Biological Applications of Near-field Fluorescence Microscopy

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Chromosomes and single fluorophores are studied using an aperture type near-field scanning optical microscope with tuning fork shear force feedback. Fluorescence in situ hybridisation (FISH) labels on repetitive and single copy probes on human metaphase chromosomes are imaged with a width of 80 nm, allowing their localisation with nanometer accuracy, in direct correlation with the simultaneously obtained topography. The single fluorophores exhibit dynamics on 10 ms to 100 seconds timescale.

INTRODUCTION

Near-field Scanning Optical Microscopy (NSOM) is a true optical microscopic technique allowing fluorescence, absorption, reflection and polarisation contrast with the additional advantage of nanometer lateral resolution, unlimited by diffraction (1). Thus "electron microscopic" resolution is obtained with "optical" contrast and operation at ambient conditions. In the present paper recent advances in biological applications of this new technique are described.

NSOM based on metal coated adiabatically tapered fibres, combined with shear force feedback and operated in illumination mode, is currently the most versatile and efficient NSOM arrangement, because of its true localisation of the optical interaction and its various optical contrast possibilities (fluorescence, polarisation, etc.) and its sensitivity down to the single molecular level. We have constructed a combined near-field fluorescence / shear force microscope, featuring photon counting, two channel polarisation sensitive detection and tuning fork force detection.

In this paper we present advances towards the molecular level in near-field fluorescence imaging of genetic material.

FLUORESCENCE *IN SITU* HYBRIDISATION OF HUMAN CHROMOSOMES

The sensitive of *in situ* hybridised DNA, on the basis of fluorescence labels or enzyme generated dyes, has promoted fluorescence *in situ* hybridisation (FISH) to one of the major cytogenetic detection methodologies for human genetics. FISH enables direct visualisation of gene sequences in a fluorescence microscope, allowing rapid localisation of genomic DNA fragments in morphologically preserved metaphase chromosomes. A resolution better than 10^6 basepair can be obtained, yet the localisation of the fluorescence labels is fundamentally limited to ~ 300 nm by diffraction in conventional fluorescence microscopy, while localisation of the numerous closely linked genes requires mapping at higher resolution. Putman *et al* (2) pioneered the potential of atomic force microscopy in the detection of morphological *in situ* hybridisation labels, however with the loss of the sensitivity and multiplicity of fluorescence detection. In this paper we present applications of "aperture" NSOM to

genetic sequencing of human metaphase chromosomes, using single copy and repetitive FISH labels, demonstrating identification of specific DNA sequences, with single fluorophore sensitivity (3). The images are accompanied by the simultaneously acquired force image, enabling direct comparison of the optical contrast with the sample topography on nanometer scale.

PREPARATION

Two types of repetitive DNA probes, specific for chromosome #1, were used: the satellite III probe pUC-1.77, that recognises the near-centromeric region (1q12), and the probe p1-79, which is specific for the telomere region of the short arm, with insert sizes of 1.77 kb and 0.90 kb, respectively. The biotinated probes were indirectly detected by cyanine (CY-3, an orange fluorescent dye). The fluorescence quantum efficiency of CY-3 is high (> 0.8) both in buffer and air. The human metaphase chromosome spreads were prepared on microscope cover slips and air-dried for imaging purposes.

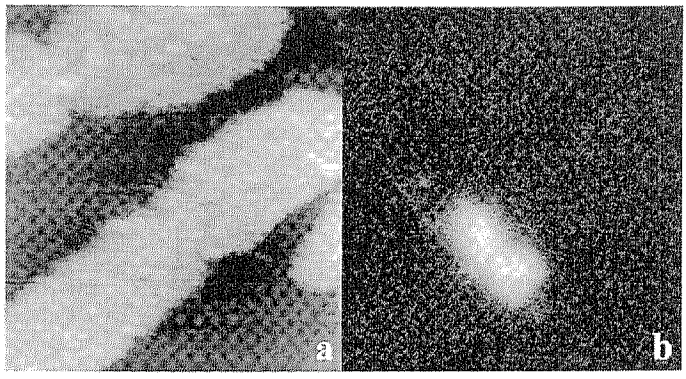

Figure 1: A 7 x 7 μm^2 scan of human metaphase chromosome #1:
(a) Shear force topographic image;
(b) Corresponding near field fluorescence image showing fluorescence in situ hybridisation labels: mainly CY3 fluorescence at centromeric area.

IMAGING

Figures 1 and 2 show corresponding topography (left) and near-field fluorescence (right) *in situ* hybridisation of human chromosome #1 (1,3): pUC1.77 hybridised to 1q12 in the centromeric area; and p1-79 hybridised to 1p36 in the telomeric region, respectively. The images are scanned with 35 nm pixel size, 40 ms/pixel scan speed, with green excitation at the 521 nm Kr^+-line. In the shear force images the piezo feedback signal is displayed with slight horizontal high spatial frequency filtering. The metaphase chromosomal structure is resolved with details as small as 40 nm and height up to ~ 150 nm. The corresponding near field optical images display the orange fluorescence at $\lambda > 550$ nm, using BG39 and KV550 Schott filters. The p1-79 probes are visualised as distinct substructure in the telomere region with at least 5 probes in each chromatide (maximum 700 counts/pixel). Lateral resolution is typically 80 nm in the fluorescence images.

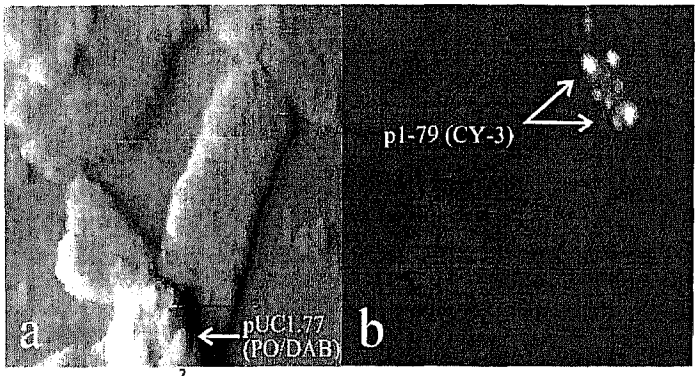

Figure 2: 7 x 7 μm² scan of human metaphase chromosome #1 (3):
(a) Shear force image, high pass filtered in horizontal direction showing the topographic pUC1.77 (PO/DAB) label (2);
(b) corresponding near field fluorescence image showing the fluorescent p1-79 (CY-3) label and slight auto-fluorescence.

DECTECTION OF SINGLE FLUOROPHORES

Recently the sensitivity of near-field fluorescence detection has reached the *single molecular* level (3,4). A preliminary result is presented in figure 3. Fluorescent Rhodamine 6G molecules have been adsorbed to a glass substrate with a sub-monolayer coverage (~ 1 molecule/μm²), so that individual molecules can be distinguished. The molecules have been traced during half an hour: in the third presented scan molecule 'a' stops fluorescing abrupt, similarly molecule 'b' in the last scan: discrete photo-dissociation, visible as a "half" molecule. Molecule 'c' is dark with varying intervals (10 ms – 1 second), an indication for radiationless decay caused by quenching or trapping in a dark state due to a molecular conformational change. The different intensity per molecule is a direct consequence of the molecular orientation relative to the polarisation direction of the incident excitation field in the near-field probe. Thus single molecules are localised with nanometer accuracy and followed in time, showing molecular mobility, re-orientation, and ultimately photo-dissociation.

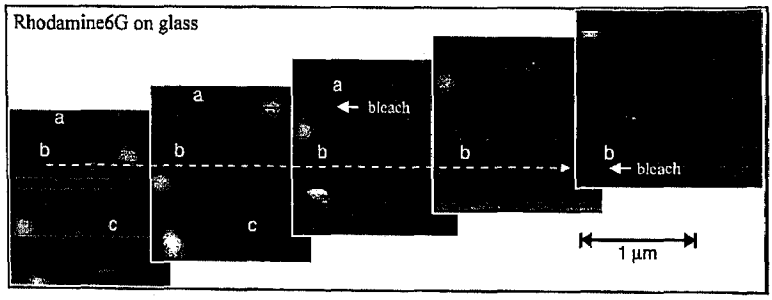

Figure 3: 1.5 x 1.5 μm² scan of fluorescent single Rhodamine 6G molecules

CONCLUSION

Near field optical microscopy of biological surfaces offers fluorescence, polarisation and absorption contrast with a lateral optical resolution typically 20 to 100 nm, depending on the arrangement used, far beyond the diffraction limit, with single molecular sensitivity. In all cases the high-resolution image is combined with a topographic force image, which facilitates co-localisation.

Clearly this unique combination of high resolution, specific optical contrast and operation at ambient conditions offers many potential new directions, especially in molecular biology and chemistry.

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