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High-Resolution Analysis of the 3D Organization of Human Metaphase Chromosomes

Stefan Thalhammer, Pietro Gobbi, Mirella Falconi, Giovanni Mazzotti, and Wolfgang M. Heckl

1. Introduction

The detailed understanding of the nuclear cell functions requires an accurate knowledge of the spatial organization of their structures. For many years the study of human metaphase chromosomes was conducted with light microscopy after staining protocols disturbing the native chromosomal structure. The approach by scanning electron microscopy (SEM) provides higher resolution as compared with light microscopy and permits surface analysis of the chromosomal structure, which cannot be adequately obtained from transmission electron microscopy. Nevertheless, to obtain high resolution in SEM observations, the use of a high electron accelerating voltage (up to 30 kV) is required ***(1)***. With these experimental conditions, the sputter coating or a conductive staining of the samples is generally required ***(1,2)***. Both procedures allow electron-charging dispersion from the sample but may obscure fine details and produce sample alterations ***(3)***.

The field emission in lens scanning electron microscope (FEISEM; *see* **Fig. 1A**) represents a special kind of SEM, fitted with a cold cathode field emission electron gun ***(4,5)***, that can operate at low accelerating voltage with reduced electron charging of the sample. In fact, the low-voltage and low-current electron beam of the FEISEM together with a liquid nitrogen anti-contamination device in correspondence to the specimen area and an "in lens" assembly of the electron-optic column allows for high-resolution imaging of the biological sample without any conductive staining or metal coating. Nevertheless, contamination of the specimen is highly reduced as compared to conventional SEM ***(4)***.

The sample location between the objective pole piece limits the dispersion of the secondary electrons collected by the magnetic field of the lens. To sum-

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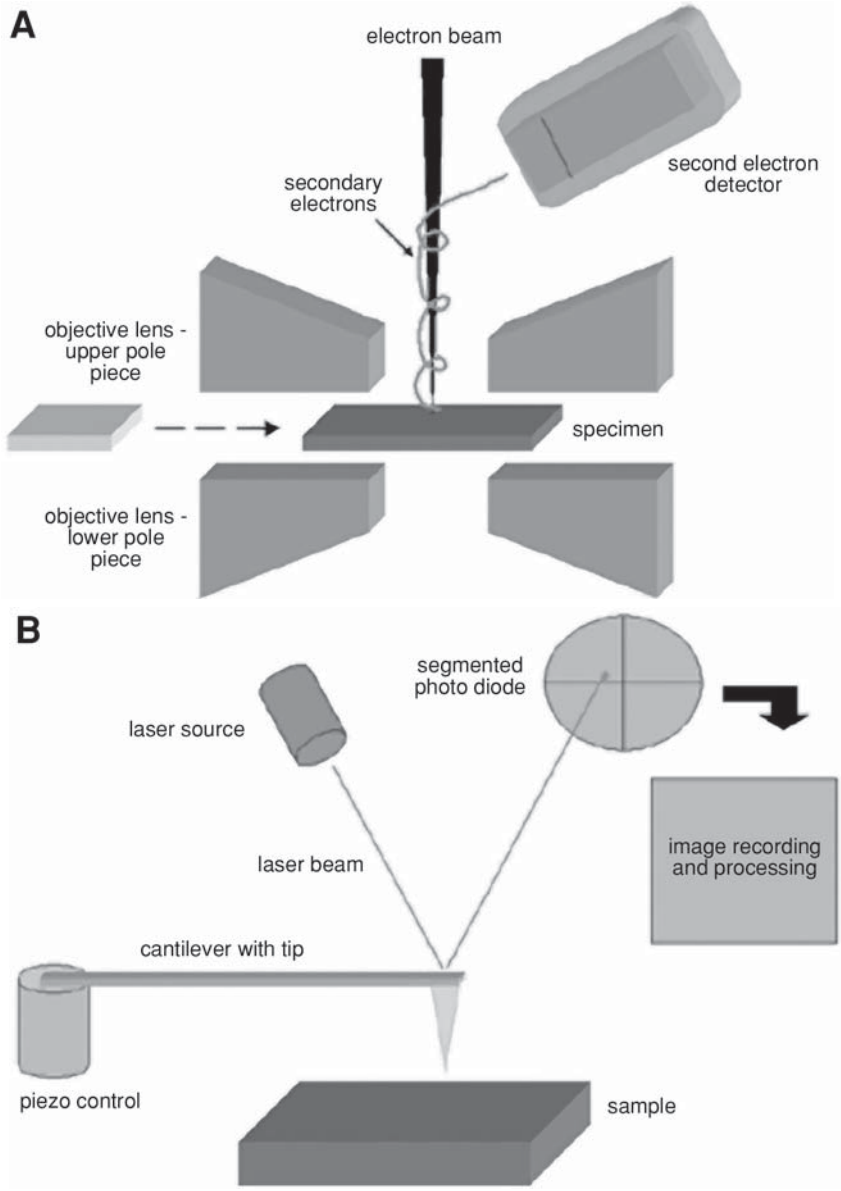

Fig. 1. **(A)** Schematic setup of a FEISEM: the specimen is brought between the upper and lower pole piece; the secondary electron beam moves in a spiral motion to a detector. **(B)** Schematic setup of an AFM with beam-bounce detection: the laser beam is deflected from the back of the cantilever according to the topographic properties of the sample. The laser beam displacement is measured with a segmented photo diode, recorded, and processed line by line.

marize, these characteristics allow the observation of uncoated biological samples with a higher resolution as compared with conventional SEM ***(6–9)***.

Since its invention in 1986, the use of the atomic force microscopy (AFM) has become a standard technique on various biological applications, including chromosomes ***(10–12)***, not requiring, especially in contact mode analysis, any particular treatment of the sample. The AFM allows imaging of chromosomes in ambient as well as in physiological conditions but with lower resolution compared with electron microscopy approaches ***(13–15)***. The schematic setup of an atomic force microscope is shown in **Fig. 1B**.

The combination of two different technical approaches shows a high correlation of the respective morphological information, both in normal and treated samples. The high-resolution potential of the FEISEM, together with the possibility to observe hydrated samples and/or to nanomanipulate the specimen with the AFM, confirms morphological data and offers an enhanced information on their biological significance ***(16)***. **Table 1** summarizes the necessary conditions of the different microscopes for the observation of biological samples.

The methods described here are aimed at producing the best possible samples obtainable from a starting material of peripheral blood lymphocytes to the final correlative observations using FEISEM and AFM.

2. Materials

1. 3× 6 mm ITO glass (Indium Thin Oxide) with 100 Ω surface resistance
2. AFM cantilevers: stiff cantilevers: spring constant, c = 0.3 N/m, nominal tip radius, $r < 10$ nm (Dr. Olaf Wolter GmbH, Germany).
3. Metaphase chromosomes: Human whole blood; Gibco Chromosome Medium 1A (Gibco/BRL); colchicine (10 µg/mL; Sigma); 0.075 *M* KCl (hypotonic solution); methanol/acetic acid (3:1); andalcoholic series (25, 50, 70, 90, and 100%).
4. GTG banding: 0.05% trypsin solution (Difco), 5% Giemsa staining solution, and 1X phosphate-buffered saline (PBS).
5. CBG banding: 0.2 *N* HCl; 5% $Ba(OH)_2$; 2X SSC buffer; and 5% Giemsa staining solution.
6. Protein digestion: Protease K (10 mg/mL; Roche); 3 *M* Na–acetate; 20% SDS.
7. FEISEM preparation: conductive carbon paint or colloidal graphite in Isopropanol (JEOL), and conductive tape (JEOL).

3. Methods

3.1. Cell Culture and Chromosome Preparation

1. Cultivate 0.4 mL human whole blood with 10 mL of Gibco Chromosome Medium 1A at 37°C for 72 h. Thirty minutes before preparation, the cells are arrested by adding colchicine (10 µg/mL) for 30 min.

Table 1
Comparison of Different Microscopic Techniques to Measure the Sample Topography

	Optical microscopy	SEM	FEISEM	AFM
Microscopic environment	Ambient Liquid Vacuum	Vacuum	Vacuum	Ambient Liquid Vacuum
Field depth	Small	High	Medium	Medium
Focus depth	Medium	Small	Very small	Small
Resolution	100 nm	5 nm	0.7 nm	0.1–1.0 nm
x, *y*, *z*	n/a	n/a	n/a	0.01 nm
Magnification	1x–2×10^3x	10x–10^6x	9x–10^5x	5×10^2x–10^8x
Necessary sample preparation	Low	Critical point drying or freeze-drying, metal coating	Critical point drying if necessary	Low
Necessary sample properties	Samples do not have to be completely transparent for visible light	Samples should not charge and have to be vacuum compatible	Vacuum compatibility	Samples do not have excessive changes in height in dependence to tip geometry

2. Spin down at **325*g*** for 10 min. Discharge supernantant and resuspend pellet in 10 mL of hypotonic solution containing 0.075 *M* KCl. Incubate at 37°C for 15 min (*see* **Note 1**).
3. After the incubation add two drops of the ice cold fixative containing methanol/acetic acid solution (3:1) and spin down at **325*g*** for 10 min (*see* **Note 1**).
4. Remove supernantant and resuspend pellet in 10 mL of ice-cold fixation mix.
5. Spin down at **325*g*** for 10 min and remove supernantant, and resuspend pellet in 10 mL of ice-cold fixative, repeating twice.
6. Spin down at **325*g*** for 10 min and resuspend pellet in 1 mL of fixative. Store at –20°C or proceed to **step 7**.
7. Resuspend the pellet and perform a drop fixation in the middle of the ITO-glass (*see* **Note 2**).

3.2. GTG Banding

1. Take 1 d or overnight aged chromosome preparations and incubate the slides at 37°C for 15 s in 0.05% trypsin-solution (Difco).
2. Rinse the slides briefly in PBS and staining and stain the treated slides in 5% Giemsa solution for 8 min.
3. Rinse the slides with water and allow to dry.

3.3. CBG Banding

1. For CBG banding use, 1- or 2-wk-old glass slides.
2. Incubate the slides in a 0.2 *N* HCl for 1 h at room temperature. Rinse briefly in deionized water and allow to dry.
3. After drying, treat the slides in a 5% $Ba(OH)_2$ at room temperature for 5 min, rinse them in deionized water, and pass through a alcoholic series and allow to dry.
4. After drying, incubate the slides in 2X SSC buffer (3 *M* NaCl, 300 m*M* Na–citrate) at 55°C for 1 h, followed by rinsing in deionized water and air drying.
5. Stain the slides in 5% Giemsa solution at room temperature for 45 min, rinse with water, and allow to dry.

3.4. Protein Digestion

1. After washing three times in 3:1 cold methanol: acetic acid, chromosome spreads were made by dropping the suspension onto the conductive surface of perfectly cleaned and degreased 3 × 6 mm ITO (Indium Thin Oxide) glasses. Metaphases were then air dried, dehydrated with an ethanol series (70, 90, 100%), air dried, and stored in a dry chamber until use.
2. Subsequently, the cleaning solutions are used alternatively. The treatment is a mix of 1 mL of 3 *M* Na–acetate, 20 µL of protease K (10 mg/mL) and 20 µL of 20% SDS for 2 min at 50°C.
3. After the cleaning procedure the ITO glass with the metaphases spreads is washed 2 min in distilled water, dehydrated in an ethanol series (25, 50, 70, 90, and 100%) and air dried.
4. The ITO glass is transferred to FEISEM microscopy.

3.5. FEISEM Microscopy

Cleaned and uncleaned metaphase spreads on ITO glasses are mounted onto the microscope specimen holders and observed without any conductive coating. Follow the instructions described in the microscope manual. We performed our experiments on a JEOL JSM-890 FEISEM (Jeol ltd. Japan) at 7k V accelerating voltage (1×10^{-11}. A probe current and 0° to 45° tilt angle. **Figure 2** shows two FEISEM images of metaphase chromosomes in low and high resolution. To unmount the ITO glass from the specimen holder cut the conductive glue and tape with a scalpel below the ITO glass, as shown in **Fig. 3**.

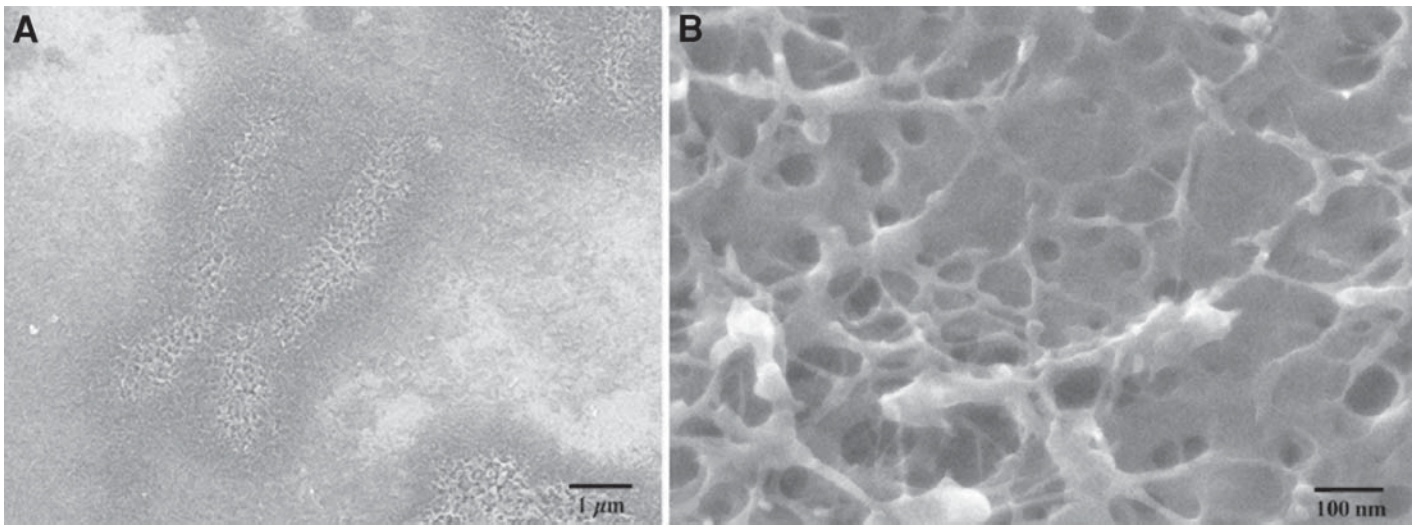

Fig. 2. FEISEM analysis of human metaphase chromosomes after protease K treatment. **(A)** The centromeric region and the chromatids are well recognizable. A dark halo surrounds the entire chromosome. Scale bar, 1 μm. **(B)** The chromosomal surface appears to be constituted of a network. Some fibrillar structures are well detectable. Scale bar, 100 nm.

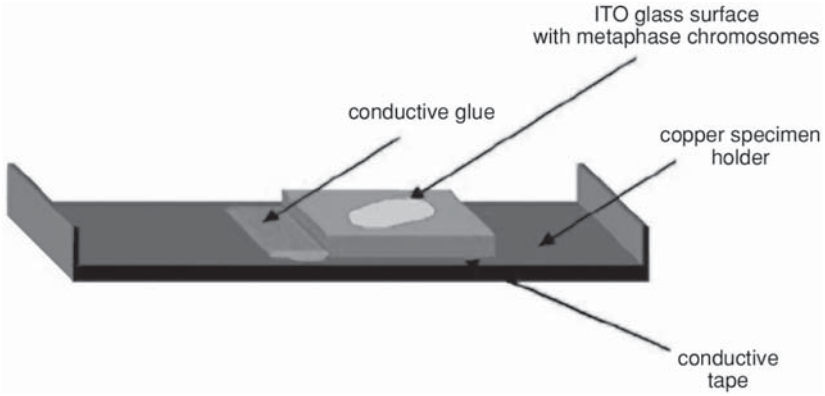

Fig. 3. Dismounting the ITO glass from the FEISEM specimen holder: to remove the ITO glass from the specimen holder, use a scalpel and cut below the conductive glue. Without any further treatment, the ITO glass can be used for AFM microscopy.

3.6. AFM

The unmounted ITO glass pieces with the metaphase chromosomes can be observed with AFM without any further treatment. Before imaging with the AFM, check the conductive side of the ITO glass (*see* **Note 2**). Please follow the recommended instructions of your AFM manual. In our experiments we used an AFM (Topometrix Explorer) with 130 µm *x*,*y*-scan range and 10 µm *z* scanner. The AFM was mounted on top of an inverted microscope to select the metaphase spreads. Observations of the human chromosomes in ambient conditions were conducted by means of stiff cantilevers in contact mode. The loading forces during AFM measurements were 10–20 nN in ambient conditions. **Figure 4** shows AFM images of metaphase chromosomes after protease K treatment. Some fibrillar structures are well detectable and the recorded chromosomal structures are comparable with the FEISEM images (*see* **Fig. 2**).

For imaging the GTG- and CBG-banded metaphase chromosomes in contact mode, we used stiff cantilevers. The loading forces during AFM measurements were 10–20 nN. The methodical properties are summarized in **Table 2**.

The scanning procedure of the AFM is controlled by the software SPMlab 3.06. The topographic and error signal image were recorded. The representation of the topographic image was done in gray scale with subsequent inversion of the image for easy comparison with known optical microscopy karyotypes. **Figure 5** shows AFM images of a GTG-banded chromosome imaged in contact mode, the corresponding error signal image, and a CBG-banded chromosome.

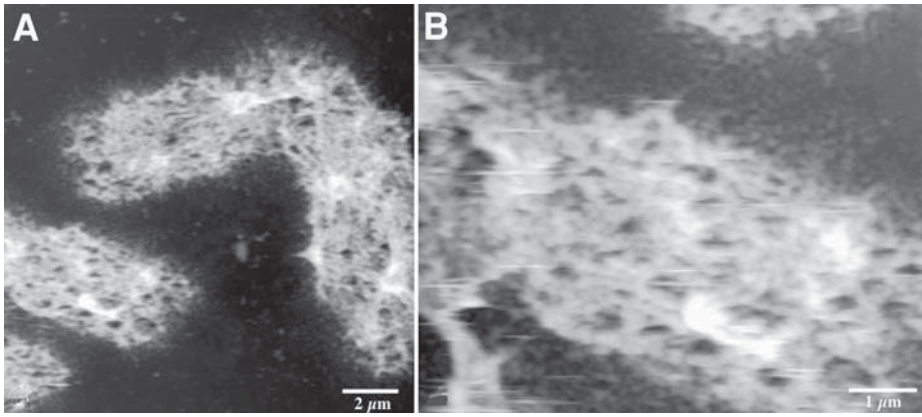

Fig. 4. The chromosomal surface presents a defined network structure after protease K treatment. Some fibrillar structures are well detectable, and the recorded chromosomal structures are comparable with the FEISEM images. Scale bar shown on Figures.

Table 2
Methodical Properties of the Different Operation Modes in AFM for High-Resolution Imaging and Manipulation of Metaphase Chromosomes

Operation mode	Contact mode	Noncontact mode	Tapping-mode
Tip loading force	Low → high	Low	Low
Contact with sample surface	Yes	No	Periodical
Manipulation of sample	Yes	No	Yes
Contamination of AFM tip	Yes	No	Yes
Microdissection	Yes	No	No

4. Notes

1. It is important to ensure that the hypotonic solution is removed from the cells immediately. By adding the fixative before spinning, the cells will be easier to resuspend. Remove all but a little of the supernatant and resuspend in the remaining solution before adding the fixative for the first time. Be sure not to leave too much hypotonic solution; this will cause a lot of cytoplasm to remain with the chromosome spreads; however, not leaving enough makes the cells difficult to resuspend.

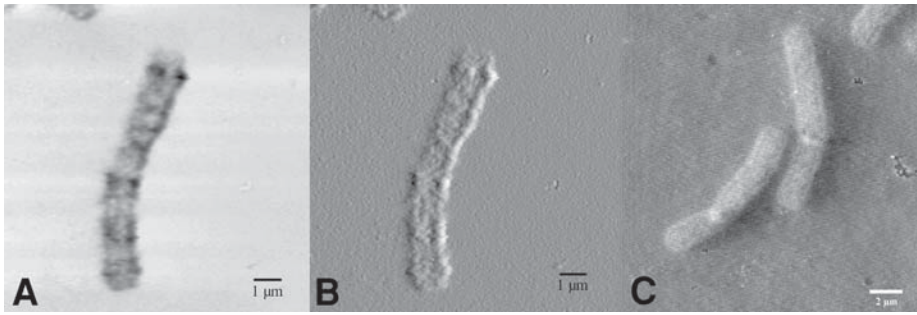

Fig. 5. **(A)** AFM image of a GTG-banded human metaphase chromosome 7. Imaging was performed in contact mode; it is a topographic image, and the single bands are well detectable. Scale bar, 1 µm. **(B)** Corresponding error signal image. **(C)** AFM image of CBG-banded human metaphase chromosomes: topographic image. Scale bar, 2 µm.

2. To check the conductive site of the ITO glass, use a voltage multimeter and make a resistance measurement. The conductive site will show a resistance, which has to be about 100 Ω.
3. To increase the contrast between sample surface and metaphase chromosomes for FEISEM microscopy a further fixation step and critical point drying can be performed. Our studies showed that this is not necessary. The fixation consists of a washing step 2 minutes in 1 × PBS buffer at room temperature, followed by a 30-min fixation in 1% glutaraldehyde in 1X PBS buffer. Please work under a hood. After a washing step in 1× PBS buffer for 2 min at room temperature the samples are fixed in 1% osmiumtetraoxide (OsO_4) in 1 × PBS buffer or in Veronal buffer (*see* **Note 4**). Wash the samples for 2 min in 1 × PBS buffer at room temperature and dehydrate the sample in an alcoholic series 70, 90, and 100% for 3 min at room temperature. Repeat the dehydration step three times and transfer the samples to critical point drying.
4. To prepare the 1% OsO_4 in 1X PBS buffer or in Veronal buffer, while working in the hood, brake two osmium crystals enclosed in a glass vessel into 100 mL of distilled water and dissolve osmium in a warm water bath for 1 d. Take an aliquot of the dissolved osmium and dilute in 1X PBS or Veronal buffer. Store the solution in a dark bottle at 4°C and fix the bottle additionally with parafilm to avoid evaporation.

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