

Fluorescence *in situ* hybridization on human metaphase chromosomes detected by near-field scanning optical microscopy

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Summary

Fluorescence *in situ* hybridization on human metaphase chromosomes is detected by near-field scanning optical microscopy. This combination of cytochemical and scanning probe techniques enables the localization and identification of several fluorescently labelled genomic DNA fragments on a single chromosome with an unprecedented resolution. Three nucleic acid probes are used: pUC1.77, p1-79 and the plasmid probe α -spectrin. The hybridization signals are very well resolved in the near-field fluorescence images, while the exact location of the probes can be correlated accurately with the chromosome topography as afforded by the shear force image.

Introduction

In situ hybridization (John *et al.*, 1969; Buongiorno-Nardelli & Amaldi, 1969; Gall & Pardue, 1969) is a cytochemical technique with important applications in (cancer) cytogenetics, cell biology, virology and oncology. The method allows the localization of genomic DNA fragments in morphologically preserved metaphase chromosomes and interphase nuclei. Nowadays, detection of DNA probes is usually done by immunofluorescence in combination with high-resolution fluorescence microscopy (Rudkin & Stollar, 1977; Bauman *et al.*, 1980; Lichter & Ward, 1990).

Technical developments of the *in situ* hybridization method are aimed at improving sensitivity, multiplicity and resolution.

1 The *sensitivity* of the hybridization procedure is defined as the smallest sequence of nucleotides detectable. Wiegant *et al.* (1993) have reported the visualization of a cDNA probe as small as 1.8 kb by indirect *in situ* hybridization methods on metaphase chromosomes. However, a dramatic increase in sensitivity is still needed to permit detection of oligonucleotide probes. If such a sensitivity can be reached, point mutation detection and allele specific hybridization becomes possible at the single cell level.

2 The *multiplicity* gives the number of different DNA target sequences that can be identified simultaneously on the basis of colour in the same object. The width of excitation and emission spectra of the fluorochromes limits the multiplicity to about 6 within one chromosome.

3 *Resolution* is the physical distance necessary between two objects in order to resolve them. Detection with conventional fluorescence microscopy limits the physical resolution to 0.3 μm . Obviously DNA resolution, i.e. the distance expressed in base pairs, is determined by the degree of condensation of DNA in the object under study. For condensed DNA in metaphase chromosomes Lichter *et al.* (1990) have reported a 3 Mb resolution, while on less condensed chromatin in interphase nuclei the DNA resolution can be as good as 50 kb (Trask *et al.*, 1989).

Both chemical and physical approaches can be followed to improve resolution of *in situ* hybridization even further. Nuclear extraction techniques, for instance, result in highly extended naked DNA loops arranged around the nuclear matrix in a halo-like structure (Wiegant *et al.*, 1992). At the level of the Watson–Crick DNA double helix 1 kb corresponds to 0.32 μm . Hence with optical microscopy a resolution of 1 kb is feasible on stretched DNA fibres. The

disadvantage of halo preparations, as well as interphase nuclei, is that the chromatin is de-condensed to such a high degree that only a very small part of the total DNA is investigated. Consequently, *in situ* hybridization to naked DNA fibres is very useful for high-resolution DNA mapping at short range. However, the relation to the physical structure and biological function of the chromosome is lost, so for chromatin organization studies an improvement of the physical resolution of *in situ* hybridization to metaphase chromosomes is required.

The physical approach involves the use of high-resolution microscopical techniques for the detection of *in situ* hybridization signals. Gold-labelled probes can be detected by electron microscopy (EM) (Hutchinson *et al.*, 1982; Hamkalo *et al.*, 1989; Fetni *et al.*, 1991, 1992) but difficulties with chromosome preparation and the lack of multiplicity are the major drawbacks of the application of EM to *in situ* hybridization. Another candidate is the atomic force microscope: Putman *et al.* (1993) have demonstrated that morphological labels such as precipitated diamine-benzidine (DAB) can be visualized as pronounced bumps on top of the chromosome structure. However, because a finite size is required for discrimination of the signal from the chromosome structure, resolution still is limited to about 200 nm. In addition, atomic force microscopy will not allow multiple 'colour' *in situ* hybridization.

A microscopical technique that combines the three demands (sensitivity, multiplicity and resolution) of *in situ* hybridization exists in the form of near-field scanning optical microscopy (NSOM) since this technique had demonstrated: (1) a detection sensitivity for fluorescence down to the single molecular level (Betzig & Chichester, 1993; Ambrose *et al.*, 1993; Xie & Dunn, 1994); (2) a spectral sensitivity identical to that obtained with normal fluorescence microscopy (Trautman *et al.*, 1994; Hess *et al.*, 1994); and (3) a physical resolution of only a few tens of nanometers (Betzig & Chichester, 1993; Betzig & Trautman, 1992). In the present paper the application of NSOM for the detection of fluorescence *in situ* hybridization (FISH) signals on human metaphase chromosomes is presented.

Materials and methods

The near-field scanning optical microscope

The principle of NSOM is the reduction of the exposed area of the sample down to sub-wavelength dimensions by illumination through a small aperture at the end of an otherwise opaque aluminium-coated, adiabatically tapered optical fibre (Fig. 1). This small light source is raster scanned closely over the surface with a shear force feedback regulation, introduced by Betzig *et al.* (1992) and Toledo-Crow *et al.* (1992), to maintain a constant tip-to-surface

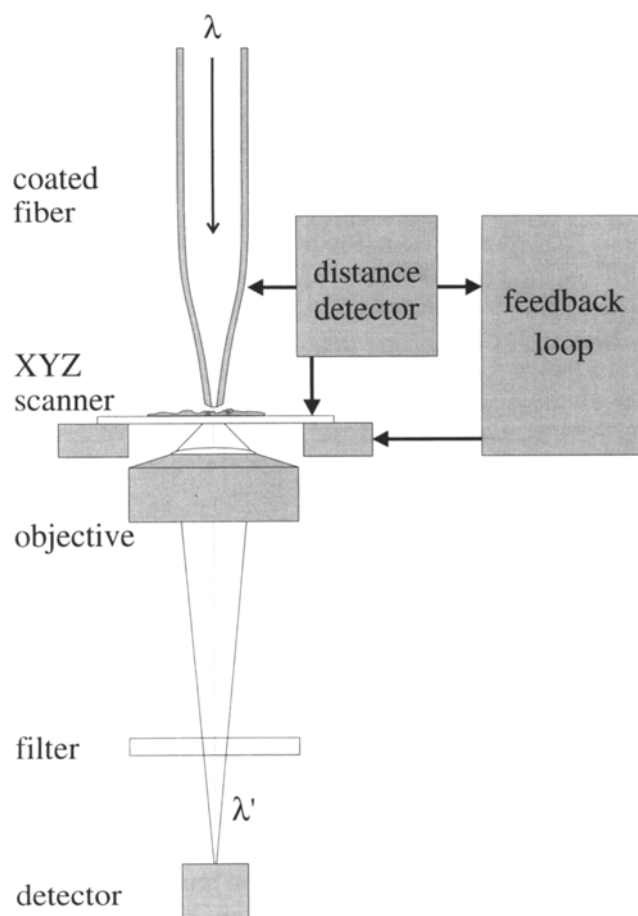


Fig. 1. Near-field scanning optical microscope. The sample is locally illuminated through the aperture at the end of the fibre, and fluorescence is detected with the inverted microscope. With a shear force feedback a constant tip-to-sample distance is maintained during scanning.

distance of only a few nanometers (Moers *et al.*, 1994, 1995). As an extra advantage of the force feedback a topographic image is obtained simultaneously with the optical image, which makes it possible to correlate the optical information with the topological structure of the sample.

For the detection of FISH signals with a near-field scanning optical microscope the aluminium-coated fibre tip, a homebuilt XYZ-scanner and the feedback system are integrated on an inverted microscope (Zeiss Axiovert 135TV). The sample is illuminated through the aperture (approximately 100 pW) while the fluorescence is collected by a Plan Neofluar (40 \times , NA = 0.75) objective. Laser light is blocked by a KV550 (Schott) optical filter, with a BG39 (Schott) filter to suppress infrared light of the shear force laser diode. The tip is aligned confocally with a photon-counting avalanche photo-diode (EG&G SPCM200) with small sensitive area (100 μ m), high quantum efficiency

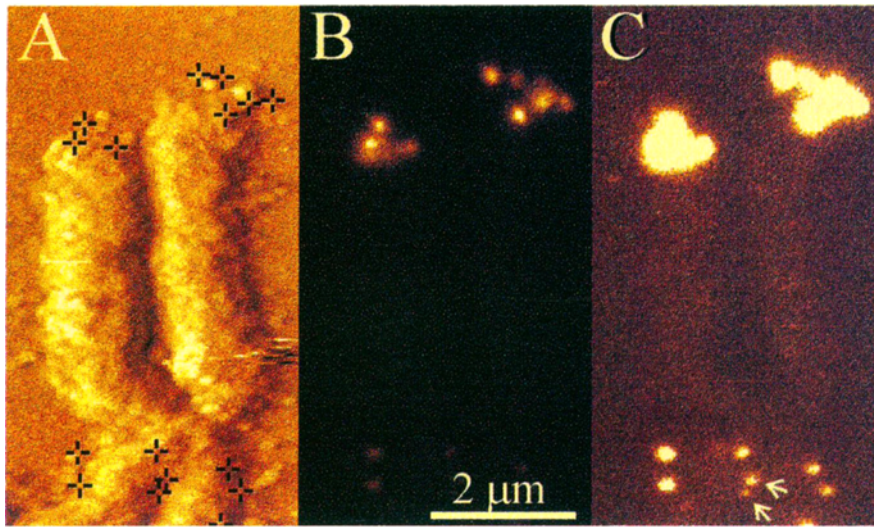


Fig. 2. Fluorescence *in situ* hybridization on human metaphase chromosome as observed using a near-field scanning optical microscope. (A) Shear force image displaying the topography of the chromosome (high-pass filtered in the horizontal direction). Markers give the exact location of the hybridization signals in the near-field fluorescence image. (B) Near-field fluorescence image obtained with green excitation ($\lambda = 521$ nm; emission > 550 nm). The telomere probe p1-79 is well resolved and shows extensive substructure. (C) similar to (B) but with contrast optimized for the specific signals at the centromeric region of the chromosome. The high dynamic range in the measurement precludes the presentation of all information within one image.

(0.59 counts photon⁻¹) and low dark current (9 counts s⁻¹). All scans are made with a pixel size of 35×35 nm², with 40 ms integration time per pixel.

Apart from collecting the fluorescence, the high-quality epifluorescence microscope plays an important role in the selection and inspection of the chromosomes from the metaphases prior to NSOM measurements.

Chromosome preparation

Routine procedures were followed for the preparation of *in situ* hybridized human metaphase chromosomes on microscope cover slips. The following recombinant DNAs for chromosome 1 were used: (1) pUC1.77, which recognizes the near centromere region (1q12) (Cooke & Hindley, 1979); (2) p1-79, for the telomere region of the short arm (1p36) of the chromosome (Buroker *et al.*, 1987); and (3) α -spectrin, a plasmid probe that hybridizes at 1q21.3 (Anderson *et al.*, 1989).

The metaphase chromosomes, obtained from phytohaemagglutinin-stimulated normal male human peripheral blood lymphocytes according to standard procedures, were pre-treated according to Wiegant *et al.* (1991a). *In situ* hybridizations with biotinylated pUC1.77, digoxigenated p1-79 and biotinylated α -spectrin were performed essentially as described by Wiegant *et al.* (1991b). Immunological detection of biotin and digoxigenin was done according to Dauwerse *et al.* (1992). The satellite III probe pUC1.77 is indirectly detected with fluorescein isothiocyanate (FITC, green fluorescence), and the p1-79 probe and the plasmid probe α -spectrin with cyanine dye (CY-3, red fluorescence).

In the same process reference samples were produced on microscope slides. While the samples for the NSOM measurements are dried in air to facilitate the shear force

feedback, the reference samples are embedded in antifading liquid (Vectorshield) supplemented with DAPI as a general counterstain and topped by cover slips. The main disadvantage of air-dried samples is the reduced fluorescence intensity of the dyes by (oxygen) quenching. In particular FITC shows a significantly lower fluorescence intensity in air than in liquid. However, we are bound to use air-dried samples because we have experienced a poor operation of our shear-force feedback on soft biological materials in liquids.

Results and discussion

Detection of FISH signals using a near-field scanning optical microscope is demonstrated in Figs. 2 and 3 showing a human metaphase chromosome 1 hybridized with two DNA probes: pUC1.77 (FITC) and p1-79 (CY3). The chromosome structure is visualized by the shear force images, Figs. 2(A) and 3(A). In the slightly high-pass filtered force images details as small as 75 nm are easily recognized. Near-field fluorescence images of the chromosome are presented in Figs. 2(B) and 3(B) with green ($\lambda = 521$ nm) and blue ($\lambda = 473$ nm) excitation, respectively.

With green excitation the p1-79 probe is detected at the telomere as a cluster of small fluorescent spots (Fig. 2B). Apart from the bright signals at the chromatid ends several weak signals are found at the centromere region of the chromosome (Fig. 2C); these are most probably due to non-specific hybridization of p1-79 in the 1q12 region. Also in conventional fluorescence microscopy some shape is recognized in the telomere probe p1-79, although it is not as clearly resolved as in these measurements.

With blue excitation the pUC1.77 probe is visualized as a homogeneous fluorescent area near the centromere of the

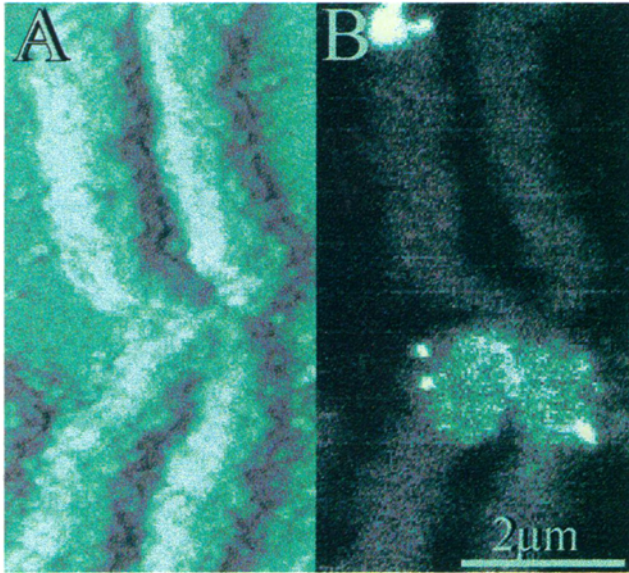


Fig. 3. Near-field fluorescence of the same chromosome as in Fig. 2 with blue excitation ($\lambda = 473$ nm): (A) shear force image (high-pass filtered in horizontal direction); (B) near-field fluorescence image revealing the near-centromere probe pUC1.77, and autofluorescence of the chromosome.

chromosome (Fig. 3B). Furthermore, CY3 signals, already seen with green excitation, are detected with blue excitation as well. As mentioned above, this is due to the low intensity of the FITC fluorescence, compared with CY3, when operated in air.

Figure 4 shows a fluograph of the reference sample obtained with a high-quality conventional fluorescence

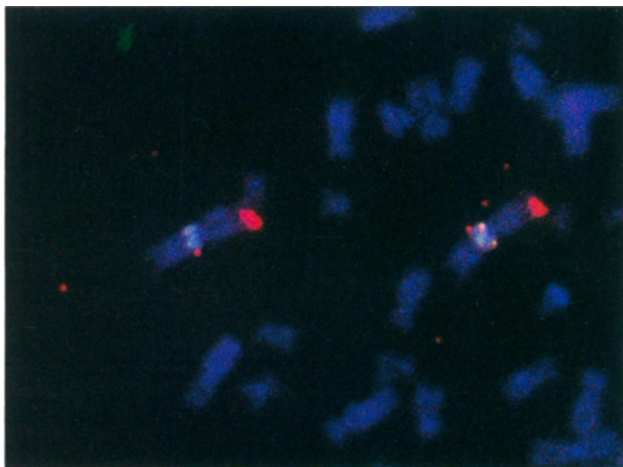


Fig. 4. Two-colour FISH of the reference sample observed by a high-quality conventional fluorescence microscope. The centromeric pUC1.77 probe (green) and the telomeric p1-79 probe (red) are easily detected on chromosome 1. The DNA is counterstained with DAPI.

microscope (Leitz DM-RBE with Plan Fluoroton 100 \times , NA = 1.3 objective). The chromosomes are clearly seen in blue (DAPI). The signals of both the centromeric probe (green) and the telomeric probe (red) are easily detected. Some substructure can be observed in the telomeric region, although comparison of the near- and far-field fluorescence images demonstrated that with near-field microscopy the FISH signals are considerably better resolved than with conventional far-field optics. As an example, the full width at half-maximum (FWHM) of the spots observed in the near-field fluorescence image Fig. 2(B) is less than 100 nm. Furthermore, signals are individually detected at a relative distance as small as 125 nm. The optical resolution in these images, therefore, is better than 100 nm. Applying the same criteria to the far-field images would yield an optical resolution of, at best, 500 nm. Furthermore, with NSOM the exact location of the signals in relation to the real chromosome structure, as it is simultaneously measured by the force microscope, can be determined with an extreme accuracy.

Interestingly, not all of the spots are actually found on the chromosome structure itself, as is learned from the comparison of optical and force images. These signals, however, can be assigned to small topographic features that are located adjacent to the chromosome. Obviously, this combination of high-resolution topographic and optical microscopy can give valuable information about chromosome structure and organization.

The sensitivity for detection of unique DNA probes is demonstrated on a metaphase chromosome sample hybridized with the probe p1-79 and the plasmid probe α -spectrin, both indirectly visualized with CY3. Figures 5 and 6 are two examples of near-field measurements showing the telomere and plasmid signals on the chromosome. In general the α -spectrin signal is detected on the external side of band 1q21.3, as in Fig. 5, but Fig. 6 shows plasmid signals on the internal side of the chromosome. An explanation might be found in the topographic image, Fig. 6(A): the chromosome appears to be twisted at the centromere. This again demonstrates the great benefit of the combination of force and optical microscopes.

Conclusions

With these results the applicability of near-field scanning optical microscopy for the detection of hybridization signals has clearly been illustrated. An excellent optical resolution is realized on metaphase chromosomes, while the combination with a force microscope reveals valuable information about the morphological structure of the chromosome. Two-colour FISH has already been accomplished, while more colours are easily feasible: the multiplicity is limited by the same properties as in conventional fluorescence

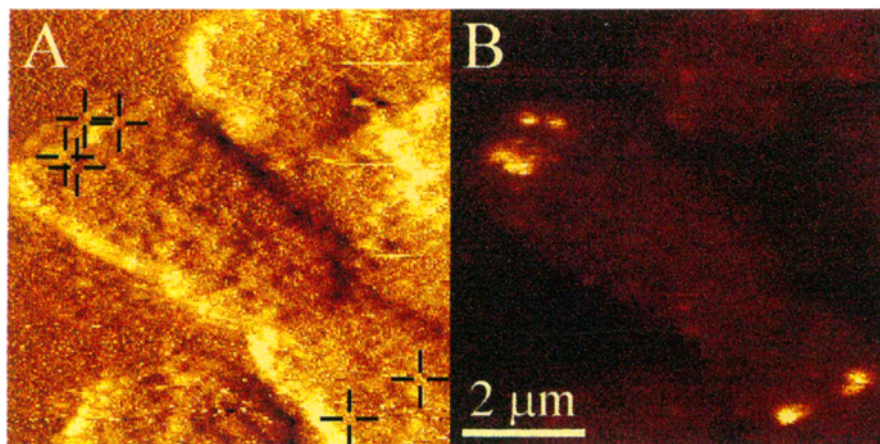


Fig. 5. Near-field fluorescence images of a human metaphase chromosome hybridized with the satellite probe p1-79 and the plasmid probe α -spectrin: (A) shear force image (high-pass filtered in horizontal direction) showing topography; (B) near-field fluorescence image revealing p1-79 at the telomere end and α -spectrin at the external side of band 1q21.3.

microscopy. Finally, we have demonstrated the sensitivity to detect single-copy genes.

Thus the near-field scanning optical microscope has good prospects for biological applications where a high optical resolution is desired, for example the detection of multiple fluorescence *in situ* hybridization signals on human metaphase chromosomes.

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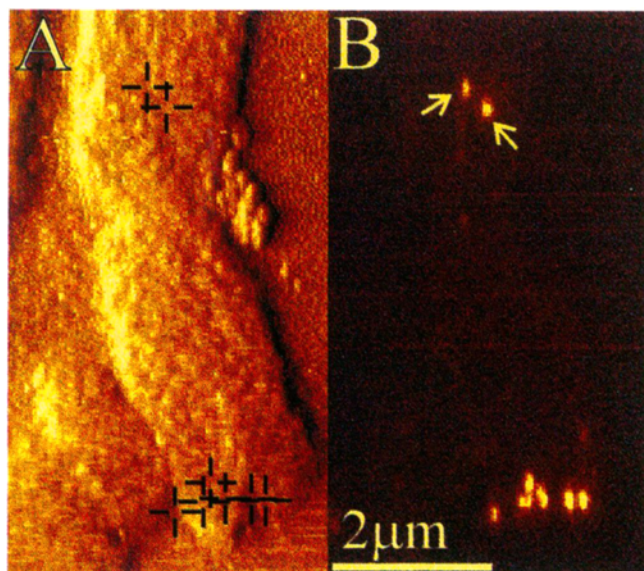


Fig. 6. Near-field fluorescence images of a human metaphase chromosome hybridized with the satellite probe p1-79 and the plasmid probe α -spectrin: (A) shear force image (high-pass filtered in horizontal direction) showing topography; (B) near-field fluorescence image again showing p1-79 at the telomere end but now α -spectrin is located at the internal side of the band. This might be caused by the twist in the chromosome visible in the force image.

W.K., A.R. and B.G.). The plasmid containing the α -spectrin was a kind gift from Dr R. V. Lebo.

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