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Biological Applications of Near-field Optical Microscopy

Optical microscopy and spectroscopy are still key techniques of observation in medicine and biology. Of course, these techniques are directly related to human sight, i.e., the high degree of development of the human eye itself: three dimensional vision, with enormous contrast and color sensitivity. In addition, the use of light has numerous advantages in itself: (i) Non-invasive, non-destructive and safe: most biological samples can be studied in their native environment (*in vivo*); (ii) All materials absorb, reflect, scatter light, and have spectroscopic states leading to a wealth of optical phenomena that all enable contrast; (iii) Spectroscopy: spectral resolution in modern spectroscopy is easily 10 Mhz at an optical frequency of 10^{15} Hz, i.e., dispersed to a fraction of 10^{-8} of the working frequency/wavelength, yielding high resolution for the chemical state, much better than accessible with e-beam or X-ray methods; (iv) Time resolution: optical detection is fast, reaching the femtosecond time domain; (v) Sensitivity: better than 1 photon/sec; (vi) Many types of sources and detectors (including the eye) are available. Optical detection works from astronomical to microscopic distances, yet, at the lower end a natural limit is encountered: the wavelength. It is fundamentally impossible to focus light to an area smaller than this size, the diffraction limit. Consequently, the spatial resolution in conventional optical microscopy is limited to about half the wavelength. This barrier, already recognized in the previous century by Abbe, has been a stimulus to develop alternative techniques such as electron and ion microscopy. Indeed, the alternatives have pushed the resolution to the atomic level (in transmission electron microscopy), however, with the loss of the previously mentioned "optical" advantages.

With the advent of scanning probe microscopy (scanning tunneling microscopy, STM[1] and especially atomic force microscopy, AFM[2]), resolution on nanometer scale has become accessible in non-vacuum conditions. Initially applica-

tion of AFM in the biological domain was limited to fixed samples in order to withstand the relatively high forces of ten to a few hundred nN[3,4]. However, with the introduction of "tapping mode" AFM[5] with operation in liquids[6,7], living biological material can be imaged almost unperturbed in its natural environment[8]. Indeed, activity of cells[8,9] and enzymes[10] has been followed in time with a wealth of ultrastructural information on even sub-nanometer scale. Yet (bio)chemical specificity in AFM is still limited. Some specificity was introduced using labels (immunogold[11,12] and enzymes[13]) based on methods developed for electron microscopy and confocal fluorescence microscopy. Recently, the intrinsic chemical specificity of AFM, contained in the very nature of the force, has become a field of study in itself. Thus "adhesion mode" AFM has been developed[14], where the difference in chemical bond strength between tip and sample is used as a contrast mechanism. By additional coating of the AFM tip with a defined molecular monolayer, the interaction can be made specific, leading to "molecular" force microscopy[15,16]. This direction in AFM has large potential, however, up to now the chemical specificity of force microscopy remains rather limited compared to optical spectroscopic methods. Thus, combination of AFM with an optical contrast mechanism is still the most powerful combination to obtain (bio)chemical specificity with nanometer resolution at biologically relevant conditions. This is the domain of near-field optical microscopy [17].

In *near-field optical microscopy*, a miniature optical probe, either a source or detector, is scanned over a sample surface at nanometer distance. Ideally the probe is of molecular dimensions and serves simultaneously as source and detector. In practice, the probe size is rather 10 to 100 nm and merely serves as a constriction to funnel an incident propagating wave to

sub-wavelength dimensions, while the outgoing far field is detected after tunneling through probe and sample. The throughput of the optical system is generally low, currently 10^{-6} at best. Therefore far field effects must be reduced in order to observe only near field interactions. The most generally applied near field optical probe consists of a small aperture (about 50 nm) at the end of a metal coated tapered optical fiber. The practical feasibility of near field optical microscopy using such an aperture probe was first demonstrated by Pohl, *et al.* [18], in 1982 with an optical super-resolution of 20 nm, even before the advent of force microscopy. However, further development of the technique has long been hampered by fabrication problems of efficient probes. Since 1990, considerable progress has been made with the achievement of adiabatic fiber pulling, as introduced by Betzig, *et al.* [19,20], and shear force feedback (Toledo-Crow, *et al.* [21], Betzig, *et al.* [22]). This combination has resulted in a reproducible, relatively efficient aperture probe, which can be operated non-destructively. Using these probes, application of near field fluorescence microscopy to biological and chemical samples has been explored by Betzig, *et al.* [23] and Moers, *et al.* [24]. An important advance in the field of molecular physics was achieved by Betzig and Chichester [25], who observed fluorescence of a single molecule using a pulled fiber probe, directly followed by single molecular spectroscopy [27], quantised luminescence [28]) and single molecular fluorescence lifetime detection [29,30]. In an alternative configuration, a purely dielectric probe is used to detect the presence of evanescent waves, occurring when the sample surface is illuminated at total internal reflection conditions [31,32]. This form of localized photon tunneling yields very high lateral resolution, down to 20 nm [31], however convincing near-field spectroscopic results have not been shown up to now. Various alternative near-field optical arrangements exist [33]. However without biological applications up to now. In this article, we discuss some recent results on biological samples (Langmuir-Blodgett films, viruses, cells, chromosomes) using combined near field optical and force microscopy, with both aperture type and dielectric probes.

1. Schematic set-up of the aperture type near-field scanning optical microscope (NSOM) with metallized adiabatically tapered fibre, scanned sample stage, shear force feedback on tip sample distance, and detection in transmission using a high NA objective.

Aperture Probe Microscopy

Microscope Set-Up

The configuration of an aperture near field scanning optical microscope (NSOM) operating in transmission is schematically shown in Fig. 1.

The near field aperture probe is fabricated by adiabatic tapering of an optical fiber using a commercially available fiber puller (Sutter P2000) and subsequent directional coating with aluminum. Thus, a 50 to 100 nm aperture is created, with surrounding aluminum for screening of the far field. The probe has a brightness of 100 pW to 1 nW when about 5 mW laser light is coupled into the fiber, i.e., an efficiency of about 10^{-7} . The incident polarization direction can be chosen using retardation and polarization plates.

For biological applications, it is important to design the near field microscope such that object glasses can be accommodated and the sample can be viewed with conventional high magnification optics for localization of a specific area of interest. Our near field optical microscope [34] is based on a Zeiss (Carl Zeiss, Oberkochen, Germany) Axiovert inverted microscope, which was chosen for its high mechanical stability. The commercial sample table was replaced by a home built table with mechanical and electrical sample translation using piezo-electric elements having a 7 μ m scan range. High numerical aperture (NA) objectives (0.75 NA dry or 1.4 NA immersion) are used for sufficient magnification and especially efficient collection of fluorescence. Several exit ports are accessible,

accommodating eyepieces, a CCD camera, and a point detector. For fluorescence detection, a dichroic mirror and long-pass filter are used, which block the excitation light. In near field operation, the probe source is confocally imaged onto the point detector. For high light levels, greater than 1 fW, a photo-multiplier tube is used in combination with a 200 μm pinhole in the image plane. For low light levels, up to 10^6 photocounts/sec, a 100 μm area photon counting avalanche photo-diode [9] is used, with about 59% quantum efficiency at a wavelength of 600 nm and about 9 dark counts/sec.

When moving the aperture probe towards the sample surface, the distance is adjusted at about 3 nm by a feedback system based on shear force detection[21,22]. Hereto the fiber probe is attached to a piezo-electric element which oscillates the fiber in the lateral direction (parallel to the sample surface) with an amplitude of about 20 to 30 nm at its resonance frequency (typically > 10 kHz). The oscillation amplitude is measured with a sensitivity of about 1 nm by illuminating the fiber with a laser diode ($\lambda = 780$ nm) and detecting the far field diffraction pattern on a split detector, where the difference signal is a measure for the fiber amplitude. On approaching the sample surface, the difference signal decreases due to "shear" forces between tip and sample, which allows the operation of a feedback loop at an effective shear force value (set point). The correction signal is fed to the z-axis piezo-electric element in the scanner and simultaneously gives the sample height.

Near field optical, shear force, and height signals are digitized and stored in a personal computer, which also generates the scan pattern and timing for photon counting.

Langmuir-Blodgett Film

A Langmuir-Blodgett film is a highly organized and oriented molecular thin film. Generally, these films serve as model systems for molecular organization in (bio)chemical membranes. For scanning probe microscopy, Langmuir-Blodgett films are interesting test samples because of their well-defined surface structure and molecular orientation. We have investigated a monolayer of diethylene glycol diamine pentacosadiynoic amide (DPDA)[24], prepared by the Langmuir-Blodgett technique. After UV polymerization, the layer is transferred to

a microscope object glass[35]. Depending on the lateral pressure during polymerization and the transfer procedure, several polymerized DPDA domains are formed, all 6 nm thick, but with a wide range of lateral dimensions. The monolayer has a strong absorption around $\lambda = 500$ nm and fluorescence at $\lambda = 550$ to 600 nm. Figure 2 shows a $4 \times 4 \mu\text{m}$ scan of the DPDA film. In the shear force image, Fig. 2a, several domains and the underlying glass substrate are visible. Figures 2b and 2c show the corresponding near field fluorescence images with mutually perpendicular directions of incident polarization. Peak value of the fluorescence intensity is about 1 fW. The fluorescence images show the high anisotropy of the polymerized diacetylenic films with about 100 nm lateral resolution; domains fluorescent for one polarization direction are dark for the perpendicular direction and vice versa. The absorption and emission dipole moments of polydiacetylenes are oriented parallel to the polymer backbone, where the orientation is uniform over each domain due to the crystallinity of the Langmuir-Blodgett film. The force image displays the topography of the monolayer domains with a lateral resolution of about 30 nm, showing some surface roughness and a few non-fluorescent structures. Comparison of these images clearly demonstrates the advantage of near field optics in combination with force microscopy. The near field optical images allow determination of the polycarbon backbone orientation and polymerization efficiency, in addition to the topography in the simultaneously recorded force image.

Virus

In order to demonstrate the high resolution potential in biological applications, one has to image objects that are not accessible by conventional optical microscopy, like viruses. Pytkki, *et al.*[36], have investigated the Tobacco Mosaic Virus (TMV) with a shear force NSOM arrangement ("Aurora" of Topometrix Corp., Santa Clara, CA, USA [37]). TMV particles of a few hundred nm in length and only 30 nm in diameter were attached to amino-silanated atomically flat mica by gluteraldehyde, and imaged after rinsing and drying. Figure 3 shows the $1.4 \times 1.4 \mu\text{m}$ shear force and bright near-field image in transmission at $\lambda = 488$ nm. Clearly, individual TMV particles are resolved in both images, with separations of less than 30 nm. The high correlation between

shear force and near-field optical image indicates that the optical contrast is mainly caused by topography. The TMV particles displays higher optical transmission than the mica substrate, caused by their height or difference in refractive index.

Cellular Surface and Skeleton

Cell observation by scanning probe microscopy has, up to now, mainly been the domain of AFM, where information

2. A $4 \times 4 \mu\text{m}$ scan of a Langmuir-Blodgett monolayer: UV polymerised diethylene glycol diamine pentacosadiynoic amide, DPDA. (a) Shear force image. (b, c) Near field fluorescence images with mutually perpendicular directions of linearly polarised excitation, showing the high anisotropy of the Lanbmuir-Blodgett film[24].

3. Tobacco Mosaic Virus macromolecules[36], $1.4 \times 1.4 \mu\text{m}$ scan area: (a) shear force image displaying topography; (b) transmission aperture NSOM image.

about cell membrane structure (pores[8], antigens[11,12], proteins[4]), mechanical properties (membrane hardness, viscoelasticity[8]) and temporal behavior of living cells (interaction, growth, formation of pseudopodia[8]), is obtained. Occasionally, sub-surface structure is obtained, e.g., the cytoskeleton can show up through the membrane in contact force microscopy[4,9]. By application of NSOM, the wealth of structural information can be extended using specific fluorescence labeling techniques, as they have been developed for (confocal) fluorescence microscopy. Thus, in addition to the topography specific cell, surface and skeletal constituents can be identified.

As an example in Fig. 4 a $14 \times 14 \mu\text{m}$ image of a rat's eye retina tissue (microtome cut) is shown, as obtained by Pylkki, *et al.*[36]. The shear force (Fig. 4a) and bright field transmission NSOM (Fig. 4b) were obtained simultaneously. Clearly,

the force image mainly shows cell surface structure, as induced by the microtome, whereas the optical image shows the tissue structure. The optical contrast in transmission is induced by a combination of topography, absorption, and refractive index, making it rather difficult to interpret.

A much more revealing and easier to interpret NSOM image is obtained when imaging in fluorescence mode. Betzig, *et al.*[23], showed the first application of near field fluorescence to observe labeled cytoskeletal actin in fixed mouse fibroblast cells. Swiss mouse 3T3 fibroblast cells were fixed in formaldehyde and air dried on a glass cover slip after fluorescent staining with rhodamine-phalloidin specific for filamentous actin. Figure 5 shows a $10 \times 10 \mu\text{m}$ image of a cellular protrusion: a flat thin lamellipodium of the 3T3 cell. Although obtained simultaneously, the shear force image (Fig. 5a) is in this case distinctly different from the near field

5. Cellular protrusion of a Swiss mouse 3T3 fibroblast cell[23], $10 \times 10 \mu\text{m}$ scan area: (a) shear force topographic image; (b) corresponding near field fluorescence image showing the stained actin in the cytoskeleton.

fluorescence image (Fig. 5b). The force image shows mainly surface topography with about 50 nm lateral resolution, whereas the fluorescence image shows the cytoskeleton organization in fine detail, with a lateral resolution of about 100 nm, well beyond confocal fluorescence microscopy. The fluorescence contrast is similar to confocal fluorescence results, thus easy to interpret, with improved resolution and almost bleach free.

Fluorescence in situ Hybridization of Human Chromosomes

Over the last decade, the technique of fluorescence *in situ* hybridization (FISH) has developed as one of the major cytogenetic detection methodologies for human genetics[38,39]. Using fluorescent labels, FISH enables direct visualization of topological or positional information of gene sequences under a fluorescence microscope. Thus, FISH allows rapid localization of genomic DNA fragments and

4. Rat's eye retina tissue[36], $14 \times 14 \mu\text{m}$ scan area: (a) shear force image showing tissue surface topography; (b) transmission aperture NSOM image showing tissue optical structure.

identification of chromosomes with a superior resolution and signal-to-noise ratio as compared to radioactive *in situ* hybridization and chromosome banding techniques. Depending on DNA condensation a resolution better than 10^6 basepairs can be obtained using (pro)metaphase chromosomes[40]. Yet the localization of the fluorescence labels is fundamentally limited by diffraction in conventional fluorescence microscopy. Recently, Putman, *et al.*[13], have shown that further improvement can be obtained using force microscopy to detect the morphological features of the *in situ* hybridization label after enhancement to 100 to 200 nm by an enzymatic cytochemical reaction. Yet fluorescence detection has the advantage of higher specificity and, moreover, multicolor labeling can be applied[40]. We show that the superior lateral resolution of near field fluorescence allows improved localization of the labels while maintaining the potential of multicolor labeling, provided the labels are sufficiently close to the chromosomal surface and the fluorescence level is still detectable.

Figure 6 shows a scan of human metaphase chromosome #1 with specific labeling of the centromeric area by *in situ* hybridization: pUC1.77 recombinant DNA hybridized to 1q12, stained with CY3 fluorophore. In the shear force image (Fig. 6a), the high spatial frequency filtered piezo feedback signal is displayed, which shows the chromosome topography with some substructure. The corresponding near field fluorescence image (Fig. 6b) displays the green fluorescence at λ greater than 570 nm, using BG39 and KV550 Schott filters, with excitation by the 521 nm Kr⁺-line. The image is 200 x 200 pixels with 25 msec/pixel. The speckled background in the fluorescence image reflects the discrete level of 0 to 3 counts/pixel. The specific labeling of the centromeric chromosome area is clearly visible with 60 to 120 counts/pixel fluorescence. Beside the chromosome are some locally fluorescent areas due to unbound fluorophore. Based on the signal level (≤ 10 counts/pixel) we estimate about 10 fluorescent molecules to be present in these spots.

Figure 7 shows another scan of a human metaphase chromosome #1, with specific labeling of the telomeric region (top of the image) of the short arm[41]: p1-79 hybridized to 1p36, again with CY3 staining. The pixel size is 35 nm. The scan speed is 40 msec/pixel. The shear force

6. A 7 x 7 μm scan of human metaphase chromosome #1: (a) Shear force image, high pass filtered in horizontal direction; (b) corresponding near field fluorescence image displaying specific labeling of the centromeric area with CY3 fluorophore by *in situ* hybridisation.

7. A 7 x 7 μm scan of human metaphase chromosome #1[41]: (a) Shear force image, high pass filtered in horizontal direction; (b) corresponding near field fluorescence image showing fluorescence *in situ* hybridisation labels: mainly CY3 fluorescence at the telomere probe p1-79 (top) and several additional probes widely distributed over the chromatides.

image (Fig. 7a) displays the well known metaphase chromosomal structure with well separated chromatides and details as small as 75 nm. The corresponding near field fluorescence image (Fig. 7b), with 521 nm Kr⁺-line excitation and λ greater than 570 nm detection, shows distinct substructure in the telomeric labels (at least 5 probes in each chromatide, with maximum 700 counts/pixel) and several isolated spots on the chromatides and in the centromeric region. The width of the fluorescence spots is less than 100 nm, while spots only 125 nm separated are individually detected. Autofluorescence can be recognized as a weak general background signal over the total chromosome area. The combined images (force and fluorescence) allow accurate determination of the

probe location on the chromosomal structure.

Photon Tunneling Microscopy

Microscope Set-Up

In photon scanning tunneling microscopy (PSTM), a sharp dielectric probe is used for local conversion of an evanescent wave into a propagating wave[42]. In our PSTM, we use a micro-fabricated silicon nitride (SiN) probe, which is commercially available (Park Scientific Instruments, Sunnyvale, CA, USA) for conventional AFM applications. For near field optical applications, the SiN probe is a suitable high-index optical structure with 20 to 50 nm apex, and transparency down to $\lambda = 290$ nm. Due to an integrated cantilever, the probe can be scanned in

8. Schematic set-up of the combined photon scanning tunneling and atomic force microscope (PSTM/AFM) based on localised frustration of total internal reflection using a micro-fabricated silicon-nitride probe[31].

close contact with a sample surface by using feedback regulation on the force interaction. Generally, the gold coating on commercial cantilevers is removed for PSTM operation. The sample is placed on a BK7 glass substrate and illuminated by a weakly focused laser beam (10 mW on about 100 μm) at an angle larger than the critical angle for total internal reflection, (see Fig. 8). The light generated by frustration of the evanescent wave at the SiN apex, about 1 nW, is collected by conventional optics. Deflection and torsion of the cantilever are detected using a standard optical beam deflection configuration. While scanning, the interaction force is kept constant by feedback of the beam deflection signal, yielding simultaneously a topographic and a near field optical image[31].

Langmuir-Blodgett Films

A UV polymerized Langmuir-Blodgett film of 10,12-pentacosadiynoic acid (PCA) was investigated[32]. After transfer to a glass substrate, these PCA films display uniform domains, with

a height of 6 nm and a wide range of lateral dimensions. The domains show strong absorption bands at $\lambda = 505$ and 555 nm, and fluorescence around $\lambda = 562$ and 640 nm, where absorption and emission dipole moments are along the highly oriented polycarbon backbone. A combined PSTM/AFM scan of a $1 \times 1 \mu\text{m}$ area PCA film is shown in Fig. 9. The AFM image (Fig. 9a) displays the z-piezo signal in feedback mode, showing mainly the monolayer topography with 6 nm height. The corresponding PSTM image (Fig. 9b) displays the fraction of the incident p-polarized light at $\lambda = 514$ nm, which is coupled out via the SiN probe. Monolayer domains are clearly visible in the PSTM image, with an edge steepness of 30 nm, far beyond the diffraction limit. The PSTM signal on the domains is 10% below the signal detected on the surrounding glass, which is in agreement with the measured absorption of a PCA monolayer at 514 nm by far field methods. Consequently, the PSTM contrast is mainly caused by absorption for this sample. Yet, it should be noted that the expected polari-

9. 10,12-pentacosadiynoic acid (PCA) Langmuir-Blodgett monolayer on a glass substrate, $1 \times 1 \mu\text{m}$ PSTM/AFM scan area: (a) Force image displaying the topography of the 6 nm monolayer and (b) the simultaneously obtained PSTM image displaying absorption of the excitation at $\lambda = 514$ nm. Edge steepness of the optical contrast is 30 nm[32].

zation anisotropy could not be observed. Also, the observed fluorescence turned out *not* to be confined to the probe dimensions.

Chromosomes

Metaphase Chinese hamster lung chromosomes are fixed and air-dried on an object glass. In order to locate the isolated chromosome clusters for PSTM/AFM imaging, part of the light path after the objective is split to a CCD camera for

10. Metaphase Chinese hamster lung chromosome[31], $8 \times 8 \mu\text{m}$ PSTM/AFM scan area: (a) AFM height mode; (b) AFM friction mode; (c) corresponding PSTM image.

conventional microscopy. The corresponding AFM and PSTM images of a selected area are shown in Fig. 10. The AFM image (Fig. 10a) displays the chromosome topography, with the characteristic metaphase shape, height up to about 100 nm, and some residual chromatide material surrounding the chromosome. Figure 10b shows the simultaneously recorded friction force image, with specific islands of different friction, and thus chemical composition, on the chromatides. Finally, in the corresponding PSTM image (Fig. 10c), the chromatides appear to reduce the optical coupling, which causes the chromatides to appear dark [31]. Simultaneously, scattering at the chromosome gives rise to a rather intense radiative contribution in the propagation direction of the incident light, extending beyond the actual position of the chromosome.

Conclusions

We have presented several biological applications of near field optical microscopy, in combination with force microscopy. Aperture NSOM with fluorescence detection gives (bio)chemical specificity and orientational information, in addition to the simultaneously acquired force image. This technique has large potential for DNA sequencing, molecular organization in monolayers, and study of the role of the cytoskeleton in cellular mobility in cell growth, cell migration, formation of protrusions, etc. Fluorescence NSOM gives high resolution on flat, not too deep surfaces. Fluorescence NSOM induces virtually no bleaching, as opposed to confocal fluorescence microscopy. Bright field NSOM in transmission generally yields a complicated contrast, caused by a mixture of dielectric and topographic contributions. Shear force feedback is essential in aperture NSOM operation with fibers, and operates on soft surfaces of cells and chromosomes. Ultimately, aperture NSOM is limited by low efficiency with a source brightness of typically 100 pW to 10 nW. Thus, in spectroscopic applications (fluorescence, Raman, etc.) photon noise will be a fundamental limit in the speed of imaging.

Photon tunneling in combination with force microscopy allows routine scanning with a high optical lateral resolution. However, interference effects can be dominant on surfaces which display extensive scattering. As such, the applica-

tion potential of PSTM to biological surfaces is rather limited.

Clearly, the virtues of optics, non-invasiveness, high spectral resolution, and high time resolution all apply to the near field optical domain with its high spatial resolution, which adds extensively to the potential of scanning probe microscopy.

Acknowledgments

The authors thank: Eric Betzig of AT&T Bell labs for essential achievements in this field and supplying some relevant results; Pat Moyer of Topometrix Corp. for making available his images; Wouter Kalle and Joop Wiegant of RijksUniversiteit Leiden for the preparation of *in situ* hybridized chromosomes; Uli Hoffmann and Hermann Gaub of the Technical University of Munich for the DPDA and PCA films; Ton Ruiter, Kees van der Werf, Frans Segerink, Eric Schipper, Ine Segers and Bart de Grooth for their assistance and suggestions; Alberto Diaspro for the invitation. This research is supported by the Dutch Foundation for Fundamental Research (FOM) and the European network on Near-field Optics and Nanotechnology.

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nificant results for biological applications are presented in this article.

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