Imaging of Chromosomes at Nano-Meter Scale Resolution Using Scanning Near-Field Optical/Atomic Force Microscopy*

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Summary. Topographic and fluorescent images of whole barley chromosomes stained with YOYO-1 were observed simultaneously by scanning near-field optical/atomic force microscopy (SNOM/AFM). The chromosome was relatively smooth and flat in the topographic images and no significant difference in height was present between regions of high fluorescent and low fluorescent intensity in the chromosomes. The telomeric region, labeled by fluorescence *in situ* hybridization (FISH) method, was also observed by SNOM/AFM at high resolution, and fluorescent signals of the telomeric region were clearly defined on the topographic image of chromatin fibers on the chromosome at the nano-meter scale level. Although the telomeric signals were usually visualized as a single fluorescent region at the end of sister chromatids by conventional light microscopy, they were observed separately as two fluorescent regions, less than 100–200 nm distance, using the SNOM/AFM. The SNOM/AFM offers great potential in identifying particular single gene location on chromosomes in the near future.

The fluorescence *in situ* hybridization (FISH) method currently finds wide application in the detection of

the location of a specific gene on the chromosome. Because the resolution of the conventional FISH method by light microscopy is essentially limited to the half micro-meter level, many have looked for a novel technique which would enable the visualization of a specific gene on the chromosome at the nano-meter scale level.

On the other hand, atomic force microscopy (AFM) has been used in studies of the chromosomal structure (USHIKI et al., 1996; TAMAYO and MILES, 2000; TAMOTO et al., 2000; HAUSMANN et al., 2001). Some reports (DECKERT et al., 1998; GARCIA-PARAJO et al., 1998) also showed that the scanning near-field optical microscope (SNOM), which is a microscope related to AFM, reveals a single DNA fiber at 100 nm resolution, although they could not accomplish the simultaneous collection of topographic and fluorescent images of DNA. Recently, the SNOM has been further applied to chromosome research and succeeded in obtaining simultaneous topographic and fluorescent images of the FISH-treated samples (MOERS et al., 1995, 1996). However, the resolution of the topographic image in these studies was quite limited probably because its tip motion was controlled by the shear force. To overcome this problem, IWABUCHI et al. (1997) and YOSHINO et al. (2002) applied an SNOM in the dynamic force mode, or SNOM/AFM which differed from the previous SNOM in the shear force mode to observations of chromosome structure. This instrument uses a bent optical fiber as a cantilever and a normal AFM method to control the distance between the tip and the sample surface, which allows operation of a dynamic force mode (MURAMATSU et al., 2000). We have considered the SNOM/AFM to be a good candidate for observing FISH-treated chro-

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mosome samples because a SNOM/AFM could provide more detailed information on the chromosomal structure and fluorescent intensity than that of the previous SNOM alone.

This study therefore proposes the development of a “nano-FISH” method that directly defines the location of a specific gene on the chromosome or the DNA fiber with a high resolution using a scanning near-field optical/atomic force microscope (SNOM/AFM). Theoretically, this method is expected to exceed the limited resolution of conventional FISH. We shall show the high-resolution fluorescent images of chromosomes using a SNOM/AFM and discuss an applicability of the nano-FISH method.

MATERIALS AND METHODS

Principles and description of SNOM/AFM

The SNOM/AFM (SNOAM™, Seiko Instruments Inc., Chiba) (MURAMATSU et al., 2000) was used to collect simultaneously topographic and fluorescent images of barley chromosomes (YOSHINO et al., 2002). Figure 1 describes the operating principles of SNOM as compared with conventional fluorescence microscopy. When small different dyes at intervals of less than 500 nm are located on a transparent substrate

such as a cover slip, all the dyes are excited at once because the visible light wave length is typically about 500 nm in length (Fig. 1a). However, by SNOM, if an optical fiber probe with a 100 nm aperture is used, individual dyes can be excited with the near-field light from the aperture, as if illuminated with a spotlight with a 100 nm radius (Fig. 1b). Thus, the resolution of the SNOM method surpasses the optical limitation. The range of the near-field from the probe tip is nearly equal to the diameter of the tip aperture.

The instrument used in this SNOM/AFM study was mounted on a conventional inverted light microscope (Fig. 2); a bent optical fiber probe was used for this SNOM/AFM instead of a silicon AFM probe and controlled by a dynamic force mode. The excitation light was coupled with the end of the optical probe and transmitted to the tip of the probe, where the near-field light is produced. The excited far-field light from the labeled fluorescent dye on the chromosome sample was collected by a photo-detector through the objective lens of the original light microscope. The resonant frequency of the optical fiber probe was controlled around 17 kHz (SPM 3800N, Seiko Instruments Inc.). We used a set of three piezo scanners with a xy-scan range of 20 μm (Seiko Instruments Inc.) and an excitation Ar ion laser (488 nm wavelength). The scan speed ranged from 0.14 to 0.19 Hz,

Fig. 1. Principles of the SNOM/AFM as compared with that of the standard fluorescence microscopy. **a.** Principle of standard inverted light microscopy. Four different dyes are excited at once because the wavelength of the visible light is more than about 500 nm. **b.** Principle of scanning near-field optical microscopy. Since individual dyes can be excited with the near-field light which is made by a tip aperture of about 100 nm in diameter, the individual dyes are thereby discriminated with this method.

yielding an imaging time for each frame of about 50 min. The amplitude reference (set point) was adjusted as low as possible, between -0.032 and -0.048 , so as not to damage the chromosome samples.

Using this specially designed SNOM/AFM, successful near-field optical resolution was obtained for the strongly stretched lambda-phage DNA molecules, namely, the optimum optical resolution of this system was estimated at 45 nm for the dye-intercalated single lambda-phage DNA molecules by a mean width evaluation (KIM et al., 2001).

Sample preparations

Chromosome samples of the barley (*Hordeum vulgare*, cv. Minorimugi) were prepared as follows. The barley root-tips on a cover slip (22×22 mm) were treated with an enzyme solution (2% cellulase Onozuka RS, Yakult Honsha Co., Ltd., Tokyo; 1% pectolyase Y-23, Kikkoman Corp., Noda; 7.5 mM KCl and 7.5 mM EDTA, pH 4.0), washed with distilled water, and compressed with tweezers in a methanol-acetic acid (3 : 1) solution or 45% acetic acid solution. The samples were treated with a 45% acetic acid solution at 50°C for 30 min, extensively washed with the same solution at room temperature, incubated overnight in a 45% acetic acid solution at 37°C, and washed again with a 45% acetic acid solution. The samples were then rinsed with a standard saline

citrate buffer ($1 \times$ SSC) and distilled water, and air-dried.

Fluorescence staining of the barley chromosomes

For whole staining of the chromosome, the samples were stained with fluorescence dye ($1 \mu\text{M}$ YOYO-1) at 4°C for 30 min, washed again with distilled water and air-dried. In addition, some chromosome samples were treated with YOYO-1 and methyl green as described by SAITOH and LAEMML (1994) to produce a banded pattern of the chromosome.

FISH of the telomeric region of the barley chromosome

The plant telomeric sequences $((\text{TTTAGGG})_n)$ amplified by PCR were used as a DNA probe. Using a primer pair $(5'\text{-AGGGTTT-}3')_5$ and $(5'\text{-AAACCCT-}3')_5$, PCR was performed in the absence of template DNA according to a previous protocol (IJDO et al., 1991). In order to mount the sample stage of the SNOM/AFM, chromosome samples were prepared on a cover slip to which a hydrophilic treatment was made to keep fixation of chromosome during the FISH treatment. The procedure of the FISH treatment was followed by the standard method (TAKAHASHI et al., 1989), although a small part of the method was modified for SNOM/AFM measurement as below (SHICHIRI et al., 2003). The cover slips were

Fig. 2. Schematic illustration of the SNOM/AFM. The instrument is mounted on a standard inverted light microscope. The excitation light (laser) is coupled with the end of a bent optical fiber probe and transmitted from the tip of the probe to the sample surface. A photo detector collects excited far-field light from the labeled fluorescent dye on the chromosome sample through the objective lens of the original light microscope.

Fig. 3. SNOM/AFM images of a whole barley chromosome. The barley chromosome uniformly stained with $1\ \mu\text{M}$ YOYO-1 was observed with SNOM/AFM. **a.** Topographic image of the YOYO-1 stained chromosome. The height range was 293 nm. **b.** Fluorescent image in the same portion of **a**. The fluorescent intensity was 0.6 V. **c.** Expanded topographic image of centromere region in **a**. The height range was 212 nm. The region, indicated by a *white arrow*, may not include DNA because the fluorescent image of this portion is not observed in **d**. **d.** Fluorescent image of the same portion of **c**. The fluorescent intensity was 0.3 V. No fluorescent signal was detected at the region where the debris were observed in **c**. Bars: $5\ \mu\text{m}$ (**a**, **b**), 500 nm (**c**, **d**)

rinsed with RNase ($100\ \mu\text{g}/\text{ml}$ in $1\times\text{SSC}$) at 37°C for 60 min, washed three times with $2\times\text{SSC}$ at room temperature for 5 min, and dehydrated in an ethanol series. They were incubated overnight at 65°C , denatured at 70°C for 2 min in 70% formamide in $2\times\text{SSC}$, and immediately placed in ice-cold 70% ethanol for 5 min and 100% ethanol for 5 min. After the biotinylated DNA probe was denatured at 75°C for 10 min and quenched in an ice-cold bath, the probe was hybridized to target chromosomes at 37°C for 20 to 24 h in a humid chamber. After hybridization, the cover slips were washed with 50% formamide in $2\times\text{SSC}$ at 37°C for 20 min and washed with $2\times\text{SSC}$

at room temperature for 15 min, and then washed twice with $1\times\text{SSC}$ at room temperature for 15 min. Biotin-labeled probe DNA was detected by $5\ \mu\text{g}/\text{ml}$ Alexa 488-streptavidin conjugates (Molecular Probes, Oregon, USA) in $10\ \text{mg}/\text{ml}$ bovine serum albumin (BSA) fraction V, $4\times\text{SSC}$ at 37°C for 60 min. To remove non-specifically bound Alexa-streptavidin conjugates, the cover slips were washed for 5 min each with $4\times\text{SSC}$, 0.1% TritonX in $4\times\text{SSC}$, $4\times\text{SSC}$, and $2\times\text{SSC}$, and dehydrated for 5 min each with 70% and 100% ethanol.

Fig. 4. SNOM/AFM images of the fluorescent banding-treated barley chromosome. The banding pattern was generated by the double staining of the chromosome with YOYO-1 and methyl green. **a.** Topographic image of the banded chromosome. The height range was 322 nm. **b.** Fluorescent image in the same portion of **a**. The fluorescent intensity was 2.9 V. **c.** Expanded topographic image of the centromere region in **a**. The height range was 293 nm. **d.** Fluorescent image of the same portion of **c**. The fluorescent intensity was 1.0 V. Bar: 500 nm (c, d)

RESULTS

Barely chromosomes stained with YOYO-1

Using our SNOM/AFM system, topographic and fluorescent images of YOYO-1 stained barely metaphase chromosomes were visualized simultaneously (Fig. 3). In the topographic image (Fig. 3a), sister chromatids in the chromosome at times presented problems to be differentially determined, probably due to the poor resolution induced by the tip radius of the optical probe, which was larger than the probe for conventional AFM. The thickness of the chromosome was about 200 nm and the fluorescent intensity of the chromosome was roughly correlated with the shape and height of the chromosome in the corre-

sponding topographic image (Fig. 3b).

At a higher magnification, the chromosome exhibited a granular (ca. 100 nm in diameter) appearance on their surface in the topographic image (Fig. 3c). The fluorescent information of the corresponding region revealed that the granular substances were all stained with YOYO-1 (Fig. 3d).

Cellular debris was observed around the chromosome on the substrate (Fig. 3a, c), where no fluorescent intensity was detected (Fig. 3b, d). Thus, a comparison between topographic and fluorescent images was useful for the determination of the nature of the debris; the debris containing DNA could be stained by YOYO-1, while the debris without DNA could not be fluorescent. Although the debris was closely located besides the chromosome, it could be obviously

Fig. 5. SNOM/AFM images of telomeric region of a barley chromosome labeled by FISH. The relationship between telomere FISH signals and topology of the chromosomes was investigated by SNOM/AFM. **a.** Topographic image of the telomere portion of the chromosome by SNOM/AFM (using an optical fiber as a probe). The height range was 80 nm. **b.** Fluorescent image in the same portion of **a**. The fluorescent intensity was 60 mV. **c.** Topographic image in the same portion of **a** using an AFM probe instead of an optical fiber probe. The height range was 80 nm. **d.** Superimposed images of **b** and **c**. Two telomere FISH signals were observed near the edge of the chromosomes. Bar: 1 μ m

distinguished with the DNA containing substances at the nano-meter scale.

Barley banded chromosomes

Fluorescent banding patterns of chromosomes were prepared by double staining of YOYO-1 and methyl green and observed by SNOM/AFM. In the topographic image, the chromosome treated with this banding method had a thickness of about 200 nm, and showed a rather flat and uniform surface (Fig. 4a). The overall shape of the chromosome in the fluorescent image (Fig. 4b) was similar to that of the topographic image, but the fluorescent intensity did not

always correlate to the thickness of the chromosome. Several spots of various sizes with high fluorescent intensity were observed on the banded chromosome, while the topographic image showed that the surface of the arm region was flat and there was no significant height difference between the spots and the other part of the arm.

In a closer view of the topographic image (Fig. 4c), globular structures of about 100 nm in diameter were observed on the surface of the banded chromosome – as was the case of a whole fluorescent stained chromosome (Fig. 3c). These structures are considered to correspond to the granular and/or fibrous structure of 30 to 50 nm in diameter that has

Fig. 6. High-resolution topographic (a) and fluorescent images (b) of the telomere region depicted as a rectangular area in Figure 5d. **a.** Expanded topographic image of a rectangular area in Figure 5d which was taken by a standard silicon AFM tip. The height range was about 40 nm. **b.** Telomere FISH signals on topographic image of **a.** Telomeric signals are more separated than these of the normal chromosome sample shown in Figure 5b. Bar: 100 nm

been reported in previous conventional AFM studies (HOSHI and USHIKI, 2001) because the tip radius of the optical probe used this imaging was much larger than that in the conventional AFM and this probably induced the size difference between the two images.

On the other hand, spots of approximately $0.5\ \mu\text{m}$ in diameter with high fluorescent intensity were observed on the arm and centromere region in an expanded view of the fluorescent image (Fig. 4d). In a comparison of the fluorescent images with the topographic ones, the same region revealed no apparent correlation between the thickness and fluorescent intensity of the spot area. Thus, it was evident that the high fluorescent intensity of the spots was not produced by the effect of chromosome thickness, but reflected the internal structure of the chromosome such as DNA density and/or base composition.

FISH of the telomeric region of the barley chromosome

FISH of the telomeric region was performed in the barley chromosomes. This treatment yielded clear topographic and fluorescent images of the entire barley chromosome labeled with telomeric DNA by SNOM/AFM (Fig. 5). In the topographic image (Fig. 5a), the chromosome structure was disorganized to some extent and its height was reduced during the preparation steps of FISH, such as enzyme, heat, denaturation and washing treatments. The height of the telomeric region after FISH treatments was about 40 nm. On the other hand, a telomeric signal

was detected at the end of each sister chromatid in the fluorescent image (Fig. 5b). The image was much clearer than that of conventional fluorescence microscopy. Since the topographic image obtained by the optical fiber probe did not have a resolution sufficient for analyzing such structures as chromatin fibers, the same area was imaged separately using a standard AFM tip (i.e. a rectangular cantilever) mounted on the SNOM/AFM instrument. Consequently, the structures of the area observed by the SNOM/AFM could be precisely analyzed in the standard AFM image (Fig. 5c). Superimposition of the topographic (Fig. 5c) and fluorescent images (Fig. 5b) was useful because fluorescent telomeric regions were clearly defined on the topographic image (Fig. 5d).

In high-resolution topographic images of the telomeric region with the standard tip, chromatin fibers, 30 to 50 nm in diameter, were visible on the surface (Fig. 6a). In the corresponding fluorescent images, telomeric FISH signals were clearly observed as a bright area. Comparing these two images allow us to distinguish the approximate position of the telomeric region on the chromatin fibers (Fig. 6a). In these images, one telomeric signal was sometimes observed as two fluorescent regions that could not be distinguished by conventional light microscopy.

At some sites in the specimens where the end of the barley chromosome had been stretched unexpectedly during sample preparation procedure, the structure of the telomeric region was remound or extended and the thickness was reduced (Fig. 7).

Fig. 7. FISH signals on a stretched telomeric region. **a.** Topographic image using an AFM probe mounted on the SNOM/AFM. The height range is about 85 nm. **b.** Superimposition image of the telomeric FISH signals and the topographic image of **a.** The minimum resolution between two fluorescent signals is improved from about 250 nm (Fig. 6) to 100–150 nm. Bar: 500 nm

Fig. 8. Bleaching of FISH signals on 45S rRNA area. The sample was imaged at once (**a**) and then magnified in the rectangular area and scanned twice (**b**). The signal was bleached after a total of three scans. **a.** The first scan of whole chromosome. Three signals were observed. The height range and the fluorescent intensity were 250 nm and 371 mV. **b.** The second scan of the rectangular area of **a.** Weak signals were still observed. The height range and the fluorescent intensity are 151 nm and 57 mV. **c.** The fourth scan of the same area of **b.** Although the fluorescent signal is slightly enhanced by image analysis, the signal has been bleached. Bars: 2 μ m (**a**), 1 μ m (**b**, **c**)

Bleaching of fluorescence during SNOM/AFM experiment

To investigate the bleaching effects of fluorescence during SNOM/AFM, topographic and fluorescent images of the barley chromosome hybridized with 45S rRNA probe were observed. One example is shown in Figure 8. After a total three scans of the same area, fluorescent dye was bleached and was not detected at all (Fig. 8c).

DISCUSSION

The present study has applied the SNOM/AFM to the observation of barley chromosomes treated with various fluorescence staining methods. The SNOM/AFM offers a great advantage of taking topographic and fluorescent images simultaneously during a single scanning operation. Because of the fine quality of its topographic images, the SNOM/AFM is useful for

clarifying the correlations between the chromatin fiber structures and fluorescent signals. According to NOVOTNY et al. (1995) and DE LANGE et al. (2001), the distance of the near-field from the aperture of an optical fiber probe is somewhat less than 100 nm and the thickness of the field is limited by aperture size. Although the height of the chromosome was about 200 nm, the greater part of fluorescent information coming from the chromosome was collected in our fluorescent images. This also indicates that the fluorescent dye labeled at bottom of the thick sample is not excited because its position is out of the near-field.

The present study has succeeded in obtaining fluorescent information on the stained chromosomes and banded chromosomes in their entirety. Since the banding method used in the present study is believed to stain the GC-rich queue of the DNA molecule, the bright spot regions were considered to have a high density of GG-rich DNA (SAITOH and LAEMMLI, 1994; YOSHINO et al., 2002). High fluorescent intensity was also observed at the centromere region, while the thickness in this region was lower than that in the other fluorescent portions; this indicates that DNA density of the centromere was very high probably because DNA and proteins form a highly compacted nucleo-protein complex in this region. Thus, SNOM/AFM can be recommended for use in investigating the internal structure of chromosomes.

In the study of the FISH treated chromosomes, the height of the telomeric region after FISH treatments was about 40 nm. This suggests that the relative amount of DNA in the chromosome can be calculated, because all labeled telomeric DNA probes hybridized in the telomeric region were excited in the near-field. To estimate accurately the density and amount of DNA in the telomeric region using the fluorescent intensity, the efficiency of DNA probe hybridization to the chromosome should be analyzed. The application of a peptide nucleic acid (PNA) probe should be also considered because of its high hybridization efficiency. If an improvement in the high efficiency is achieved, the quantity of telomeric DNA on the chromosome might be identified.

We have also demonstrated that the stretched chromosome is useful for analyzing the telomeric region more precisely than before. Development of mechanical and/or chemical stretching methods for chromosome structure analysis might be necessary. When the chromosome is stretched, chromosome thickness must be reduced. Such thin samples are quite suitable for the SNOM/AFM technique, since the thickness of near-field from the optical probe tip is generally estimated at only less than 100 nm when

the aperture of probe is less than 100 nm.

Bleaching is quite a serious problem for applying SNOM/AFM to biological samples. When the SNOM/AFM measurements were performed in the air, bleaching of the fluorescent dye is accelerated due to the presence of oxygen. To avoid the bleaching problem, measurement in a liquid condition is indispensable because an anti-bleaching reagent can be obtained in a liquid condition. Measurements in liquid are also anticipated since this can contribute to realizing the high resolution imaging of non-fixed or physiological materials.

In conclusions, the simultaneous topographic and fluorescent imaging of barley chromosomes at the nano-meter scale level was succeeded using SNOM/AFM. The SNOM/AFM observations of banded and non-banded chromosomes provided excellent information which would be difficult to obtain by conventional light microscopy. Especially, the SNOM/AFM was useful for the investigation of the telomeric region in relation to the chromosome structure at the chromatin fiber level. It indicated that SNOM/AFM will contribute to the detection of the exact position of genes on the chromosome or the single DNA fiber at the nano-meter scale level. In summary, the "Nano-FISH" method using SNOM/AFM has the ability to become one of the conventional tools in biological and medical fields, although further investigation of the pre-treatment of samples as well as improvement of the SNOM/AFM hardware will be necessary to establish practical uses for this technology.

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