Nanoscopic imaging of human chromosomes via a scanning near-field optical/atomic-force microscopy (SNOAM)

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Abstract

Scanning near-field optical/atomic-force microscopy (SNOAM) provided us with simultaneous topographic and fluorescence images of human chromosomes. The SNOAM uses a bent optical fiber simultaneously as a dynamic mode atomic force microscopy cantilever. Optical resolution was approximately 50–100 nm in fluorescence mode. Conventional karyotyping information was linked with SNOAM topographic analyses such as location of centromere and length of individual chromosomes. The height profile clearly indicated higher teromere regions. The SNOAM fluorescence images were different shapes from topographic images probably due to results from the combination of fluorescence dye and chromosome DNA.

Keywords: Human chromosomes; Scanning near-field optical/atomic-force microscopy; Genetic transmission

1. Introduction

There are a number of methods for investigation of the structure, function and evolution of the human genome. These include examining direct observation of normal and abnormal chromosomes following the genetic transmission of phenotypes and DNA sequence variations. Optical analyses linked with staining chromosomes have provided us with identification of physical mapping of individual chromosome. However, conventional optics had limit of resolution which was based on half of wavelength of visible light, while a scanning near-field optical microscopy (SNOM) has demonstrated that spatial resolution of only a few tens nanometers and sensitivity down to the single molecule level. This technique is analogous to scanning tunnel microscopy (STM) and atomic force microscopy (AFM). We have developed a scanning near-field optical/atomic-force microscopy we call it ‘SNOAM’ in which a method of the dynamic mode AFM was used to control the tip-to-sample separation [1–6]. The SNOAM is also superior in biological observation to other systems because this system operates excellently in liquid. It is safely applicable for observation of soft samples with great variations in height, such as cultured cells and chromosome.

Human chromosomes are only a few microns in length and hence high resolution microscopy is desired for Karyotyping and structure related function analyses. A karyotype shows the metaphase chromosomes of an individual cell, arranged in pairs and sorted according to size. There are 22 pairs of autosomal chromosomes and a pair of sex chromosomes in humans. Karyotypes are a simple way to evaluate chromosomes. Many diseases and malformations are a direct result of missing, broken or extra chromosomes. In a karyotype, we can recognize and identify many of these gross chromosomal abnormalities. When chromosomes are stained by methods that do not produce bands, they can be arranged into seven readily distinguishable groups based on descending order of size and position of the centromere.

Group 1–3(A) Large metacentric chromosomes readily distinguished from each other by size and centromere position

Group 4–5(B) Large submetacentric chromosomes which are difficult to distinguish from each other.

Group 6–12,X(C) Medium sized metacentric chromosomes The X chromosome resembles the longer chromosomes in this group. This large group is
Fig. 1. Schematic diagram of the SNOM/AFM system, The Seiko SPA700 SNOAM system (right photo).
the one, which presents major difficulty in identification of individual chromosomes without banding techniques. Group 13–15 (D) Medium-sized acrocentric chromosomes with satellites. Group 16–18 (E) Relatively short metacentric chromosome (#16) or submetacentric chromosomes (#17 and 18) Group 19–20 (F) Short metacentric chromosomes. Group 21–22,Y (G) Short acrocentric chromosomes with satellites. The Y chromosome is similar to these chromosomes, but bears no satellites.

Basic karyotype analysis is based on the result of several conferences (ISCN, International System for Human Cytogenetic Nomenclature) established in Denver, USA in 1960. Chromosome number and morphology were decided by banding or non-banding techniques. When chromosomes are stained by methods that do not produce bands, they can be arranged into seven readily distinguishable groups (A–G) based on descending order of size and position of the centromere. Usually, a metacentric chromosome’s centromere appears as a pronounced minimum in either the width or shape profile, whereas that of an acrocentric centromere is represented only by a smaller than usual gradient at one end of the profile. In this report, SNOAM system provided us with simultaneous topographic and optical images of human chromosomes.

2. Material and methods

2.1. Preparation of human chromosomes

In this research human metaphase chromosomes were imaged and identified using SNOAM. The metaphase chromosomes were derived from human B cell lymphoblastoid line RPMI1788 [7]. The cells were grown in RPMI11640 medium with 10% of fetal calf serum (FCS) at 37°C. Metaphase chromosomes were obtained after addition of colcemid (final concentration 0.05 mg/ml) which gave synchronization of the cell cycle. Synchronized cells were harvested by centrifugation (500 g, 5 min). Chromosomes were prepared by the ‘Surface-Spreading Whole-Mount Technique’ [8]. To begin with, after centrifugation, collected cells were placed on the clean surface of distilled water with a clean platinum loop. Here, the cells burst due to osmotic pressure and then spread out rapidly over the water surface. The chromosomes were transferred to a glass coverslip by contact with the surface of the water and then the sample was dried in air.

2.2. SNOAM system

The SNOAM system is as shown in Fig. 1. An optical fiber with a tapered tip was bent for using the probe as a cantilever for AFM, and the vibration amplitude of the cantilever was held constant during scanning. Due to nano scale aperture, light from laser was localized at top of fiber so call near-field light was generated. The vibration amplitude is monitored by detecting the reflection of the laser beam, which is reflected on the ground surface of the optical fiber cantilever. The probe-sample distance is controlled by decreasing the vibration amplitude to an appropriate level when the distance between the probe and the sample decrease. Laser beams (458, 488, 514.5 nm) from a multi-line Ar ion laser (max. 150 mW) were selected by polychromatic AO modulator and coupled to the optical fiber on the other side of the optical fiber probe. Signal light from the sample is collected by objective lens (typically:100 X oil immersion type) and separated by dichroic mirror to the CCD camera and detectors. Photomultiplier and Intensified-CCD (ICCD) camera with spectrometer were connected as the detectors.

3. Results and discussion

3.1. SNOAM probes

To overcome the diffraction-based resolution limit in visible light microscopy, the scanning near-field optical microscopy makes use of a strong localization of light. This localization is commonly achieved by using aperture probes. These squeeze light through a narrow metal tunnel with an aperture substantially smaller than the wavelength. This indeed strongly localizes the probing light ensuring spatial resolution of < 50 nm. However, this gain in resolution is paid for by a severe power loss since the waveguide is operated in the cut-off situation. Therefore, since the resolution is limited rather by the detector’s signal-to-noise ratio than by the aperture size, it is fundamentally important to understand what happens inside SNOM’s taper.

Fig. 2 indicated a scanning electron micrograph of a typical SNOAM probe. We have two types of optical fiber. One aluminium type, which is designed for operation in air, and one gold type which is designed for operation in water. Both are made from optical fibers which are chemically etched to make a tip, then bent by irradiation from a CO2 laser. They are then coated with a 100–200 nm thick metal and then an aperture is cut at the tip. Air operation probes are based on a 125 mm fiber optic core. The metal covering is aluminium, and resonant frequency is around 15 KHz. The Q factor is typically between 300 and 500.

3.2. SNOAM imaging and spectrum

The resolution and imaging properties of our SNOAM system is largely dependent on the probes used. Optical fiber based probes are hand made and thus will vary in properties.
Generally probes will have apertures of between 50 and 100 nm. To test the properties of any particular probe, a standard sample can be used. The standard sample we have used is a glass plate with a chequered chromium pattern on top. The evaporated chromium height is 20 nm. The chromium areas are raised and hence will give a brighter topographic AFM image. Chromium is however, opaque and hence all light created from the near field is scattered or absorbed, but not transmitted. Hence these areas are darker in the SNOM image. As a result the two images were the inverse of each other. The resolution can be obtained from the step height, which in this case is about 60 nm (Fig. 3).

To test the fluorescence measuring properties of a SNOAM system, fluorescent beads can be used. Latex beads of known size and covered with a known fluorophore is useful for fluorescence calibration. In this case, a photon counting type photomultiplier is used for fluorescence measurement instead of the analogue type photomultiplier for the transmission mode. Topographic and fluorescence images of a 100 nm fluorescence bead are shown in Fig. 4(a) and (b) where beads are scattered in PVA (poly (vinyl alcohol)) film on a glass plate. The topographic image shows the round shape of the bead and wrinkles of the film around it. The fluorescence image was observed with a 488 nm laser beam for excitation and showed the round shape of the fluorescence bead. The profile of fluorescence intensity in this figure (b) shows that the half width of the fluorescence peak of the bead is about 243 nm (Fig. 4(c)). In this case, the diameter of the bead is 100 nm and the aperture of the probe we used was 50–100 nm, therefore a width of just over 200 nm is reasonable.

SNOAM is also capable of analyzing fluorescence spectra. In this case a spectrofluorometer, consisting of a polychromator and an ICCD (Intensified Charged Couple Device) is attached in place of the photon counting head. The sensitivity of a spectrofluorometer is not as high as that for a photon counting head. Thus the aperture has to be bigger and hence the spatial resolution is lower.

In Fig. 5 the fluorescence spectra was obtained from 100 nm, fluorescent latex beads. The fluorescent dye on the spheres was fluorescein, which has excitation and emission wavelengths of 488 and 515 nm, respectively.

The graphs show fluorescence spectrographs when the probe tip was positioned around the fluorescence bead in 100 nm steps. In this experiment the spectrum window is limited to a range of 515–600 m because the system has a dichroic mirror of 500 nm, a long wave pass filter of 515 nm...
to cut the excitation light, and a short wave pass filter of 600 nm to cut the laser beam of the optical lever (670 nm); The fluorescence peak weakens with increasing distance from the bead and shows a background signal only at a position of 300 nm from the bead center.

3.3. Chromosome imaging

Chromosomes provide the basic material for a large proportion of genetic investigations, from the construction of gene maps and models of chromosome organization to
the investigation of gene function. Chromosome make highly condensed structure which consists of nucleosome fiber, whose component is chromatin consisted of histon protein and DNA strand. The size of each structure is shown here. Conventional AFM is possible to obtain topographic image with 2 nm resolution. Resolution of our SNOAM system is around 50 nm, however, optical information can be simultaneously obtained.

Fig. 6 shows a typical topographic image of a human metaphase chromosomes which are recognized as three different types of chromosomes. Dynamic mode AFM (DFM) image displaying the topography of human metaphase chromosomes on a glass coverslip in air. The image area is multiply 10 by 10 mm. The dark-brown (umber) to white scale of the image is 80 nm. The height information in cyclic contact mode is calibrated just as for the height information in the conventional contact mode. Fig. 7 shows height profiles of each chromosome. From a morphological view of the chromosome (b) in Fig. 7(a) same as previous picture, its length was measured approximately 8–9 mm, and a centromere was observed on one-side of the chromosome. The candidate of the chromosome (b) is #4 or #5 in group C. Similarly, chromosome (a) in Fig. 7(b) was also judged to be part of group E or F, and numbered 16,19, or 20 because it was about 5–6 mm in length and had a metacentric centromere (Fig. 7(b)). Chromosome (c) was designated a Y chromosome because of an acrometric centromere and its length, at approximately 3–4 mm (Fig. 7(c)). This estimation is based on topographic images of SNOAM system.

Fig. 6. Dynamic mode AFM (DFM) image displaying the topography of human metaphase chromosomes.

Fig. 7. (a) Cross section profile of chromosome (a) in Fig. 6; (b) Cross section profile of chromosome (b) in Fig. 6; (c) Cross section profile of chromosome (c) in Fig. 6 Scale bar: 1 μm. Filled triangle sign indicates a position of centromere of chromosome.
3.4. Fluorescence imaging of chromosome

Fig. 8 shows the fluorescent chromosomes stained with SYBR\textsuperscript{TM} Green I corresponding to the topographic images of chromosome (b). SYBR green I (excitation at 497 nm, emission at 520 nm) was used as a fluorescent intercalater combined with DNA strands. The fluorescence images were detected at 520 nm emission with 488 nm Argon laser beam excitation. SYBR\textsuperscript{TM} Green I is the most sensitive stain available for detection of nucleic acids. Less than 20 pg of double stranded DNA can be detected in a single band of a SYBR\textsuperscript{TM} Green I-stained gel.

Topographic images clearly indicated duplicate structure on the metaphase chromosome, while the fluorescence images were a somewhat different shape. This probably results from the combination of SYBR\textsuperscript{TM} Green I and chromosome DNA. SYBR\textsuperscript{TM} Green I is thought to bind non-covalently to the phosphate backbone of the DNA molecule.

Atomic force images have some artefacts (Fig. 7), however, they can be corrected by comparison with fluorescence image. The resolution of conventional far-field optical microscopy is theoretically over half of wavelength. Although electron microscopy provides better resolution it cannot allow observation without preparative procedures such as fixation, staining and vacuum evaporation. Fluorescence spectra obtained from the nanoscopic area were available for nano-scale FISH technique [9,10] (data not shown). SNOAM -based nanoscopic physical mapping of target genes will be realized on each chromosome linked with sequence of human genes [11].

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