was fused with hexa-histidine tag (HR23B-His), was overexpressed in the *Escherichia coli* strain BL21 (DE3) and purified as described [233]. Human XPC protein was expressed in the High Five cell

line by infection with a recombinant virus vFB1.FlagXPC. The cell extract was sequentially purified on Heparin-Sepharose, anti-FLAG M2 agarose and HisTrap as described in Ref. [234] with some modifications. To avoid XPC protein aggregations in vitro, purified HR23B-His proteins were added to cell extract before purifications.

6.2.2.2 *Rad4/Rad23*

The yeast Rad4 protein is also basic, with an isoelectric point of 7.93 [232]. The truncated Rad4/Rad23 heterodimer used in this work has a molecular mass of 97 kDa [27] and was a kind gift of Jun-Hyun Min and Nikola P. Pavletich, Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York 10021, USA. The Rad4 protein is His-tagged and contains from amino acid (aa) 101 to aa 632 and is truncated to remove 100 amino-terminal and 122 carboxy-terminal residues, which are highly susceptible to limited proteolysis and are presumably unstructured or loosely folded. The Rad23 (aa1-398) has a deletion between aa 135 and aa 229 which is replaced with a thrombin cleavage site. The protein expression and purification follows the same protocol published in Ref. [27]. Briefly, the Rad4/Rad23 complex was co-expressed in the Hi5 insect cells, and purified sequentially using His-Select Nickel agarose resin (Sigma-Aldrich), anion exchange chromatography (Source Q, Amersham, Piscataway, NJ), cation exchange (Source Q, Amersham) and gel-filtration (Superdex200, Amersham) chromatography. The complex was concentrated by ultrafiltration in 5mM bis-trispropane-HCl (BTP-HCl) (pH = 6.8), 800mM NaCl, 10mM dithiothreitol (DTT).

6.2.2.3 *XPC/Alexa488-HR23B*

XPC/Alexa488-HR23B protein complex was kindly provided by Dr. W. Vermeulen and Dr. H. Lans, Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, Netherlands. Human XPC protein was fused to a FLAG-epitope at the C-terminus and expressed in the insect cell line Sf9. Recombinant XPC-FLAG protein was purified using anti-FLAG M2 affinity gel (Sigma-Aldrich). HR23B protein was fused to the (His)6-tag and produced by overexpression in E. coli strain BL21(DE3). Recombinant HR23B-His was purified using Ni-NTA chromatography (Qiagen, Valencia, CA). HR23B protein contains one C-terminal cysteine residue which was used to covalently attach Alexa Fluor 488 C5 maleimide (Invitrogen) according to the manufacturer's protocol. To reconstitute the XPC/HR23B complex, HR23B-His-Alexa488 was added in excess to the XPC insect cell lysate, after which the proteins in complex were purified using anti-FLAG M2 affinity gel (Sigma-Aldrich).

6.2.3 Preparation of DNA substrates

Four DNA substrates were used in DPB assay in this study as shown in Fig. 6-1. Alexa488 dye labeled 55 bps double-stranded DNA (Alexa488-55-bp-dsDNA) was made by annealing equimolar amounts of the commercial 5' Alexa488 dye labeled

Fig. 6-1 DNA substrates used for combined FCS and SFA measurements. (a) Alexa488-55-mer-ssDNA: Alexa488 dye labeled 55-mer oligonucleotide; (b) Alexa488-55-bp-dsDNA: Alexa488 dye labeled 55 bps double-stranded DNA substrate; (c) Alexa488-B5-DNA: Alexa488 dye labeled 55-bp dsDNA containing a 5 unpaired base pairs bubble; (d) Alexa488-AAF-DNA: Alexa488 dye labeled double-stranded 44 bps DNA substrate containing a single AAF adduct. F represents the fluorescent labeling – Alexa488 dye attached to a 5'-end of DNA.

55-mer single-stranded oligonucleotide (Alexa488-55-mer-ssDNA) to its complementary strand. For the preparation of Alexa488 dye labeled 55-bp double-stranded DNA containing a 5 unpaired base pairs bubble (Alexa488-B5-DNA) in Fig. 6-1, equimolar amount of Alexa488-55-mer-ssDNA in Fig. 6-1 and its complementary strand with five unpaired bases were hybridized.

Aromatic amino and nitro compounds are potent carcinogens and are found that can exert toxic effects by reacting with DNA following metabolic activation. N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (AAF) is one the important adducts which has been widely used in the studies of the mechanisms of DNA repair and mutagenesis [235]. AAF modified 44-mer oligonucleotide was a kind gift of Jung-Eun Yeo and Orlando D. Schärer, Stony Brook University, NY, USA, and it was prepared as described in Ref. [235] and used for the preparation of Alexa488 dye labeled double-stranded 44 bps DNA substrate containing an AAF adduct (Alexa488-

Fig. 6-2 Electrophoresis of DNA substrates. Samples were run on two 10% PAA gel in TBE buffer (90 mM Tris-Borate, 2 mM EDTA, pH 8.0). Gels were illuminated with blue light 488 ± 25 in combination with emission detection at 535 ± 25 nm to visualize the Alexa488 dye dye label.

Panel (a): Lane 1, Alexa488-44-mer-ssDNA; Lane 2, Alexa488-AAF-DNA; Lane 3, Alexa488-55-mer-ssDNA;

Panel (b): Lane 1, Alexa488-B5-DNA; Lane 2, Alexa488-55-bp-dsDNA; Lane 3, Alexa488-55-mer-ssDNA.

AAF-DNA in Fig. 6-1) by synthesizing with equimolar amount of its complementary strand.

Except for AAF modified 44-mer oligonucleotide, all oligonucleotide products were purchased from DNA Technology A/S (Risskov, Denmark). All commercial oligonucleotide products were requested reverse phase high performance liquid chromatography (RP-HPLC) purification. The quality of all resulting DNA products was checked by 10% polyacrylamide (PAA) gel electrophoresis as shown in Fig. 6-2, and the results were in good agreement with our expectation.

Plasmid DNA pUC19 (2.7 kb) was purified using the Plasmid Maxi Kit from Qiagen. UV-damaged plasmid DNA was obtained by irradiation with germicidal lamps at 254 nm and a fluence rate of $10 \text{ Js}^{-1}\text{m}^{-2}$ for 5 min.

6.2.4 XPC/HR23B and DNA binding reactions for gel mobility shift assay

For the gel mobility shift assay in Fig. 6-3, DPB reaction followed the same method as used by K. Sugawara [218]. Three 10 μl binding reaction solutions each consists of 0.7 μM XPC/HR23B complex and 0.33 μM DNA substrate (Alexa488-55-mer-ssDNA, Alexa488-55-bp-dsDNA and Alexa488-B5-DNA, respectively) in the binding reaction buffer (40 mM Hepes, 140 mM KCl, 10 mM MgCl_2 , 1 mM DTT, 5% glycerol, 0.01% Triton X-100 and 1 mg/ml BSA). The binding reactions were first performed at 30 °C for half an hour and then the reactions were chilled on ice. 2.5% glutaraldehyde was added to the reaction solutions to fix the resulting DNA-protein complexes immediately before the gel mobility shift assay.

6.2.5 Protein titration binding reaction

Protein titration binding reaction experiments for the combined FCS and SFA analyses in this work were performed in the binding reaction buffer (10 mM Tris-HCl, pH = 7.4, 300 mM NaCl, 0.02% TX100, 2% glycerol). 30 μl binding reaction solutions each consists of 4 nM DNA substrate, 1 $\times$ binding reaction buffer, and a certain amount of XPC/HR23B complex, Rad4/Rad32 complex or XPC/Alexa488-HR23B. The binding reactions were performed at 30°C with stirring for an hour to achieve equilibrium condition.

6.2.6 Competition binding reaction for XPC/HR23B complex

A benefit of competition experiment is the ability to determine binding affinities without the requirement of labeling the ligand under study. DNA-protein competition binding reactions were performed in the same binding reaction buffer as used in binding reaction experiments (section 6.2.5) for the combined FCS and SFA analyses. 30 μl binding reaction solutions each consists of 4 nM DNA probes, 64 nM XPC/HR23B complex, 1 $\times$ binding reaction buffer, and a certain amount of DNA competitor. The binding reactions were performed at 30°C with stirring for an hour.

6.2.7 Single-molecule fluorescence confocal microscope

We have constructed a single-molecule fluorescence confocal microscope (SFCM) for the combined FCS and SFA analyses in confocal volume of the sample solution. The main configuration of this SFCM has been described elsewhere [67, 68], and a recent development of on-line quantitative FCS analysis has been introduced in Ref. [69]. Here we describe a further development of SFA on the SFCM. The SFCM consisted of an Ar⁺/Kr⁺ mixed gas laser (Coherent, Innova 70 spectrum, Santa Clara, CA) and an inverted optical microscope placed on a vibration isolation optical table. A water immersion objective Zeiss C-Apochromat 63×, NA=1.20, working distance = 0.24 mm) was used. Linearly polarized laser lines 488nm was used to excite Alexa488 dye in this work. After a prism-based laser line selection and separation [67], excitation light passes a beam expander, a Glan-Thompson polarizer, a low-reflection/high-transmission (LRHT, Reflection 5%, Transmission 95%) beam splitter and finally enter the objective. A diaphragm with a diameter of ~3 mm was used in between the beam expander and the Glan-Thompson polarizer to confine the excitation beam, which offers underfilling-objective excitation profile to avoid depolarization effect [158, 159] of the high NA objective in SFA measurement. The same objective is used to collect emitted fluorescent light and reflected light, >95% of which passes through the beam splitter HRLTBS, one or more notch filters to remove the reflected excitation light, a biconvex lens and a 15 μm diameter pinhole towards two avalanche photodiodes (APDs), in between which a polarizing beam splitter (Model: 05FC16PB.3, Newport, Irvine, CA) is used to split fluorescent light into its polarized components.

The on-line quantitative FCS analysis [69] allows us directly monitoring the molecule diffusion in aqueous solution with an enough number of measurements from one droplet of sample solution for good statistics to avoid experimental artifact since FCS technology suffers from high sensitivity to microscope configuration [119, 123] and the size and shape of the molecules in sample solution. The outputs from both APDs are used to determine ACF in FCS by

$$g(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} - 1 \quad (6-2)$$

and SFA by

$$r = \frac{(I_p - I_{p,bk}) - G(I_s - I_{s,bk})}{(I_p - I_{p,bk}) + 2G(I_s - I_{s,bk})} \quad (6-3)$$

where $I = I_p + I_s$; the terms I_p and I_s are the fluorescent intensity parallel and perpendicular to the excitation polarization as selected using the Glan-Thompson polarizer; G accounts for different transmission and detection efficiencies of the imaging system at parallel and perpendicular polarization [157], and $I_{p,bk}$ and $I_{s,bk}$ are average background intensity from the APDs, respectively, and separately measured in the binding buffer solution without fluorescent sample. The G -factor may be

obtained by excitation of sample with first vertical and then horizontal polarization [157].

6.2.8 Data analysis

6.2.8.1 One-site binding model

For DPB reaction, a simple one-site binding model [236]

$$D_f + P_f \rightleftharpoons B, \text{ with } \theta = \frac{[B]}{[D_T]} = \frac{[P_T] - [B]}{K + [P_T] - [B]} \quad (6-4)$$

was used in this work, where D_f , P_f and B represent free DNA, free protein and protein-DNA binding complex, respectively, and $[B]$, $[D_T]$ and $[P_T]$ are the total concentrations of DPB complex, fluorescently labeled DNA and protein, respectively. θ and K are binding degree and dissociation constant, respectively. The DPB complex concentration $[B]$ can be evaluated by

$$[B] = \frac{1}{2} \left([D_T] + [P_T] + K - \sqrt{([D_T] + [P_T] + K)^2 - 4[D_T][P_T]} \right). \quad (6-5)$$

6.2.8.2 Competition binding model

Competition titration experiments are useful in this work for two reasons: one is to disclose specificity of DNA repair proteins for different labeled DNA substrates and the other is to determine the binding affinity of unlabeled DNA competitor. Historically, a model [237] of the competitor (or inhibitor) for one-site ligand-receptor binding has been derived to be

$$\frac{[B]}{[D_T]} = \frac{[P_T] - [B]}{K(1 + [C_T]/K_C) + ([P_T] - [B])} \quad (6-6)$$

where $[B]$, $[C_T]$, $[D_T]$ and $[P_T]$ represent the total concentrations of DPB complex, competitor, fluorescently labeled DNA and protein, respectively. K and K_C are dissociation constants for the fluorescently labeled DNA probe and the unlabeled competitor, respectively. The DPB complex concentration $[B]$ can be determined by

$$[B] = \frac{1}{2} \left(\Xi - \left(\Xi^2 - 4[D_T][P_T] \right)^{\frac{1}{2}} \right). \quad (6-7)$$

where $\Xi = K[C_T]/K_C + [D_T] + [P_T] + K$. As indicated in Eq. (6-6), the dissociation constant of unlabeled DNA competitor can be determined if the dissociation constant K for labeled DNA substrates is already known. In this work, K is determined according to Eq. (6-4) by protein titration experiment.

6.2.8.3 Binding model for FCS

The measured ACF from Eq. (6-2) is fit to the freely diffusing model [198]:

$$g(\tau) = \frac{1}{N} \cdot \frac{1}{1 + \frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1 + \left(\frac{r_0}{z_0}\right)^2 \frac{\tau}{\tau_D}}} \quad (6-8)$$

where the parameters N , τ_D , r_0 and z_0 represent the average number of molecules in confocal observation volume, molecular TDT, lateral and axial widths of the focal volume, respectively. The parameters r_0 and z_0 of confocal volume size was determined by a separate calibration FCS experiment was performed on 10 nM Rhodamine 6G in Milli-Q water. If the sample solution has two classes of components, for example, typically free and bound fluorescently labeled molecules in binding reaction solution, the measured TDT τ_D is actually the mixture of the TDTs of both components. Here let us consider the relationship between the TDT that a molecule stays in the focal volume and the translational diffusion coefficient D , which is independent on the particular used setup,

$$D = \langle r_0 \rangle^2 / 4\tau_D. \quad (6-9)$$

where r_0 is lateral radius of the focal volume and highly depends on the setup used. For two-component system, if the diffusion constants of both components are D_1 and D_2 , respectively, the measured diffusion constant from one-component freely diffusing model with Eq. (6-8) should be the average of D_1 and D_2 weighted by the population of the components, which is

$$D = (N_1 D_1 + N_2 D_2) / (N_1 + N_2) \quad (6-10)$$

where N_1 and N_2 represent the average molecule numbers (or concentrations) of both components in focal volume, respectively. Since both components share an identical confocal volume in one experiment, Using Eq. (6-9) and Eq. (6-10) can be rewritten as

$$\tau_D = \tau_{fD} \tau_B ([D_T] + [B]) / (\tau_{fD} [B] + \tau_B ([D_T] - [B])) \quad (6-11)$$

where τ_{fD} and τ_B are the TDTs for free fluorescently labeled DNA probes and reaction product, *i.e.*, DPB complex, in focal volume. In Eq. (6-11), the DPB complex concentration $[B]$ is obtained from Eq. (6-5) for protein titration curve fitting or from Eq. (6-7) for competition titration curve fitting, respectively. For a FCS analysis, protein titration curve fitting yields three parameters: dissociation constant K of DNA repair proteins to a given DNA substrate, and TDTs τ_{fD} and τ_B for the free and bound DNA probes, respectively, while competition titration curve fitting gives dissociation constant K_c of DNA repair protein to a given competitor as well as both τ_{fD} and τ_B as for protein titration curve fitting. In competition titration experiments, the resulting τ_{fD} higher than that experimentally determined for the free DNA probe will indicate the presence of non-specific binding (as in chemistry) of the protein to the DNA probe.

6.2.8.4 Binding model for SFA

Fluorescent molecules involved in binding assays will exist in only one of two states: bound or free. In general, the bound and free states of the fluorescent molecule will each have a unique SFA value – high for the bound state and low for the free state. The data analysis for SFA was used the same methods as proposed by Hey, *et al.* [40, 238]. The average values of SFA against the protein concentration were fitted to the simple one-site binding model (Eq. (6-4)) by using the SFA additivity equation:

$$r = \frac{[B]}{[D_T]} r_B + \frac{([D_T] - [B])}{[D_T]} r_{fD} \quad (6-12)$$

where r_B and r_{fD} are the SFAs of DPB complex and free DNA, respectively. The DPB complex concentration $[B]$ in Eq. (6-12) is taken from Eq. (6-4) for protein titration curve fitting or from Eq. (6-7) for competition titration curve fitting, respectively. For SFA analyses, three parameters can be obtained from protein titration curve fitting - dissociation constant K of DNA repair proteins to a given DNA substrate, and SFAs r_{fD} and r_B for the free and bound DNA probes, respectively, while competition titration curve fitting gives dissociation constant K_c of DNA repair protein to a given competitor as well as both r_{fD} and r_B as for protein titration curve fitting. The same as for FCS, the resulting r_{fD} in competition titration experiments higher than that experimentally determined for the free DNA probe indicates the presence of non-specific binding of the protein to the DNA probe.

6.2.8.5 Non-linear least squares fitting for titration curves

The protein titration and competition binding curves were non-linear least squares fitted with custom programs in Matlab (The Mathworks, Natick, MA). The experimental data points and error bars on each curve shown in the figures were mean and standard deviation that obtained from 25 10-second intensity time trace measurements. As shown in the figures (Fig. 6-4 – Fig. 6-8), titration curves for both TDt in FCS and SFA can be well-fitted to the one-site binding model (Eq. (6-4)) or the competition binding model (Eq. (6-6)) in combination with Eq. (6-11) for FCS analyses or Eq. (6-12) for SFA. However, if a concave-up curve with an x -asymptote observed in competition binding curve the presence of non-specific binding may be elucidated and consequently the fitting results may be affected. In chemistry, non-specific binding is widely defined as binding that is not displaceable by an excess of unlabeled ligand and often results from the presence of multiple classes of binding sites [239], so it can show specificity of the binding sites on different DNA substrates for the DNA repair protein. The non-specific binding in competition titration experiment can result in inaccurate estimation of the binding affinity for the DNA competitor and increase in the fitted values of TDt τ_{fD} and SFA r_{fD} of free fluorescent DNA probe. However, due to the lack of knowledge of the non-specific bindings for all DNA substrates and DNA repair proteins, it is still difficult to have a proper model to quantitatively describe the specificity of a given DNA substrate.

6.2.9 Combined FCS and SFA analyses

FCS and SFA analyses in the SFCM follow the same procedure as described in Ref. [69]. Briefly, 8 μ l sample solution was applied to a TFOS-treated hydrophobic coverslip to avoid the molecular adsorption to the glass surface. 25 10-second fluorescence intensity traces are sequentially measured for FCS analysis to determine the TDt and simultaneously SFA analysis for the fluorescent molecular probes in the sample solution with good statistics. There was no significant change in the fluorescence intensity observed in all binding reaction solutions for titration experiments. The average of SFA is normally able to get stable much faster than that of TDt in the focal volume measured by FCS. The average SFA and TDt of the Alexa488 dye were not altered upon the addition of the maximum amount of NER proteins in this study.

6.3 RESULTS

6.3.1 Validation of SFA measurements

For SFA, G-factor in Eq. (6-3) was estimated to be 1.02, using reference fluorophores, by comparative measurements of SFA carried out in a commercial spectrofluorometer Fluoromax-4 (HORIBA Jobin Yvon Inc, Edison, NJ) with polarization capabilities and on our SFCM. In these calibration experiments, 10 nM free fluorescent molecules including enhanced green fluorescent protein (eGFP), R6G and Alexa488 dye in aqueous solution were excited by 488 nm laser light. We obtained the SFAs of 0.023 ± 0.001 and 0.028 ± 0.001 (Table 6-4) for Alexa488 using the spectrofluorometer and the SFCM, respectively, and 0.32 ± 0.001 and 0.31 ± 0.01 for eGFP, which is in agreement with those values reported in Ref. [163, 240]. As a result of these experiments, depolarization effect [158, 159] due to the high NA objective is negligible by using an underfilling-objective excitation beam in the SFCM.

6.3.2 Gel mobility shift assay of XPC/HR23B and DNA binding reactions

Similar to the observation for the binding of Rad4/Rad23 complex [27], XPC/HR23B complex has been found substantially binding affinity for undamaged DNA substrate [39, 40, 218, 241]. To illustrate the binding of XPC/HR23B complex to damaged and undamaged double-stranded DNA and single-stranded DNA, three 0.33 μ M DNA substrates, Alexa488-55-mer-ssDNA, Alexa488-55-bp-dsDNA, and Alexa488-B5-

Fig. 6-3 mobility shift result of XPC/HR23B binding to three DNA substrates: Alexa488-55-mer-ssDNA (Lane 1), Alexa488-55-bp-dsDNA (Lane 2) and Alexa488-B5-DNA (Lane 3). The binding reactions were run on a 6% PAA gel in a 4 °C cooled room. See the text for details.

DNA (shown in Fig. 6-3), were performed binding reaction experiments with 0.7 μ M XPC/HR23B complex as described previously (section 6.2.4). Gel mobility shift assay was carried out on a 6% nondenaturing PAA gel in a 4 °C cooled room. The Gel mobility shift results shown in Fig. 6-3 indicate that XPC/HR23B complex can bind to all three DNA substrates and the specificity of XPC/HR23B binding to these DNA substrates is not apparently distinct.

6.3.3 Binding of XPC/HR23B to DNA substrates

XPC/HR23B complex has been shown to preferentially bind DNA substrates containing various types of damages [40, 218]. To characterize the properties of the binding of the XPC/HR23B complex to different fluorescently labeled DNA substrates, we performed protein titrations by using the combined FCS and SFA. The bindings of the fluorescent DNA substrates by the XPC/HR23B complex were studied by monitoring the increases of the TDt in FCS and the SFA that results from the formation of DNA-protein complexes. Fig. 6-4 shows typical protein titration curves that were obtained for the XPC/HR23B complex to four fluorescently labeled DNA substrates, Alexa488-55-bp-dsDNA (Fig. 6-4(a) and Fig. 6-4(e)), Alexa-B5-DNA (Fig. 6-4(b) and Fig. 6-4(f)), Alexa488-AAF-DNA (Fig. 6-4(c) and Fig. 6-4(g)) and Alexa488-55-mer-ssDNA (Fig. 6-4(d) and Fig. 6-4(h)) as shown in Fig. 6-4. These protein titration curves were separately fit to one site binding model of Eq. (6-4) in combination with Eq. (6-11) for TDt (Fig. 6-4(a) – Fig. 6-4(d)) in FCS or Eq. (6-12) for SFA (Fig. 6-4(e) – Fig. 6-4(h)), respectively. The binding affinity (inverse of dissociation constant) and other fitting parameters of interest for each DNA substrate in Fig. 6-1 are given in Table 6-1. As shown in Table 6-1, the binding affinities of the XPC/HR23B complex determined by SFA for the double-stranded DNA with and without 5 unpaired base pairs bubble and single-stranded oligonucleotides are in good agreement with those measured with a spectrofluorometer by T. Hey, *et al.* [40]. This provides another convincing evidence for the validation of our SFA measurement based on the SFCM.

Table 6-1 Binding affinity and one-site binding model fitting parameters of the XPC/HR23B complex to four DNA substrates in protein titration experiments.

DNA substrate	FCS			SFA		
	K (nM)	τ_B (μ s)	τ_{fD} (μ s)	K (nM)	τ_B	τ_{fD}
Alexa488-55-bp-dsDNA	2.2	1012	403	8.6	0.23	0.07
Alexa488-B5-DNA	0.8	942	428	3.7	0.23	0.07
Alexa488-AAF-DNA	1.8	602	265	5.3	0.20	0.05
Alexa488-55-mer-ssDNA	6.1	896	282	10.6	0.22	0.05

As shown in Fig. 6-4, upon binding of XPC/HR23B complex the TDts of fluorescently labeled DNA probes increase from 419 ± 20 μ s to 942 ± 59 μ s for Alexa488-B5-DNA and from 417 ± 13 μ s to 957 ± 68 μ s for the Alexa488-55-bp-dsDNA, respectively, and the SFA from 0.073 ± 0.001 to 0.216 ± 0.003 for the

Fig. 6-4 Combined FCS (a-d) and SFA (e-h) analyses of XPC/HR23B binding to Alexa488-55-bp-dsDNA ((a) and (e)), Alexa488-B5-DNA ((b) and (f)), Alexa488-AAF-DNA ((c) and (g)) and Alexa488-55-mer-ssDNA ((d) and (h)). 4 nM DNA was titrated with the XPC/HR23B complex in the binding reaction buffer. The protein titration curves for the experimental TDT τ_D and SFA r were separately fit to one-site binding model (lines) of Eq. (6-4) in combination with Eq. (6-11) for FCS for Eq. (6-12) for SFA, respectively.

Alexa488-B5-DNA and from 0.075 ± 0.001 to 0.211 ± 0.002 for Alexa488-55-bp-dsDNA, respectively. Titration binding curve fitting analyses in Fig. 6-4(a) and Fig. 6-4(e) yielded the dissociation constants of 2.2 nM by FCS and 8.6 nM by SFA for

the Alexa488-55-bp-dsDNA (Table 6-1), while the same analyses for both curves in Fig. 6-4(b) and Fig. 6-4(f) give the dissociation constants of 0.8 nM by FCS and 3.7 nM by SFA for the Alexa488-B5-DNA (Table 6-1). Although the determined dissociation constants for both Alexa488-55-bp-dsDNA and Alexa488-B5-DNA by SFA are very close to those for 36-bp double-stranded DNAs with and without six-nucleotide bubble, the measured TDts in Fig. 6-4(a) and Fig. 6-4(b) and SFAs in Fig. 6-4(e) and Fig. 6-4(f) for both DNA probes are nearly the same. This shows that XPC/HR23B complex has substantial binding affinity to both DNA substrates, which is consistent with the result of gel mobility shift assay in Fig. 6-3.

In the titration of XPC/HR23B complex binding to the damaged 44-bp double-stranded DNA substrates, Alexa488-AAF-DNA, containing a single AAF adduct, the initial TDt and SFA were measured to be $276 \pm 14 \mu\text{s}$ and 0.056 ± 0.001 , respectively, and both values increased with the protein addition and reached to $583 \pm 40 \mu\text{s}$ and 0.180 ± 0.002 , respectively at protein concentration of 64 nM in binding reaction solution. Fig. 6-4(c) and Fig. 6-4(g) show the protein titration experimental results for the Alexa488-AAF-DNA, and curve fitting analyses turned out the dissociation constants of 1.8 nM by FCS and 5.3 nM by SFA for the Alexa488-55-bp-dsDNA (Table 6-1).

Another DNA substrate used in the protein titration experiment with the XPC/HR23B complex is Alexa488-55-mer-ssDNA. The same observation as the results from DNA substrates electrophoresis in Fig. 6-2 was found by FCS that the mobility of Alexa488-55-mer-ssDNA (molecular mass: ~18 kDa) is even lower than that of Alexa488-AAF-DNA (molecular mass: ~29 kDa). The initial TDt of free Alexa488-55-mer-ssDNA is $304 \pm 9 \mu\text{s}$. This suggest the dynamic behavior of individual DNA molecules is not only governed by hydrodynamic interactions according to Stokes-Einstein relation, but the molecular conformation (or chain connectivity for DNA molecules in this case) is also another important factor [111, 112]. In contrast, the initial SFA of Alexa488-55-mer-ssDNA is 0.048 ± 0.002 , so for all four free DNA substrates, the SFA does not completely agree with the FCS analysis. A detailed comparison of both technologies for molecular dynamics is in the section Discussion of this chapter. With the addition of the XPC/HR23B complex in the binding reaction solution, the measured TDt and SFA reached to $801 \pm 72 \mu\text{s}$ and 0.203 ± 0.003 , respectively, for the Alexa488-55-mer-ssDNA at protein concentration of 64 nM. Fig. 6-4(d) and Fig. 6-4(h) show the protein titration experimental results for the Alexa488-55-mer-ssDNA, and curve fitting analyses gave the dissociation constants of 6.1 nM by FCS and 10.6 nM by SFA for the Alexa488-55-bp-dsDNA (Table 6-1). The XPC/HR23B complex was shown high affinity for single-stranded DNA which is consistent with the observations by gel mobility shift assay [41, 241].

An important issue of interest in all studies on the function of the DNA repair proteins has been their specificity for damaged and undamaged DNA. The determined

dissociation constants in Table 6-1 differ slightly for all four DNA substrates from the protein titration experiments in Fig. 6-4, but these protein titration curves were found too close to distinguish with each other if they are normalized, and thus they do not exhibit apparent specificity for all four DNA substrates interacted with the XPC/HR23B complex. To address the damage recognition ability of XPC/HR23B complex, competition titration experiments were employed to clarify the specificity of XPC/HR23B complex to damaged DNA substrates.

6.3.4 Competition titration for XPC/HR23B binding specificity

Competition titrations have been used in either gel mobility shift assay [218] or SFA [40] for the study of the specificity of XPC/HR23B complex for damaged and undamaged DNA substrates, and it has been extensively used in chemistry to compare the binding affinity difference between ligand probe and competitor. If XPC/HR23B preferentially binds to a given DNA probes, which may produce non-specific binding and competition binding curve (e.g., Fig. 6-5 – Fig. 6-7) become concave-up with an x -asymptote when using an excess of unlabeled competitor. Besides the study of XPC/HR23B complex binding specificity, competition titration experiments are able to determine the binding affinity of unlabeled DNA competitor by using the competition binding model of Eq. (6-6). In these experiments, Alexa488-55-bp-dsDNA, Alexa488-B5-DNA and Alexa488-AAF-DNA were fluorescent labeled probes and challenged with either unlabeled 55-mer oligonucleotide, which was the complementary stand of the Alexa488-55-mer-ssDNA in Fig. 6-1, or UV-irradiated plasmid DNA. As shown in the Fig. 6-5 to Fig. 6-7, one binding reaction solution for each DNA substrate has no competitor in the preparation of all competition titration experiments, and it was used as standard marker for internal calibration in both FCS and SFA analyses by comparing with the results in the protein titration experiment mentioned above. Nearly the same experimental fluorescence intensity, TDt and SFA as those obtained from the protein titration experiments for these standard markers ensured good comparability of the experimental parameters for a given DNA substrate from both protein titration experiments and competition titration experiment. The competition titration curves in Fig. 6-5 to Fig. 6-7 were well-fit to the competition binding model of Eq. (6-6) in combination with Eq. (6-11) for FCS analyses or Eq. (6-12) for SFA, and the fitting results are given in Table 6-2. As mentioned previously

Table 6-2 Binding affinity of unlabeled DNA competitors in the competition titrations of the XPC/HR23B complex to various DNA substrates

Competitor	DNA substrate	FCS			SFA		
		K_c (nM)	τ_B (μ s)	τ_{fD} (μ s)	K_c (nM)	τ_B	τ_{fD}
Unlabeled 55-mer oligonucleotide	Alexa488-55-bp-dsDNA	7.6	1030	449	10.6	0.25	0.08
	Alexa488-B5-DNA	2.6	921	537	9.0	0.23	0.10
	Alexa488-AAF-DNA	7.2	552	286	12.1	0.20	0.06
UV-irradiated plasmid	Alexa488-55-bp-dsDNA	0.5	1004	399	0.9	0.24	0.07
	Alexa488-B5-DNA	0.3	937	415	0.6	0.22	0.08

(see sections 6.2.8.3 and 6.2.8.4), except the presence of non-specific binding, the binding affinity of the competitor DNA substrate can be accurately determined by the competition titration curve fitting.

Fig. 6-5 show the competition titration curves for the binding of 64 nM XPC/HR24B complex to two 4 nM fluorescent labeled DNA substrates, Alexa488-55-bp-dsDNA and Alexa488-B5-DNA by addition of the unlabeled single-stranded 55-mer oligonucleotide. In Fig. 6-5, until the oligonucleotide competitor was used in a very high concentration of 0.96 μ M, the measured TDts (Fig. 6-5(a)) of 475 ± 24 μ s for the Alexa488-55-bp-dsDNA and 557 ± 33 μ s for the Alexa488-B5-DNA as well as the experimental SFA (Fig. 6-5(b)) of 0.087 ± 0.002 for the Alexa488-55-bp-dsDNA and 0.106 ± 0.003 μ s for the Alexa488-B5-DNA showed that both DNA probes were still not completely free in the competition binding reaction. Curve fitting analyses (Table 6-2) on both Fig. 6-5(a) and Fig. 6-5(b) yielded $\tau_{fD} = 449$ μ s, $\tau_B = 1030$ μ s, and $K_c = 7.6$ nM by FCS and $r_{fD} = 0.08$, $r_B = 0.25$, and $K_c = 10.6$ nM by SFA for the Alexa488-55-bp-dsDNA, while those parameters for the Alexa488-B5-DNA are $\tau_{fD} = 537$ μ s, $\tau_B = 921$ μ s, and $K_c = 2.6$ nM by FCS and $r_{fD} = 0.10$, $r_B = 0.23$, and $K_c = 9.0$ nM by SFA. For the Alexa488-B5-DNA both r_{fD} and τ_{fD} for the free DNA probe from the curve fitting analyses are much higher than those ($\tau_{fD} = 419$ μ s and $r_{fD} = 0.073$) experimentally determined from the protein titration experiments, which indicates a strong non-specific binding affinity of the XPC/HR23B complex to the Alexa488-B5-DNA compared to the competitor single-stranded oligonucleotide. In contrast, the XPC/HR23B complex shows little non-

Fig. 6-5 Competition of the binding of 64 nM XPC/HR23B complex to two 4 nM fluorescently labeled DNA substrates, Alexa488-55-bp-dsDNA (triangles) and Alexa488-B5-DNA (circles), by addition of the unlabeled single-stranded 55-mer oligonucleotide competitor. The experimental TDt τ_D (a) and SFA r (b) were fit to the competition binding model (lines) in combination with Eq. (6-11) for FCS or Eq. (6-12) for SFA, Eq. (6-6), respectively. Note that the x-axis is in logarithm scale.

Fig. 6-6 Competition of the binding of 64 nM XPC/HR23B complex to two 4 nM DNA substrate Alexa488-AAF-DNA by addition of the unlabeled single-stranded 55-mer oligonucleotide competitor. The experimental TDT τ_D (a) and SFA r (b) were fit to the competition binding model (lines) of Eq. (6-6) in combination with Eq. (6-11) for FCS or Eq. (6-12) for SFA, respectively.

specific binding to the Alexa488-55-bp-dsDNA in Fig. 6-5, and both r_{fD} and τ_{fD} for the free DNA probe from the curve fitting analyses are very close to those ($\tau_{fD} = 417 \mu s$ and $r_{fD} = 0.07$) experimentally determined from the protein titration experiments. Besides the derivation of the specificity of the XPC/HR23B complex to both DNA probes, due to the lack of non-specific binding, competition titrations for the Alexa488-55-bp-dsDNA probe yielded very accurate dissociation constants $K_c = 7.6 \text{ nM}$ by FCS and $K_c = 10.6 \text{ nM}$ by SFA for the oligonucleotide competitor in the curve fitting analyses. Both resulting dissociation constants for the oligonucleotide are nearly the same as those obtained for the Alexa488-55-mer-ssDNA from the protein titration experiments, so the Alexa488-55-bp-dsDNA can be used as a good fluorescently labeled probe to study the binding affinity of unlabeled DNA substrates by competition titration.

Another DNA substrate used to competition titration experiment challenging with the unlabeled 55-mer oligonucleotide is the Alexa488-AAF-DNA, containing a single AAF adduct. Fig. 6-6 shows the competition titration curves for both FCS (Fig. 6-6(a)) and SFA (Fig. 6-6(b)). In this competition titration experiment, the XPC/HR23B complex concentration is 64 nM. As in Fig. 6-6, when the oligonucleotide competitor concentration in the binding reaction was high to $0.96 \mu M$, the measured TDT of $309 \pm 16 \mu s$ and SFA of 0.082 ± 0.002 show that part of the Alexa488-AAF-DNA probes was still bound by the XPC/HR23B complex. However, curve fittings to competition binding model yielded $\tau_{fD} = 286 \mu s$, $\tau_B = 552 \mu s$, and $K_c = 7.2 \text{ nM}$ by FCS in Fig. 6-6(a) and $r_{fD} = 0.06$, $r_B = 0.20$, and $K_c = 12.1 \text{ nM}$ by SFA in Fig. 6-6(b). Both r_{fD}

and τ_{fD} for the free DNA probe from the curve fitting analyses are very close to those ($\tau_{fD} = 276 \mu\text{s}$ and $r_{fD} = 0.056$) experimentally determined from the protein titration experiments, which indicates both titration curves in Fig. 6-6 are actually downhill even at 0.96 nM oligonucleotide competitor concentration and they can converge to the experimental values for the free Alexa488-AAF-DNA. Therefore, it should conclude that there is no non-specific binding affinity of the XPC/HR23B complex to the Alexa488-AAF-DNA compared to the oligonucleotide competitor. As it can be expected, from this competition titration experiment for the Alexa488-AAF-DNA probe and the oligonucleotide competitor, the resulting competitor dissociation constants are also nearly the same as those obtained from the protein titration experiments for the Alexa488-55-mer-ssDNA and from the competition titration experiment for the Alexa488-55-bp-dsDNA probe and the oligonucleotide competitor.

Fig. 6-7 Competition of the binding of 64 nM XPC/HR23B complex to two 4 nM DNA substrates, Alexa488-55-bp-dsDNA (triangles) and Alexa488-B5-DNA (circles), by addition of the unlabeled UV irradiated plasmid DNA competitor. The experimental TDT τ_D (a) and SFA r (b) were fit to the competition binding model (lines) of Eq. (6-6) in combination with Eq. (6-11) for FCS or Eq. (6-12) for SFA, respectively.

Compared with the Alexa488-B5-DNA, the binding affinity of the XPC/HR23B complex to the Alexa488-AAF-DNA is weaker but is slightly better than to the Alexa488-55-bp-dsDNA, which is consistent with the observations by gel mobility shift assay [218]. As early suggested by Batty *et al.* [39] and Sugasawa *et al.* [41], a feasible mechanism for XPC/HR23B complex binding to DNA damage by detecting the local helix distortion that an adduct confers by inducing improper base pairing and circumventing a lesion of several unpaired base pairs bubble.

As learnt previously from the competition titrations for both the Alexa488-55-bp-dsDNA and the Alexa488-B5-DNA, non-specific binding in competition titration experiment indicates the specificity of the XPC/HR23B complex to the damaged DNA substrate - Alexa488-B5-DNA. However, it is a question whether the binding affinity of the competitor used in competition binding reaction influences the non-

specific binding of the protein to a given DNA probe. To address this question, competition titrations for both the Alexa488-55-bp-dsDNA and the Alexa488-B5-DNA were repeated by using a stronger competitor – UV irradiated plasmid DNA, which was found very high binding affinity to be bound by XPC/HR23B complex [40]. Fig. 6-7 shows the experimental results including four competition titration curves for the Alexa488-55-bp-dsDNA (triangles) and the Alexa488-B5-DNA (circles) separately by FCS (Fig. 6-7(a)) and SFA (Fig. 6-7(b)). As expected, challenging by UV irradiated plasmid DNA, the experimental TDts in Fig. 6-7(a) and SFAs in Fig. 6-7(b) of both DNA probes dramatically decreases with the addition of the competitor. At the UV irradiated plasmid DNA concentration of 0.32 μM , the measured TDts of $416 \pm 20 \mu\text{s}$ for the Alexa488-55-bp-dsDNA and $424 \pm 23 \mu\text{s}$ for the Alexa488-B5-DNA as well as the experimental SFA of 0.073 ± 0.002 for the Alexa488-55-bp-dsDNA and 0.079 ± 0.001 for the Alexa488-B5-DNA show that the XPC/HR23B complex hardly bound to both DNA probes. Curve fittings analyses on both Fig. 6-7(a) and Fig. 6-7(b) yielded $\tau_{fD} = 399 \mu\text{s}$, $\tau_B = 1004 \mu\text{s}$, and $K_c = 0.5 \text{ nM}$ by FCS and $r_{fD} = 0.07$, $r_B = 0.24$, and $K_c = 0.9 \text{ nM}$ by SFA for the Alexa488-55-bp-dsDNA, while those parameters for the Alexa488-B5-DNA are $\tau_{fD} = 415 \mu\text{s}$, $\tau_B = 937 \mu\text{s}$, and $K_c = 0.3 \text{ nM}$ by FCS and $r_{fD} = 0.08$, $r_B = 0.22$, and $K_c = 0.6 \text{ nM}$ by SFA (Table 6-2). For both the DNA probes, the parameters r_{fD} and τ_{fD} for the free DNA probe determined from the curve fitting analyses are very close to those experimentally determined from the protein titration experiments, which shows no non-specific binding of the XPC/HR23B complex to both the Alexa488-55-bp-dsDNA and the Alexa488-B5-DNA in the presence of the competitor – UV irradiated plasmid DNA. Among all DNA substrates used in this work, highest binding affinity in Table 6-2 was found from the UV irradiated plasmid DNA by curve fitting analyses for Fig. 6-7. Therefore, it can be concluded from this experiment that non-specific binding effect may disappear for a given DNA-protein binding system when a strong competitor with a high binding affinity to the receptor is used in a competition titration. However, with the increase of the amount of competitor in the competition binding reaction, the TDt and SFA of the Alexa488-B5-DNA probe decreases apparently slower than those of the Alexa488-55-bp-dsDNA does. This again shows the higher specificity of XPC/HR23B complex binding to the Alexa488-B5-DNA substrate.

6.3.5 Binding of Rad4/Rad23 to dsDNA and damaged bubble DNA

The other NER protein Rad4 is the yeast XPC orthologue and has been found similar functions to human XPC in nucleotide excision repair [27]. Protein titrations using the combined FCS and SFA techniques were also used to characterize the binding affinity of Rad4/Rad23 protein complex to the Alexa488-55-bp-dsDNA and the Alexa488-B5-DNA. The experimental protein titration curves are shown in Fig. 6-8 separately for FCS (Fig. 6-8(a)) and SFA (Fig. 6-8(b)). With the increase of the Rad4/Rad23

Fig. 6-8 Combined FCS (a) and SFA (b) analyses of Rad4/Rad23 binding to DNA substrates, Alexa488-55-bp-dsDNA (triangles) and Alexa488-B5-DNA (circles). 4 nM DNA substrate was titrated with the Rad4/Rad23 complex in the binding reaction buffer. The experimental TDT τ_D and SFA r were separately fit to one-site binding model (lines) of Eq. (6-4) in combination with Eq. (6-11) for FCS or Eq. (6-12) for SFA, respectively.

complex concentration in binding reaction, in Fig. 6-8(a) the TDT of fluorescently labeled DNA probes increase from $400 \pm 14 \mu\text{s}$ to $537 \pm 17 \mu\text{s}$ for Alexa488-B5-DNA and from $399 \pm 12 \mu\text{s}$ to $512 \pm 20 \mu\text{s}$ for the Alexa488-55-bp-dsDNA, respectively, and in Fig. 6-8(b) the SFA from 0.074 ± 0.001 to 0.163 ± 0.001 for the Alexa488-B5-DNA and from 0.071 ± 0.001 to 0.161 ± 0.002 for Alexa488-55-bp-dsDNA, respectively. The curve fitting analyses on Fig. 6-8 yielded dissociation constants of 79.1 nM for the Alexa488-55-bp-dsDNA and 66.0 nM for the Alexa488-B5-DNA by FCS and 85.3 nM for the Alexa488-55-bp-dsDNA and 68.8 nM for the Alexa488-B5-DNA by SFA (Table 6-3). Similar to the observation with the XPC/HR23B complex, the binding affinity of Rad4/Rad23 complex was not found significant discrimination for both DNA substrates. This shows that Rad4/Rad23 complex has substantial binding affinity to both DNA substrates, which is consistent with the observations by Min et al. [27]. However, it can conclude that the truncated Rad4/Rad23 complex has >10-fold less binding affinity to the DNA substrates than the XPC/HR23B.

Corresponding to the smaller molecular mass of the Rad4/Rad23 complex, for both the bound DNA probes, the TDT τ_B and SFA r_B were found in Table 6-3 by curve fitting much smaller than those obtained in Table 6-1 from the protein titration experiments with XPC/HR23B complex.

Table 6-3 Binding affinity and one-site binding model fitting parameters of the Rad4/Rad23 complex to damaged and undamaged double-stranded DNA substrates in protein titration experiments.

DNA substrate	FCS			SFA		
	K (nM)	τ_B (μs)	τ_{fD} (μs)	K (nM)	r_B	r_{fD}
Alexa488-55-bp-dsDNA	79.1	554	395	85.3	0.18	0.07
Alexa488-B5-DNA	66.0	562	401	68.8	0.18	0.07

6.3.6 Binding of XPC/Alexa488-HR23B to UV-irradiated plasmid DNA

The protein HR23B was labeled with Alexa488 dye in this work and used in binding reaction between XPC/Alexa488-HR23B complex and UV irradiated plasmid DNA to indicate whether the HR23B is released after the XPC/HR23B complex bound to DNA damage site. In the bind reaction solution, 5 nM XPC/Alexa488-HR23B was reacted with 320 nM UV irradiated plasmid DNA in the binding reaction buffer described in the Materials and Methods (see section 6.2.5). As usual, the binding reactions were performed at 30°C with stirring for an hour to achieve equilibrium condition. The excess amount of UV irradiated plasmid DNA in solution was used to decrease as much as possible the population of the binding complexes that multiple protein complexes bound to one single plasmid DNA. As unraveled in Fig. 6-7, the XPC/HR23B complex possesses very high binding affinity to the UV irradiated plasmid DNA, and this amount of the DNA substrate can produce the entire XPC/Alexa488-HR23B complexes bound. For the free XPC/Alexa488-HR23B complex, the Tdt τ_D and SFA has been separately measured to be $523 \pm 25 \mu\text{s}$ and 0.206 ± 0.003 (Table 6-4), respectively. For FCS, only two different results can be expected when examining the binding reaction product: one is for a smaller τ_D obtained to indicate that the Alexa488-HR23B is released from the complex and the other for a larger τ_D indicate that the Alexa488-HR23B is still bound after the XPC/Alexa488-HR23B complex targets to DNA. The experimental results are given in Table 6-4. As a control, the free Alexa488 dye dissolved in the DNA-protein binding reaction buffer was determined $\tau_D = 73 \pm 2 \mu\text{s}$ and SFA $r = 0.028 \pm 0.001$. Slightly larger Tdt obtained for the free dye is resulted from the use of the underfilling-objective excitation (see section 6.2.7) [123]. As shown in Table 6-4, the XPC/Alexa488-HR23B complex binding to the UV irradiated plasmid DNA yielded $\tau_D = 1518 \pm 232 \mu\text{s}$ by FCS and SFA $r = 0.207 \pm 0.003$. The same experiments were repeated three times, and all of them led to a conclusion that the lower mobility of the binding complex by FCS indicated that Alexa488-HR23B protein was not released from the XPC protein bound with the DNA, while the SFA kept unaltered after the binding complex formation. This can be explained by the physical principles of both techniques – FCS and SFA as discussed in the following (section 6.4.1.1).

Table 6-4 Experimental Tdt τ_{fD} and SFA of the XPC/Alexa488-HR23B complex that is free or bound to UV-irradiated plasmid DNA

DNA substrate	τ_D (μs)	SFA
Alexa488 dye	73 ± 2	0.028 ± 0.001
Free XPC/Alexa488-HR23B complex	523 ± 25	0.206 ± 0.003
Bound XPC/Alexa488-HR23B complex	1518 ± 232	0.207 ± 0.003

6.4 DISCUSSION

6.4.1 Comparison of both FCS and SFA techniques

6.4.1.1 Global translational motion and local rotational motions

As learnt from Fig. 6-4 to Fig. 6-8, SFA responds to the formation of DNA-protein complex in almost the same manner as the TDt by FCS does. However, this failed when the fluorescent label is shifted to the HR23B protein (Table 6-4). Intriguingly, this presents a comparative study of the FCS and SFA techniques. As well known [4], For fluorescently labeled macromolecules, FCS in principle measures their TDt of Brown motion, which provide unique information about the average translational mobility of the molecules, whereas SFA responds to depolarization through two factors: global rotational motion (Brownian tumbling) of the molecules in addition to the local rotational motion(s) of the fluorescent labels. Macromolecular Brownian motion is influenced by the size and shape of molecules and rigidity of various molecular environments. For DNA-protein binding, FCS can be expected to always show response to the binding complex formation, which must result in the change of the size and shape of the molecules. However, for a large macromolecule (e.g. 183 kDa XPC/HR23B complex), global rotational motion of the molecules is much slow and contributes less to the fluorescence depolarization and thus SFA is governed by local rotational motion(s) of the fluorescent labels on the molecules. This is supposed to be exactly the case for the XPC/Alexa488-HR23B complex, since the SFA of 0.206 (Table 6-4) obtained for the free XPC/Alexa488-HR23B complex exhibits the slow Brownian rotational motion of the complex. As schematically illustrated in Fig. 6-9, because XPC protein is positively charged, and both HR23B protein and DNA molecule are negatively charged in the binding reaction buffer (see section 6.2.4), the DNA molecule is supposed to be bent or wrap on the XPC protein after the DPB complex formation. Therefore, if the fluorescent dye is attached to one end of the DNA molecule, its local rotational motion(s) on the binding complex is confined when the fluorescently labeled end of the DNA molecule closes or sticks to the XPC protein. Alternatively, the local rotational motion(s) of the fluorescent dye on the HR23B protein is not expected to be influenced in the binding complex. In this way, FCS and SFA in the work yield complementary information for overall molecular

Fig. 6-9 A schematic illustration of the binding complex of a XPC/HR23B complex to a DNA molecule in which the Alexa488 dye separately fuses to one end of the DNA molecule or the HR23B protein. The XPC protein possesses positive charge in the binding reaction buffer, but the DNA molecule is negatively charged. Therefore, the DNA molecule is supposed to wrap up the XPC protein.

mobility and the state of the fluorescent label on molecule. However, a lesson is therefore that SFA (or fluorescence polarization) technique actually suffers from the risk that it cannot always accurately report the binding formation due to the influence of the local rotational motion(s) of fluorescence label, although it has been extensively used in the DNA-protein binding study [4]. This also gives implications that the binding affinities obtained by FCS in all cases are higher (smaller dissociation constants in Table 6-1 to Table 6-3) than those by SFA.

6.4.1.2 Resolution

The applications using combined FCS and SFA techniques in this chapter give best opportunity to compare the characteristic features, e.g., resolution, sensitivity, *etc.*, of both techniques. In accordance with usual practice, here we express “resolution” as the relative error of experimental data of a physical parameter, *i.e.*, TDt in FCS or SFA, under investigation. The resolutions of FCS and SFA techniques are large different. Compared with FCS, SFA showed very high resolution (much smaller relative errors in Fig. 6-4 to Fig. 6-8) in both protein titration experiment and competition titration experiment. Fig. 6-10 shows a comparison of the resolutions for both techniques. The analyses in Fig. 6-10 were used one fluorescent intensity time trace data set including 25 10-second traces that measured in one experimental measurement in 5 nM XPC/Alexa488-HR23B solution (see Table 6-4). The intensity time traces each were cut and only the foremost part of each was used for the

Fig. 6-10 Comparison of the resolutions of SFA ((a) and (b)) and FCS ((c) and (d)). The SFA (a) and the TDts (c) were calculated by using foremost part of the time traces each, which length is indicated on x-axis. See the text for the methods of error bars in (a) and (c) and relative errors of SFA (b) and TDt (d).

calculations of the SFA in Fig. 6-10(a) and the TDts in Fig. 6-10(b), both of which were plotted against the length (duration) of the used part of each time trace. Error bars in Fig. 6-10 (a) and Fig. 6-10 (c) are the standard deviation in 25 analytical results, and relative errors are determined by dividing the standard deviation with the mean value of each parameter (SFA in Fig. 6-10(b) and TDt in Fig. 6-10(d)). Clearly, for both FCS and SFA, the resolution is highly dependent on the length of the fluorescent intensity time trace but the SFA has much higher resolution than the FCS under the same experimental conditions. For example, using 1-second time traces, the relative error for SFA is 0.024, which is 12 times better than that ($= 0.283$) for the TDt by FCS. Increasing the length of experimental intensity time traces results in the improved resolutions for both techniques. As shown in Fig. 6-10(d), the relative errors of the TDts by FCS seems still to be able to be improved even using 10-second intensity time traces; in contrast, The relative errors of the SFA became stable after the length of the intensity time trace increases to 3 seconds. Using 10-second intensity time traces, the relative error of SFA is 0.015, which is still 3 times lower than that ($= 0.048$) for TDts by FCS. Although from Fig. 6-10 SFA shows much lower relative error and thus much higher resolution than FCS does, SFA obtained from sub-second intensity time traces is found to suffer from poor resolution (relative error 0.435 in Fig. 6-10(a) for 1 ms fluorescent intensity time traces) as well. This makes SFA image noisy and special image processing methods are thus required as described in Chapter 7. 25 autocorrelation curves shown in Fig. 6-11(a) were calculated by using the 1-second foremost part of the 10-second time traces used in Fig. 6-10. In comparison with the curves in Fig. 6-11(a) calculated using the full length of intensity time traces, the decrease of the length of intensity time traces caused poor smoothness of the curves,

Fig. 6-11 Two set of ACFs (open circles) and their fitting (lines) obtained from 25 10-second fluorescent intensity time traces used in Fig. 6-10. The curves in Fig. 6-11(a) were calculated by using the 1-second foremost part of the time traces each while those in Fig. 6-11(b) using the full length of time traces each.

which can be another source of FCS artifact. This shows that FCS measurements required enough long stable intensity time traces to achieve reliable results for the determination of τ_D , which makes FCS technique hardly possible for imaging.

6.4.1.3 Sensitivity to significant fluorescent intensity bursts

As mentioned above, FCS needs more number of measurements than SFA for each sample solution to achieve a relatively stable average τ_D and good statistical analysis. Sparse significant fluorescent intensity bursts in fluorescent intensity time trace often mean complex sample molecule system (e.g., molecular aggregations) or fluorescent contaminations in solution. In this case, FCS can produce very complex ACFs that cannot be simply fitted to the current available models (see section 2.4.4 in Chapter 2).

Fig. 6-12 Representative intensity time traces in the binding reaction solution (a) of the XPC/Alexa488-HR23B complex to the UV-irradiated plasmid DNA and the free XPC/Alexa488-HR23B complex solution (b). The bin time is 1 ms. (c) Photon counting histogram for Fig. 6-12(a) (black) and Fig. 6-12(b) (light grey). Note that the y-axis is in logarithm scale.

This is actually because FCS is more sensitive to the significant individual event (large burst in the intensity time trace) in the fluorescent intensity time trace. To illustrate this characterization, one representative example is from the above binding study of the XPC/Alexa488-HR23B complex to the UV-irradiated plasmid DNA. As given in Table 6-4, the relative error of the measured τ_D ($1518 \pm 232 \mu\text{s}$) for the binding complex by FCS is much higher than that for SFA (0.206 ± 0.003). For the ease of comparison, Fig. 6-12 shows two representative intensity time traces separately from the binding reaction solution (Fig. 6-12(a)) for the XPC/Alexa488-HR23B complex binding to UV-irradiated plasmid DNA and from the free XPC/Alexa488-HR23B complex solution (Fig. 6-12(b)) and their photon counting

histogram (PCH) (Fig. 6-12(c)). Comparing both the intensity time traces in Fig. 6-12(a) and in Fig. 6-12(b) and the PCHs in Fig. 6-12(c) shows only very less population of the binding complexes each of which was involved multiple XPC/Alexa488-HR23B complexes. This is due to the excess amount of the UV-irradiated plasmid DNA molecules challenging with only limited amount of the XPC/Alexa488-HR23B complex. However, as given in Table 6-4, FCS was strongly affected while SFA kept stable. This can also be expected from the technological strategies used for both FCS in Eq. (6-2) and SFA in Eq. (6-3). As manifested in Eq. (6-2), the high bursts in the intensity trace have much more contribution than the smaller ones by multiplying themselves at a short time lag τ when calculating ACF for FCS.

6.4.1.4 Additivity of diffusion constants and SFAs

The linear combination of diffusion constants in Eq. (6-10) or SFA in Eq. (6-12) of two components was used in the data analyses for FCS and SFA, respectively, but Eq. (6-10) is physically valid and Eq. (6-12) remains discussion as clarified in Ref. [40, 238] as well. However, with the exception of the case that the XPC/Alexa488-HR23B binds to the UV irradiated plasmid DNA (Table 6-4), our results showed the assumption that the average SFA in two-component system is the linear combination of the SFA of each component is a good approximation by comparing with FCS analyses in our study. This means the rotational and the translational motions of the fluorescently labeled DNA and DNA-protein complex are all slowed down in a similar way, and the additivity of SFAs from multiple components is thus approximately valid. Nevertheless, SFA was found for many molecules not to follow the TDt in the same manner in this work. Therefore, the use of SFA technique alone for the study of DPB is susceptible to the fluorescent label position and the experimental results may not be always correct. The influence of the local rotational motion(s) on SFA is an important lesson as previously interpreted (see section 6.4.1.1).

6.4.2 Translational diffusion time of binding complex

According to the Stokes-Einstein relation, the TDt τ_D of spherical macromolecules (molecular mass: m) can be predicted by

$$\tau_D / \tau_D^M = (m / m^M)^{\frac{1}{3}} \quad (6-13)$$

where τ_D^M , and m^M represent the TDt and molecular mass of the fluorescent molecule marker used in the FCS calibration experiment. Eq. (6-13) is apparently useful to determine the number of molecules of interest in a single binding complex. However, as was often in the FCS analyses in this work, the violation of the Stokes-Einstein relation was found. For instance, if Alexa488 was used as the internal calibration marker ($\tau_D^M = 73 \mu s$ in Table 6-4), the expected TDts for the Alexa488-55-bp-dsDNA and the binding complex of a single XPC/HR23B complex to a single the Alexa488-

55-bp-dsDNA are, respectively, 282 μ s and 511 μ s, both of which are significantly smaller than those experimental values in Table 6-1. For the experimental TDT $\tau_B = 1012 \mu$ s of the binding complex of the XPC/HR23B complex to Alexa488-55-bp-dsDNA, it requires 9-10 protein complexes bound to a single DNA molecule which total length is approximately 18 nm. Apparently this is hardly possible since the XPC/HR23B complex must have a hydrodynamic radius of > 3 nm (which is for 30 kDa eGFP). The Stokes-Einstein relation is derived for spherical macromolecules and based on hydrodynamic assumptions which supposed that (a) the solution is homogeneous and isotropic, and (b) the solute is so highly diluted that no solute-solute interactions can be expected. Although in this work proteins or DNA molecules were in nanomolar concentration, the binding reaction buffer contains 2% glycerol and 0.02% (0.23 mM) TX100, which is used to stabilize protein functionality [69] and has been tested no interactions with both protein and DNA molecules. Therefore, the binding reaction solution is actually a molecular crowding environment and the assumption (b) for the derivation of the Stokes-Einstein relation is highly susceptible to validation. In addition, as mentioned previously, the molecular dynamics in aqueous solution is not only governed by hydrodynamic interactions, but also strongly depends on the molecular conformation which exact contribution is hardly explicit for a given binding complex in most cases. A most obvious example is that the dynamic behavior of DNA molecules in solution is controlled by hydrodynamic interactions and chain connectivity [111, 112].

6.4.3 Multiple binding sites on a single molecule

It is often an interesting question how many binding sites on a single molecule. As mentioned above, due to the potentially complicated molecular confirmation and thus the failure of the Stokes-Einstein relation, this question is not able to precisely answer by FCS. Although all the titration curves in this work were found well fit to the one-site binding model (Eq. (6-4)), the experimental TDTs at high ligand (DNA repair protein) concentration suggest multiple binding sites on a single DNA molecule.

A few trials on the other multiple independent binding sites model shows that the multiple independent binding sites model often suffers from loss of physical sense, because the total number of molecules in one binding complex is not constant and actually highly dependent on the ratio of protein and DNA concentrations in the binding reaction buffer. For the same reason, it is not practical as well for a simple scheme to determine the number of the binding sites on a single DNA by using fluorescently labeled protein complex, e.g., the XPC/Alexa488-HR23B complex, and unlabeled DNA substrates to do binding reactions and estimate the number of the fluorescently labeled protein complex on a single binding complex through the fluorescent intensity time trace and PCH like in Fig. 6-12. As indicated by the protein titration reactions (Fig. 6-4 for the XPC/HR23B complex and Fig. 6-8 for the

Rad4/Rad23 complex), to saturate all binding sites on a single DNA molecule requires significant excess amount of the protein complexes, which produce large population of the free fluorescent labeled protein complex in the binding reaction and hardly make precise analysis to answer the question by the used techniques.

6.4.4 Mechanism of XPC/HR23B binding to DNA

The function of HR23B protein in the XPC/HR23B complex in GG-NER is still unclear, but it has been found to be able to stabilize XPC protein and thereby enhance NER activity [52]. In this work, the Alexa488-HR23B alone was found not to bind to the UV-irradiated plasmid DNA under conditions where XPC/HR23B complex does (data not shown). This suggests that protein XPC rather than HR23B is actually responsible for the recognition of DNA damage. Similar observations were reported in yeast. The Rad4 and Rad23 proteins are the yeast homolog of the human XPC and HR23B, respectively. In yeast, Rad23 without Rad4 does not bind to DNA under conditions where Rad4/Rad23 complex does [242]. HR23B is essential for XPC function *in vitro* maybe because it increases the solubility of XPC and prevents XPC protein from aggregations.

A multistep model for GG-NER mechanism has been proposed [2, 3]. Many NER factors are involved in the model and the ordered arrival and release of each factor results in the elimination of the DNA damage. XPC/HR23B was indentified *in vitro* and *in vivo* as the first factor that targets damaged DNA [26, 39]. After the XPC/HR23B complex bound to the DNA damage site, it is highly speculated that a few NER factors including XPA, RPA, TFIIH, and XPC/HR23B form a complex at the damaged DNA site [2], and XPC/HR23B complex is released from the damage site before the premature dual incision complex with the arrival of XPG. Although fluorescently labeling all NER factors remains difficult, the combined FCS and SFA techniques are in principle possible to explicate the multistep model and describe the architecture of the NER mechanism. In this work, we have experimentally confirmed that HR23B is not released from XPC/HR23B complex when the XPC/HR23B complex binds to DNA (section 6.3.6).

The binding mechanism of the DNA damage recognition proteins, human XPC/HR23B complex and yeast Rad4/Rad23 complex, to DNA damage sites is not completely clear. Scanning force microscopy revealed that the XPC/HR23B complex induces a bend in DNA upon binding and this is stabilized at the site of damage [243, 244], and suggest that XPC protein attracts DNA helical chain upon binding which supports the schematic model for the binding complex of XPC/HR23B complex and a short DNA molecule in Fig. 6-9. A thermodynamically biased recognition mechanism has been proposed that XPC/HR23B complex probes DNA damage sites by sensing the thermodynamic instability of the helical distortions [245]. However, it still remains unelucidated how XPC/HR23B complex finds a lesion in the genome, e.g.,

Does XPC/HR23B complex bind to DNA damage in a random distributive fashion or by processive scanning of large genome segments?

6.5 CONCLUSIONS

NER proteins, human XPC/HR23B and yeast Rad4/Rad23, binding to various DNA substrates was studied by the combined FCS and SFA techniques. When the fluorophore label, Alexa488 dye, is fused at one end of DNA, the protein titration curves showed that both SFA and Tdt in FCS increased with protein concentration in binding reaction solution. The protein titration curves were fitted to single-site binding model and the binding affinities of XPC/HR23B and Rad4/Rad23 to the DNA substrates were determined. The results showed that the binding affinity of the XPC/HR23B to the used DNA substrates can be ten-time higher than that of Rad4/Rad23, and FCS was found more sensitive to the formation of binding complex than SFA. When the fluorophore label was transfer to XPC/HR23B complex, FCS can exactly find the formation of binding complex but SFA failed. This indicates that the fluorophore label experienced much faster local rotational motion(s) on the surface of XPC/HR23B complex than the global rotation of a binding complex. Competition titrations of XPC/HR23B binding to fluorescently labeled DNA substrates were performed and the binding reaction solutions were examined by SFA and FCS. The experimental results revealed that XPC/HR23B complex preferentially binds to damaged DNA substrates with a few improperly pairing bases. FCS and SFA on the binding reactions of the XPC/Alexa488-HR23B complex to the UV-irradiated plasmid DNA revealed that HR23B protein does not release from the protein complex when XPC/HR23B binds to DNA molecule *in vitro*. The combined FCS and SFA techniques showed a promised perspective to explicate the multistep model and describe the architecture of the NER mechanism.

Chapter 7

Multiparameter Fluorescence Imaging in Single Cells

ABSTRACT

In this chapter, we show the capability of multiparameter fluorescence imaging microscopy in single cells based on a single-molecule fluorescence confocal microscope. The multiparameter fluorescence imaging, including fluorescent intensity imaging, fluorescent spectral imaging and steady-state fluorescence anisotropy (SFA) imaging, was performed in single living XP4PA cells to study the activity of the DNA repair protein XPC tagged with green fluorescent protein in the nucleus. Fluorescent intensity imaging and fluorescent spectral imaging can be used to precisely localize fluorescent molecules in cells, while SFA imaging may localize the molecular interactions by which the fluorescent molecules' rotational motion is disturbed yielding changes in SFA. The advantages and problems for SFA imaging in cells are addressed and discussed. The feasibility of fluorescence correlation spectroscopy (FCS) in cells was strongly limited by fluorescent background intensity variations arising from the autofluorescence, photobleaching of the limited number of fluorescence molecules, and complicated internal cellular motions. These limitations of FCS in cells are presented and potential use of other methods for fluorescent time trace analysis is suggested.

7.1 INTRODUCTION

Nucleotide excision repair (NER) in the eukaryotic cell is a versatile DNA repair pathway that is responsible for removing a wide variety of helix-distorting base lesions, such as ultraviolet light (UV) induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4) pyrimidone photoproducts (6-4PPs) as well as many bulky adducts induced by numerous chemical compounds in cells. Impaired NER activity is associated with several rare autosomal recessive disorders in human, e.g., xeroderma pigmentosum (XP), Cockayne syndrome (CS) and *et al.* [16, 246]. Mammalian NER can be accomplished through two distinctive pathways, known as global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). As indicated from both two names, GG-NER activity works throughout the entire genome, whereas TC-NER action is performed within active transcriptional regions. For the UV-induced lesions, repair of 6-4PP is fast throughout the genome and is mainly by GG-NER, while removal of CPD is relatively slow by GG-NER but can be accelerated from the transcribed strand of expressed genes through TC-NER [247]. NER is a multistep process involving recognition and verification of DNA damage, opening up of the DNA helix around the lesion, dual incision and subsequent excision of the oligonucleotide containing the DNA lesion followed by and resynthesis of the gap and ligation of the residual nick [1, 23, 217]. Although the mechanical multi-steps of both GG-NER and TC-NER are largely overlapped, the initial actions of both NER pathways are quite different. In TC-NER, RNA polymerase II stalls at damage sites and triggers the repair reaction [248], whereas in GG-NER proteins bind to damage sites and initialize repair activity. Many evidence shows that XP group C (XPC) protein plays an essential role in damage recognition step of GG-NER [3, 26, 42]. As revealed in Chapter 6, purified XPC-HR23B complex has been found high affinity *in vitro* for undamaged and damaged single and double-strand DNA [39, 218, 241], but it has also been shown a preferential binding to the DNA molecules with small bubble structures and even without a lesion as shown by competition titration experiments in Chapter 6 (and Ref. [218]) as well as other damaged DNA molecules with various other induced lesions [40, 241].

In order to study the distribution and dynamics of XPC/HR23B complex within single living cells, XPC proteins in cell were tagged with green fluorescent protein (GFP). A single-molecule fluorescence confocal microscope (SFCM) was developed with the capability of multiparameter fluorescence imaging microscopy including confocal fluorescent intensity imaging spectroscopy [249], fluorescence steady-state anisotropy spectroscopy (SFA) [250-252], fluorescence correlation spectroscopy (FCS) [107, 127, 253-255], and fluorescent spectral imaging [256-258] in single living cells. This technique makes it possible to gather as much spectroscopic information as possible from the fluorophores in cells. The primary characteristics

exhibited by a fluorophore are its spectrum, intensity, fluorescence lifetime and anisotropy. These spectroscopic properties of the fluorophores are often found to be dependent on the local molecular environment and can give direct insight on molecular distribution, dynamics and interactions. Therefore, it is apparently useful to develop an extension of confocal fluorescence technology to achieve a combined multiparameter fluorescence imaging technique for a variety of distinct and complementary fluorescent microscopies. Fluorescent intensity imaging allows us to study the distribution properties of GFP fused XPC/HR23B complex (GFP-XPC/HR23B) in single cells, and fluorescent spectral imaging is required for fluorophore identification, which is particularly useful in the presence of the autofluorescence background in cells. Based on the SFCM, combined FCS and SFA (Chapter 6) have been successfully applied to study the binding properties of DNA repair proteins, human XPC/HR23B complex and yeast Rad4/Rad23 complex, to various damaged and undamaged DNA substrates *in vitro*, and particularly SFA manifested much lower variance than FCS, which makes SFA technique more suitable for cell imaging with a reasonable temporal resolution. Perhaps for this reason, SFA imaging has been widely used to study biological and biophysical problems [259-262], since the first polarization-sensitive imaging in red blood cell membrane by Axelrod in 1979 [263]. In contrast, as learnt from the combined FCS and SFA for DNA-protein binding *in vitro* in Chapter 6, FCS is accompanied by a higher variance and statistically requires very long intensity time trace measurements to achieve stable values of average number and translational diffusion time of molecules in the confocal focal volume. The limitations of FCS in cells are discussed.

7.2 MATERIALS AND METHODS

7.2.1 Cell culture

The cell strains, which were originally XPC-defective XP4PA fibroblasts and transfected by XPC-GFP fusion construct using a liposome-based method several years ago, were kindly provided by Dr. Hannes Lans and Dr. Wim Vermeulen in Medical Genetics Center, Department of Cell Biology and Genetics, Erasmus MC, Rotterdam, the Netherlands. The cell culture was used the same method as described in Ref. [264]. Briefly, all cell strains used in this work were cultured in RPMI + Hepes (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 400 µg/ml G418 (Sigma-Aldrich, St. Louis, MO) and antibiotics, and maintained in a humidified 5% CO₂, 37 °C incubator.

7.2.2 UV irradiation

Prior to irradiation, medium was aspirated and kept at 37 °C. XP4PA cells were rinsed with PBS (Invitrogen) and exposed to global UV irradiation with germicidal lamps at 254 nm and a fluence rate of 10 Js⁻¹m⁻² for 5 min. Subsequently, the cells were

returned to the culture medium, and finally microscopic measurements were performed within half an hour.

7.2.3 Multiparameter fluorescence imaging microscopy

The SFCM was constructed for the multiparameter fluorescence imaging microscopy in single live cells. The main configuration of this SFCM has been presented elsewhere [67, 68], and the SFCM has been further developed with the on-line quantitative FCS, which has been used for the study of soluble protein stability in Chapter 5, and the combined FCS and SFA techniques for DNA repair proteins binding to DNA in Chapter 6. In both Chapters 5-6, FCS and SFA were performed on-line in the fluorescent intensity time traces measured in sample solutions at a fixed position or by sequentially scanning a few preset positions. Consequently, after the recent developments, the fluorescent intensity imaging, spectral imaging and SFA imaging are, in principle, ready for the multiparameter fluorescence imaging applications in cells within the SFCM. In this work, linearly polarized laser line 488nm was used to excite GFPs in the XP4PA cells and a water immersion objective Zeiss C-Apochromat 63×, NA=1.20, working distance = 0.24 mm) was used for microscopic imaging. The SFA is formally evaluated by

$$r = \frac{I_p - GI_s}{I_p + 2GI_s} \quad (7-1)$$

where I_p and I_s are the fluorescent intensity parallel and perpendicular to the excitation polarization as selected using the Glan-Thompson polarizer in the excitation path of the SFCM; G accounts for different transmission and detection efficiencies of the imaging system at parallel and perpendicular polarization [265] and it was the same for all pixels in a SFA image due to the scanning motion using a piezo nano-positioning stage (P-740.20 50×50μm², Physik Instrumente, Waldbronn, Germany). Prior to cell imaging, calibrations for SFA were performed in fluorescent dye solutions and enhanced GFP solution as described previously in Chapter 6.

The excitation power used for fluorescent intensity imaging, SFA imaging and FCS is 10 μW (or 8 kW/ cm²), but for fluorescent spectral measurements it is 100 μW by flipping away a 10× attenuator in the optical excitation path of the SFCM.

7.2.4 Multiparameter fluorescence imaging measurements in cells

One day prior to microscopic multiparameter fluorescence imaging measurements, XP4PA cells were transferred onto Lab-Tek™ Chambered Coverglass (NUNC™, Rochester, NY), which is a cell culture vessel with a borosilicate coverslip as the cell growth surface and allows direct microscopic measurements in the SFCM, and kept in cell culture conditions. Before microscopic experiments, cell culture medium was aspirated and was replaced with PBS to avoid the fluorescent background from the medium.

7.2.5 Image processing and data analysis

A custom computer program was written in Labview (National Instruments, Austin, TX) to control the fluorescence microscope piezo nano-positioning stage and record fluorescent amplitude time traces or spectra in the SFCM. The recorded fluorescent intensity or fluorescent spectra data allow many other off-line mathematical analysis techniques. SFA imaging and the combined single point FCS and SFA measurements in cells were performed in on-line mode. Other custom programs based on Matlab (The Mathworks, Natick, MA) and Labview platforms were used for off-line image processing.

In a SFA image, it is difficult to analyze the statistical properties of the SFA values at each pixel. In Chapter 2, we have discussed the resolution of SFA in solution, and found that reliable SFA requires long intensity time traces, but this is often not practical for cell imaging. SFA imaging in cells need each pixel measurement as soon as possible to avoid internal nucleus motion of a living cell or photobleaching of the limited number of fluorescent molecules in the confocal focal volume in a cell. Therefore, the intensity data acquisition time for each pixel of an image is usually in the order of milliseconds, and consequently the SFA value at each pixel suffers from high risk of statistical error. To achieve statistically reliable SFA in a cell image, the SFA values at the pixels that have the same fluorescent intensity were averaged and the average SFA is then plotted as a function of the pixel fluorescent intensity. This resulting SFA r versus fluorescent intensity I curve (r - I curve) can give relatively reliable determination of SFA of GFP-XPC in cell nuclei.

In fluorescent spectral imaging, the background correction of fluorescent spectra was done by subtracting the average background spectrum without the laser excitation.

7.3 RESULTS

The representative multiparameter fluorescence images measured in four living human XP4PA cells are shown in Fig. 7-1 to Fig. 7-7. Fig. 7-1 to Fig. 7-4 are the fluorescent intensity images, SFA images and their fluorescent spectral images for the two normal cells while those images in two UV-irradiated cells are given in Fig. 7-5 to Fig. 7-7.

7.3.1 Distribution of GFP-XPC in mammalian nuclei

The distribution of GFP-XPC in living human XP4PA cells was studied by fluorescence intensity imaging and fluorescent spectral imaging in the SFCM. The representative images for two single normal living XP4PA cells are given in Fig. 7-1 to Fig. 7-4. Fig. 7-1(a) and Fig. 7-3(a) are, respectively, the fluorescent intensity images of both single cells and their fluorescent spectral images are given in Fig. 7-2 and Fig. 7-4, respectively. Although some relatively weak intensity light spots can be seen around the cell nuclei in Fig. 7-1(a) and Fig. 7-3(a), fluorescent spectral images

Fig. 7-1 (a) Fluorescent intensity image from one single XP4PA cell. Image size is $25 \times 25 \mu\text{m}^2$, 256×256 pixels, dwell time 1 ms/pixel. The colorbar is shown along the x-axis of Fig. 7-1(c) (b) The SFA image for the cell in Fig. 7-1(a). The colorbar is shown along the y-axis of Fig. 7-1(c) (c) The *r-I* curve (solid line) derived from Fig. 7-1(a) and Fig. 7-1(b) (see the text for details) as well as the pixel intensity histogram (dots) for Fig. 7-1(a). Scale bars in Fig. 7-1 (a) and Fig. 7-1 (b): $5 \mu\text{m}$.

(Fig. 7-2 and Fig. 7-4) reveals that all the light spots out of the cell nuclei are brighter in the fluorescent spectral images (Fig. 7-2(b) and Fig. 7-4(b)) in the spectral range of 600–750 nm, and thus they are actually autofluorescence. The same observations were also found from the UV-irradiated cell in Fig. 7-5 to Fig. 7-6. Fig. 7-5(a) shows the fluorescent intensity image for the UV-irradiated cell and its fluorescence spectral image is given in Fig. 7-6, where many light spots around cell nucleus can be seen from both figures, Fig. 7-6(a) and Fig. 7-6(b) in the spectral ranges of 500 – 550 nm and 600 – 750 nm, respectively. The spectra (e.g., Fig. 7-6) of those spots were found over a broad range from 500 nm to 700 nm, and thus were actually from the autofluorescence. In conclusion, as expected, the GFP-XPC proteins were strictly found in cell nuclei, which is the same as the previous observations by D. Hoogstraten [264].

7.3.2 Autofluorescence

In the fluorescent spectral imaging experiments, the autofluorescence intensity amplitude in cells differed from cell to cell and also from different observation positions in a single cell. The spectra (Fig. 7-2(b) and Fig. 7-6(b)) of autofluorescence span a large wavelength range from 500 nm to 700 nm, which includes the GFP emission spectrum, and thus cannot be suppressed by using optical band pass filters.

In contrast to the intensity imaging, spectral imaging allows spectrally resolving spatial events.

7.3.3 SFA imaging in cells

SFA imaging was performed in the living XP4PA cells simultaneously with the

Fig. 7-2 (a-b) Fluorescent spectral images in the spectral ranges of 500-550 nm (a) and 600-750 nm (b) from the single XP4PA cell which fluorescent intensity image is given in Fig. 7-1(a). Image size is $30 \times 30 \mu\text{m}^2$, 128×128 pixels, spectral data acquisition time 2 ms/pixel. **(c-e)** Three frames of representative spectra were measured from the points 1-3 in Fig. 7-2(a-b), respectively. **(f)** The average fluorescent spectrum calculated from the entire fluorescent spectra over all pixels of the image. Scale bars in Fig. 7-2 (a) and Fig. 7-2 (b): 5 μm.

fluorescence intensity imaging. Fig. 7-1(b) and Fig. 7-3(b) are two representative examples of the SFA images for the normal XP4PA cells, which fluorescent intensity images are shown in Fig. 7-1(a) and Fig. 7-3(a), respectively, while for the UV-irradiated XP4PA cells in Fig. 7-5(a) and Fig. 7-7(a), their SFA images are given in

Fig. 7-3 (a) Fluorescent intensity image from one single XP4PA cell. Image size is $25 \times 25 \mu\text{m}^2$, 256×256 pixels, dwell time 1 ms/pixel. The colorbar is shown along the x-axis of Fig. 7-3(c). (b) The SFA image for the XP4PA cell in Fig. 7-1(a). The colorbar is shown along the y-axis of Fig. 7-3(c). (c) The *r-I* curve (solid line) derived from Fig. 7-3(a) and Fig. 7-3(b) as well as the pixel intensity histogram (dots) for Fig. 7-3(a). Scale bars in Fig. 7-3(a) and Fig. 7-3(b): $5 \mu\text{m}$.

Fig. 7-5(b) and Fig. 7-7(b), respectively. Following each SFA image, an *r-I* curve is given for statistically reliable SFA values.

For the normal cells, the SFA images in Fig. 7-1(b) and Fig. 7-3(b) show homogeneously distributed SFA with higher values for the GFP-XPC proteins in the cell nuclei, and this is in a good agreement with the expected SFA value for the natural SFA of GFP as well as the slow motion of the GFP-XPC due to the interaction with DNA [264]. The *r-I* curve, the solid line in Fig. 7-1(c), is the plot of SFA in Fig. 7-1(b) versus pixel intensity in Fig. 7-1(a) (see section 7.2.5), while the histogram of the pixel intensity in Fig. 7-1(a) is also given (dot line) in Fig. 7-1(c). In Fig. 7-1(c), statistically stable SFA can be found at a number of fluorescent intensity values if there is a large population of the pixel fluorescent intensity. For example, the histogram of pixel fluorescent intensity shows a large population at a pixel intensity 73 cts/pixel which was measured from out of the cell nucleolus and has an average SFA of 0.084 ± 0.007 , which corresponds to the expected fast rotational motion of autofluorescence molecules in the cell. From the fluorescent intensity of 300 cts/pixel to its maximum 1058 cts/pixel in Fig. 7-1(c), the average SFA is 0.321 ± 0.014 , which is nearly the same as the SFA 0.31 ± 0.01 of eGFP in solution (see Chapter 6). For the SFA image shown in Fig. 7-3(b) for the other normal living cell, the *r-I* curve and the pixel intensity histogram is given in Fig. 7-3(c). The average SFA in the pixel fluorescent intensity range (mostly in the cell nucleus) of 500 cts/pixel to 2238

Fig. 7-4 (a-b) Fluorescent spectral images in the spectral ranges of 500-550 nm (a) and 600-750 nm (b) from the single XP4PA cell used for Fig. 7-3. Image size is $20 \times 20 \mu\text{m}^2$, 128×128 pixels, spectral data acquisition time 2 ms/pixel. (c-e) Three frames of representative spectra were measured from the points 1-3 in Fig. 7-4(a), respectively. (f) A background spectrum (dash line) measured at point 4 in Fig. 7-4(a-b) and the average fluorescent spectrum (solid line) calculated from the entire fluorescent spectra over all pixels of the image. Scale bars in Fig. 7-4(a) and Fig. 7-4(b): $5 \mu\text{m}$.

cts/pixel is 0.320 ± 0.015 . In Fig. 7-3(c), highest frequency of pixel intensity is 82 cts/pixel, where the SFA is 0.089 ± 0.015 . Most of the image pixels where intensities are on the slopes in the r - I curves in Fig. 7-1(c) and Fig. 7-3(c) were found at the edge of the cell nuclei, where perhaps multiple components (GFP-XPC in the cell nuclei and others by autofluorescence) separately contributing different SFA were involved in the confocal focal volume.

After UV irradiation, most of the fluorescent intensity images of the XP4PA cells became lower intensity which was likely caused by the photobleaching induced by UV light. Fig. 7-5(b) and Fig. 7-7(b) are two representative SFA images from two UV-irradiated cells. Using the same analysis method above for the normal XP4PA cells, from the r - I curve in Fig. 7-5(c) the average SFA in the pixel fluorescent intensity range of 300 cts/pixel to 1000 cts/pixel is 0.330 ± 0.029 , and the SFA is

Fig. 7-5 (a) Fluorescent intensity image from one single UV-irradiated XP4PA cell. Image size is $22 \times 22 \mu\text{m}^2$, 256×256 pixels, dwell time 1 ms/pixel. The colorbar is shown along the x-axis of Fig. 7-5(c). (b) The SFA image for the XP4PA cell in Fig. 7-5(a). The colorbar is shown along the y-axis of Fig. 7-5(c). (c) The *r-I* curve (solid line) derived from Fig. 7-5(a) and Fig. 7-5(b) as well as the pixel intensity histogram (dots) for Fig. 7-5(a). Scale bars in Fig. 7-5(a) and Fig. 7-5(b): $5 \mu\text{m}$.

0.099 ± 0.030 at the pixel intensity of 27 cts/pixel with highest frequency in the pixel intensity histogram; in Fig. 7-7(c) the average SFA in the pixel fluorescent intensity range of 80 cts/pixel to 336 cts/pixel is 0.351 ± 0.044 , and the SFA is 0.071 ± 0.022 at the pixel intensity of 26 cts/pixel with highest frequency in the pixel intensity histogram. Unlike the *r-I* curves for the normal cells in Fig. 7-1(c) and Fig. 7-3(c), in both the *r-I* curves (Fig. 7-5(c) and Fig. 7-7(c)), SFA is not homogeneously distributed at higher pixel intensity which perhaps was caused by GFP-XPC locally bound to the DNA damage site.

Although the amplitudes of SFA differ from the normal to the UV-irradiated cells, they are not significantly different for GFP-XPC throughout the whole cell for a single cell and also from cell to cell. This is because the rotational correlation time of GFP is longer than its fluorescence lifetime [266], and thus the SFA does not increase strongly due to a decreased rotational motion upon interaction of GFP-XPC bound to DNA.

Fig. 7-6 (a-b) Fluorescent spectral images in the spectral ranges of 500-550 nm (a) and 600-750 nm (b) from the single UV-irradiated XP4PA cell used for Fig. 7-6(a). Image size is $20 \times 20 \mu\text{m}^2$, 128×128 pixels, spectral data acquisition time 2 ms/pixel. (c-e) Three frames of representative spectra were measured from the points 1-3 in Fig. 7-6(a-b), respectively. (f) The average fluorescent spectrum calculated from the entire fluorescent spectra over all pixels of the image. Scale bars in Fig. 7-6(a) and Fig. 7-6(b): 5 μm .

7.3.4 Single point FCS and SFA in cells

Since FCS was first proposed in the 1970s [85-87], FCS was little used [267-270] for the measurements in living cells mainly because FCS requires long measurement times. FCS in cells for the determinations of both concentration and translational diffusion time for fluorescent molecules has at least two limitations [127, 128] that were found in this work as well: (1) the unknown shape and size of the confocal detection volume varying in cells with the local refractive index; (2) background variations arising from the autofluorescence, photobleaching of the limited number of fluorescence molecules, and complicated internal cellular motions. For a long time fluorescent intensity time trace measurement, SFA can be affected as well by the increased contribution from the autofluorescence background due to the

Fig. 7-7 (a) Fluorescent intensity image from one single XP4PA cell. Image size is $22 \times 22 \mu\text{m}^2$, 256×256 pixels, dwell time 1 ms/pixel. The colorbar is shown along the x-axis of Fig. 7-7(c). **(b)** The SFA image for the XP4PA cell in Fig. 7-7(a). The colorbar is shown along the y-axis of Fig. 7-7(c). **(c)** The *r-I* curve (solid line) derived from Fig. 7-7(a) and Fig. 7-7(b) as well as the pixel intensity histogram (dots) for Fig. 7-7(a). Scale bars in Fig. 7-7(a) and Fig. 7-7(b): $5 \mu\text{m}$.

photobleaching of the fluorescent molecules in cell. To address these problems, Fig. 7-8 shows the experimental results from a representative combined FCS and fluorescence measurement in a living XP4PA cell with 26 sequential 30-second fluorescent intensity time traces at one fixed point. As shown in Fig. 7-8(a), the SFA decreases with measurement time and fluorescent intensity (Fig. 7-8(b)) in the cell. This can be understood that the number of local GFP-XPC in the cell nucleus was limited and simultaneously multiple autofluorescent components existed there and contributed to the fluorescence, but after photobleaching due to a long excitation, GFP-XPC contributed less and less to fluorescent intensity and thus the SFA. For the ease of demonstration, Fig. 7-8(c) gives the first frame of fluorescent intensity time trace which shows an obvious fluorescent photobleaching trace and significantly influences the auto-correlation function (ACF, blue squares in Fig. 7-8(d)) in FCS. This then again proves the interpretation in Chapter 6 that the ACFs in FCS are susceptible to the high bursts (significant fluorescent intensity by large and slow molecule clusters) in the intensity time traces. In contrast with the common FCS technique on cells [267, 269] that shows only one typical ACF for analysis, in the measurement for Fig. 7-8 we acquired 26 sequential 30-second fluorescent intensity time traces at one fixed point in the living XP4PA cell by using the on-line quantitative FCS. All 26 intensity traces were separately performed auto-correlation

analyses and the resulting ACFs are shown in Fig. 7-8(d). The profiles of the ACFs in Fig. 7-8 observably vary from one to one and completely differ from those obtained from the FCS measurement on fluorescent molecules in aqueous solutions (Chapter 6). This is because the ACF profile of the fluorescence intensity fluctuation in focal volume represents the instantaneous average of the diffusion by all molecules in various conformational states, but it is weighted by the number of molecules in each of those states. Fig. 7-8(d) is only one representative example of the common on-line FCS measurements on cells, and all on-line FCS measurements in cells gave a set of similarly complicated ACFs, which suggests that the shape and size and mobility of molecules in cell vary in a very broad range and are hardly possible to describe by two- or three-component systems. As shown in Fig. 7-8(d), although all ACFs can be

Fig. 7-8 A combined FCS and SFA measurement with 26 sequential 30-second fluorescent intensity time traces at one fixed point in one normal living XP4PA cell. (a) SFA decreases with measurement times; (b) Average fluorescent intensity decreases with measurement times due to the photobleaching of limited number of fluorescent molecules in the cell; (c) The first 30-second fluorescent intensity time trace for the combined FCS and SFA analyses; (d) The ACFs (colored circles) and their multi-component 3D diffusion model fitting (colored lines) (The top blue squares curve was derived from the first fluorescent intensity time trace in Fig. 7-8(c)).

best fit to the two-components three-dimensional model [267], the resulting translational diffusion times were found almost over a full time scale from 100 μ s to 1 second. Therefore, we may conclude that auto-correlation analysis is not a perfect method on cells, and other more reliable time trace analysis methods should be expected for this application to extract the information about the frequency and waist width of the bursts in a long time trace but, of course most importantly, it should be able to be performed efficiently by using modern computers. However, surprisingly there has already been a review [96] claiming a bright future for FCS on cells but without any attention to these practical problems.

7.4 DISCUSSION AND PERSPECTIVE

7.4.1 Rotational mobility of GFP-XPC in cells by SFA

Although the measured SFA in normal (Fig. 7-1(c) and Fig. 7-3(c)) and UV irradiated living XP4PA cells (Fig. 7-5(c) and Fig. 7-7(c)) are very close as described previously, Fig. 7-5(c) and Fig. 7-7(c) show non-uniformly distributed SFA of GFP-XPC in cell nuclei after UV irradiation, which seems to be able to lead to the expected finding that GFP-XPC locally binds to the DNA damage site. To confirm that this observation is the nature, a new function for a local UV irradiation is expected for the SFCM, and it should provide us an ability to locally irradiate one single cell and SFA images can afterwards be performed to find local higher SFA indicating the slow mobility of the GFP-XPC at the UV irradiated position.

7.4.2 Fluorescent label for SFA imaging in cells

Compared with FCS, SFA technique in this work shows a promising future for the applications in cells. However, as mentioned before, GFP has an intrinsic rotational correlation time longer than its fluorescence lifetime [266], and its SFA is not strongly dependent on its rotational motion used to predict the interaction of protein XPC binding to DNA. Ideally, for the study of molecular interaction, SFA requires fluorescent labels have a longer fluorescence lifetime and a proper position on the molecules where their rotational motion can be efficiently affected by the molecular interactions under study (see Chapter 6).

7.4.3 Perspectives for fluorescent intensity time trace analysis

As discussed previously for Fig. 7-8(d), the ACF in FCS represents the instantaneous average of the fluorescence intensity fluctuation in focal volume by the diffusion of all molecules in various conformational states, but is weighted by the number of molecules in each of those states, which are diverse in cells and vary with time. As a result, the auto-correlation analysis is disturbed (Fig. 7-8(d)). However, the fluorescent intensity time trace contains a wealth of information that auto-correlation cannot fully extract, so other data processing analysis methods for fluorescent intensity time traces are expected to extract more information from a long time trace. The proposed method should be able to efficiently analyze the frequency and waist width of the fluorescent intensity bursts and be developed with proper models for various applications. For instance, power spectrum [271] is the Fourier transform of an ACF and it can give the strength of the variations as a function of frequency, and thus is possible to offer exact solution for the population of the intensity bursts as a function of their waist widths. However, power spectrum analysis requires a complete ACF calculated point-to-point over a broad time scale, which is a significantly time-consuming work for a common commercial personal computer. In contrast, the simple correlation analysis in FCS only needs a limited number of points (770 points from 12.5 μ s to 10 seconds for each curve in Fig. 7-8(d)) on an ACF.

7.5 CONCLUSIONS

We show the ability of the SFCM for multiparameter fluorescence imaging for single cells and the technique was applied to single living GFP-XPC expressing XP4PA cells for the study of the interactions between DNA repair protein GFP-XPC and DNA in cell nuclei. Fluorescent intensity imaging and fluorescent spectral imaging revealed that GFP-XPC proteins only present in cell nuclei and the light spots out of the cell nuclei are mostly autofluorescence. SFA imaging exhibits a promising future for the study of the molecular activities in cells, and in this work, a non-uniform distribution of SFA of GFP-XPC proteins in cell nuclei after UV irradiation seems to be able to be related to the GFP-XPC local interaction with the DNA at damage site. It was found that fluorescent label should be carefully chosen and prepared to optimize the SFA imaging in cells. The limitations of FCS on cells are presented, and other potential data analysis methods are suggested for the fluorescent intensity time trace analysis.

Chapter 8

Nanometer Single-molecule Colocalization of Nucleotide Excision Repair Protein complexes XPC/Alexa488-HR23B Binding to Cy5 Labeled DNA

ABSTRACT

In cells, a variety of DNA repair pathways maintain genome stability to prevent oncogenesis. Nucleotide excision repair (NER) in eukaryotes is a versatile DNA repair system capable of removing a wide range of DNA lesions that distort one strand of the DNA double helix. The xeroderma pigmentosum group C (XPC) protein, which is in complex with HR23B in vivo, has a central role in recognizing lesions and initiating global-genome NER. Here we present a nanometer-precision single-molecule colocalization (NSC) technique in total internal reflection fluorescence (TIRF) microscopy to study the binding of Alexa Fluor 488 dye (Alexa488) labeled XPC/HR23B to Cy5 dye labeled damaged and undamaged double-stranded DNA substrates. NSC analyses of TIRF images taken from DNA bound by XPC/HR23B reveal that XPC/HR23B bound to DNA is more flexible than XPC/HR23B aspecifically bound to coverslip surfaces. Furthermore, we found that DNA bound by XPC/HR23B had a shorter length than free DNA molecules, indicating that XPC/HR23B binding induces a conformation change in DNA. XPC/HR23B complexes were found to bind with an average experimental accuracy of ~6 nm near the artificial damage site in DNA.

CHAPTER

8

8.1 INTRODUCTION

Nucleotide excision repair (NER) is a particularly important DNA repair mechanism by which the cell can prevent unwanted mutations by removing the vast majority of UV-induced DNA damage, mostly in the form of cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4PP) [1]. Inborn genetic mutations of NER proteins can result in several photosensitive and/or cancer-prone human diseases such as xeroderma pigmentosum and cockayne's syndrome [18]. NER can be divided into two subpathways – global genomic (GG-NER) and transcription coupled NER (TC-NER) that differ only in their recognition of helix-distorting DNA damage [23]. In TC-NER, the elongating RNA Polymerase II was found stalled at the site of a lesion and serving as the damage recognition signal [36–38]. In GG-NER xeroderma pigmentosum group C (XPC) protein plays a central role in recognizing lesions and recruiting other NER proteins to start the GG-NER machine [218]. Recognition of the distortions in the DNA double helix leads to the removal of a short single-stranded DNA segment that includes the lesion, creating a single-strand gap in the DNA, and subsequent resynthesis of the gap by DNA polymerase by using the undamaged strand as a template [2, 23].

XPC protein accumulates to DNA damage sites *in vivo* [42], initiating GG-NER, and is required for the subsequent accumulation of the other NER proteins [42]. XPC protein exists *in vivo* as a heterotrimeric complex with one of the two mammalian homologs of *S. cerevisiae* Rad23p (HR23A or HR23B) [43] and centrin 2 [44, 45]. XPC/HR23B complex was found to be sufficient for the complementation of XPC deficiency in a cell-free repair reaction [43] and reconstitution of the GG-NER reaction *in vitro* [272, 273]. Furthermore, purified XPC/HR23B complex was found to be the first factor for initiation of GG-NER *in vitro* [26] and to bind with high specificity and affinity to a variety of DNA lesions and especially to the local helix distortion including improper base pairing [26, 40, 218, 274], but it is also found not to be sufficient to complete NER progress [218]. XPC/HR23B complex binding to DNA damage locally melts the DNA duplex and potentially promotes recruitment of other NER proteins [51]. Single-molecule imaging by scanning force microscopy revealed that XPC-HR23B complex binds preferentially to the damage site on DNA molecules and induces a bend in DNA after binding [243, 244].

In order to study the molecular mechanism of XPC/HR23B complex binding to DNA, we fluorescently labeled XPC/HR23B complex and DNA substrate, separately, and employed fluorescence single-molecule spectroscopy to image the binding of XPC/HR23B to DNA. According to the classical diffraction optics theory [180], the resolution in light microscopy, *i.e.*, the two closest points that can be distinctly discriminated, is ~200 nm. Therefore, direct observation and precise detection of single-molecules in biological complex remains challenging using light microscopy.

The resolution can be improved by alternative technical approaches. For example, scanning electron microscopy [275-277], transmission electron microscopy [278, 279], scanning tunneling microscopy (STM) [280-282], near-field scanning optical microscopy (NSOM) [186, 283] and atomic force microscopy (AFM) [284-286] have been used to image nanostructure materials. However, all these techniques have their own limitations for use in biomolecular applications. The greatest obstacle to examine biological materials using electron microscopy is the non-physiological conditions to which specimens must be exposed, such as drying to be used in a very high vacuum. STM requires conductive or rigid surfaces of biomolecules and it relies on the quantum mechanics property of the molecules to generate an image. In NSOM, the resolution and sensitivity of a near-field microscope strongly depend on the quality of the probe aperture. For AFM, measurements of the lateral shape and size of the molecules is often limited by AFM tip size, while determination of the height of the molecules is relatively reliable. Particularly for kinetics study of biomolecular systems, these techniques often suffer from poor temporal resolution due to the use of the scanning strategy. It is difficult or sometimes impossible with those methods to investigate dynamic complexes under native biomolecular conditions and with chemical specificity. In this chapter we will show that far field fluorescence microscopy does allow a combination of these requirements with molecular nanometric localization precision.

Nanometer-precision single-molecule localization (NSL) in fluorescent single-molecule image is powerful technique to study biological systems at nanometer scale. Classical diffraction optics theory [180] predicts that the point spread function of an ideal wide-field circular optical system is an Airy disk pattern, but in practice it is more tractable to be fitted to a two-dimensional (2D) Gaussian with established relationship between the parameters of both functions as in Chapters 3-4. If a sufficient number of photons are collected from a single fluorescent molecule that is being imaged, fitting the image to a 2D Gaussian function yields nanometer-precision localization of the centroid position as in Chapters 3-4 or Ref. [165, 167, 169, 189]. As disclosed by all experimental and theoretical studies [165, 167, 169, 189], >10-nm localization accuracy, which was the standard deviation of the resulting centroid coordinates of a fluorescent molecule, can be achieved under normal experimental conditions by this technique. It follows that this technique can bridge the gap between near- or far field imaging and fluorescence resonance energy transfer (FRET), since FRET is limited to the applications for measuring distance change of < 10 nm between two fluorescent molecules [287]. The NSL technique has been extensively used to study the stepping mechanism of molecular motors [288], such as myosin V [169], kinesin [189], myosin VI [168, 289], dynein [290]. In these studies a single molecule was tracked in time sequence imaging. Here we present the nanometer-precision single-molecule colocalization (NSC) technique was developed to measure

distances between multiple single-molecules residing within a diffraction-limited spot. This technique requires spatially overlapping single-molecule images to be distinguishable either spectrally [173] or by sequential photobleaching [172] of each individual fluorescent molecule. The NSC technique has been applied before to study the stepping mechanism of myosin V [291, 292] and rapidly map DNA [170, 172].

In this work, XPC/HR23B complexes and DNA substrates were labeled with Alexa Fluor 488 dye (Alexa488) and Cy5 dye, respectively. Their binding reaction products were specifically immobilized onto protein-resistant coverslip surfaces. We performed single-molecule imaging using a home-built total internal reflection fluorescence (TIRF) microscope to study the confined diffusion of molecules in single binding complexes. The resulting TIRF images were analyzed using the NSL and NSC technique to resolve, the positions of the dyes fused to the protein and DNA molecules. The results enable the determination of the location of the protein on the DNA template and the change in the length of DNA upon protein binding.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Biotinylated polyethylene glycol amine (biotin-PEG-NH₂; product No. 12141-0895), CH₃O-PEG-NH₂ (product No.12-750-2) and (3-aminopropyl)dimethylethoxysilane (APDMES; product No. SIA0603.0) were purchased from PolyPure (Oslo, Norway), Rapp Polymere (Tübingen, Germany) and Gelest (Morrisville, PA), respectively. Poly(acrylic acid) sodium salts (PAA; average molecular mass, 15 000; product No. 416037) were purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin (product No. S888) was purchased from Invitrogen (Carlsbad, CA). Milli-Q water had a resistance of 18 MΩ and was further purified by filtration over a 0.2 μm filter. High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) and used in all buffers. Microscope immersion oil (product No.16212) used was obtained from Cargille Labs (Cedar Grove, NJ).

8.2.2 Preparation of XPC/Alexa488-HR23B

XPC/Alexa488-HR23B was prepared as described in section 6.2.2.3 in Chapter 6.

8.2.3 Preparation of DNA substrates

Two DNA substrates were used in the DNA-protein binding (DPB) assays in this

- (a) F-5'-TCCCCCGGGCTGCACTCATCAACATCCTTGTTCCTCACAATCAATAAGCTTATC-3' - biotin
AGGGGGCCCGACGTGAGTAGTTGTAGGAACAAGGAAGTGTAGTTATTGCAATAG
- (b) F-5'-TCCCCCGGGCTGCACTCATCAACATCC^{TGT}CCTTCACAATCAATAAGCTTATC-3' - biotin
AGGGGGCCCGACGTGAGTAGTTAGG^{TGT}GGAAGTGTAGTTATTGCAATAG

Fig. 8-1 DNA substrates. (a) Cy5-dsDNA: Cy5 dye labeled 55 bps dsDNA substrate; (b) Cy5-B5-DNA: Cy5 dye labeled 55-bp dsDNA containing a 5 unpaired bps bubble. F represents the fluorescent label – Cy5 dye attached to a 5'-end of DNA. The 3'-end of all DNA substrates is labeled with a biotin.

study as shown in Fig. 8-1. Cy5 dye labeled 55 base pairs (bps) double-stranded DNA (Cy5-dsDNA) was made by annealing equimolar amounts of the commercial 5' Cy5 dye labeled 55-mer single-stranded oligonucleotide (Cy5-55-mer-ssDNA) to its complementary strand. The Cy5 dye labeled 55-bp double-stranded DNA containing a 5 unpaired bps bubble (Cy5-B5-DNA) was prepared by hybridizing equimolar amounts of Cy5-55-mer-ssDNA and its complementary strand containing five unpaired bases.

All oligonucleotide products were purified using reverse phase high performance liquid chromatography (RP-HPLC). Production of the DNA duplexes was checked by 10% polyacrylamide (PAA) gel electrophoresis (data not shown) and found to be in accordance with expectation.

8.2.4 DNA-protein binding reaction

DNA and XPC/Alexa488-HR23B binding reactions were performed at 30°C for one hour in a binding reaction buffer (10 mM Tris-HCl, pH = 7.4, 300 mM NaCl, 0.02% Triton X-100), of which 20 μ l contained 4 nM DNA substrate and 25 nM XPC/Alexa488-HR23B.

8.2.5 Streptavidin functionalized protein-resistant surface

Normal coverslip surface has natural negative charges and plenty of silanol groups and therefore are very sticky to many soluble proteins and especially for DNA repair protein XPC that has a basal isoelectric point of 9.54. Fig. 8-2 represents the schematic experimental strategy of molecule immobilization on coverslip surfaces for the TIRF imaging of XPC/Alexa488-HR23B complexes and Cy5 labeled DNA molecules after binding reactions. To prevent aspecific and excessive single molecule surface adsorption, coverslip surfaces were pre-treated using a monolayer of CH₃O-PEG with inlaid sparse streptavidin-biotin-PEG groups to immobilize biotin-labeled DNA molecules, as well as to pacify the remaining surface.

Pretreatment was according to a method based on the protocol of Wazawa et al. [193] and described in Chapter 4. Briefly, glass coverslips (24 mm $\times$ 60 mm $\times$ 0.17 mm) were first cleaned by dipping into 65% nitric acid for more than one day, washed using milli-Q water, and dried using N₂ gas. Next, the coverslips were sequentially treated using 2% APDMES in ethanol, 0.01 mM PAA, and 1 mM CH₃O-PEG-NH₂ in the presence of 0.01 mM biotin-PEG-NH₂. As a result, the coverslips were coated with multiple layers of APDMES/PAA/CH₃O-PEG&biotin-PEG. Finally, the coverslips were carefully rinsed and dried again for temporary storage in a cleaned desiccator at room temperature (20-22°C). Before use, a biotin functionalized surface coverslip was treated with 0.2 mg/ml streptavidin in 25 mM Tris-HCl buffer (pH = 7.5) for 15 minutes, and again carefully rinsed and dried.

Fig. 8-2 Representation of the experimental strategy of the NSC technique to study the binding of XPC/Alexa488-HR23B to Cy5 labeled DNA substrate. (a) One end of the DNA molecule was labeled with biotin and immobilized on a streptavidin functionalized protein-resistant coverslip surface, while the other end was labeled with a Cy5 dye. DsDNA has a persistence length of 50-nm, and thus the 55-bp dsDNA can be considered to be a rigid rod with a length of ~18 nm. The Cy5 dye can freely diffuse in solution in a semispherical space with a radius R_D that equals to the end-to-end length of the DNA molecule. The positions of the Cy5 dye was determined by the NSC technique and found to be distributed in a circular area (diffusion area) originating at the position of the immobilized end of the DNA molecule. The diffusion area center was obtained by averaging all positions of the Cy5 dye and the distance (DtA, for distance to average) from each position to the diffusion area center was measured. (b) XPC/Alexa488-HR23B binds to the DNA molecule. Depending on the position R_P of the binding site on the DNA molecule, the diffusion area of the XPC/Alexa488-HR23B complex is different from that of the DNA molecule. (c) An alternative model for Fig. 8-2(b) – the DNA molecule is bent as a result of XPC/Alexa488-HR23B binding.

8.2.6 TIRF microscope

Single-molecule imaging was performed using a home-built prism-based TIRF microscope as schematically depicted in Fig. 8-3 with some technical modifications based on the previous configuration described in Chapter 4. It was constructed on an optical table (Newport, Irvine, CA) on the basis of a Zeiss Axiovert 135 TV microscope (Oberkochen, Germany) equipped with a 100× water immersion (numerical aperture 1.20, Leica, Wetzlar, Germany) objective. An Ar⁺ laser (Coherent, Innova 70, Santa Clara, CA) was used to supply 488 nm laser to excite Alexa488 dyes

Fig. 8-3 Schematic diagram of TIRF microscope: (1) Ar⁺/Kr⁺ Laser (single wavelength 488 nm), (2) Ar⁺/Kr⁺ Laser (single wavelength 647 nm), (3) shutters, (4) attenuators, (5) optical fiber couplers, (6) 488 nm single-mode optical fiber with a 50/50 fused fiber coupler, (7) beam collimator, (8) lens, (9) BK7 glass prism, (10) microscope translation stage, (11) objective, (12) tube lens, (13) rotatable mirror, (14) holographic notch filter for 488 nm laser, (15) holographic notch filter for 647 nm laser, (16) bandpass filter HQ525/50, (17) bandpass filter FF01-695/75, (18) Andor EMCCD DU-897, (19) laser stop, (20) beam collimator for (21) Epi-illumination, (22) top coverslip – streptavidin functionalized coverslip, and (23) bottom coverslip – a cleaned coverslip, (24) 76×26 mm microscope side (thickness 1 mm).

and the other Ar⁺/Kr⁺ mixed gas laser (Coherent, Innova 70 spectrum, Santa Clara, CA) to supply 647 nm laser for the excitation of Cy5 dyes. Both lasers were working in single wavelength mode. In front of each laser source, a shutter was installed and automatically controlled to avoid unnecessary excitation. Both laser lines were separately coupled to two input ends of a 488 nm single mode 50/50 optical fiber coupler (FFS-B88K61XX3-SFO953, SIFAM, UK); the output laser light through another end of the fiber coupler was routed through a beam collimator and a convex lens, and finally focused into the prism at the back focal plane of the objective. The

excitation powers for 488 nm and 647 nm lasers in front of the prism were 3 mW and 6 mW, respectively, and the laser spot size was an elliptical shape of approximate $50\ \mu\text{m} \times 100\ \mu\text{m}$. In the fluorescence detection path, two holographic notch filter (Kaiser Optical Systems) were used to attenuate the Rayleigh scattering of the excitation beam. Two optical bandpass filters, HQ525/50 (Chroma Technology, Rockingham, VT) and FF01-695/75 (Semrock, Rochester, NY) were separately used for passing the fluorescence emission detection of Alexa488 dye or Cy5 dye, respectively, in front of a back illuminated EMCCD camera (DU-897; 512×512 pixels; pixel size $16 \times 16\ \mu\text{m}^2$; single photon sensitivity > 90% quantum efficiency; Andor, South Windsor, CT) cooled to $-85\ ^\circ\text{C}$. Andor Solis software was used for camera control and image acquisition platform. Baseline clamping was used to ensure stable background baseline value across a kinetic series of images. The EM gain was fixed to 20 in this work. A red light source on top of the prism provided epi-illumination of the excitation area. A foldable mirror could be inserted in the detection path to guide the image signal to a video camera for rough alignment of objective focal plane.

In all experimental measurements, laser excitation at 647 nm in combination with the use of the FF01-695/75 bandpass filter in front of the EMCCD camera was first used to excite and detect Cy5 dye labeled DNA molecules, and next laser excitation at 488 nm and the HQ525/50 bandpass filter were used for exciting and imaging XPC/Alexa488-HR23B. The FF01-695/75 bandpass filter was removed from the fluorescent detection path after finishing excitation with 647 nm laser beam.

CHAPTER

8

8.2.7 Sample preparation for microscopic measurements

Binding reaction product was first $10\times$ diluted in the binding reaction buffer. $\sim 100\ \mu\text{l}$ of resulting solution (0.4 nM DNA and 2.5 nM XPC/Alexa488-HR23B) was applied to a streptavidin functionalized surface coverslip for about half an hour. The coverslip surface was then washed using $\sim 200\ \mu\text{l}$ of binding reaction buffer and carefully placed on a microscope slide with the functionalized surface on top. In between the coverslip and the microscope slide, a droplet of microscope immersion oil was applied. $\sim 100\ \mu\text{l}$ of binding reaction buffer was added on the coverslip surface. Next, the coverslip was covered with another normal cleaned coverslip. Two pieces of double-sticky tapes (thickness $\sim 50\ \mu\text{m}$) were used in between both coverslips at the short edges to form a chamber for sample solution. Finally the sample was placed on the microscope translation stage with the glass slide on top to support the optical prism as shown in Fig. 8-3. A droplet of microscope immersion oil was applied in between the prism and the microscope side. Laser evanescent wave was created at the bottom of the top coverslip to excite fluorescently labeled molecules immobilized on the streptavidin functionalized surface.

8.2.8 Image acquisition and processing

As schematically depicted in Fig. 8-2, DNA molecules were fused on one end with a Cy5 dye, which could freely diffuse in solution. On the other end, DNA molecules were immobilized on the streptavidin functionalized coverslip surface through biotin binding. HR23B protein, which forms a dimer with the DNA binding XPC protein, was fluorescently labeled with a single Alexa488 dye. The positions of both Cy5 and Alexa488 dyes on the images can be determined. For each dye, a position was obtained from each different frame of TIRF images. The subsequent positions were projected in a 2D image, such as Fig. 8-6(a) and Fig. 8-9(a). The immobilization of DNA restricts the diffusion to a confined area, which is defined in Fig. 8-2. The centre of the diffusion area was obtained by averaging the centroid coordinates, and the distance (DtA, for “distance to average”) from each position to the centre of diffusion area was determined. For a single dsDNA molecule alone, if its total length is less than the ~50 nm persistence length of dsDNA [293], the DtAs are constrained by its end-to-end length [172, 291]. If the Cy5 labeled DNA molecule and the XPC/Alexa488-HR23B complex binds together, they appear in two chromatically separated TIRF images in which their centroids were colocalized and mapped to the same pixel.

The images from the Andor EMCCD camera were acquired with the Andor Solis software. The projected pixel size on the camera was determined to be $100 \text{ nm} \times 100 \text{ nm}$ by imaging a standard optical grating with known feature size. The image acquisition time was set to 0.2 seconds, and for multiple kinetic frames of TIRF imaging, each frame was acquired sequentially with a time interval of 2 seconds. In this work, for each dye, 100 frames of TIRF images were sequentially acquired in a field of view (FOV) during 200 seconds to generate a TIRF movie.

A custom Matlab (The Mathworks, Natick, MA) program was used to read and display TIRF movies, plot fluorescent intensity time trace curves at given pixels, indentify single fluorophores and (co-)localize the positions of a single fluorophore one frame by one frame. Single point fluorescent intensity time trace was measured for the molecule of interest at the pixel where the fluorescent emission intensity showed maximum amplitude in the first frame.

For NSL, a small square region of interest (SROI, typically 30×30 pixels) where only one bright spot appeared as close to the center as possible was chosen for all frames. A threshold was set for each TIRF image to be around 1/3 of the maximum pixel intensity of all frames to exclude the frames in which the fluorophore was photobleached or few photons were collected. NSL was only performed for frames with peak pixel intensity above the threshold. In NSL, the positions of a molecule in a TIRF movie were first determined by fitting the SROIs in all frames to 2D Gaussian profile using the full least squares fitting (FLSF) as described in Chapter 3, and then averaged to determine the diffusion area center as defined in Fig. 8-2. The theoretical

localization accuracy was determined by using the analytical expression, *i.e.*, Eq. (3-5), for the FLSF in Chapter 3.

For the NSC (colocalization) of the Cy5 and Alexa488 labels in single binding complexes, the program can automate to locate the peak intensity pixels (PIPs) in the first frames of both chromatically separated TIRF movies obtained in the same FOV. If two PIPs are found at the same position in both Cy5- and Alexa488 TIRF images, the corresponding molecules can be considered to be a molecule pair (DNA-protein pair, DPP) in a binding complex. NSL was separately performed on each molecule in a DPP in both Cy5- and Alexa488 TIRF movies. Consequently the diffusion area centers were determined for each dye in the DPP, and the distance between the diffusion area centers of the two dyes was then calculated by using the distance determination method as previously used in Chapter 4 to further confirm the colocalization of a DPP. In addition the DtAs for both dyes in each DPP of interest were measured to investigate the binding properties of XPC/Alexa488-HR23B complex to DNA substrate.

8.3 RESULTS

8.3.1 Single-molecule TIRF imaging

The coverslip surface used for TIRF imaging was coated with a monolayer of streptavidin-biotin-PEG and CH₃O-PEG to specifically immobilize DNA tagged with biotin and to increase the biocompatibility of glass coverslip surface, which is important to minimize the non-specific surface adsorption effect on the DNA-protein interaction system. As shown in Fig. 8-4 and Fig. 8-7, the resulting coverslip surfaces markedly prevented protein adhesion since only a sparse distribution of XPC/Alexa488-HR23B complexes were observed. Complexes appeared as single light spots distributed randomly on the surfaces, which perhaps caused by ineluctable non-uniformed defects of the CH₃O-PEG layer in the surface preparation. Similar observations were also made using Cy3 dye labeled positively charged protein – lysozyme (Isoelectric point pI = 11.1) using the same coverslip surfaces [193].

The representative experimental results for the binding product of XPC/Alexa488-HR23B to Cy5-dsDNA are given in Fig. 8-4 to Fig. 8-6, and for the binding product of XPC/Alexa488-HR23B to Cy5-B5-DNA are given in Fig. 8-7 to Fig. 8-10.

8.3.2 Photostability of Cy5 and Alexa488 dyes for NSL

To visualize DNA and XPC/HR23B, we made use of Cy5 and Alexa488 as the fluorescent tracers, respectively. Although we did not use the oxygen-scavenging system as previously reported for imaging Cy3 dyes [169, 172, 189, 294], both the Cy5 and Alexa488 dyes exhibited excellent photostability as shown in Fig. 8-5 and Fig. 8-8. Both dyes could be imaged for a few minutes under non-saturating illumination conditions. In a TIRF movie, more than 50 frames per molecule could be

acquired with an average of ~ 8000 counts per frame, which is sufficient for theoretical localization accuracy better than 3 nm with the background noise standard deviation of 7 counts per frame as previously studied in Chapters 3-4.

8.3.3 XPC/Alexa488-HR23B and Cy5-dsDNA

XPC/Alexa488-HR23B complex was mixed with a solution of undamaged DNA substrate Cy5-dsDNA (see Materials and methods). The TIRF images for the binding reaction products hardly showed any reaction binding product – DPPs as expected from the low affinity of XPC/HR23B for undamaged DNA. Two representative TIRF images of the XPC/Alexa488-HR23B complexes and the Cy5-dsDNAs separately in binding reaction solution are shown in Fig. 8-4. Both images in Fig. 8-4 were the first frames of two TIRF movies which were obtained in the same FOV in the TIRF microscope. Three fluorescent molecules were labeled in Fig. 8-4(a) and Fig. 8-4(b) as shown in the figures and their fluorescent intensity time traces were determined (Fig. 8-5). For comparison, two TIRF movies were separately obtained in the same field of view of Fig. 8-4 without laser excitation. Two background intensity time traces were obtained at two arbitrary pixels from two movies and are plotted (squares on lines) in Fig. 8-5(a) and Fig. 8-5(b). In Fig. 8-5(b), when the Cy5 dyes photobleached, fluorescent intensity dropped to the background level, showing that the background noise from the laser excitation was extremely low during TIRF imaging.

Fig. 8-4 Representative TIRF images of the binding reaction product of XPC/Alexa488-HR23B (a) and Cy5-dsDNA (b). The average excitation intensity was $\sim 0.4 \text{ kW/cm}^2$ for the 488 nm laser and $\sim 0.8 \text{ kW/cm}^2$ for the 647 nm laser. Image size is $50 \times 50 \text{ } \mu\text{m}^2$, 512×512 pixels. The image acquisition time was 200 ms/frame with 1.8 seconds delay between two frames. A total of 100 frames were acquired in the same FOV for XPC/Alexa488-HR23B and Cy5-dsDNA, each of which the first frames are separately shown in Fig. 8-5 (a) and Fig. 8-5(b). As shown in the figures, no DNA-protein pair was found. 6 molecules were separately labeled, of which intensity time traces are shown in Fig. 8-4.

Fig. 8-5 Fluorescent intensity time traces measured at the pixels of 6 molecules labeled in Fig. 8-4 gave peak amplitudes of fluorescent emission in the first frames of images. As control, the laser excitation was blocked and two background intensity time traces (squares on lines) were measured at two arbitrary pixels of a TIRF images acquired in the same field of view for Fig. 8-4.

Next, NSL analysis was performed on the single-molecules labeled in Fig. 8-4(a) and Fig. 8-4(b). The average total number of counts of the Cy5-dsDNA molecule labeled R3 was found to be 10,281 counts per frame. The average background noise standard deviation was 7 counts per frame in the NSL. However, experimental localization accuracy was 13 nm, which is much larger than the theoretically expected localization accuracy of 2 nm. Probably, this difference results from the confined diffusion of Cy5-dsDNA as schematically depicted in Fig. 8-2(a). The positions of molecule R3 in the first 50 frames of images were resolved in the NSL and all centroid coordinates were averaged to determine the centre of the diffusion area. The position diagram and the histogram of the DtAs of molecule R3 are shown in Fig.

Fig. 8-6 (a) The distances (DtAs) of the Cy5 dyes on the single Cy5-dsDNA molecule R3 in Fig. 8-4 to its diffusion area center (see Fig. 8-2) determined by averaging all centroid coordinates of the Cy5 dyes in NSL. The dashed line circle originating at the origin of the coordinates has a radius of the average DtA = 16 nm. (b) The histogram of the DtAs in Fig. 8-6(a). (c) The average DtAs of the fluorophores labeled in Fig. 8-4. Minus error bars were ignored for clarity.

8-6(a) and Fig. 8-6(b), respectively. The average DtA of the Cy5 dye attached on the single Cy5-dsDNA molecule was found to be 16 ± 8 nm, which is very close to the length of ~ 18 nm for a 55-bp dsDNA. The same analysis was performed on all six fluorophores images labeled in Fig. 8-4, and the average DtAs of all Cy5 and Alexa488 labels were plotted in Fig. 8-6(c).

As shown in Fig. 8-6(c), the average DtAs for the Alexa488 dyes (G1-G3 in Fig. 8-4(a)) on the XPC/Alexa488-HR23B complexes were confined within 5 nm, which indicated that the XPC/Alexa488-HR23B complexes were non-specifically immobilized on the surface by local surface adsorption. In contrast, Fig. 8-6(c) shows that the average DtAs for the Cy5 dyes (R1-R3 in Fig. 8-4(b)) labeled on the Cy5-dsDNA molecules are much larger, and as expected they are very close to the length of ~ 18 nm for a 55-bp dsDNA.

8.3.4 XPC/Alexa488-HR23B and Cy5-B5-DNA

To visualize XPC/Alexa488-HR23B binding to damaged DNA, we mixed the XPC/Alexa488-HR23B solution with a solution of Cy5-B5-DNA. Fig. 8-7 shows two representative TIRF images which are the first frames in two TIRF movies made from the same FOV. In Fig. 8-7(a), the image of XPC/Alexa488-HR23B is presented and in Fig. 8-7(b) the image of Cy5-B5-DNA. Twenty fluorescent events in Fig. 8-7 were labeled as shown in the figures, of which seven coincident XPC/Alexa488-HR23B and Cy5-B5-DNA pairs are labeled G1 to G7 and R1 to R7, respectively. The fluorescent intensity time traces of three Alexa488 molecules and three Cy5 molecules are presented in Fig. 8-8(a) and Fig. 8-8(b), respectively.

NSL analyses were performed separately for the Cy5 molecule and Alexa488 molecule in each DPP. For example, in the NSL analyses for the DPP R1-G1 in Fig. 8-7, average total numbers of counts per frame were determined to be 9,593 for the Cy5 dye and 9184 for the Alexa488 dye, respectively. The average background noise standard deviations were 8 counts for the Cy5 dye image and 7 counts for the Alexa488 dye image, respectively. The experimental localization accuracies were 9 nm for the Cy5 dye and 6 nm for the Alexa488 dye, which are much larger than the theoretically expected localization accuracy of 2 nm for both dyes. We hypothesize that this difference results from the confined diffusion of the two dyes in the binding complex as illustrated in Fig. 8-2(b) or in Fig. 8-2(c). For the R1-G1 pair, the positions in the first 50 frames of the Cy5 dye images and in the total 100 frames of the Alexa488 dye images were resolved, after which the diffusion area center for each dye was determined. Fig. 8-9(a) shows the position diagram for both fluorescent molecules. The histograms of the DtAs of the Cy5 and Alexa488 molecules are shown in Fig. 8-9(b) and Fig. 8-9(c), respectively. As shown in Fig. 8-9(a), the diffusion area centers of both dye molecules in the DPP G1-R1 were found at different positions. For all other six DPPs, the position diagrams are presented in Fig. 8-10. Also for the

two fluorescent molecules in these DPPs, it was observed that the diffusion area centers were in different positions (Fig. 8-10). The distance between the diffusion area

Fig. 8-7 Representative TIRF images of the binding reaction product of XPC/Alexa488-HR23B (a) and Cy5-B5 DNA (b) were obtained in the same FOV. The experimental conditions and the image dimensions are the same as those for Fig. 8-4. Seven pairs (DPPs) of XPC/Alexa488-HR23B and Cy5-B5 molecules, from G1-R1 to G7-R7 as labeled in the figures, colocalized within a few nanometer distances as shown in Fig. 8-7(e), which indicates that the XPC/Alexa488-HR23B complexes were bound to the Cy5-B5 molecules. In Fig. 8-7(e), minus error bars were ignored for clarity. Fig. 8-7(c) and Fig. 8-7(d) show the expanded images of the corresponding areas marked in Fig. 8-7(a) and Fig. 8-7(b), respectively. Six uncoupled molecules are labeled G8-10 in Fig. 8-7(a) and R8-10 in Fig. 8-7(b) for comparison in the NSL analysis.

Fig. 8-8 Fluorescent intensity time traces measured at the pixels of 6 molecules in Fig. 8-7 gave peak amplitudes of fluorescent emission in the first frames of images.

centers of the two dye molecules in each DPP in Fig. 8-7 was determined by using the method described in the section “Image acquisition and processing”, and the results are presented in Fig. 8-7(e). For the DPP G1-R1, the distance between the diffusion area centers of both dye molecules is determined to be 4 nm. Furthermore, Fig. 8-7(e) shows that the two dye molecules in DPPs colocalized within <12 nm.

In Fig. 8-9(a), it shows that the average DtA of the Alexa488 dye was 8 ± 4 nm, which suggest that the XPC/Alexa488-HR23B complex binds at the middle of the DNA molecule where an artificial damage site existed. Similar results were also obtained for the other Alexa488 dyes (G1-G7) in the DPPs. As shown in Fig. 8-9(d), the average DtAs for the XPC/Alexa488-HR23B complexes (G1-G7) bound to Cy5-B5-DNA were in the range of 8-11 nm, which is observably larger than those of non-specifically adsorbed XPC/Alexa488-HR23B complexes (G8-G10). Probably the mobility of the XPC/Alexa488-HR23B complexes (G1-G7) bound to DNA increased strongly with respect to non-specifically adsorbed XPC/Alexa488-HR23B complexes (G8-G10). We hypothesize that the increase in mobility reflects the binding of XPC/Alexa488-HR23B to the damage site in the middle of Cy5-B5-DNA.

In addition to the DtAs of Alexa488, Fig. 8-9(a) shows the DtAs of the Cy5 dye in the DPP G1-R1. We found that the average DtA was 12 ± 6 nm, which is noticeably less than the average DtAs of free, unbound Cy5 dyes on Cy5-dsDNA (R1-R3 in Fig. 8-6(c)) and Cy5-B5-DNA (R8-R10 in Fig. 8-9(e)). Similar results were obtained for all seven Cy5-B5-DNA molecules, R1-R7, which possibly reflects shortening of DNA when XPC/Alexa488-HR23B binds to it. This suggests that XPC/HR23B binding to DNA induces a conformational change that shortens the length of the DNA.

Fig. 8-9 (a) The DtAs of the Cy5 dye (red) and the Alexa488 dye (green) in the DPP R1-G1 formed by the single Cy5-B5 DNA molecule R1 and the single XPC/Alexa488-HR23B complex G1 in Fig. 8-7. The origin of coordinates is at the diffusion area center of the Cy5 dye. The red dashed line circle originating at origin of coordinates has a radius of average DtA = 12 nm for the Cy5 dye, and the green one for the Alexa488 dye has a radius of average DtA = 8 nm and originates at its diffusion area center. (b) The histogram of the DtAs for the Cy5 dye in Fig. 8-9(a). (c) The histogram of the DtAs of the Alexa488 dye in Fig. 8-9(b). (d-e) The average DtAs of the fluorophores of all 20 labeled molecules in Fig. 8-7. Minus error bars were ignored for clarity.

8.4 DISCUSSION

8.4.1 Photophysical properties of Cy5 and Alexa488 dyes

Single fluorophores have the natural signature of almost constant emission intensity terminating with abrupt photobleaching, if the fluorescent intensity acquisition time is much longer than their “blinking” period. The fluorescing time of a fluorophore depends on many molecular photophysical properties and external factors, such as excitation intensity and buffer composition (e.g., oxygen in solution for Cy3 dye as reported in Ref. [169, 172, 189, 294]). If a few fluorophores reside in a diffraction-limited fluorescent light spot, photobleaching of any one of the fluorophores gives a

Fig. 8-10 The DtAs of the Cy5 dye (red) and the Alexa488 dye (green) in the DPPs, R2-G2 to R7-G7 shown in Fig. 8-7, are shown in Fig. 8-10(a) to Fig. 8-10(f), respectively. The origins of coordinates are at the diffusion area center of the Cy5 dye.

sudden step decrease in fluorescent intensity time trace on the spot, which has been used for localizing multiple single-molecules with nanometer-precision [172].

In the experiments depicted in Fig. 8-5(b) and Fig. 8-8(b), the Cy5 dyes were found to emit fluorescence for more than 1.7 minutes. Some molecules were even emitting throughout the entire imaging time of 3.3 minutes under continuous 647 nm laser excitation with an intensity of $\sim 0.8 \text{ kW/cm}^2$. Therefore, it was possible to obtain at least 50 frames of images in a FOV, which is sufficient for statistical analyses in the NSL. If the laser excitation was blocked during the delay in between two frames of images, single Cy5 dyes were found to fluoresce even longer. Importantly, almost all Cy5-dsDNA molecules in Fig. 8-4(b) and Fig. 8-7(b) were found to be single-molecules, possibly due to the use of streptavidin functionalized protein-resistant coverslip surfaces, the use of detergent Triton X-100 (see Chapter 2) and a very low concentration (0.4 nM) of DNA molecules in the binding reaction solutions.

Compared with the Cy5 dyes, the Alexa488 dyes showed even better photostability. As shown in Fig. 8-5(a) and Fig. 8-8(a), when the Alexa488 dyes were excited using a continuous 488 nm laser at excitation intensity of $\sim 0.4 \text{ kW/cm}^2$, fluorescence was observed for 3.3 minutes without photobleaching. Under the same conditions, we have observed the single molecules of Alexa488 dyes to fluoresce for more than 10 minutes. Although in the images (Fig. 8-4(a) and Fig. 8-7(b)) of XPC/Alexa488-HR23B complexes there was no photobleaching observed as signature of single-molecules, we believe that the fluorescent spots represent single-molecules because the original XPC/Alexa488-HR23B complex sample solution was verified to contain no protein aggregates under the same binding reaction condition as in Chapter 6. Low excitation powers were used throughout this work since more photons are obtained from individual molecules, albeit at a slower rate. The signal to noise ratio (SNR) of the single-molecule images was improved under these conditions.

8.4.2 Theoretical localization in NSL

NSL analysis of single Cy5 dyes demonstrated that thousands of counts can be collected from a single Cy5 dye in one frame of TIRF image, which easily offers 2-8 nm theoretical localization accuracy as previously studied in Chapters 3-4. For example, an average of 9593 counts and 3866 counts per frame were separately obtained from the two Cy5 dyes labeled R1 and R4 in Fig. 8-7(d), respectively, which lead to the theoretical localization accuracies of 2 nm and 4 nm, respectively. In the NSL, the standard deviation of the background noise (the fitting residues in the NSL) was 7 counts. Similar to the TIRF images of Cy5 labeled single DNA molecules, thousands of counts could be collected from a single Alexa488 dye in one image, readily yielding 2-5 nm theoretical localization accuracy. For example, an average of 9184 counts and 4300 counts were separately obtained per frame from the two Alexa488 dyes labeled G1 and G3 in Fig. 8-7(c), respectively. From these counts, the theoretical localization accuracies were calculated to be 2 nm and 4 nm, respectively, with a background noise standard deviation of 7 counts.

8.4.3 DNA length measurement

As described previously for the DNA molecules which were not bound by XPC/Alexa488-HR23B, the average DtAs (see R1-R3 in Fig. 8-6(c) and R8-R10 in Fig. 8-9(e)) were found to be ~18 nm long using 55-bp dsDNA. This suggests that less than 150-bp dsDNA molecules can be generally used as length standard to verify the precision of distance determination using NSL microscopy because these short DNA molecules are considered to be rigid rods [293]. These results are in good agreement with previous studies [172, 291], and prove that we used the similar experimental conditions. However, as shown in Fig. 8-6(a), some positions of the Cy5 dye on the Cy5-dsDNA molecule R3 were found much farther away than 18 nm to its diffusion area centre, an observation which was previously reported [172, 291] as well. An explanation for this observation was provided by Churchman *et al.* [291]. Briefly, the localization accuracy of 2 nm still allows a small population of 6-10 nm errors between actual and determined centroid coordinates of the fluorophore under study. As shown in Fig. 8-2(a), when the DNA molecule is parallel to the coverslip surface and the Cy5 label lies on the circumference of the circle originating at its diffusion area centre, 24-28 nm or even longer DtA can result from a large localization error but with small probability. Another possible source for the larger DtA of the Cy5 label can result from a mobile diffusion area centre instead of a static one. As shown in Fig. 8-2, we assume that the Cy5-dsDNA molecule diffusion is confined around a static point, i.e., the diffusion area centre, where one end of the Cy5-dsDNA molecule is immobilized on the surface via biotin-streptavidin binding. However, the 52 kDa tetrameric protein, streptavidin, which size is less than 5 nm [295], forms a protomer which binds a biotin on the coverslip surface and is in connection with a biotin fused to the DNA molecule. Therefore, the diffusion area centre of the Cy5 label on the DNA molecule in Fig. 8-2 may not be an ideal point but vary in a small area as a function of the position of the Cy5 label. Consequently a DtA that is larger than the length of the DNA molecule is expected.

8.4.4 Binding model of protein XPC/Alexa488-XPC to DNA

The results (Fig. 8-7(e), Fig. 8-9(a) and Fig. 8-10) from the XPC/Alexa488-HR23B and Cy5-B5-DNA binding reaction products show that the centers of the diffusion areas of the two dyes in each DPP do not coincide. This reflects their different positions in the complexes as well as a difference in the radial diffusion rotation axes. This could be caused by a bending in the DNA molecule as shown in Fig. 8-2(c). We concluded that NSC reveals that XPC binding to DNA induces a conformational change, which is in line with the previous studies by using combined FCS and fluorescence anisotropy techniques in Chapter 6 as well as by Janićijević *et al.* using scanning atomic force microscopy (AFM) [243]. This conclusion is further supported by the observation (Fig. 8-9(e)) that the average DtAs of the Cy5 dyes (R1-R7) in the

DPPs were shorter, suggesting that the binding of XPC to DNA shortens the end-to-end distance, possibly due to bending of the DNA molecule. More detailed studies will be necessary to further elucidate how DNA lesions are being recognized by XPC/HR23B and to investigate what exactly happens to DNA on a molecular scale after binding.

8.4.5 Binding degree of XPC/HR23B complex to DNA substrates

XPC/HR23B complex preferentially binds to damaged DNA substrates with a local helix distortion including improper base pairing as in Chapter 6. It was determined by FCS in Chapter 6 that the dissociation constants of XPC/HR23B complex bound to Alexa488 labeled 55-bp undamaged Alexa488-dsDNA and damaged Alexa488-B5-DNA are determined to be 2.2 nM and 0.8 nM, respectively. Only a small population of the bound complexes were found in the TIRF images for the binding reaction products of XPC/Alexa488-HR23B complex and Cy5-B5. This agrees with the observation by the Jančićević *et al* for the binding reaction solution of XPC/HR23B complex and damaged DNA containing a defined cholesterol lesion. This can be interpreted by the specific binding properties of XPC/HR23B complex to DNA. According to the one-site binding model used in Chapter 6, the binding degrees θ_D and θ_P of protein and DNA in DPB reaction are defined to be

$$\theta_D = \frac{[B]}{[D_T]}; \theta_P = \frac{[B]}{[P_T]} \quad (8-1)$$

where $[B]$, $[D_T]$ and $[P_T]$ represent the total concentrations of DPB complex, fluorescently labeled DNA and protein, respectively. K is dissociation constant. The DNA-protein complex concentration $[B]$ can be evaluated by

$$[B] = \frac{1}{2} \left([D_T] + [P_T] + K - \sqrt{([D_T] + [P_T] + K)^2 - 4[D_T][P_T]} \right) \quad (8-2)$$

In this work, $[D_T] = 4$ nM and $[P_T] = 25$ nM in the initial binding reaction solution and thus we can expect $\theta_D = 96\%$ and $\theta_P = 15\%$. Before the molecule immobilization onto the coverslip surface, $10\times$ dilution of the binding reaction product will result in $\theta_D = 73\%$ and $\theta_P = 12\%$, respectively. However, after the incubation of a $10\times$ diluted binding reaction solution on the coverslip for molecule immobilization, the solution in between two coverslips was replaced with the binding reaction to avoid the too much free fluorescent molecules contributing to the background noise, and thus the resulting solution suffered from even more low concentrations of both protein and DNA molecules and thus potentially much lower binding degree on the coverslip surface.

8.5 CONCLUSIONS

We have used the NSL technique in TIRF microscopy for the study of NER protein XPC/HR23B binding to damaged and undamaged dsDNA substrates. The DNA molecules, which were not bound by XPC/Alexa488-HR23B, were found to have an average DtA which was in very good agreement with the expected end-to-end length

of the DNA. This is also consistent with previous experimental reports [172, 291]. Due to the extremely low concentrations of XPC/Alexa488-HR23B and Cy5 labeled DNA used in this work, we found hardly any binding of XPC/Alexa488-HR23B to undamaged DNA and only a small population of XPC/Alexa488-HR23B bound to damaged DNA substrate. In the binding complexes of XPC/Alexa488-HR23B to DNA, the diffusion areas of both dyes in each binding complex were found to be centered at different positions, while the average DtAs of the Cy5 dyes were much shorter than the expected end-to-end length of a 55-bp dsDNA molecule. This suggests that the binding of XPC/HR23B induces bending of the DNA molecule. Furthermore, the average DtAs of the Alexa488 dyes suggests that XPC/Alexa488-HR23B binds near the middle of Cy5-B5-DNA, where a 5-base unpaired bubble is situated as an artificial damaged site. A comparison between aspecifically surface bound XPC/Alexa488-HR23B molecules and specific DNA-bound revealed a larger mobility of the latter, suggesting realistic complex dynamics. We conclude that the NSC technique as implemented in this study of DNA-protein complex formation is a powerful method to investigate complex mobility and intra-molecular distances. Based on these findings, we expect that TIRF microscopy will be very helpful to further dissect the precise molecular mechanism by which DNA damages are detected and repaired.

Chapter 9

General Discussion and Prospects

In this chapter, quantitative fluorescence microscopy techniques are discussed in view of further developments and future applications in the study of DNA repair and other biological or chemical systems.

9.1 QUANTITATIVE ON-LINE FCS

The quantitative on-line fluorescence correlation spectroscopy (FCS) [69] presented in this thesis has been demonstrated to be a well established technique within the laser scanning single-molecule fluorescence confocal microscope (SFCM). It can reliably provide relevant information from complicated biological and chemical systems, particularly for the study of molecular binding interactions, e.g., nucleotide excision repair protein complex XPC-HR23B binding to DNA in Chapter 6. As demonstrated in Chapter 2, one of most important advantages of the quantitative on-line FCS is the avoidance of FCS artifacts [117-120, 123, 126] which bias the molecular diffusion properties measured by a traditional FCS technique due to the influence of optical and photophysical effects.

In Chapter 2 we present another application of the quantitative on-line FCS for the study of the interfacial interactions between the fluorescent molecules in solution and the different glass surfaces. It was found that the diffusion properties of molecules in solution and at glass-solution interface can be pronouncedly different. At surface-solution interface, two classes of interactions, i.e., surface-molecule interaction (surface adsorption or repulsion) and water-molecule interaction (relate to molecule solubility) play decisive roles on molecule diffusion in many physical, chemical and biological processes. The study of molecule diffusions at an interface are often found to be more complicated, because there are multiple components (e.g., solid surface with charges or not, water solution, molecules under investigation, ions in solution, *et al.*) involved in multiple interactions. The classical treatment of ions at an interface and in solution is the Poisson-Boltzmann (PB) theory [296, 297]. In spite of oversimplifying assumptions it can describe well univalent ions not too close to the interface for low electrolyte concentrations (millimolar). However, as well known [298, 299], one striking deficiency of the PB theory is that it produces the same results for the ions of the same valence, which is against many experimental observations that revealed pronounced differences between different ions. Traditionally, on the basis of the effects on the solubility of proteins and small organic molecules in water, ions can be arranged in Hofmeister series [300] which is a classification of ions in order of their ability to change water structure. The diversity of the interfacial effects [55-63, 301, 302] observed by different techniques can be a direct consequence of the subtle interplay of electrostatics, dispersion forces, thermal motion, polarization, fluctuations, hydration, *etc.*, and it thus is an apparent challenge for the generation of a general theory. The most useful information for the assessment of theories is knowledge of the molecular interactions and distributions at a surface-water interface. After some technical improvements (mainly a function of precisely autopoisoning along *z*-axis, i.e., optical path), the quantitative on-line confocal FCS can be the most important method to construct the interfacial architecture of molecular interactions

since it is, to the best of our knowledge, a unique technique that can measure molecular mobility both in solution and at interface. In contrast, the TIRF FCS technique [303-309] based on the total internal reflection fluorescence microscopy can only perform FCS measurements at surface (< 200 nm depth into solution).

FCS has been used for the study of the molecular properties for micellar system to determine the formation, critical micelle concentration, aggregation number and hydrodynamic size in surfactant systems [310-312]. Polymeric micelles are nano-sized or micro-sized particles that are made up of polymer chains and are usually spontaneously formed by self-assembly in a liquid, generally as a result of hydrophobic or ion pair interactions between polymer segments, and they typically have a so-called core-shell structure [313]. The core of the micelles, which is either the hydrophobic part or the ionic part of the polymers, can contain small molecules such as therapeutic drugs, while the shell provides interactions with the solvent and make the drugs thereby stable in the liquid. Synthetic polymers are highly versatile in terms of composition and architecture [313]. One of their applications is for drug delivery in pharmaceutical and scientific area. We have already used the quantitative on-line confocal FCS to study the thermosensitive gel-containing polymersomes (hydrosomes) that are potential novel carriers for anticancer drugs and proteins and were prepared by incorporating poly(N-isopropylacrylamide) hydrogels into poly(ethylene glycol)-b-poly(d,l-lactide) polymersomes [314]. As discussed in Chapter 2 and Chapter 6, the translational diffusion time measured by FCS represents the instantaneous average residence time of all molecules in various conformational states (shape, size, *etc.*), but is weighted solely by the number of molecules in each of those states, and it has preferentially high sensitivity to the prominent fluorescent intensity bursts indicating very large molecules travelling through confocal focal volume. However, in general the shape and size of micelles are significantly different from one another, and therefore the determined molecular diffusion times for the micelles systems suffers from very large standard deviations. In this case, it would be more useful to extract more detailed statistic information for all conformational states and their corresponding population from fluorescent intensity time traces by some other mathematical data analysis methods.

The quantitative on-line FCS was also found to be a remarkably convenient method to qualify protein conjugates for protein labeling experiments. Most protein labeling methods involve one of four common target strategies: 1) the most common target is primary amines primarily on lysine residues that in general are abundant on the surface of proteins; 2) the second most common target is sulfhydryls, which are present in oxidized form as disulfide bonds in proteins and are less abundant than primary amines, so targeting them results in more specific conjugates; 3) Carboxyls, like primary amines, are abundant and easily accessible. However, they do not react as readily as amines and coupling to them requires the cross-linker 1-Ethyl-3-(3-

dimethylaminopropyl) carbodiimide, hydrochloride salt (*ab.* EDC); 4) Carbohydrate moieties are present on glycoproteins and they must first be oxidized to create reactive aldehydes for labeling reaction. After protein labeling reaction, purifying the labeled proteins is very important for the removal of free dyes in the reaction product, and the purification efficiency can be readily tested by using the quantitative on-line FCS with only ~50 femtomole proteins in a few minutes. Compared with the commonly used technique, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the advantage of the quantitative on-line FCS is obvious in this application.

Recently confocal correlation spectroscopy has been extendedly developed to measure the particle size of nonfluorescent nanoparticles [315, 316] based on resonance light scattering [317].

9.2 QUANTITATIVE ON-LINE FCCS

The extension of FCS, fluorescence cross-correlation spectroscopy (FCCS), has been applied to investigate for irreversible association kinetics of DNA renaturation [318], real-time enzyme kinetics [319, 320], aggregation of prion proteins [321], and direct quantification of mRNA expression [322]. The SFCM [69] is apparently also suited to perform quantitative on-line FCCS. However, two fluorescent species under FCCS investigation should not have a large spectral overlap in the fluorescence emission spectra, which leads to cross talk in the correlation signal that is hard to correct [323]. This problem can be solved by using proper fluorescent labels for FCCS. The main limitation in the FCCS applications for protein-DNA interactions is the binding degrees for both protein and DNA molecules. As discussed in Chapter 8, the binding degrees for protein and DNA molecules are $\theta_P = 15\%$ and $\theta_D = 96\%$, respectively, in the binding reaction solution which contained 4 nM Cy5-B5 and 25 nM XPC/Alexa488-HR23B complex. Therefore, more than 85% free fluorescently labeled protein and DNA molecules in solution contribute to the background intensity fluctuation, which make hardly possible to perform FCCS to exactly study the binding events of such a low population.

9.3 TWO PHOTON FCS

Two-photon excitation (2PE) for laser scanning fluorescence confocal microscope was first demonstrated by Denk *et al.* [324] as an elegant solution to achieve intrinsic three-dimensional resolution. The process of two-photon excitation (2PE) occurs by simultaneous absorption of two low-energy photons of the excitation by a molecule that is then excited to an energy state that is equal to the sum of the energies of the two absorbed photons. If the excited-state bands for fluorescence emission are reached for the molecule, the fluorescence is emitted. For the 2PE, simultaneous absorption of two photons should be completed within $\sim 10^{-15}$ seconds, which is the appropriate uncertainty-principle time scale, and both photons must affect a single molecular cross-section area of $\sigma_{1P} = 10^{-16} \text{ cm}^2$. Therefore, 2PE requires high

instantaneous photon flux densities of the order of 10^{31} photons/cm², and two-photon cross section of a molecule is approximately $10^{-16} \times 10^{-16} \times 10^{-15} = 10^{-47}$ cm⁴s [325]. For 2PE the detection focal volume becomes the square of the illumination profile, which provide a highly restricted spatial excitation volume without further adjustment in the absence of confocal pinholes [326]. Compared with one-photon excitation (1PE), 2PE was reported to be able to minimize FCS artifacts by spatially restricting focal volume [123] as well as molecular fluorescence photobleaching and thus preserve long-term signal acquisition in cells for intercellular FCS measurements [123, 327].

9.4 FLUORESCENCE ANISOTROPY

As demonstrated in Chapter 2, Chapter 6 and Chapter 7, steady-state fluorescence anisotropy (SFA) was used for the study of protein-DNA binding in nucleotide excision repair system. Compared with FCS technique, SFA has less sensitivity to individual events in measurements *in vitro* (see Chapter 2 and Chapter 6) and it also suits intracellular measurements (see Chapter 7). However, as disclosed in Chapter 6, SFA was found dependent on fluorescent labeling strategy and may become irresponsive to the formation of protein-DNA binding complex. In this case, a combination of both FCS and SFA has turned out to be very useful to provide complementary information for a ligand-receptor binding system.

9.5 TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY

A prism-based total internal reflection fluorescence (TIRF) microscope was presented in Chapter 4 and Chapter 8 with the applications of nanometer-precision single-molecule localization (NSL) in this thesis. Compared with laser scanning fluorescence confocal (LSFC) microscope, obvious advantages in TIRF microscopy for NSL are as follows: 1) high temporal resolution allows fast kinetic image acquisition and simultaneous observation for many molecular events in a large field of view of microscope; 2) localization accuracy can be theoretically predicted and is dependent on Poisson distributed photon noise and normally Gaussian distributed background noise from camera. In contrast, the position precision of piezo stage in LSFC microscope is dependent on scanning step size and can induce additional localization error for NSL; 3) Very thin excitation volume of the order of 100 nm depth (it is ~ 1.2 μ m for LSFC microscope) is ideal for capturing high resolution images with very high signal to noise ratio, but it can also be a drawback for the study of molecular interaction especially in TIRF-FCS [303-309] since surface-molecule interaction or other interfacial effects (see Chapter 2) can play a significant role at glass-solution interface and thus has to be minimized or eliminated prior to the actual experiments for real molecular interaction systems.

Current software platform of our TIRF microscope is commercial Andor Solis, which only has basic function for image acquisition from the two EMCCD cameras.

For the further applications, new software for automatically controlling microscope would be expected for a high throughput screening and an online coincidence analysis.

9.6 DNA REPAIR

Cellular DNA is susceptible to continual attack, by both reactive species inside cells and environmental agents. DNA damage may lead to heritable mutation and genetic instability and consequently oncogenesis [16]. Fortunately, genomic DNA appears to be chemically stable, and its genetic integrity and stability are generally well preserved. This is attributed to some 130 gene products that can be distinguished in humans [328]. Five DNA repair pathways including direct damage reversal, base excision repair, nucleotide excision repair (NER), mismatch repair, and double-strand break repair has been characterized [16, 17] and NER is considered as the most versatile DNA repair system and one of the major human DNA repair pathways [3]. In NER, two different subpathways, the general global genome NER (GG-NER) and transcription-coupled NER (TC-NER), were proposed to remove the DNA damage from the entire genome and from actively transcribed strand [36, 329], respectively. In GG-NER, six core NER factors, XPC/HR23B, transcription factor II H (TFIIH), XPA, replication protein A (RPA), XPG and XPF-ERCC1, work together to remove the damaged oligonucleotide [3]. Despite the complexity of GG-NER, its molecule mechanism has been now understood in considerable detail. Like RNA polymerase II gene transcription and DNA replication, the molecular mechanism of NER is a complex and multistep process (see Fig. 1-5) [2, 3]. The DNA damage is first recognized by XPC/HR23B complex [26], and then from damage recognition to DNA dual incision, the XPC/HR23B complex first recruits and positions TFIIH to allow the XPB and XPD helicases of the TFIIH to unwind the damaged DNA [3, 42, 51] around the lesion [3]. TFIIH does not interact with the damaged DNA without XPC/HR23B [3]. XPC/HR23B and TFIIH can sequentially bind to damaged DNA in the absence of adenosine-5'-triphosphate (ATP), but without ATP, TFIIH cannot unwind DNA and other dual incision factors including XPA, RPA, XPG and XPC-ERCC1 cannot be recruited to the damaged DNA [2, 3]. However, many questions on overall NER molecular mechanisms are still unanswered. For instance, what is the temporal and spatial organization of all NER proteins in each step of NEP process? How are they assembled to a DNA damage site? These kinds of questions require answers both *in vivo* and *in vitro*. In this thesis, we focused our attention on the fluorescence single-molecule microscopic study *in vitro* for only the first initial step, damage recognition, in NER. The used fluorescence microscopic techniques, the combined quantitative on-line FCS and SFA techniques in Chapter 6 as well as the nanometer-precision single-molecule localization in TIRF microscopy in Chapter 8, showed a promised perspective to explicate the molecular mechanism models in detail for NER and other

DNA repair systems and finally expatiate on the architecture of all DNA repair machineries.

Summary

DNA is the carrier of our genetic information. Damage to the DNA is constantly inflicted by a large number of agents; it can alter the coding sequence of DNA and lead to mutations or cancer in mammals, or produce DNA lesions which can be deleterious to the cell by interfering with normal cellular transactions, such as DNA replication or transcription. There are various DNA repair pathways including base excision repair, nucleotide excision repair (NER), mismatch repair, double-strand breaks repair, etc., to counteract the adverse effect by removing lesions from DNA. The importance of DNA repair is the maintenance of the genomic integrity for all organisms.

The work described in this thesis is for the project – “In vitro and in vivo studies of the architecture of nucleotide excision repair complexes” granted by the Human Frontiers in Science Program (HFSP) RGP0007/2004-C. The goal of this project was the study of damage recognition, complex assembly and mechanism of human NER using designed photoreactive and fluorescent NER substrates and a number of biochemical, biophysical and cell biological techniques. The whole project was carried out in several international laboratories in close collaboration. Our objective is to develop and use new instrumentation and methods for the quantitative fluorescent nanospectroscopic techniques to study: 1) the composition of the NER complex at various stages of the NER process, 2) the assembly-order of NER proteins on DNA substrates, 3) the precise location of different proteins in NER complexes, and 4) the kinetics and dynamics of DNA-protein interactions. Finally, we have developed and used single-molecule fluorescence confocal microscopy and total internal reflection fluorescence (TIRF) microscopy for this project. The instruments and experimental methods were continuously improved as our insight and knowledge increased.

In **Chapter 2**, the developments of a single-molecule fluorescence confocal microscope (SFCM) are presented. The SFCM has been extended with many functions and it now allows performing fluorescent intensity imaging, steady-state fluorescent anisotropy (SFA) imaging, fluorescent spectral imaging, and single-point on-line quantitative fluorescence correlation spectroscopy (FCS) and SFA. Most experiment measurements and data processing can be performed on-line. In this chapter, we demonstrate the single-molecule sensitivity of the SFCM and in Chapter 7 we demonstrated all functions of the SFCM in the multiparameter fluorescence spectroscopic study on single living XP4PA cells. FCS and SFA techniques are known to be susceptible to give artifacts since they are very sensitive to many factors. Therefore, the artifact sources are carefully studied for both techniques and various strategies are proposed and applied to avoid artificial results in actual measurements.

On-line quantitative single-point FCS and SFA have been used to study the soluble protein stability in Chapter 5 and the binding interaction between the NER protein complexes, human XPC/HR23B and yeast Rad4/Rad23, and DNA substrates in Chapter 6. Importantly, we found in quantitative on-line FCS measurements that the molecular dynamic behavior at glass-solution interface can be significantly different from those in solution. Sample fluorescent molecules were observed aggregating at glass-solution interface but not in solution even after suppressing surface adsorption effect. In Chapter 2, several experiments were designed and performed to study and interpret the interfacial aggregation phenomenon.

Chapters 3 and 4 present the study on theoretical and experimental nanometer-precision single-molecule localization (NSL) accuracy. The results provide the groundwork for the advanced application of NSL technique in Chapter 8 for the nanometer single-molecular colocalization of NER protein complexes, Alexa Fluor 488 (Alexa488) dye labeled XPC/Alexa488-HR23B heterodimer, binding to Cy5 dye labeled DNA. In chapter 3, a full treatment of all relevant factors which limit NSL accuracy has been investigated and a new extension to the NSL accuracy theory has been developed. The relationships between two-dimensional Gaussian parameters and Airy pattern parameters have been presented, since the Gaussian function is commonly used to describe the Airy pattern in far field microscopy. Chapter 4 provides experimental evidence for the theoretical study on the NSL accuracy in Chapter 3 by using a TIRF microscope imaging water soluble quantum dot 655 Streptavidin Conjugates (QDot655) immobilized on biotin-PEG functionalized coverslip surfaces. The experiment QDot655 localization accuracies are very close to the theoretical predictions from the adapted localization accuracy formulas in Chapter 3. With distinguishing feature resided in the different dynamic light emitting properties, absolute distance measurements between two QDot655 nanoparticles were demonstrated and a distance of 15 ± 5 nm, as an example, was determined between two “identical” QDot655 nanoparticles.

A study on soluble protein stability in fluorescence microscopy is presented in **Chapter 5**. At the beginning of the project, we found in the NER proteins and DNA substrates binding reaction experiments that the non-ionic surfactant buffer component is crucial to achieve stable experimental results and high DNA-protein binding affinity by quantitative on-line FCS. Surprisingly, more carefully designed experiments with other soluble proteins, fluorescent proteins (FPs) and restriction enzymes, proved that these soluble proteins also favor a molecular crowding environment in the presence of relatively high concentration non-ionic surfactant. In Chapter 5, quantitative on-line FCS experiments revealed that FP concentration decreases in highly dilute FPs solutions enclosed by molecule-adsorption protected interfaces and this suggested that the loss of protein functionality was probably caused by denaturation of the FPs. In contrast, the typical lifetime of protein functionality in

highly dilute, approximately single-molecule per femtoliter solutions can be dramatically extended more than thousand-fold by adding compounds with surfactant behavior. However, quantitative on-line FCS experiments show that no direct interactions between the surfactant and the fluorescent proteins. These observations suggest that soluble biomolecules can extend an influence over much larger distances than suggested by their actual volume. This provides the useful information for optimizing the buffer condition for the DNA-protein binding reaction experiments in Chapters 6 and 8.

Chapter 6 shows a detailed study on the bindings of NER protein complexes, XPC/HR23B and Rad4/Rad23, to damaged and undamaged DNA substrates by using combined FCS and SFA techniques. With combined FCS and SFA techniques, molecule translational and rotational diffusion can be simultaneously measured. FCS and SFA were performed in protein titration solutions and to determine the protein binding affinity for various DNA substrates while those in competition titration solutions to determine the substrate specificity as well as the protein binding affinity for the competitor DNA substrates. The competition titration experiments revealed that XPC/HR23B complex preferentially binds to damaged DNA substrates with a few improperly pairing bases. For XPC/HR23B heterodimer, HR23B protein was fluorescently labeled with Alexa488 dye. FCS and SFA was performed in the binding reaction solutions of the XPC/Alexa488-HR23B complex to the UV-irradiated plasmid DNA, and the results revealed that HR23B protein does not release from the protein complex when XPC/HR23B binds to DNA molecule *in vitro*. This shows that a promised perspective to explicate the multistep model and describe the architecture of the NER mechanism by the combined FCS and SFA techniques.

Multiparameter fluorescence imaging in single living XP4PA cells in **Chapter 7** is to demonstrate all functions of the SFCM. In this chapter, single XP4PA cells were studied by fluorescent intensity imaging, fluorescent spectral imaging, SFA imaging and single-point FCS and SFA for the activity of the DNA repair protein XPC tagged with green fluorescent protein (GFP) in the cells. XPC proteins were localized only in nuclei by fluorescent intensity imaging and fluorescent spectral imaging. In cells, autofluorescence was characterized by fluorescent spectral imaging and forms a technique barrier that limits the applications of FCS and SFA in the cells. The autofluorescence components have a smaller SFA and translational diffusion time (TDt) than GFP-XPC in cells. The existence of the autofluorescence requires the measurement time as short as possible because GFP molecules have only a limited amount in cell and will be rapidly photobleached in a long-time measurement and consequently the measured signal is dominated by the autofluorescence. SFA imaging can be measured in a very short time and may find the molecular interactions while FCS commonly requires a long measurement time and was thus strongly limited by fluorescent background intensity variations arising from the autofluorescence and

complicated internal cellular motions. Potential use of other methods for fluorescent time trace analysis is discussed and suggested in this chapter.

We have shown that NSL enables distance measurements on the molecular scale in Chapters 3 and 4. The goal of this technique is to measure the architecture of molecular complex such as protein and DNA in NER complex through the distances between fluorescent labels on different components in the complex. Finally, nanometer-precision single-molecule colocalization (NSC) technique was carried out for the study on Alexa488 labeled NER protein complex XPC/Alexa488-HR23B binding to Cy5 dye labeled DNA substrates and the results are presented in **Chapter 8**. In the applications, we immobilized the DNA molecules that were bound or not bound with XPC/Alexa488-HR23B complexes on streptavidin functionalized protein-resistant coverslip surfaces via one biotin-labeled end of the DNA molecules. NSL analyses proved that the XPC/Alexa488-HR23B complex binding to DNA exhibit higher mobility due to the participation in the DNA molecule confined diffusion than those non-specially immobilized ones. XPC/Alexa488-HR23B complexes were precisely localized at the artificial damage site of DNA molecules in the DNA-protein binding complexes. NSL analyses also determined shorter lengths of DNA molecules in DNA-protein binding complexes than that of free DNA molecules and this suggests that XPC/Alexa488-HR23B binding induces bending in DNA molecules.

In conclusion, the developed quantitative nanospectroscopic techniques presented in this thesis enabled us to end the work off with a flourish. The quantitative on-line FCS and SFA are excellent techniques to determine the kinetics and dynamics of DNA-protein interactions and the assembly-order of NER proteins on DNA substrates in NER complex as well as the composition of the NER complex at various stages of the NER process. NSL or NSC is most useful for the precise location of different proteins in NER complexes. All these techniques provide a bright future for the study of the architecture of NER machinery as well as other biological system of interest.

Samenvatting

DNA is de drager van onze genetische informatie. Een groot aantal agentia kunnen voortdurend schade toebrengen aan DNA waardoor de genetische code veranderd kan worden. Dit kan leiden tot mutaties en kanker in zoogdieren, of ontbrekend DNA hetgeen schadelijk kan zijn voor de cel door beïnvloeding van de normale cellulaire activiteit, zoals replicatie en transcriptie. Er bestaan verscheidene DNA reparatie mechanismes zoals “base excision repair”, “nucleotide excision repair” (NER), “mismatch repair”, “double-strand breaks repair”, enzovoort, die erop gericht zijn om de schade op het niveau van DNA te corrigeren. Het belang van “DNA repair” is het onderhoud van de kwaliteit van het genoom voor het behoud van alle organismen.

Het werk beschreven in dit proefschrift is tot stand gekomen in het kader van het project “In vitro and in vivo studies of the architecture of nucleotide excision repair complexes”, dat toegekend is door het “Human Frontiers in Science Program” (HFSP) RGP0007/2004-C. Het doel van dit project was het onderzoek naar schadeherkenning, complexopbouw en het mechanisme van menselijk NER door gebruik te maken van ontworpen fotoreactieve en fluorescente NER substraten en een aantal biochemische-, biofysische- en cel-biologische technieken. Het gehele project werd uitgevoerd in verschillende internationale laboratoria in nauwe samenwerking. Ons doel is het ontwikkelen en toepassen van nieuwe instrumentatie en methoden voor de kwantitatieve fluorescente nanospectroscopie van: 1) de samenstelling van NER complexen in verschillende stadia van het NER proces, 2) de volgorde van binding van NER eiwitten op DNA substraten, 3) de locatie van verschillende eiwitten in NER complexen en 4) de kinetiek en dynamica van DNA-eiwit interactie. We hebben een “één-molecuul fluorescentie confocale microscoop” en “totale interne reflectie fluorescentie microscoop” ontwikkeld en gebruikt in dit project. De instrumentatie werd voortdurend verbeterd naarmate onze kennis en inzichten toenamen.

In **hoofdstuk 2**, wordt de ontwikkeling van een “één-molecuul fluorescentie confocale microscoop” (SFCM) gepresenteerd. De SFCM werd uitgebreid met vele functies en stelt ons nu in staat om “fluorescentie intensiteit microscopie”, “statische fluorescentie anisotropie” (SFA) microscopie, fluorescentie spectrale microscopie en puntsgewijs, doorlopend kwantitatieve “fluorescentie correlatie spectroscopie” (FCS) en SFA te combineren. Het grootste deel van de gegevensverwerking kan tijdens de meting uitgevoerd worden. In dit hoofdstuk demonstreren we één-molecuul gevoeligheid van de SFCM en in hoofdstuk 7 demonstreren we alle mogelijkheden van de SFCM in een multi-parameter fluorescentie spectroscopische studie van levende XP4PA cellen. Van FCS en SFA is het bekend dat ze gevoelig zijn voor vele factoren. Om die reden zijn de foutbronnen van beide technieken zorgvuldig

bestudeerd en verschillende strategieën zijn voorgesteld en toegepast om problemen in de uiteindelijke metingen te voorkomen. “On-line” kwantitatieve FCS en SFA zijn gebruikt om de stabiliteit van oplosbare eiwitten te bestuderen in hoofdstuk 5 en ook de binding tussen NER eiwit complexen, humaan XPC/HR23B en gist Rad4/Rad23, en DNA substraten in hoofdstuk 6. Van belang is de waarneming dat de moleculaire dynamica in de glas-oplossing tussenfase zeer kan verschillen van die in oplossing. Fluorescente moleculen aggregaerden aan het oppervlak maar niet in oplossing, zelfs na het onderdrukken van oppervlakte adsorptie effecten. In hoofdstuk 2 worden verscheidene experimenten beschreven om het gedrag van moleculen in de tussenfase te begrijpen.

Hoofdstukken 3 en 4 presenteren theoretische en experimentele ontwikkelingen om op de nanometer schaal nauwkeurige metingen te doen aan de positie van moleculen (nanometer single-molecule localization (NSL)). De resultaten bieden de basis voor de geavanceerde toepassing van NSL in hoofdstuk 8 voor nanometer één-molecuul colocalisatie van NER eiwit complexen, Alexa Fluor488 (Alexa488) kleurstof gemerkt XPC/Alexa488-HR23B heterodimer dat bindt aan Cy5-gemerkte DNA. In hoofdstuk 3 wordt een volledige behandeling gegeven van alle belangrijke factoren, die de NSL nauwkeurigheid beperken en werd een nieuwe uitbreiding aan de bestaande NSL theorie beschreven. De relaties tussen 2-dimensionale Gaussische bundel parameters en Airy bundel parameters worden gepresenteerd omdat de Gaussische functie veelvuldige wordt gebruikt om een Airy bundel te beschrijven in verre veld microscopie. Hoofdstuk 4 biedt experimentele ondersteuning voor de theoretische studie van de NSL nauwkeurigheid in hoofdstuk 3 door gebruik te maken van TIRF microscopie afbeeldingen van water oplosbare “quantum dot” 655 bedekt met streptavidine conjugaten (Qdot655), die geïmmobiliseerd waren op biotine-PEG gefunctionaliseerde dekglas oppervlakken. De experimentele Qdot655 localisatie nauwkeurigheid liggen zeer dicht bij de theoretische voorspellingen van de nieuwe formules voor de localisatie nauwkeurigheid uit hoofdstuk 3. Door gebruik te maken van verschillen in de dynamische emissie van verschillende Qdot655 deeltjes werden absolute afstandmetingen van 15 ± 5 nm gedemonstreerd tussen twee “identieke” Qdot’s.

Een studie aan de stabiliteit van oplosbare eiwitten in fluorescentie microscopie werd gepresenteerd in **hoofdstuk 5**. In het begin van het project werd in de bindingsexperimenten van NER eiwitten en DNA substraten gevonden dat toevoeging van een niet-ionogene oppervlakte-actieve component aan de buffer van wezenlijk belang was voor de verkrijging van stabiele experimentele resultaten en een hoge DNA-eiwit bindingsconstante met behulp van kwantitatieve, on-line FCS. Verrassend was dat met zorgvuldig ontworpen experimenten met andere fluorescente eiwitten (FP’s) en restrictie enzymen aangetoond werd dat ook deze moleculen de voorkeur gaven aan een omstandigheid van hogere moleculaire concentratie zoals bereikt werd

door niet-ionogene oppervlakte-actieve stoffen toe te voegen. In hoofdstuk 5 toonden kwantitatieve on-line FCS experimenten aan dat een afname van de FP concentratie optrad in zeer verdunde FP oplossingen, zelfs als deze omgeven waren door oppervlakken die adsorptie van moleculen minimaliseerden en dit suggereerde dat het verlies van eiwit functionaliteit waarschijnlijk veroorzaakt werd door denaturatie van de FP's. Daartegenover staat dat de levensduur van eiwit functies in sterk verdunde, ongeveer 1 eiwit-molecuul per femtoliter, oplossingen enorm, 1000-voudig verlengd kon worden door verbindingen toe te voegen met oppervlakte activiteit. Echter, kwantitieve on-line FCS liet zien dat er geen directe interactie tussen de oppervlakte-actieve moleculen en de eiwitmoleculen zichtbaar was. Deze waarneming suggereerde dat oplosbare biomoleculen over veel grotere afstanden invloed op elkaar uitoefenen dan gesuggereerd door hun eigenlijke volume. Deze waarneming bood bruikbare informatie om de buffer condities voor de DNA-eiwit bindingsexperimenten in hoofdstuk 6 en 8 te optimaliseren.

Hoofdstuk 6 presenteert een gedetailleerde studie over de binding van NER eiwit complexen XPC/HR23B en Rad4/Rad23 met beschadigde en onbeschadigde DNA substraten door gebruik te maken van FCS en SFA technieken. Met de gecombineerde FCS en SFA techniek kunnen de moleculaire verplaatsing en draaiing gelijktijdig gemeten worden. FCS en SFA werden uitgevoerd in eiwit titratie experimenten om de affiniteit van verschillende eiwitten te bepalen en in competitie experimenten om substraat specificiteit en eiwit bindingsaffiniteit te bepalen voor de concurrerende DNA substraten. De competitie experimenten toonden aan dat XPC/HR23B bij voorkeur bindt aan beschadigd DNA met een klein aantal niet-gepaarde DNA-basen. Heterodimeer XPC/HR23B was fluorescent gemerkt met Alexa488. FCS en SFA werden uitgevoerd in de oplossing waar XPC/Alexa488-HR23B een bindingsreactie heeft met UV-beschadigd plasmide DNA en de resultaten toonden dat HR23B niet verdween uit het eiwit-DNA complex in vitro. Dit toont aan dat FCS en SFA succesvol gebruikt kunnen worden om de architectuur van multistep mechanisme zoals NER te onderzoeken.

Multi-parameter fluorescentie afbeeldingen van levende XP4PA cellen in **hoofdstuk 7** demonstreren de mogelijkheid om cel onderzoek te doen met de opstelling. In dit hoofdstuk werden enkelvoudige XP4PA cellen bestudeerd met fluorescentie intensiteit microscopie, fluorescentie spectrale microscopie, SFA microscopie en 1-punts FCS en SFA om de activiteit van het DNA reparatie eiwit XPC gemerkt met “green fluorescent protein” (GFP) te bepalen. XPC was alleen aanwezig in de kernen van de cellen zoals aangetoond met fluorescentie intensiteit microscopie en fluorescentie spectrale microscopie. In cellen werd de autofluorescentie gekarakteriseerd door fluorescentie spectrale microscopie. Autofluorescentie vormt een beperking voor de toepassing van FCS en SFA in cellen. De autofluorescentie heeft een kleinere SFA en translatie diffusie tijd (TDt) dan GFP-

XPC in cellen. Het bestaan van de autofluorescentie maakt het noodzakelijk om de meettijd zo kort mogelijk te maken omdat er maar een beperkte hoeveelheid GFP is per cel, dat snel gebleekt zal worden in een langdurig experiment. Als gevolg zal het gemeten signaal gedomineerd worden door autofluorescentie. SFA microscopie kan in korte tijd gemeten worden en zou informatie kunnen geven over moleculaire interacties, terwijl FCS gewoonlijk een lange meettijd vereist. FCS in cellen wordt gelimiteerd door variaties in de autofluorescente achtergrond en gecompliceerde intracellulaire beweging. Andere methoden voor analyse van de fluorescentie tijd evolutie worden in dit hoofdstuk voorgesteld en bediscussieerd.

We hebben aangetoond in hoofdstuk 3 en 4 dat NSL afstandsmetingen op de moleculaire schaal mogelijk maakt. Het doel van deze techniek is om de architectuur van moleculaire complexen zoals die in NER/DNA complexen te meten door de afstand te bepalen tussen de fluorescente labels op verschillende componenten in het complex. In **hoofdstuk 8** worden de mogelijkheden van deze techniek geïllustreerd door afstanden te meten tussen Cy5 op DNA en Alexa488 op XPC/Alexa488-HR23B. Voor dit doel werd DNA, via een biotine molecuul, gekoppeld aan een oppervlak gefunctionaliseerd met streptavidine. NSL analyse toonde aan dat DNA gebonden XPC/Alexa488-HR23B een hogere mobiliteit heeft dan niet-specifiek gebonden XPC/Alexa488-HR23B als gevolg van de (beperkte) diffusie van DNA, dat aan een zijde gebonden is aan het oppervlak. XPC/Alexa488-HR23B werd gelocaliseerd op de schade in de DNA-eiwit complexen. NSL toonde tevens aan dat DNA moleculen in DNA-eiwit complexen korter waren dan vrij DNA en dit suggereert dat XPC/Alexa488-HR23B een bocht induceert in DNA.

Concluderend kan gezegd worden dat de kwantitatieve nanospectroscopische technieken, die gepresenteerd zijn in dit proefschrift, succesvol toegepast zijn. De kwantitatieve on-line FCS en SFA techniek zijn uiterst geschikt om kinetiek en dynamica van DNA-eiwit interacties en de volgorde van eiwit binding in complexen te onderzoeken. NSL is geschikt om de locatie op nanometer schaal van eiwitten in complexen te meten. Deze technieken bieden ruime mogelijkheden voor verdere exploratie van de architectuur van NER complexen.

内容简介

脱氧核糖核酸（DNA）是遗传信息的载体。DNA 的生物学功能是贮存决定物种性状的几乎所有蛋白质和 RNA 分子的全部遗传信息，是编码和设计生物有机体在一定的时空中有序地转录基因和表达蛋白完成定向发育的所有程序，因而 DNA 能初步确定了生物独有的性状和个性以及和环境相互作用时所有的应激反应。正是由于 DNA 的这些重要作用，在细胞中维持 DNA 的结构稳定性和复制的准确性都是极为重要的。

在细胞核中，许多来自环境的外在因素和细胞内的化学物质都能破坏 DNA 的化学结构。造成 DNA 破损的环境因素很多，通常紫外光和很多高能辐射都能破坏 DNA 结构，例如，紫外线可以产生胸腺嘧啶二聚体(cyclobutane pyrimidine dime，简称 CPD) 和 pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) 两种基本类型的 DNA 结构破损。另外在细胞内部新陈代谢过程中也会产生很多有害化学物质改变 DNA 的结构。根据破损的 DNA 结构，DNA 损坏的种类有很多，包括各种 DNA 碱基结构或者序列的变化。事实上在构成一个生命体的大量的细胞中，DNA 破损一直都在不断地发生。它们平均一天可以对一个细胞中的 DNA 分子结构造成多达 100 万次不同程度的破坏，尽管这些破损只能占单条人类 DNA 具有的大约 30 亿个碱基对中极少的部分，但是这些破损足以产生严重的基因变异，影响细胞的功能，并引发癌症。

如上所述，在细胞核中的 DNA 结构会经常被各种因素破坏，然而正常的细胞对多种多样的 DNA 破损都具有高效快捷的修复功能。DNA 修复（DNA repair）是细胞对 DNA 受损伤后的一种反应，这种反应可以使破损的 DNA 结构恢复原样。正是这些正常细胞具有的基本的 DNA 修复功能使得存储在 DNA 上的基因信息能够保持完整准确。目前，研究发现在正常细胞中主要有五种基本的 DNA 修复方式：同源重组（Homologous recombination，简称 HRR），非同

源末端连接（Nonhomologous End Joining，简称 NHEJ），核苷酸切除修复（Nucleotide Excision Repair，简称 NER），碱基切除修复（Base Excision Repair，简称 BER）和错配修复（Mismatch repair，简称 MMR）。对不同的 DNA 损伤细胞可以有不同的修复反应，选择不同的修复方式。

NER 通过能够识别，切除和修复很多受到损坏不正常的 DNA 结构。一般地它是以识别 DNA 损伤开始，然后在损伤的单链上切除包括破损碱基在内的 24-30 碱基序列，最后在 DNA 聚合酶和 DNA 连接酶的作用下以另一根正常的单链为模板，合成新的互补链将缺口连接起来。NER 对在非转录状态和转录状态的 DNA 对损伤的识别有所不同，前者被称为全基因组核苷酸切除修复（Global Genome NER，简称 GGNER），后者被称为 DNA 转录偶联核苷酸切除修复（Transcription-coupled NER，简称 TCNER）。目前的研究人类细胞中的 GGNER 需要通过 XPC/HR23B 蛋白组合实现 DNA 损伤的识别，进而激活整个 GGNER 的修复过程，这期间要有大约 30 多种蛋白质的共同参与才能完成。TCNER 被激活通常是由于 DNA 损伤阻挡了 RNA 聚合酶 II，RNA 转录停止。除了对损伤的识别方式不同，GGNER 和 TCNER 的后续步骤都是一样的。NER 系统失效会产生几种癌症，包括 Xeroderma pigmentosum (XP)，Cockayne's syndrome (CS) 和 Trichothiodystrophy (TTD)。

本论文是使用定量的纳米光学显微技术对细胞中核苷酸切除修复 NER 机制中对 DNA 损伤识别机理进行的细胞学和单分子生物学研究。全文九章。第一章是对研究的历史，目标和技术的介绍；第二章阐述对单分子共聚焦荧光显微镜技术的发展和改进；第三章是对纳米光学定位技术的理论研究；第四章是对第三章的理论结果的实验证实；第五章使用单分子共聚焦显微镜研究水溶生物蛋白的寿命；第六章利用荧光相关光谱（FCS）和稳态荧光各向异性（SFA）技术较为详细的研究人类 XPC/HR23B 蛋白组合和酵母 Rad4/Rad23 蛋白组合与正常的 DNA 模板和带有损伤的 DNA 模板的相互作用。第七章使用遗传修饰（Genetic modification）技术，用绿色荧光蛋白标记 XP4PA 活细胞中的 XPC 蛋白，然后使用单分子共聚焦荧光显微镜对单个 XP4PA 活细胞中 XPC 的活动进

行观察研究。第八章是利用第三章和第四章的纳米光学定位技术较为详细的研究人类 XPC/HR23B 蛋白组合于正常 DNA 模板和带有损伤的 DNA 模板的相互作用。第九章是对未来技术发展的建议和展望。

项目全部工作和完成论文历时四年，几乎全部的工作都是在荷兰 Twente 大学科学技术系生物物理学工程组完成。与本研究项目合作的其他五个研究小组有：1) 荷兰鹿特丹 Erasmus 医学研究中心的细胞和基因研究实验室 Wim Vermeulen 副教授领导的小组；2) 美国纽约 Stony brook 大学药理学系 Orlando D. Schärer 副教授的实验室；3) 俄罗斯 Novosibirsk 州大学的化学生物学和基础药理学研究所的生物酶的生物化学实验室 Olga I. Lavrik 博士的小组；4) 日本 Saitama 市物理与化学研究所 Sugawara 教授的实验室；5) 美国纽约 Memorial Sloan-Kettering 癌症研究中心 Nikola P. Pavletich 教授的实验室。

REFERENCES

1. Orlando, D. S. "Chemistry and Biology of DNA Repair". *Angew. Chem. Int. Ed.* 42:2946-2974 (2003).
2. Mocquet, V., J. P. Laine, T. Riedl, Z. Yajin, M. Y. Lee, and J. M. Egly. "Sequential recruitment of the repair factors during NER: The role of XPG in initiating the resynthesis step". *EMBO J.* 27:155-167 (2008).
3. Riedl, T., F. Hanaoka, and J. M. Egly. "The comings and goings of nucleotide excision repair factors on damaged DNA". *EMBO Journal* 22:5293-5303 (2003).
4. Lakowicz, J. R. *Principles of fluorescence spectroscopy*. Springer, New York.(2006).
5. Webb, W. W. "Applications of fluorescence correlation spectroscopy". *Q. Rev. Biophys.* 9:49-68 (1976).
6. Perrin, F. "Polarisation de la lumière de fluorescence. Vie moyenne des molécules dans l'état excité". *J. Phys. Radium* 7:390-401 (1926).
7. Bolger, R., T. E. Wiese, K. Ervin, S. Nestich, and W. Checovich. "Rapid screening of environmental chemicals for estrogen receptor binding capacity". *Environ. Health Perspect.* 106:551-557 (1998).
8. Lundblad, J. R., M. Lurance, and R. H. Goodman. "Fluorescence polarization analysis of protein-DNA and protein-protein interactions". *Mol. Endocrinol.* 10:607-612 (1996).
9. Ozers, M. S., J. J. Hill, K. Ervin, J. R. Wood, A. M. Nardulli, C. A. Royer, and J. Gorski. "Equilibrium binding of estrogen receptor with DNA using fluorescence anisotropy". *J. Biol. Chem.* 272:30405-30411 (1997).
10. Wu, P., M. Brasseur, and U. Schindler. "A high-throughput STAT binding assay using fluorescence polarization". *Anal. Biochem.* 249:29-36 (1997).
11. Bolger, R., and W. Checovich. "A new protease activity assay using fluorescence polarization". *Biotechniques* 17:585-589 (1994).
12. Bolger, R., and D. Thompson. "A quantitative RNase assay using fluorescence polarization". *Am. Biotechnol. Lab.* 12:113-116 (1994).
13. Lodish H, B. A., Matsudaira P, Kaiser CA, Krieger M, Scott MP, Zipursky SL, Darnell J. . In *Mol. Biol. Cell*. W. Freeman, editor, New York, NY. 963 (2004).
14. Browner, W. S., A. J. Kahn, E. Ziv, A. P. Reiner, J. Oshima, R. M. Cawthon, W.-C. Hsueh, and S. R. Cummings. "The genetics of human longevity". *The American Journal of Medicine* 117:851-860 (2004).
15. Hasty, P., J. Campisi, J. Hoeijmakers, H. van Steeg, and J. Vijg. "Aging and Genome Maintenance: Lessons from the Mouse?". *Science* 299:1355-1359 (2003).
16. Hoeijmakers, J. H. J. "Genome maintenance mechanisms for preventing cancer". *Nature* 411:366-374 (2001).
17. Friedberg, E. C. "DNA damage and repair". *Nature* 421:436-440 (2003).
18. Lehmann, A. R. "DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy". *Biochimie* 85:1101-1111 (2003).
19. Nance, M. A., and S. A. Berry. "Cockayne Syndrome: Review of 140 cases". *Am. J. Med. Genet.* 42:68-84 (1992).
20. Van Den Boom, V., N. G. J. Jaspers, and W. Vermeulen. "When machines get stuck - Obstructed RNA polymerase II: Displacement, degradation or suicide". *Bioessays* 24:780-784 (2002).
21. Itin, P. H., A. Sarasin, and M. R. Pittelkow. "Trichothiodystrophy: Update on the sulfur deficient brittle hair syndromes". *J. Am. Acad. Dermatol.* 44:891-920 (2001).
22. Vermeulen, W., E. Bergmann, J. Auriol, S. Rademakers, P. Frit, E. Appeldoorn, J. H. J. Hoeijmakers, and J. M. Egly. "Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder". *Nat. Genet.* 26:307-313 (2000).
23. Gillet, L. C. J., and O. D. Schärer. "Molecular mechanisms of mammalian global genome nucleotide excision repair". *Chemical Reviews* 106:253-276 (2006).
24. Asahina, H., I. Kuraoka, M. Shirakawa, E. H. Morita, N. Miura, I. Miyamoto, E. Ohtsuka, Y. Okada, and K. Tanaka. "The XPA protein is a zinc metalloprotein with an ability to recognize various kinds of DNA damage". *Mutation Research - DNA Repair* 315:229-237 (1994).
25. Reardon, J. T., and A. Sancar. "Molecular Anatomy of the Human Excision Nuclease Assembled at Sites of DNA Damage". *Mol. Cell. Biol.* 22:5938-5945 (2002).

26. Sugasawa, K., J. M. Y. Ng, C. Masutani, S. Iwai, P. J. van der Spek, A. P. M. Eker, F. Hanaoka, D. Bootsma, and J. H. J. Hoeijmakers. "Xeroderma Pigmentosum Group C Protein Complex Is the Initiator of Global Genome Nucleotide Excision Repair". *Mol. Cell* 2:223-232 (1998).
27. Min, J.-H., and N. P. Pavletich. "Recognition of DNA damage by the Rad4 nucleotide excision repair protein". *Nature* 449:570-575 (2007).
28. Reardon, J. T., and A. Sancar. "Recognition and repair of the cyclobutane thymine dimer, a major cause of skin cancers, by the human excision nuclease". *Genes Dev.* 17:2539-2551 (2003).
29. Wakasugi, M., and A. Sancar. "Order of Assembly of Human DNA Repair Excision Nuclease". *J. Biol. Chem.* 274:18759-18768 (1999).
30. Bohr, V. A., C. A. Smith, D. S. Okumoto, and P. C. Hanawalt. "DNA repair in an active gene: Removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall". *Cell* 40:359-369 (1985).
31. Mellon, I., G. Spivak, and P. C. Hanawalt. "Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene". *Cell* 51:241-249 (1987).
32. Leadon, S. A., and P. K. Cooper. "Preferential repair of ionizing radiation-induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome". *Proc. Natl. Acad. Sci. U. S. A.* 90:10499-10503 (1993).
33. Venema, J., L. H. F. Mullenders, A. T. Natarajan, A. A. Van Zeeland, and L. V. Mayne. "The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA". *Proc. Natl. Acad. Sci. U. S. A.* 87:4707-4711 (1990).
34. Tang, J., and G. Chu. "Xeroderma pigmentosum complementation group E and UV-damaged DNA-binding protein". *DNA Repair* 1:601-616 (2002).
35. Venema, J., A. Van Hoffen, V. Karcagi, A. T. Natarajan, A. A. Van Zeeland, and L. H. F. Mullenders. "Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes". *Mol. Cell. Biol.* 11:4128-4134 (1991).
36. Fouteri, M., and L. H. F. Mullenders. "Transcription-coupled nucleotide excision repair in mammalian cells: Molecular mechanisms and biological effects". *Cell Res.* 18:73-84 (2008).
37. Citterio, E., W. Vermeulen, and J. H. J. Hoeijmakers. "Transcriptional healing". *Cell* 101:447-450 (2000).
38. Hanawalt, P. C., J. M. Ford, and D. R. Lloyd. "Functional characterization of global genomic DNA repair and its implications for cancer". *Mutation Research/Reviews in Mutation Research* 544:107-114 (2003).
39. Batty, D., V. Rapić-Otrin, A. S. Levine, and R. D. Wood. "Stable binding of human XPC complex to irradiated DNA confers strong discrimination for damaged sites". *J. Mol. Biol.* 300:275-290 (2000).
40. Hey, T., G. Lipps, K. Sugasawa, S. Iwai, F. Hanaoka, and G. Krauss. "The XPC-HR23B Complex Displays High Affinity and Specificity for Damaged DNA in a True-Equilibrium Fluorescence Assay". *Biochemistry (Mosc.)* 41:6583-6587 (2002).
41. Sugasawa, K., Y. Shimizu, S. Iwai, and F. Hanaoka. "A molecular mechanism for DNA damage recognition by the xeroderma pigmentosum group C protein complex". *DNA Repair* 1:95-107 (2002).
42. Volker, M., M. J. Mone, P. Karmakar, A. van Hoffen, W. Schul, W. Vermeulen, J. H. J. Hoeijmakers, R. van Driel, A. A. van Zeeland, and L. H. F. Mullenders. "Sequential Assembly of the Nucleotide Excision Repair Factors In Vivo". *Mol. Cell* 8:213-224 (2001).
43. Masutani, C., K. Sugasawa, J. Yanagisawa, T. Sonoyama, M. Ui, T. Enomoto, K. Takio, K. Tanaka, P. J. Van Der Spek, D. Bootsma, J. H. J. Hoeijmakers, and F. Hanaoka. "Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23". *EMBO J.* 13:1831-1843 (1994).
44. Araki, M., C. Masutani, M. Takemura, A. Uchida, K. Sugasawa, J. Kondoh, Y. Ohkuma, and F. Hanaoka. "Centrosome Protein Centrin 2/Caltractin 1 Is Part of the Xeroderma Pigmentosum Group C Complex That Initiates Global Genome Nucleotide Excision Repair". *J. Biol. Chem.* 276:18665-18672 (2001).
45. Nishi, R., Y. Okuda, E. Watanabe, T. Mori, S. Iwai, C. Masutani, K. Sugasawa, and F. Hanaoka. "Centrin 2 Stimulates Nucleotide Excision Repair by Interacting with Xeroderma Pigmentosum Group C Protein". *Mol. Cell. Biol.* 25:5664-5674 (2005).
46. Yokoi, M., C. Masutani, T. Maekawa, K. Sugasawa, Y. Ohkuma, and F. Hanaoka. "The xeroderma pigmentosum group C protein complex XPC-HR23B plays an important role in the recruitment of transcription factor IIH to damaged DNA". *J. Biol. Chem.* 275:9870-9875 (2000).
47. Dip, R., U. Camenisch, and H. Naegeli. "Mechanisms of DNA damage recognition and strand discrimination in human nucleotide excision repair". *DNA Repair* 3:1409-1423 (2004).

48. Fitch, M. E., S. Nakajima, A. Yasui, and J. M. Ford. "In Vivo Recruitment of XPC to UV-induced Cyclobutane Pyrimidine Dimers by the DDB2 Gene Product". *J. Biol. Chem.* 278:46906-46910 (2003).
49. Shivji, M. K. K., A. P. M. Eker, and R. D. Wood. "DNA repair defect in xeroderma pigmentosum group C and complementing factor from HeLa cells". *J. Biol. Chem.* 269:22749-22757 (1994).
50. Mu, D., D. S. Hsu, and A. Sancar. "Reaction Mechanism of Human DNA Repair Excision Nuclease". *J. Biol. Chem.* 271:8285-8294 (1996).
51. Tapias, A., J. Auriol, D. Forget, J. H. Enzlin, O. D. Scharer, F. Coin, B. Coulombe, and J. M. Egly. "Ordered Conformational Changes in Damaged DNA Induced by Nucleotide Excision Repair Factors". *J. Biol. Chem.* 279:19074-19083 (2004).
52. Ng, J. M. Y., W. Vermeulen, G. T. J. Van der Horst, S. Bergink, K. Sugawara, H. Vrieling, and J. H. J. Hoeijmakers. "A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein". *Genes Dev.* 17:1630-1645 (2003).
53. Okuda, Y., R. Nishi, J. M. Y. Ng, W. Vermeulen, G. T. J. van der Horst, T. Mori, J. H. J. Hoeijmakers, F. Hanaoka, and K. Sugawara. "Relative levels of the two mammalian Rad23 homologs determine composition and stability of the xeroderma pigmentosum group C protein complex". *DNA Repair* 3:1285-1295 (2004).
54. Ortolan, T. G., L. Chen, P. Tongaonkar, and K. Madura. "Rad23 stabilizes Rad4 from degradation by the Ub/proteasome pathway". *Nucleic Acids Res.* 32:6490-6500 (2004).
55. Crocker, J. C., and D. G. Grier. "Microscopic measurement of the pair interaction potential of charge-stabilized colloid". *Phys. Rev. Lett.* 73:352-355 (1994).
56. Kepler, G. M., and S. Fraden. "Attractive potential between confined colloids at low ionic strength". *Phys. Rev. Lett.* 73:356-359 (1994).
57. Larsen, A. E., and D. G. Grier. "Like-charge attractions in metastable colloidal crystallites". *Nature* 385:230-233 (1997).
58. Gomez-Guzman, O., and J. Ruiz-Garcia. "Attractive interactions between like-charged colloidal particles at the air/water interface". *J. Colloid Interface Sci.* 291:1-6 (2005).
59. Oettel, M., A. Dominguez, and S. Dietrich. "Attractions between charged colloids at water interfaces". *Journal of Physics Condensed Matter* 17:L337-L342 (2005).
60. Ellis, J. S., H. G. Abdelhady, S. Allen, M. C. Davies, C. J. Roberts, S. J. B. Tendler, and P. M. Williams. "Direct atomic force microscopy observations of monovalent ion induced binding of DNA to mica". *J. Microsc.* 215:297-301 (2004).
61. Han, Y., and D. G. Grier. "Confinement-Induced Colloidal Attractions in Equilibrium". *Phys. Rev. Lett.* 91:038302 (2003).
62. Nikolaides, M. G., A. R. Bausch, M. F. Hsu, A. D. Dinsmore, M. P. Brenner, C. Gay, and D. A. Weitz. "Electric-field-induced capillary attraction between like-charged particles at liquid interfaces". *Nature* 420:299-301 (2002).
63. Nikolaides, M. G., A. R. Bausch, M. F. Hsu, A. D. Dinsmore, M. P. Brenner, C. Gay, and D. A. Weitz. "Capillary attraction (communication arising): Like-charged particles at liquid interfaces". *Nature* 424:1014-1014 (2003).
64. Adamson, A. W. "The Gentle Force of Entropy". *Science* 280:655c- (1998).
65. Kestenbaum, D. "PHYSICS: Gentle Force of Entropy Bridges Disciplines". *Science* 279:1849- (1998).
66. Dinsmore, A. D., A. G. Yodh, and D. J. Pine. "Entropic control of particle motion using passive surface microstructures". *Nature* 383:239-242 (1996).
67. Kassies, R., A. Lenferink, I. Segers-Nolten, and C. Otto. "Prism-based excitation wavelength selection for multicolor fluorescence coincidence measurements". *Appl. Opt.* 44:893-897 (2005).
68. Segers-Nolten, G. M. J., C. Wyman, N. Wijgers, W. Vermeulen, J. H. J. Hoeijmakers, A. T. M. Lenferink, J. Greve, and C. Otto. "Scanning confocal fluorescence microscopy for single molecule analysis of nucleotide excision repair complexes". *Nucleic Acids Research* 30:4720-4727 (2002).
69. Zhang, D., H. Lans, W. Vermeulen, A. Lenferink, and C. Otto. "Quantitative Fluorescence Correlation Spectroscopy Reveals a 1000-Fold Increase in Lifetime of Protein Functionality". *Biophys. J.* 95:3439-3446 (2008).
70. Ambrose, W. P., P. M. Goodwin, J. H. Jett, A. Van Orden, J. H. Werner, and R. A. Keller. "Single Molecule Fluorescence Spectroscopy at Ambient Temperature". *Chem. Rev.* 99:2929-2956 (1999).
71. Hausteil, E., and P. Schuille. "Single-molecule spectroscopic methods". *Curr. Opin. Struct. Biol.* 14:531-540 (2004).

72. Kelso, D. M. "Biomedical applications of single molecule detection". In *Proceedings of SPIE - The International Society for Optical Engineering*. 206-212 (1997).
73. Kumbhakar, M., S. Nath, T. Mukherjee, J. P. Mittal, and H. Pal. "Single-molecule detection in exploring nanoenvironments: An overview". *Journal of Photochemistry and Photobiology C: Photochemistry Reviews* 5:113-137 (2004).
74. Michalet, X., and S. Weiss. "Single-molecule spectroscopy and microscopy". *Comptes Rendus Physique* 3:619-644 (2002).
75. Moerner, W. E., and D. P. Fromm. "Methods of single-molecule fluorescence spectroscopy and microscopy". *Rev. Sci. Instrum.* 74:3597-3619 (2003).
76. Nie, S., D. T. Chiu, and R. N. Zare. "Probing individual molecules with confocal fluorescence microscopy". *Science* 266:1018-1021 (1994).
77. Orrit, M., and J. Bernard. "Single pentacene molecules detected by fluorescence excitation in a p-terphenyl crystal". *Phys. Rev. Lett.* 65:2716-2719 (1990).
78. Plakhotnik, T., E. A. Donley, and U. P. Wild. "Single-molecule spectroscopy". In *Annu. Rev. Phys. Chem.* 181-212 (1997).
79. Rigler, R., M. Orrit, and T. Basche. "Single molecule spectroscopy nobel conference lectures: Preface". In *Springer Ser. Chem. Phys.* v-ix (2001).
80. Walter, N. G. "Future of biomedical sciences: Single molecule microscopy". *Biopolymers* 85:103-105 (2007).
81. Walter, N. G., C. Y. Huang, A. J. Manzo, and M. A. Sobhy. "Do-it-yourself guide: How to use the modern single-molecule toolkit". *Nature Methods* 5:475-489 (2008).
82. Xie, X. S. "Single-Molecule Spectroscopy and Dynamics at Room Temperature". *Acc. Chem. Res.* 29:598-606 (1996).
83. Xie, X. S., and J. K. Trautman. "Optical studies of single molecules at room temperature". *Annu. Rev. Phys. Chem.* 49:441-480 (1998).
84. Dickson, R. M., A. B. Cubitt, R. Y. Tsien, and W. E. Moerner. "On/off blinking and switching behaviour of single molecules of green fluorescent protein". *Nature* 388:355-358 (1997).
85. Magde, D., E. L. Elson, and W. W. Webb. "Fluorescence correlation spectroscopy. II. An experimental realization". *Biopolymers* 13:29-61 (1974).
86. Elson, E. L., and D. Magde. "Fluorescence correlation spectroscopy. I. Conceptual basis and theory". *Biopolymers* 13:1-27 (1974).
87. Magde, D., E. Elson, and W. W. Webb. "Thermodynamic Fluctuations in a Reacting System - Measurement by Fluorescence Correlation Spectroscopy". *Phys. Rev. Lett.* 29:705-708 (1972).
88. Boukari, H., and D. L. Sackett. "Fluorescence Correlation Spectroscopy and Its Application to the Characterization of Molecular Properties and Interactions". *Methods Cell Biol.* 84:659-678 (2008).
89. Haustein, E., and P. Schwill. "Fluorescence correlation spectroscopy: Novel variations of an established technique". *Annu. Rev. Biophys. Biomol. Struct.* 36:151-169 (2007).
90. Hwang, L. C., and T. Wohland. "Recent advances in fluorescence cross-correlation spectroscopy". *Cell Biochem. Biophys.* 49:1-13 (2007).
91. Bulseco, D. A., and D. E. Wolf. "Fluorescence Correlation Spectroscopy: Molecular Complexing in Solution and in Living Cells". *Methods Cell Biol.* 81:525-559 (2007).
92. Mikuni, S., and M. Kinjo. "Fluorescence correlation spectroscopy and fluorescence cross correlation spectroscopy for cell biology". *Tanpakushitsu Kakusan Koso.* 51:1998-2005 (2006).
93. Berland, K. M. "Fluorescence correlation spectroscopy: a new tool for quantification of molecular interactions". *Methods Mol. Biol.* 261:383-398 (2004).
94. Blom, H., L. Kastrup, and C. Eggeling. "Fluorescence fluctuation spectroscopy in reduced detection volumes". *Current Pharmaceutical Biotechnology* 7:51-66 (2006).
95. Hess, S. T., S. Huang, A. A. Heikal, and W. W. Webb. "Biological and Chemical Applications of Fluorescence Correlation Spectroscopy: A Review". *Biochemistry (Mosc.)* 41:697-705 (2002).
96. Kim, S. A., and P. Schwill. "Intracellular applications of fluorescence correlation spectroscopy: Prospects for neuroscience". *Curr. Opin. Neurobiol.* 13:583-590 (2003).
97. Kinjo, M. "Single molecule detection using fluorescence correlation spectroscopy". *Tanpakushitsu Kakusan Koso.* 44:1431-1438 (1999).
98. Noda, I. "Advances in two-dimensional correlation spectroscopy". *Vib. Spectrosc* 36:143-165 (2004).
99. Symonds, G. "Fluorescence correlation spectroscopy". *Today's Life Science* 11:16-18 (1999).
100. Takahashi, Y., R. Sawada, K. Ishibashi, S. Mikuni, and M. Kinjo. "Analysis of cellular functions by multipoint fluorescence correlation spectroscopy". *Current Pharmaceutical Biotechnology* 6:159-165 (2005).

101. Thompson, N. L., A. M. Lieto, and N. W. Allen. "Recent advances in fluorescence correlation spectroscopy". *Curr. Opin. Struct. Biol.* 12:634-641 (2002).
102. Widengren, J., and R. Rigler. "Fluorescence correlation spectroscopy as a tool to investigate chemical reactions in solutions and on cell surfaces". *Cellular and molecular biology (Noisy-le-Grand, France)* 44:857-879 (1998).
103. Zhang, P., and J. Ren. "Advances in fluorescence correlation spectroscopy and its applications in single molecule detection". *Fenxi Huaxue* 33:875-880 (2005).
104. Krichinsky, O., and G. Bonnet. "Fluorescence correlation spectroscopy: the technique and its applications". *Rep. Prog. Phys.* 65:251-297 (2002).
105. Webb, W. W. "Fluorescence correlation spectroscopy: Inception, biophysical experimentations, and prospectus". *Appl. Opt.* 40:3969-3983 (2001).
106. Wahl, M., I. Gregor, M. Patting, and J. Enderlein. "Fast calculation of fluorescence correlation data with asynchronous time-correlated single-photon counting". *Opt. Express* 11:3583-3591 (2003).
107. Vukojevic, V., A. Pramanik, T. Yakovleva, R. Rigler, L. Terenius, and G. Bakalkin. "Study of molecular events in cells by fluorescence correlation spectroscopy". *Cellular and Molecular Life Sciences* 62:535-550 (2005).
108. Hansen, J. P., and I. R. McDonald. *Theory of Simple Liquids*. Academic Press, New York, NY.(2006).
109. Wachsmuth, M., W. Waldeck, and J. Langowski. "Anomalous diffusion of fluorescent probes inside living cell investigated by spatially-resolved fluorescence correlation spectroscopy". *J. Mol. Biol.* 298:677-689 (2000).
110. Shusterman, R., T. Gavrinov, and O. Krichinsky. "Internal dynamics of superhelical DNA". *Phys. Rev. Lett.* 100:098102 (2008).
111. Petrov, E. P., T. Ohrt, R. G. Winkler, and P. Schwill. "Diffusion and Segmental Dynamics of Double-Stranded DNA". *Phys. Rev. Lett.* 97:258101-258104 (2006).
112. Shusterman, R., S. Alon, T. Gavrinov, and O. Krichinsky. "Monomer Dynamics in Double- and Single-Stranded DNA Polymers". *Phys. Rev. Lett.* 92:048303 (2004).
113. Brister, P. C., K. K. Kuricheti, V. Buschmann, and K. D. Weston. "Fluorescence correlation spectroscopy for flow rate imaging and monitoring - Optimization, limitations and artifacts". *Lab on a Chip - Miniaturisation for Chemistry and Biology* 5:785-791 (2005).
114. Fogarty, K., and A. Van Orden. "Two-Beam Fluorescence Cross-Correlation Spectroscopy for Simultaneous Analysis of Positive and Negative Ions in Continuous-Flow Capillary Electrophoresis". *Anal. Chem.* 75:6634-6641 (2003).
115. Kuricheti, K. K., V. Buschmann, and K. D. Weston. "Application of fluorescence correlation spectroscopy for velocity imaging in microfluidic devices". *Appl. Spectrosc.* 58:1180-1186 (2004).
116. Lenne, P. F., D. Colombo, H. Giovannini, and H. Rigneault. "Flow profiles and directionality in microcapillaries measured by fluorescence correlation spectroscopy". *Single Molecules* 3:194-200 (2002).
117. Nagy, A., J. Wu, and K. M. Berland. "Characterizing observation volumes and the role of excitation saturation in one-photon fluorescence fluctuation spectroscopy". *Journal of Biomedical Optics* 10:044015-044019 (2005).
118. Nagy, A., J. Wu, and K. M. Berland. "Observation Volumes and γ -Factors in Two-Photon Fluorescence Fluctuation Spectroscopy". *Biophys. J.* 89:2077-2090 (2005).
119. Enderlein, J., I. Gregor, D. Patra, and T. Dertinger. "Art and artifacts of fluorescence correlation spectroscopy". In *Imaging, Manipulation, and Analysis of Biomolecules and Cells: Fundamentals and Applications III*. SPIE, San Jose, CA, USA. 167-174 (2005).
120. Gregor, I., D. Patra, and J. Enderlein. "Optical saturation in fluorescence correlation spectroscopy under continuous-wave and pulsed excitation". *ChemPhysChem* 6:164-170 (2005).
121. Davis, L. M., G. Shen, and D. A. Ball. "Saturation effects in fluorescence correlation spectroscopy". In *Progress in Biomedical Optics and Imaging - Proceedings of SPIE*. 128-137 (2005).
122. Nishimura, G., and M. Kinjo. "Systematic Error in Fluorescence Correlation Measurements Identified by a Simple Saturation Model of Fluorescence". *Anal. Chem.* 76:1963-1970 (2004).
123. Hess, S. T., and W. W. Webb. "Focal Volume Optics and Experimental Artifacts in Confocal Fluorescence Correlation Spectroscopy". *Biophys. J.* 83:2300-2317 (2002).
124. Berland, K., and G. Shen. "Excitation saturation in two-photon fluorescence correlation spectroscopy". *Appl. Opt.* 42:5566-5576 (2003).

125. Marrocco, M. "Fluorescence correlation spectroscopy: Incorporation of probe volume effects into the three-dimensional Gaussian approximation". *Appl. Opt.* 43:5251-5262 (2004).
126. Davis, L. M., and G. Shen. "Accounting for triplet and saturation effects in FCS measurements". *Current Pharmaceutical Biotechnology* 7:287-301 (2006).
127. Elson, E. L. "Fluorescence Correlation Spectroscopy Measures Molecular Transport in Cells". *Traffic* 2:789-796 (2001).
128. Kudryavtsev, V., S. Felekyan, A. K. Wozniak, M. Konig, C. Sandhagen, R. Kuhnemuth, C. A. M. Seidel, and F. Oesterhelt. "Monitoring dynamic systems with multiparameter fluorescence imaging". *Analytical and Bioanalytical Chemistry* 387:71-82 (2007).
129. Widengren, J., and C. A. M. Seidel. "Manipulation and characterization of photo-induced transient states of Merocyanine 540 by fluorescence correlation spectroscopy". *PCCP* 2:3435-3441 (2000).
130. Haupts, U., S. Maiti, P. Schwille, and W. W. Webb. "Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy". *Proc. Natl. Acad. Sci. U. S. A.* 95:13573-13578 (1998).
131. Widengren, J., U. Mets, and R. Rigler. "Photodynamic properties of green fluorescent proteins investigated by fluorescence correlation spectroscopy". *Chem. Phys.* 250:171-186 (1999).
132. Widengren, J., B. Terry, and R. Rigler. "Protonation kinetics of GFP and FITC investigated by FCS - Aspects of the use of fluorescent indicators for measuring pH". *Chem. Phys.* 249:259-271 (1999).
133. Schwille, P., S. Kummer, A. A. Heikal, W. E. Moerner, and W. W. Webb. "Fluorescence correlation spectroscopy reveals fast optical excitation-driven intramolecular dynamics of yellow fluorescent proteins". *Proc. Natl. Acad. Sci. U. S. A.* 97:151-156 (2000).
134. Heikal, A. A., S. T. Hess, G. S. Baird, R. Y. Tsien, and W. W. Webb. "Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: Coral red (dsRed) and yellow (Citrine)". *Proc. Natl. Acad. Sci. U. S. A.* 97:11996-12001 (2000).
135. Heikal, A. A., S. T. Hess, G. S. Baird, R. Y. Tsien, and W. W. Webb. "Erratum: Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: Coral red (dsRed) and yellow (Citrine) (Proceedings of the National Academy of Sciences of the United States of America (October 24, 2000) 97:22 (11996-12001))". *Proc. Natl. Acad. Sci. U. S. A.* 97:14831 (2000).
136. Schenk, A., S. Ivanchenko, C. Rocker, J. Wiedenmann, and G. U. Nienhaus. "Photodynamics of Red Fluorescent Proteins Studied by Fluorescence Correlation Spectroscopy". *Biophys. J.* 86:384-394 (2004).
137. Huang, Z., D. Ji, S. Wang, A. Xia, F. Koberling, M. Patting, and R. Erdmann. "Identification of triplet state and photoisomerization of Cy5 by means of ensemble and single-molecule measurements". In *Ultrasensitive and Single-Molecule Detection Technologies*. SPIE, San Jose, CA, USA. 60920Y-60926 (2006).
138. Huang, Z., D. Ji, S. Wang, A. Xia, F. Koberling, M. Patting, and R. Erdmann. "Spectral identification of specific photophysics of Cy5 by means of ensemble and single molecule measurements". *J. Phys. Chem. A* 110:45-50 (2006).
139. Goulding, D., and J. P. Hansen. "Attraction between like-charged colloidal particles induced by a surface: A density-functional analysis". *Europhys. Lett.* 46:407-413 (1999).
140. Levin, Y. "When do like charges attract?". *Physica A: Statistical Mechanics and its Applications* 265:432-439 (1999).
141. Dufresne, E. R., T. M. Squires, M. P. Brenner, and D. G. Grier. "Hydrodynamic coupling of two brownian spheres to a planar surface". *Phys. Rev. Lett.* 85:3317-3320 (2000).
142. Squires, T. M., and M. P. Brenner. "Like-charge attraction and hydrodynamic interaction". *Phys. Rev. Lett.* 85:4976-4979 (2000).
143. Allahyarov, E., I. D'Amico, and H. Lowen. "Effect of geometrical confinement on the interaction between charged colloidal suspensions". *Physical Review E - Statistical Physics, Plasmas, Fluids, and Related Interdisciplinary Topics* 60:3199-3210 (1999).
144. Lowen, H., E. Allahyarov, and I. D'Amico. "Effective interaction between confined colloids: Repulsion or attraction?". *Prog. Colloid Polym. Sci.* 115:367-370 (2000).
145. Allahyarov, E., I. D'Amico, and H. Lowen. "Attraction between like-charged macroions by coulomb depletion". *Phys. Rev. Lett.* 81:1334-1337 (1998).
146. Ha, B. Y., and A. J. Liu. "Counterion-mediated attraction between two like-charged rods". *Phys. Rev. Lett.* 79:1289-1292 (1997).
147. Neu, J. C. "Wall-Mediated Forces between Like-Charged Bodies in an Electrolyte". *Phys. Rev. Lett.* 82:1072-1074 (1999).

148. Taboada-Serrano, P., S. Yiaccoumi, and C. Tsouris. "Electrostatic surface interactions in mixtures of symmetric and asymmetric electrolytes: A Monte Carlo study". *J. Chem. Phys.* 125 (2006).
149. Molnar, F., and J. Rieger. "'Like-charge attraction' between anionic polyelectrolytes: Molecular dynamics simulations". *Langmuir* 21:786-789 (2005).
150. Bai, Y., R. Das, I. S. Millett, D. Herschlag, and S. Doniach. "Probing counterion modulated repulsion and attraction between nucleic acid duplexes in solution". *Proc. Natl. Acad. Sci. U. S. A.* 102:1035-1040 (2005).
151. Qiu, X., L. W. Kwok, H. Y. Park, J. S. Lamb, K. Andresen, and L. Pollack. "Measuring inter-DNA potentials in solution". *Phys. Rev. Lett.* 96:1-4 (2006).
152. Angelini, T. E., H. Liang, W. Wriggers, and G. C. L. Wong. "Like-charge attraction between polyelectrolytes induced by counterion charge density waves". *Proc. Natl. Acad. Sci. U. S. A.* 100:8634-8637 (2003).
153. According to the product instruction (product No. PA25001) of Amersham Biosciences, Piscataway, NJ, United States.
154. Chandler, D. "Interfaces and the driving force of hydrophobic assembly". *Nature* 437:640-647 (2005).
155. Atkins, P., and J. d. Paula. *Physical Chemistry*. W. H. Freeman, New York, NY (2001).
156. Varnai, P., and K. Zakrzewska. "DNA and its counterions: A molecular dynamics study". *Nucleic Acids Res.* 32:4269-4280 (2004).
157. Siegel, J., K. Suhling, S. Leveque-Fort, S. E. D. Webb, D. M. Davis, D. Phillips, Y. Sabharwal, and P. M. W. French. "Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): Imaging the rotational mobility of a fluorophore". *Rev. Sci. Instrum.* 74:182-192 (2003).
158. Axelrod, D. "Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization". *Biophys. J.* 26:557-573 (1979).
159. Ha, T., T. A. Laurence, D. S. Chemla, and S. Weiss. "Polarization Spectroscopy of Single Fluorescent Molecules". *J. Phys. Chem. B* 103:6839-6850 (1999).
160. Axelrod, D. "Fluorescence polarization microscopy". *Methods Cell Biol.* 30:333-352 (1989).
161. Richards, B., and E. Wolf. "Electromagnetic Diffraction in Optical Systems. II. Structure of the Image Field in an Aplanatic System". *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences* 253:358-379 (1959).
162. Wolf, E. "Electromagnetic Diffraction in Optical Systems. I. An Integral Representation of the Image Field". *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences* 253:349-357 (1959).
163. Davey, A. M., R. P. Walvick, Y. Liu, A. A. Heikal, and E. D. Sheets. "Membrane Order and Molecular Dynamics Associated with IgE Receptor Cross-Linking in Mast Cells". *Biophys. J.* 92:343-355 (2007).
164. Hess, S. T., E. D. Sheets, A. Wagenknecht-Wiesner, and A. A. Heikal. "Quantitative analysis of the fluorescence properties of intrinsically fluorescent proteins in living cells". *Biophys. J.* 85:2566-2580 (2003).
165. Thompson, R. E., D. R. Larson, and W. W. Webb. "Precise Nanometer Localization Analysis for Individual Fluorescent Probes". *Biophys. J.* 82:2775-2783 (2002).
166. Lagerholm, B. C., L. Averett, G. E. Weinreb, K. Jacobson, and N. L. Thompson. "Analysis Method for Measuring Submicroscopic Distances with Blinking Quantum Dots". *Biophys. J.* 91:3050-3060 (2006).
167. Snyder, G. E., T. Sakamoto, J. A. Hammer, III, J. R. Sellers, and P. R. Selvin. "Nanometer Localization of Single Green Fluorescent Proteins: Evidence that Myosin V Walks Hand-Over-Hand via Telemark Configuration". *Biophys. J.* 87:1776-1783 (2004).
168. Yildiz, A., H. Park, D. Safer, Z. Yang, L.-Q. Chen, P. R. Selvin, and H. L. Sweeney. "Myosin VI Steps via a Hand-over-Hand Mechanism with Its Lever Arm Undergoing Fluctuations when Attached to Actin". *J. Biol. Chem.* 279:37223-37226 (2004).
169. Yildiz, A., J. N. Forkey, S. A. McKinney, T. Ha, Y. E. Goldman, and P. R. Selvin. "Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization". *Science* 300:2061-2065 (2003).
170. Gordon, M. P., T. Ha, and P. R. Selvin. "Single-molecule high-resolution imaging with photobleaching". *Proc. Natl. Acad. Sci. U. S. A.* 101:6462-6465 (2004).
171. Park, H., G. T. Hanson, S. R. Duff, and P. R. Selvin. "Nanometre localization of single ReAsH molecules". *J. Microsc.* 216:199-205 (2004).
172. Qu, X., D. Wu, L. Mets, and N. F. Scherer. "Nanometer-localized multiple single-molecule fluorescence microscopy". *Proc. Natl. Acad. Sci. U. S. A.* 101:11298-11303 (2004).

173. Lacoste, T. D., X. Michalet, F. Pinaud, D. S. Chemla, A. P. Alivisatos, and S. Weiss. "Ultra-high-resolution multicolor colocalization of single fluorescent probes". *Proc. Natl. Acad. Sci. U. S. A.* 97:9461-9466 (2000).
174. Hanninen, P. "Light microscopy (communication arising): Beyond the diffraction limit". *Nature* 419:802-802 (2002).
175. Stelzer. "Contrast, resolution, pixelation, dynamic range and signal-to-noise ratio: fundamental limits to resolution in fluorescence light microscopy". *J. Microsc.* 189:15-24 (1998).
176. Stelzer, E. H. K. "Light microscopy: Beyond the diffraction limit?". *Nature* 417:806-807 (2002).
177. Bobroff, N. "Position measurement with a resolution and noise-limited instrument". *Rev. Sci. Instrum.* 57:1152-1157 (1986).
178. Ober, R. J., S. Ram, and E. S. Ward. "Localization Accuracy in Single-Molecule Microscopy". *Biophys. J.* 86:1185-1200 (2004).
179. Bevington, P. R., and D. K. Robinson. *Data Reduction and Error Analysis for the Physical Sciences*. McGraw-Hill, New York.(1969).
180. Born, M., and E. Wolf. *Principles of Optics* Cambridge Univ. Press, Cambridge, UK.(1997).
181. Cheezum, M. K., W. F. Walker, and W. H. Guilford. "Quantitative comparison of algorithms for tracking single fluorescent particles". *Biophys. J.* 81:2378-2388 (2001).
182. Kubitscheck, U., O. Kuckmann, T. Kues, and R. Peters. "Imaging and Tracking of Single GFP Molecules in Solution". *Biophys. J.* 78:2170-2179 (2000).
183. Lewin, L. *Dilogarithms and associated functions*. Macdonald, London.(1958).
184. The derivation was performed with the help of Wolfram Mathematica 6 (Wolfram Research, Champaign, IL, United States).
185. Willig, K. I., S. O. Rizzoli, V. Westphal, R. Jahn, and S. W. Hell. "STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis". *Nature* 440:935-939 (2006).
186. Betzig, E., and R. J. Chichester. "Single Molecules Observed by Near-Field Scanning Optical Microscopy". *Science* 262:1422-1425 (1993).
187. Hess, S. T., T. P. K. Girirajan, and M. D. Mason. "Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy". *Biophys. J.* 91:4258-4272 (2006).
188. Shroff, H., C. G. Galbraith, J. A. Galbraith, H. White, J. Gillette, S. Olenych, M. W. Davidson, and E. Betzig. "Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes". *Proc. Natl. Acad. Sci. U. S. A.* 104:20308-20313 (2007).
189. Yildiz, A., M. Tomishige, R. D. Vale, and P. R. Selvin. "Kinesin Walks Hand-Over-Hand". *Science* 303:676-678 (2004).
190. Gao, X., and S. Nie. "Molecular profiling of single cells and tissue specimens with quantum dots". *Trends Biotechnol.* 21:371-373 (2003).
191. Michalet, X., F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir, and S. Weiss. "Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics". *Science* 307:538-544 (2005).
192. Lidke, K. A., B. Rieger, T. M. Jovin, and R. Heintzmann. "Superresolution by localization of quantum dots using blinking statistics". *Opt. Express* 13:7052-7062 (2005).
193. Wazawa, T., Y. Ishizuka-Katsura, S. Nishikawa, A. H. Iwane, and S. Aoyama. "Grafting of Poly(ethylene glycol) onto Poly(acrylic acid)-Coated Glass for a Protein-Resistant Surface". *Anal. Chem.* 78:2549-2556 (2006).
194. According to the product instruction (product No. Q10151MP) of Invitrogen, Carlsbad, CA, United States.
195. Ehrenberg, M., and R. Rigler. "Rotational brownian motion and fluorescence intensify fluctuations". *Chem. Phys.* 4:390-401 (1974).
196. Rigler, R., and E. S. Elson. *Fluorescence Correlation Spectroscopy: Theory and Applications: V. 65* (Springer Series in Chemical Physics). Springer-Verlag Berlin and Heidelberg GmbH & Co. K, Heidelberg, Germany.(2001).
197. Schwille, P. "Fluorescence correlation spectroscopy and its potential for intracellular applications". *Cell Biochem. Biophys.* 34:383-408 (2001).
198. Aragón, S. R., and R. Pecora. "Fluorescence correlation spectroscopy as a probe of molecular dynamics". *J. Chem. Phys.* 64:1791-1803 (1976).
199. Tsien, R. Y. "The green fluorescent protein". *Annu. Rev. Biochem.* 67:509-544 (1998).
200. Shaner, N. C., R. E. Campbell, P. A. Steinbach, B. N. G. Giepmans, A. E. Palmer, and R. Y. Tsien. "Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein". *Nat Biotech* 22:1567-1572 (2004).
201. Roberts, R. J., M. Belfort, T. Bestor, A. S. Bhagwat, T. A. Bickle, J. Bitinaite, R. M. Blumenthal, S. K. Degtyarev, D. T. F. Dryden, K. Dybvig, K. Firman, E. S. Gromova, R. I. Gumport, S. E.

- Halford, S. Hattman, J. Heitman, D. P. Hornby, A. Janulaitis, A. Jeltsch, J. Josephsen, A. Kiss, T. R. Klaenhammer, I. Kobayashi, H. Kong, D. H. Kruger, S. Lacks, M. G. Marinus, M. Miyahara, R. D. Morgan, N. E. Murray, V. Nagaraja, A. Piekarowicz, A. Pingoud, E. Raleigh, D. N. Rao, N. Reich, V. E. Repin, E. U. Selker, P.-C. Shaw, D. C. Stein, B. L. Stoddard, W. Szybalski, T. A. Trautner, J. L. Van Etten, J. M. B. Vitor, G. G. Wilson, and S.-y. Xu. "A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes". *Nucl. Acids Res.* 31:1805-1812 (2003).
202. Robson, R. J., and E. A. Dennis. "The size, shape, and hydration of nonionic surfactant micelles. Triton X-100". *J. Phys. Chem.* 81:1075-1078 (1977).
203. Franks, F. "Protein stability: the value of 'old literature'". *Biophys. Chem.* 96:117-127 (2002).
204. Ernst, J. A., R. T. Clubb, H. X. Zhou, A. M. Gronenborn, and G. M. Clore. "Demonstration of positionally disordered water within a protein hydrophobic cavity by NMR". *Science* 267:1813-1817 (1995).
205. Cheung, M. S., A. E. Garcia, and J. N. Onuchic. "Protein folding mediated by solvation: Water expulsion and formation of the hydrophobic core occur after the structural collapse". *Proc. Natl. Acad. Sci. U. S. A.* 99:685-690 (2002).
206. Fernandez, A., and H. A. Scheraga. "Insufficiently dehydrated hydrogen bonds as determinants of protein interactions". *Proc. Natl. Acad. Sci. U. S. A.* 100:113-118 (2003).
207. Fernandez, A., and L. R. Scott. "Adherence of Packing Defects in Soluble Proteins". *Phys. Rev. Lett.* 91:018102 (2003).
208. Pal, S. K., J. Peon, B. Bagchi, and A. H. Zewail. "Biological Water: Femtosecond Dynamics of Macromolecular Hydration". *J. Phys. Chem. B* 106:12376-12395 (2002).
209. Pal, S. K., J. Peon, and A. H. Zewail. "Biological water at the protein surface: Dynamical solvation probed directly with femtosecond resolution". *Proc. Natl. Acad. Sci. U. S. A.* 99:1763-1768 (2002).
210. Kuntz, I. D., Jr., and W. Kauzmann. "Hydration of proteins and polypeptides". *Adv. Protein, Chem.* 28:239-345 (1974).
211. Cheng, Y.-K., and P. J. Rossky. "Surface topography dependence of biomolecular hydrophobic hydration". *Nature* 392:696-699 (1998).
212. Smolin, N., A. Oleinikova, I. Brovchenko, A. Geiger, and R. Winter. "Properties of Spanning Water Networks at Protein Surfaces". *J. Phys. Chem. B* 109:10995-11005 (2005).
213. Teeter, M. M., A. Yamano, B. Stec, and U. Mohanty. "On the nature of a glassy state of matter in a hydrated protein: Relation to protein function". *Proc. Natl. Acad. Sci. U. S. A.* 98:11242-11247 (2001).
214. Nandi, N., K. Bhattacharyya, and B. Bagchi. "Dielectric Relaxation and Solvation Dynamics of Water in Complex Chemical and Biological Systems". *Chem. Rev.* 100:2013-2046 (2000).
215. Bray, D. In *Cell Movements: From Molecules to Motility*. Garland Science Taylor & Francis group, London. 303 (2000).
216. Friedberg E.C., G. C. Walker, and W. Siede. *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.(1995).
217. de Laat, W. L., N. G. J. Jaspers, and J. H. J. Hoeijmakers. "Molecular mechanism of nucleotide excision repair". *Genes Dev.* 13:768-785 (1999).
218. Sugawara, K., T. Okamoto, Y. Shimizu, C. Masutani, S. Iwai, and F. Hanaoka. "A multistep damage recognition mechanism for global genomic nucleotide excision repair". *Genes Dev.* 15:507-521 (2001).
219. Foldes-Papp, Z. "Fluorescence fluctuation spectroscopic approaches to the study of a single molecule diffusing in solution and a live cell without systemic drift or convection: A theoretical study". *Current Pharmaceutical Biotechnology* 8:261-273 (2007).
220. Sakudo, A., I. Nakamura, K. Ikuta, and T. Onodera. "Recent developments in prion disease research: Diagnostic tools and in vitro cell culture models". *J. Vet. Med. Sci.* 69:329-337 (2007).
221. Van Orden, A., and J. Jung. "Fluorescence correlation spectroscopy for probing the kinetics and mechanisms of DNA hairpin formation". *Biopolymers* 89:1-16 (2008).
222. LiCata, V. J., and A. J. Wowor. "Applications of Fluorescence Anisotropy to the Study of Protein-DNA Interactions". *Methods Cell Biol.* 84:243-262 (2008).
223. Eccleston, J. F., S. R. Martin, and M. J. Schilstra. "Rapid Kinetic Techniques". *Methods Cell Biol.* 84:445-477 (2008).
224. Piston, D. W., and M. A. Rizzo. "FRET by Fluorescence Polarization Microscopy". *Methods Cell Biol.* 85:415-430 (2008).
225. Tramier, M., and M. Coppey-Moisán. "Fluorescence Anisotropy Imaging Microscopy for Homo-FRET in Living Cells". *Methods Cell Biol.* 85:395-414 (2008).

226. Small, E. W., and I. Isenberg. "Hydrodynamic properties of a rigid molecule: rotational and linear diffusion and fluorescence anisotropy". *Biopolymers* 16:1907-1928 (1977).
227. Belford, G. G., R. L. Belford, and G. Weber. "Dynamics of Fluorescence Polarization in Macromolecules". *Proc. Natl. Acad. Sci. U. S. A.* 69:1392-1393 (1972).
228. Kinosita, K., Jr., A. Ikegami, and S. Kawato. "On the wobbling-in-cone analysis of fluorescence anisotropy decay". *Biophys. J.* 37:461-464 (1982).
229. Vogel, H., and F. Jahnig. "Fast and slow orientational fluctuations in membranes". *Proc. Natl. Acad. Sci. U. S. A.* 82:2029-2033 (1985).
230. Restall, C. J., R. E. Dale, E. K. Murray, C. W. Gilbert, and D. Chapman. "Rotational diffusion of calcium-dependent adenosine-5'-triphosphatase in sarcoplasmic reticulum: A detailed study". *Biochemistry (Mosc)*. 23:6765-6776 (1984).
231. Bialik, C. N., B. Wolf, E. L. Rachofsky, J. B. Ross, and W. R. Laws. "Dynamics of Biomolecules: Assignment of Local Motions by Fluorescence Anisotropy Decay". *Biophys. J.* 75:2564-2573 (1998).
232. Henning, K. A., C. Peterson, R. Legerski, and E. C. Friedberg. "Cloning the *Drosophila* homolog of the xeroderma pigmentosum complementation group C gene reveals homology between the predicted human and *Drosophila* polypeptides and that encoded by the yeast RAD4 gene". *Nucleic Acids Res.* 22:257-261 (1994).
233. Masutani, C., M. Araki, K. Sugawara, P. J. Van Der Spek, A. Yamada, A. Uchida, T. Maekawa, D. Bootsma, J. H. J. Hoeijmakers, and F. Hanaoka. "Identification and characterization of XPC-binding domain of hHR23B". *Mol. Cell. Biol.* 17:6915-6923 (1997).
234. Sugawara, K., C. Masutani, A. Uchida, T. Maekawa, P. J. Van Der Spek, D. Bootsma, J. H. J. Hoeijmakers, and F. Hanaoka. "hHR23B, a human Rad23 homolog, stimulates XPC protein in nucleotide excision repair in vitro". *Mol. Cell. Biol.* 16:4852-4861 (1996).
235. Gillet, L. C. J., J. Alzeer, and O. D. Scharer. "Site-specific incorporation of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) into oligonucleotides using modified 'ultra-mild' DNA synthesis". *Nucleic Acids Res.* 33:1961-1969 (2005).
236. Lauffenburger, D. A., and J. J. Linderman. *Receptors: Models for binding, trafficking and signaling*. Oxford University Press, London, UK.(1993).
237. Yung Chi, C., and W. H. Prusoff. "Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction". *Biochem. Pharmacol.* 22:3099-3108 (1973).
238. Hey, T., G. Lipps, and G. Krauss. "Binding of XPA and RPA to Damaged DNA Investigated by Fluorescence Anisotropy". *Biochemistry (Mosc)*. 40:2901-2910 (2001).
239. Mendel, C. M., and D. B. Mendel. "'Non-specific' binding. The problem, and a solution". *Biochem. J* 228:269-272 (1985).
240. Rocheleau, J. V., M. Edidin, and D. W. Piston. "Intrasequence GFP in Class I MHC Molecules, a Rigid Probe for Fluorescence Anisotropy Measurements of the Membrane Environment". *Biophys. J.* 84:4078-4086 (2003).
241. Reardon, J. T., D. Mu, and A. Sancar. "Overproduction, Purification, and Characterization of the XPC Subunit of the Human DNA Repair Excision Nuclease". *J. Biol. Chem.* 271:19451-19456 (1996).
242. Guzder, S. N., P. Sung, L. Prakash, and S. Prakash. "Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA". *J. Biol. Chem.* 273:31541-31546 (1998).
243. Janicijevic, A., D. Ristic, and C. Wyman. "The molecular machines of DNA repair: Scanning force microscopy analysis of their architecture". *J. Microsc.* 212:264-272 (2003).
244. Janicijevic, A., K. Sugawara, Y. Shimizu, F. Hanaoka, N. Wijgers, M. Djurica, J. H. J. Hoeijmakers, and C. Wyman. "DNA bending by the human damage recognition complex XPC-hHR23B". *DNA Repair* 2:325-336 (2003).
245. Gunz, D., M. T. Hess, and H. Naegeli. "Recognition of DNA adducts by human nucleotide excision repair. Evidence for a thermodynamic probing mechanism". *J. Biol. Chem.* 271:25089-25098 (1996).
246. Friedberg, E. C. "How nucleotide excision repair protects against cancer". *Nat Rev Cancer* 1:22-33 (2001).
247. Van Hoffen, A., J. Venema, R. Meschini, A. A. Van Zeeland, and L. H. F. Mullenders. "Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts". *EMBO Journal* 14:360-367 (1995).

248. Tornaletti, S., and P. C. Hanawalt. "Effect of DNA lesions on transcription elongation". *Biochimie* 81:139-146 (1999).
249. Sieberg, D., C. M. Roth, and D. P. Herten. "Single molecule fluorescence spectroscopy - Approaches towards quantitative investigations of structure and dynamics in living cells". In *Ultrasensitive and Single-Molecule Detection Technologies*. SPIE, San Jose, CA, USA. 609202-609205 (2006).
250. Tramier, M., and M. Coppey-Moisan. "Fluorescence Anisotropy Imaging Microscopy for Homo-FRET in Living Cells". In *Methods in Cell Biology*. 395-414 (2008).
251. Bader, A. N., E. G. Hofman, P. Van Bergen En Henegouwen, and H. C. Gerritsen. "Confocal time-resolved fluorescence anisotropy imaging". In *Progress in Biomedical Optics and Imaging - Proceedings of SPIE*, (2007).
252. Bader, A. N., E. G. Hofman, P. M. P. Van Bergenen Henegouwen, and H. C. Gerritsen. "Imaging of protein cluster sizes by means of confocal time-gated fluorescence anisotropy microscopy". *Optics Express* 15:6934-6945 (2007).
253. Bulseco, D. A., and D. E. Wolf. "Fluorescence Correlation Spectroscopy: Molecular Complexing in Solution and in Living Cells". In *Methods in Cell Biology*. 525-559 (2007).
254. Levin, M. K., and J. H. Carson. "Fluorescence correlation spectroscopy and quantitative cell biology". *Differentiation* 72:1-10 (2004).
255. Kim, S. A., K. G. Heinze, and P. Schuille. "Fluorescence correlation spectroscopy in living cells". *Nature Methods* 4:963-973 (2007).
256. DaCosta, R. S., B. C. Wilson, and N. E. Marcon. "Fluorescence and spectral imaging". *TheScientificWorldJournal* 7:2046-4071 (2007).
257. Schwartz, S. "Spectral imaging evolves to meet biomedical needs". *Laser Focus World* 41:87-91 (2005).
258. Haraguchi, T., T. Shimi, T. Koujin, N. Hashiguchi, and Y. Hiraoka. "Spectral imaging fluorescence microscopy". *Genes to Cells* 7:881-887 (2002).
259. Dix, J. A., and A. S. Verkman. "Mapping of fluorescence anisotropy in living cells by ratio imaging. Application to cytoplasmic viscosity". *Biophys. J.* 57:231-240 (1990).
260. Gough, A. H., and D. L. Taylor. "Fluorescence anisotropy imaging microscopy maps calmodulin binding during cellular contraction and locomotion". *Journal of Cell Biology* 121:1095-1107 (1993).
261. Blackman, S. M., C. E. Cobb, A. H. Beth, and D. W. Piston. "The orientation of eosin-5-maleimide on human erythrocyte band 3 measured by fluorescence polarization microscopy". *Biophysical Journal* 71:194-208 (1996).
262. Bigelow, C. E., D. L. Conover, and T. H. Foster. "Confocal fluorescence spectroscopy and anisotropy imaging system". *Optics Letters* 28:695-697 (2003).
263. Axelrod, D. "Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization". *Biophysical Journal* 26:557-573 (1979).
264. Hoogstraten, D. "The spatio-temporal orgnization of DNA-repair: A live cell study". In (PhD dissertation). Erasmus Medical Center, Rotterdam, the Netherlands. Chapter 8, 129 (2003).
265. Siegel, J., K. Suhling, S. Leveque-Fort, S. E. D. Webb, D. M. Davis, D. Phillips, Y. Sabharwal, and P. M. W. French. "Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): Imaging the rotational mobility of a fluorophore". *Review of Scientific Instruments* 74:182-192 (2003).
266. Schaffler, J., A. Volkmer, C. Eggeling, V. Subramaniam, G. Striker, and C. A. M. Seidel. "Identification of Single Molecules in Aqueous Solution by Time-Resolved Fluorescence Anisotropy". *J. Phys. Chem. A* 103:X1-336 (1999).
267. Brock, R., M. A. Hink, and T. M. Jovin. "Fluorescence correlation microscopy of cells in the presence of autofluorescence". *Biophys. J.* 75:2547-2557 (1998).
268. Brock, R., and T. M. Jovin. "Fluorescence correlation microscopy (FCM)-fluorescence correlation spectroscopy (FCS) taken into the cell". *Cellular and molecular biology (Noisy-le-Grand, France)* 44:847-856 (1998).
269. Brock, R., and T. M. Jovin. "Fluorescence correlation microscopy (FCM): Fluorescence correlation spectroscopy (FCS) in cell biology". In *Springer Ser. Chem. Phys.* 132-161 (2001).
270. Brock, R., G. Vamosi, G. Vereb, and T. M. Jovin. "Rapid characterization of green fluorescent protein fusion proteins on the molecular and cellular level by fluorescence correlation microscopy". *Proc. Natl. Acad. Sci. U. S. A.* 96:10123-10128 (1999).
271. Press, W. H., B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling. ""Power Spectra Estimation Using the FFT" and "Power Spectrum Estimation by the Maximum Entropy (All Poles) Method."

- ". In *Numerical Recipes in FORTRAN: The Art of Scientific Computing*, 2nd ed. Cambridge University Press, Cambridge, England. 542-551 and 565-569 (1992).
272. Mu, D., C. H. Park, T. Matsunaga, D. S. Hsu, J. T. Reardon, and A. Sancar. "Reconstitution of human DNA repair excision nuclease in a highly defined system". *J. Biol. Chem.* 270:2415-2418 (1995).
273. Aboussekhra, A., M. Biggerstaff, M. K. K. Shivji, J. A. Vilpo, V. Moncollin, V. N. Podust, M. Protic, U. Hubscher, J. M. Egly, and R. D. Wood. "Mammalian DNA nucleotide excision repair reconstituted with purified protein components". *Cell* 80:859-868 (1995).
274. Kusumoto, R., C. Masutani, K. Sugawara, S. Iwai, M. Araki, A. Uchida, T. Mizukoshi, and F. Hanaoka. "Diversity of the damage recognition step in the global genomic nucleotide excision repair in vitro". *Mutation Research - DNA Repair* 485:219-227 (2001).
275. Elias, A. L., J. A. Rodriguez-Manzo, M. R. McCartney, D. Golberg, A. Zamudio, S. E. Baltazar, F. Lopez-Urias, E. Munoz-Sandoval, L. Gu, C. C. Tang, D. J. Smith, Y. Bando, H. Terrones, and M. Terrones. "Production and characterization of single-crystal FeCo nanowires inside carbon nanotubes". *Nano Lett.* 5:467-472 (2005).
276. Yan, H., A. Saiani, J. E. Gough, and A. F. Miller. "Thermoreversible protein hydrogel as cell scaffold". *Biomacromolecules* 7:2776-2782 (2006).
277. Pease, R. F. W., and W. C. Nixon. "High resolution scanning electron microscopy". In *Advances in Imaging and Electron Physics*. 195-205 (2004).
278. Fu, A., C. M. Micheel, J. Cha, H. Chang, H. Yang, and A. P. Alivisatos. "Discrete nanostructures of quantum Dots/Au with DNA". *J. Am. Chem. Soc.* 126:10832-10833 (2004).
279. Muller, S. A., and A. Engel. "Biological scanning transmission electron microscopy: Imaging and single molecule mass determination". *Chimia* 60:749-753 (2006).
280. Mikkelsen, A., N. Skold, L. Ouattara, M. Borgstrom, J. N. Andersen, L. Samuelson, W. Seifert, and E. Lundgren. "Direct imaging of the atomic structure inside a nanowire by scanning tunnelling microscopy". *Nature Materials* 3:519-523 (2004).
281. Mukhopadhyay, R. "Molecular level structural studies of metalloproteins/metalloenzymes by scanning tunnelling microscopy: Scopes and promises". *Curr. Sci.* 84:1202-1210 (2003).
282. Williams, P. M., M. S. Cheema, M. C. Davies, D. E. Jackson, and S. J. B. Tendler. "Biological Applications of Scanning Tunneling Microscopy". In *Methods Mol. Biol. Humana Press*, Totowa. 1064-3745 (1994).
283. de Lange, F., A. Cambi, R. Huijbens, B. de Bakker, W. Rensen, M. Garcia-Parajo, N. van Hulst, and C. G. Figdor. "Cell biology beyond the diffraction limit: near-field scanning optical microscopy". *J. Cell Sci.* 114:4153-4160 (2001).
284. Gould, S., O. Marti, B. Drake, L. Hellemans, C. E. Bracker, P. K. Hansma, N. L. Keder, M. M. Eddy, and G. D. Stucky. "Molecular resolution images of amino acid crystals with the atomic force microscope". *Nature* 332:332-334 (1988).
285. Bahatyrova, S., R. N. Frese, C. A. Siebert, J. D. Olsen, K. O. Van Der Werf, R. Van Grondelle, R. A. Nijderman, P. A. Bullough, C. Otto, and C. N. Hunter. "The native architecture of a photosynthetic membrane". *Nature* 430:1058-1062 (2004).
286. Van Raaij, M. E., I. M. J. Segers-Nolten, and V. Subramaniam. "Quantitative morphological analysis reveals ultrastructural diversity of amyloid fibrils from α -synuclein mutants". *Biophys. J.* 91:L96-L98 (2006).
287. Weiss, S. "Fluorescence spectroscopy of single biomolecules". *Science* 283:1676-1683 (1999).
288. Park, H., E. Toprak, and P. R. Selvin. "Single-molecule fluorescence to study molecular motors". *Q. Rev. Biophys.* 40:87-111 (2007).
289. Park, H., A. Li, L.-Q. Chen, A. Houdusse, P. R. Selvin, and H. L. Sweeney. "The unique insert at the end of the myosin VI motor is the sole determinant of directionality". *Proc. Natl. Acad. Sci. U. S. A.* 104:778-783 (2007).
290. Reck-Peterson, S. L., A. Yildiz, A. P. Carter, A. Gennerich, N. Zhang, and R. D. Vale. "Single-Molecule Analysis of Dynein Processivity and Stepping Behavior". *Cell* 126:335-348 (2006).
291. Churchman, L. S., Z. Okten, R. S. Rock, J. F. Dawson, and J. A. Spudis. "Single molecule high-resolution colocalization of Cy3 and Cy5 attached to macromolecules measures intramolecular distances through time". *Proc. Natl. Acad. Sci. U. S. A.* 102:1419-1423 (2005).
292. Warshaw, D. M., G. G. Kennedy, S. S. Work, E. B. Krementsova, S. Beck, and K. M. Trybus. "Differential labeling of myosin V heads with quantum dots allows direct visualization of hand-over-hand processivity". *Biophys. J.* 88:L30-L32 (2005).
293. Franca, L. T. C., E. Carrilho, and T. B. L. Kist. "A review of DNA sequencing techniques". *Q. Rev. Biophys.* 35:169-200 (2002).

294. Adachi, K., R. Yasuda, H. Noji, H. Itoh, Y. Harada, M. Yoshida, and K. Kinoshita Jr. "Stepping rotation of F1-ATPase visualized through angle-resolved single-fluorophore imaging". *Proc. Natl. Acad. Sci. U. S. A.* 97:7243-7247 (2000).
295. Hendrickson, W. A., A. Pahler, J. L. Smith, Y. Satow, E. A. Merritt, and R. P. Phizackerley. "Crystal Structure of Core Streptavidin Determined from Multiwavelength Anomalous Diffraction of Synchrotron Radiation". *Proc. Natl. Acad. Sci. U. S. A.* 86:2190-2194 (1989).
296. Gouy, G. "Constitution of the electric charge at the surface of an electrolyte". *Journal de Physique (France)* 9:14 (1910).
297. Chapman, D. L. "A contribution to the theory of electrocapillarity". *Philos. Mag.* 25:475-481 (1913).
298. Koelsch, P., P. Viswanath, H. Motschmann, V. L. Shapovalov, G. Brezesinski, H. Mohwald, D. Horinek, R. R. Netz, K. Giewekemeyer, T. Salditt, H. Schollmeyer, R. von Klitzing, J. Daillant, and P. Guenoun. "Specific ion effects in physicochemical and biological systems: Simulations, theory and experiments". *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 303:110-136 (2007).
299. Jungwirth, P., and B. Winter. "Ions at aqueous interfaces: From water surface to hydrated proteins". In *Annu. Rev. Phys. Chem.* 343-366 (2008).
300. Hofmeister, F. "Zur Lehre von der Wirkung der Salze - Zweite Mittheilung". *Archiv fur Experimentelle Pathologie und Pharmakologie* 24:247-260 (1888).
301. Rau, D. C., and V. A. Parsegian. "Direct measurement of the intermolecular forces between counterion-condensed DNA double helices. Evidence for long range attractive hydration forces". *Biophys. J.* 61:246-259 (1992).
302. Rau, D. C., and V. A. Parsegian. "Direct measurement of temperature-dependent solvation forces between DNA double helices". *Biophys. J.* 61:260-271 (1992).
303. Harlepp, S., J. Robert, N. C. Darron, and D. Chatenay. "Subnanometric measurements of evanescent wave penetration depth using total internal reflection microscopy combined with fluorescent correlation spectroscopy". *Appl. Phys. Lett.* 85:3917-3919 (2004).
304. Hassler, K., M. Leutenegger, P. Rigler, R. Rao, R. Rigler, M. Gotsch, and T. Lasser. "Total internal reflection fluorescence correlation spectroscopy (TIR-FCS) with low background and high count-rate per molecule". *Opt. Express* 13:7415-7423 (2005).
305. Hassler, K., P. Rigler, H. Blom, R. Rigler, J. Widengren, and T. Lasser. "Dynamic disorder in horseradish peroxidase observed with total internal reflection fluorescence correlation spectroscopy". *Opt. Express* 15:5366-5375 (2007).
306. Sonesson, A. W., H. Blom, K. Hassler, U. M. Elofsson, T. H. Callisen, J. Widengren, and H. Brismar. "Protein-surfactant interactions at hydrophobic interfaces studied with total internal reflection fluorescence correlation spectroscopy (TIR-FCS)". *J. Colloid Interface Sci.* 317:449-457 (2008).
307. Starr, T. E., and N. L. Thompson. "Total internal reflection with fluorescence correlation spectroscopy: Combined surface reaction and solution diffusion". *Biophys. J.* 80:1575-1584 (2001).
308. Thompson, N. L., T. P. Burghardt, and D. Axelrod. "Measuring surface dynamics of biomolecules by total internal reflection fluorescence with photobleaching recovery or correlation spectroscopy". *Biophys. J.* 33:435-454 (1981).
309. Thompson, N. L., and B. L. Steele. "Total internal reflection with fluorescence correlation spectroscopy". *Nature Protocols* 2:878-890 (2007).
310. Hink, M. A., A. van Hoek, and A. J. W. G. Visser. "Dynamics of Phospholipid Molecules in Micelles: Characterization with Fluorescence Correlation Spectroscopy and Time-Resolved Fluorescence Anisotropy". *Langmuir* 15:992-997 (1999).
311. Yu, L., M. Tan, B. Ho, J. L. Ding, and T. Wohland. "Determination of critical micelle concentrations and aggregation numbers by fluorescence correlation spectroscopy: Aggregation of a lipopolysaccharide". *Anal. Chim. Acta* 556:216-225 (2006).
312. Zettl, H., Y. Portnoy, M. Gottlieb, and G. Krausch. "Investigation of Micelle Formation by Fluorescence Correlation Spectroscopy". *J. Phys. Chem. B* 109:13397-13401 (2005).
313. Discher, D. E., and F. Ahmed. "Polymersomes". In *Annual Review of Biomedical Engineering*. 323-341 (2006).
314. Lee, J. S., W. Zhou, D. Zhang, C. Otto, and J. Feijen. "Hydrosomes, novel thermosensitive gel-containing polymersomes". In *10th European Symposium on Controlled Drug Delivery (April 2-4, 2008) Noordwijk aan Zee, the Netherlands*. p140 (2008).

315. Wang, K., X. Qiu, C. Dong, and J. Ren. "Single-molecule technology for rapid detection of DNA hybridization based on resonance light scattering of gold nanoparticles". *ChemBioChem* 8:1126-1129 (2007).
316. Kuyper, C. L., K. L. Budzinski, R. M. Lorenz, and D. T. Chiu. "Real-time sizing of nanoparticles in microfluidic channels using confocal correlation spectroscopy". *J. Am. Chem. Soc.* 128:730-731 (2006).
317. Pasternack, R. F., and P. J. Collings. "Resonance light scattering: A new technique for studying chromophore aggregation". *Science* 269:935-939 (1995).
318. Schwille, P., F. J. Meyer-Almes, and R. Rigler. "Dual-color fluorescence cross-correlation spectroscopy for multicomponent diffusional analysis in solution". *Biophys. J.* 72:1878-1886 (1997).
319. Kettling, U., A. Koltermann, P. Schwille, and M. Eigen. "Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy". *Proc. Natl. Acad. Sci. U. S. A.* 95:1416-1420 (1998).
320. Koltermann, A., U. Kettling, J. Bieschke, T. Winkler, and M. Eigen. "Rapid assay processing by integration of dual-color fluorescence cross-correlation spectroscopy: High throughput screening for enzyme activity". *Proc. Natl. Acad. Sci. U. S. A.* 95:1421-1426 (1998).
321. Bieschke, J., A. Giese, W. Schulz-Schaeffer, I. Zerr, S. Poser, M. Eigen, and H. Kretzschmar. "Ultrasensitive detection of pathological prion protein aggregates by dual-color scanning for intensely fluorescent targets". *Proc. Natl. Acad. Sci. U. S. A.* 97:5468-5473 (2000).
322. Camacho, A., K. Korn, M. Damond, J. F. Cajot, E. Litborn, B. Liao, P. Thyberg, H. Winter, A. Honegger, P. Gardellin, and R. Rigler. "Direct quantification of mRNA expression levels using single molecule detection". *J. Biotechnol.* 107:107-114 (2004).
323. Thews, E., M. Gerken, R. Eckert, J. Zapfel, C. Tietz, and J. Wrachtrup. "Cross talk free fluorescence correlation spectroscopy in live cells". *Biophys. J.* 89:2069-2076 (2005).
324. Denk, W., J. H. Strickler, and W. W. Webb. "Two-photon laser scanning fluorescence microscopy". *Science* 248:73-76 (1990).
325. Goppert-Mayer, M. "Uber elementarakte mit zwei quantensprungen". *Ann. Phys.* 9:273-294 (1931).
326. Sandison, D. R., D. W. Piston, R. M. Williams, and W. W. Webb. "Quantitative comparison of background rejection, signal-to-noise ratio, and resolution in confocal and full-field laser scanning microscopes". *Appl. Opt.* 34:3576-3588 (1995).
327. Schwille, P., U. Haupts, S. Maiti, and W. W. Webb. "Molecular Dynamics in Living Cells Observed by Fluorescence Correlation Spectroscopy with One- and Two-Photon Excitation". *Biophys. J.* 77:2251-2265 (1999).
328. Wood, R. D., M. Mitchell, J. Sgouros, and T. Lindahl. "Human DNA repair genes". *Science* 291:1284-1289 (2001).
329. Mullenders, L. H. F., and M. Berneburg. "Photoimmunology and nucleotide excision repair: Impact of transcription coupled and global genome excision repair". *Journal of Photochemistry and Photobiology B: Biology* 65:97-100 (2001).

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Roche Y., Zhang D, Segers-Nolten GMJ, Vermeulen W, Wyman C, Sugasawa K, J. Hoeijmakers JHJ, Otto C. "Fluorescence Correlation Spectroscopy of the binding of nucleotide excision repair protein XPC-hHr23B with DNA substrates". *J. Fluoresc.*, in press, doi: 10.1007/s10895-008-0390-6.

Conference presentation:

Zhang, D., R. Vermeij, A. Lenferink, I. Segers-Nolten, C. Blum, V. Subramaniam, and C. Otto. 2007. Applications of fluorescence correlation spectroscopy. In Annual Dutch meeting on Molecular and Cellular Biophysics 2007 (1-2 Oct 2007), Veldhoven, the Netherlands.

Conference posters:

Zhang, D., R. Vermeij, A. Lenferink, I. Segers-Nolten, C. Blum, V. Subramaniam, and C. Otto. 2007. Elimination of adsorptive behaviour of biomolecules at the glass-solution interface in fluorescence correlation spectroscopy. In *Eur. Biophys. J.* 36 (6th EBSA & British Biophysical Society Congress July 14 - 18 2007). Springer Berlin / Heidelberg, Imperial College, London, U.K. P-503.

Zhang, D., A. Lenferink, W. L. Lans, I. Segers-Nolten, V. Subramaniam, W. Vermeulen, and C. Otto. 2007. Nucleotide excision repair in fluorescence correlation spectroscopy. In *Advanced Macromolecular Systems Across the Length Scales*, 26Apr2007, Enschede, the Netherlands.

Zhang, D., A. Lenferink, I. Segers-Nolten, V. Subramaniam, and C. Otto. 2007. Monitoring protein stability in fluorescence correlation spectroscopy. In *10th Conference on Methods and Applications of Fluorescence* (9-13 Sep 2007), Salzburg, Austria.

Zhang, D., H. Lans, A. Lenferink, W. Vermeulen, D. S. Orlando, K. Sugawara, J.-H. Min, N. P. Pavletich, and C. Otto. 2008. Combined Fluorescence Correlation Spectroscopy and Fluorescence Anisotropy on Nucleotide Excision Repair. In *Annual Dutch meeting on Molecular and Cellular Biophysics 2008* (29-30 Sep 2008), Veldhoven, the Netherlands.

Lee, J. S., W. Zhou, D. Zhang, C. Otto, and J. Feijen. 2008. Hydrosomes, novel thermosensitive gel-containing polymersomes. In *10th European Symposium on Controlled Drug Delivery* (April 2-4, 2008) Noordwijk aan Zee, the Netherlands. p140.

Curriculum Vitae

Author name: Dianwen Zhang

Birth: December 29, 1976 in Jilin, P. R. China

Education:

- *BSc.*: Changchun Institute of Optics and Fine Mechanics of China (1995-1999).
- *MSc.*: Changchun Institute of Optics, Fine Mechanics and Physics of Academy of Science of China (1999-2002).

Working Experience:

- January 2002 – August 2002, **Project Officer**, School of Electrical and Electronic Engineering, Nanyang Technological University, Singapore.
- August 2002 – June 2003, **Research Associate**, School of Electrical and Electronic Engineering, Nanyang Technological University, Singapore.
- June 2003 – November 2004, **Research Associate**, Singapore and University of Washington Alliance, Biomedical Engineering Research Center, Nanyang Technological University, Singapore.
- November 2004 – November 2008, **AIO PhD student**, Biophysical Engineering Group, MESA⁺ Institute for Nanotechnology, Faculty of Science and Technology, University of Twente, the Netherlands.
- November 2008 – present, **Post-Doc**, Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, the Netherlands.

Awards and Honors

Granted ‘**Liu Yongling**’ Special Scholarship by Chinese Academy of Sciences in 2003.

Key publications before 2005:

1. D. W. Zhang, X.-C. Yuan, “Entangled Double-Helix Phase”, *Opt. Lett.* 28, 1864-1866 (2003).
2. D. W. Zhang, X. -C. Yuan, “Optical doughnut for optical tweezers”, *Opt. Lett.* 28, 740-742 (2003).
3. D. W. Zhang, X. -C. Yuan, S. C. Tjin, and S. Krishnan, “Rigorous time domain simulation of momentum transfer between light and microscopic particles in optical trapping.” *Opt. Express* 12, 2220 (2004).
4. D. W. Zhang, X. -C. Yuan, N. Ngo, and P. Shum. "Fast Hankel transform and its application for studying the propagation of cylindrical electromagnetic fields". *Opt. Express* 10, 521 (2002).

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