6

Corneal Tissue Observed by Atomic Force Microscopy

Stylliani Lydataki, Miltiadis K. Tsilimbaris, Eric S. Lesniewska, Alain Bron, and Iannis G. Pallikaris

1. Introduction

The cornea is the transparent avascular part of the anterior segment of the eye and consists of a stratified nonkeratinizing squamous epithelium, a stromal dense connective tissue layer, and an endothelium facing the anterior chamber. The cornea contributes largely to the intraocular refraction of the light. Damage can impair its tissue transparency and lead to loss of vision. Significant diseases, such as corneal dystrophies, keratoconus, and refractive errors, are related to the structure and integrity of the cornea.

In conventional scanning electron microscopy studies, the corneal surface appears like a mosaic consisting of three types of cells, as it can be deduced from their electron reflex and size ***(1–4)***. The apical membrane of these cells is covered by the tear film. The inner corneal surface, facing the anterior chamber of the eye, is the apical membrane of the endothelium, which forms a monolayer of polygonal cells responsible for maintaining the state of relative deturgescence of the stroma through active transport ***(5–10)***. The stromal layer consists of regularly arranged dense connective tissue constituting 90% of the corneal thickness. It comprises sheets of lamellae of highly ordered collagen fibrils, embedded in a matrix of proteoglycans, and keratocytes. The former are interspersed between the lamellae, forming an interlinking network throughout the cornea ***(11–13)***.

AFM has been recently introduced with success in the research of corneal surfaces and components ***(11,14–16)***. Compared with other forms of microscopy used in corneal study, AFM offers several advantages: it can reach very high magnifications with high resolution, it requires minimal tissue preparation, and it is able to image samples in aqueous environments, thus permitting images to be obtained under conditions that resemble the tissue's native environment. Additional advantages include the possibility of dynamic in vivo

From: *Methods in Molecular Biology, vol. 242: Atomic Force Microscopy: Biomedical Methods and Applications*
Edited by: P. C. Braga and D. Ricci © Humana Press Inc., Totowa, NJ

study of biological processes and the capability of characterizing the nanomechanical properties of relatively smooth surfaces. Limitations of the method include the relatively small scan sizes and scan speeds and difficulties in imaging very soft biological samples. Because of such limitations, the AFM is currently used either as an investigational tool or as an adjuvant to other microscopic techniques. In long term, however, it has the potential to evolve in a unique multipotential instrument for the study of the morphology and mechanical properties of various biological tissues ***(17)***.

This chapter describes the methodology used to study the surface of the cornea in albino New Zealand rabbits and in humans. We describe the procedures necessary in rabbits to study the normal epithelial and endothelial surfaces as well as the corneal stroma after mechanical and excimer laser ablation. Samples were imaged in balanced salt solution (BSS) both fresh and after fixation in glutaraldehyde. We studied in humans the endothelial surface of two corneal buttons received after corneal transplantation for endothelial dystrophy. The tissue was imaged in BSS after fixation in glutaraldehyde.

2. Materials

2.1. Tissue Collection and Preparation

1. Rabbit corneas: New Zealand albino rabbits with 3–4 kg body weight.
2. Human corneas: Transplant recipient corneal button.
3. Anesthesia solution: 10 mg/kg xylazine hydrochloride + 10 mg/kg ketamine hydrochloride.
4. Proparacaine drops.
5. Operating microscope.
6. Surgical blades.
7. Excimer laser.
8. Surgical instruments for enucleation and corneal dissection.
9. Precision wipe paper.
10. Rinsing and observation solution (Alcon Laboratories, Fort Worth, TX).
11. Solutions for enzymatic preparation: 30 mU/mL neuraminidase in phosphate buffer solution (Sigma Chemical Co., St. Louis, MO); 30 mU/mL hyaluronidase in phosphate buffer solution (Sigma).
12. Fixative solution: glutaraldehyde, 2.5% buffered solution, pH 7.3, at 4°C.
13. Buffer solution for fixative preparation: 0.2 *M* stock solution of sodium cacodylate, pH 7.3, kept at 4°C.
14. Euthanasia solution: sodium pentobarbital.

2.2. Microscopy Equipment

1. AFM (Nanoscope IIIa, Digital Instruments, Veeco Inst., Santa Barbara, CA), including an optical viewing system and image analysis software.
2. Piezo-electric scanners, 12–150 μm.

3. V-shaped silicon nitride tips with a spring constant of 10 mN/m (Microlever; Park Scientific Instruments, Sunnyval, CA).
4. Magnetic stainless-steel punches.
5. Epoxy glue.
6. Fine forceps for tissue transfer and manipulation.

3. Methods

3.1. Tissue Collection (see *Notes 1–5)*

3.1.1. Rabbit Cornea

3.1.1.1. Anesthesia

The animals are anesthetized with a subcutaneous injection of xylazine and ketamine. Additional topical anesthesia with proparacaine drops is used to anesthetize the cornea.

3.1.1.2. Stromal Ablation

The anesthetized animal is placed under the operating microscope. Mechanical ablation is performed using a sharp surgical blade, and the anterior one third of the cornea is dissected taking care not to penetrate the cornea. Excimer laser ablation is performed following a standard protocol for myopia correction; a myopic correction of three diopters is aimed.

3.1.1.3. Euthanasia

Animals are euthanized by an injection of sodium pentobarbital overdose delivered via a peripheral ear vein.

3.1.1.4. Enucleation

The eye globes are carefully enucleated as soon as possible after death. Special care is taken not to contaminate the corneal surface with blood and not to touch or stress the tissue during manipulation. Eyes that will be imaged fresh are placed in BSS solution. For eyes that are going to be examined fixed, the fixation process described in the next paragraph is followed.

3.1.2. Human Corneas

The recipient corneal buttons from patients undergoing corneal transplantation are collected.

3.2. Fixation Process

3.2.1. Rabbit Eyes

Immediately after enucleation, the eye globes are placed into fixative solution. After 30 min and while the eye globe is still in the solution, a hole is

opened 6 mm behind the limbus to allow penetration of the fixative solution in the interior of the eye. The fixative solution is replaced with freshly prepared solution. The eyes are kept overnight in the solution at 4°C before AFM observation.

3.2.2. Human Corneal Buttons

Immediately after trephination, the recipient button is placed into fixative solution. The eyes are kept overnight in the solution at 4°C before AFM observation.

3.3. Preparation of Corneal Specimens

Handle all cornea specimens withfine instruments under microscopic observation, paying attention not to distort the tissue during manipulations such as cutting, transportation, and gluing

3.3.1. Rabbit Corneas

This step is performed immediately after enucleation in eyes that are going to be imaged fresh. Fixed eyes are processed after completion of the fixation. The anterior part of the eye is cut away and the cornea is freed from the underlying iris, cilliary body, and lens. The tissue is trimmed near the sclerocorneal limbus and it is dissected in two semicircular pieces.

Corneal specimens are transferred to magnetic stainless-steel punches and are fixed with epoxy glue. Specimens are maintained with the surface that is going to be examined upwards. Before transfer, the excess of solution is absorbed from the seating side by using a precision wipe paper. After transfer to the magnetic punches all specimens are covered with BSS solution and placed under the micoscope. For corneas that will be observed after enzymatic treatment the process described below is followed prior to transfer to the punches.

3.3.2. Human Corneas

The corneal button is dissected in two semicircular pieces. Corneal specimens are transferred to magnetic stainless-steel punches and are fixed with epoxy glue. Specimens are maintained with the surface that is going to be examined upwards. After transfer to the magnetic punches, all specimens are covered with BSS solution and placed under the microscope.

3.3.3. Enzymatic Preparation

The cornea freed from the underlying iris, cilliary body, and lens is immersed in neuraminidase or hyaluronidase enzymatic solution with the surface to be examined directed upwards. The dishes containing the enzymatic solutions are closed and kept at 37°C for 30 min. After the completion of this time, they are

removed from the solution and rinsed gently with BSS for 5 min to remove the excess of enzyme and the enzymatic digestion products. After that the specimens are transferred to magnetic punches.

3.4. AFM Imaging (see Notes 6–15)

3.4.1. Image Aquisition

1. The area of interest is chosen using the optical microscope attached to the viewing window of the AFM. The central area at a distance of some millimeters from the specimen's edges is considered the area most appropriate for observation.
2. Imaging starts using large scanning areas, when possible. Large scanning areas provide information about the general topography of the sample and allow for the selection of flat regions without defects for small-scale imaging. For imaging of areas from 20–100 µm (**Fig. 1**) a 100-µm scanner is used. For smaller areas ranging from 10–0.2 µm, high resolution can be achieved with a 12-mm scanner (**Fig. 2**).
3. To obtain good images, the force curve needs to be corrected repeatedly. In fresh tissue the adhesion of the surface glycocalyx sugars to the microscope tip, results in fuzzy images. In these sample it is often difficult to achieve a good forces-vs-distance curve and several tries are necessary until satisfactory images are acquired (**Fig. 3A**). Imaging of fixed tissue is considerably easier because the surface glycocalyx is removed during the fixation process (**Fig. 3B**).
4. The scan rate ranges between 0.5 and 10 Hz, depending on the scan size. Small frequencies are used to scan large areas (**Fig. 4**) and vice versa.
5. Imaging forces of not more than 100 pN are used. High forces are applied only as a means to mechanically remove the surface layer that adheres to the tip.
6. Images are obtained with a resolution 512 × 512 pixels of trace and retrace collecting data. Three types of images can be obtained during the contact mode imaging:
   a. In height images the color-coded contrast refers to the spatial variation of the Z-height of the tip (**Figs. 3** and **4**).
   b. In deflection images the contrast differences of the surface refer to the spatial variation of the strength of the probe–specimen interaction (**Fig. 5**).
   c. In lateral force microscopy or friction images, information concerning the friction on the surface f the specimen during the movement of the tip is displaced. However, interpretation and analysis of the later images of the cornea remains difficult.

3.4.2. Image Analysis

1. For a better presentation, height images are processed using a plane-fit adjustment, when the sample surface is not perpendicular to the scanner's *z*-axis.
2. To evaluate the surface structure, sections on the height images are used that present the profile of the surface. These sections are indispensable when features like protrusions, particles, holes, fibrils, and so on have to be measured. The sections are performed on raw data images. Zooming is necessary when small features of large-scanning images have to be measured.

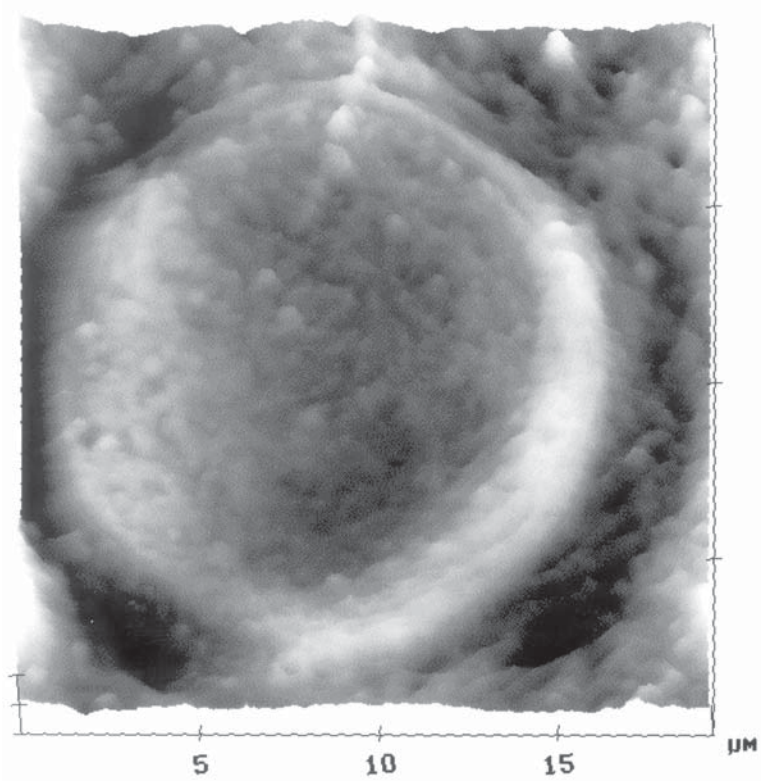
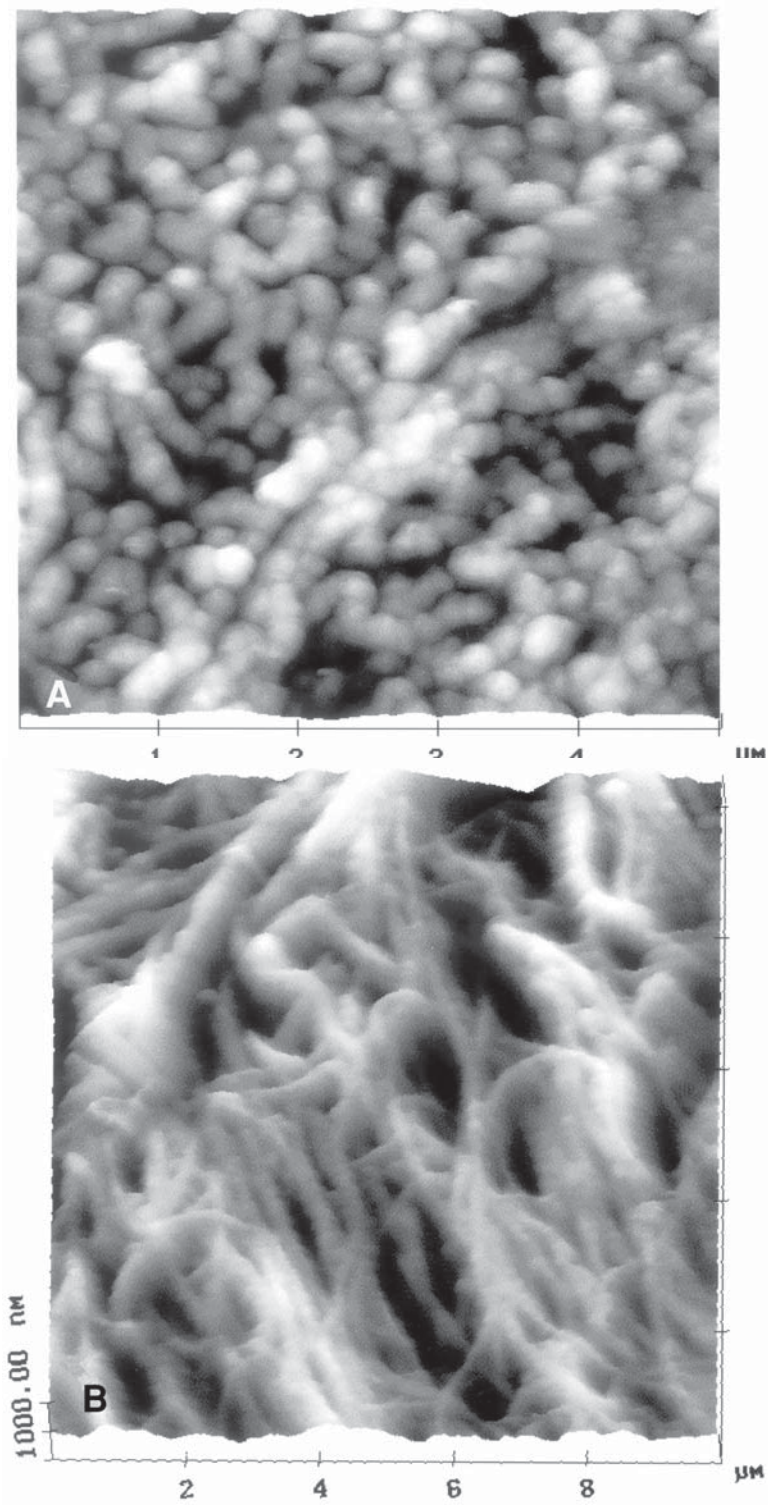


Fig. 1. Low-force contact-mode AFM image. Human corneal endothelium from a patient with corneal endothelial dystrophy who underwent corneal transplantation. The recipient corneal button was studied with AFM. 20-μm scan range; 1.5-Hz scan rate; scanning force <100 pN.

3. Quantitative data are acquired after the measurement of several morphological characteristics. The meta-analysis tools provided by the system's software facilitate for the calculation of statistical and topographic parameters. These include the ratio of the length along the longer axis over the height of measured structures as well as the measurement of surface roughness. Such quantitative analysis gives more precise information about the morphology of the surface.

Fig. 2. *(opposite)* Low-force contact-mode AFM images. **(A)** Height image of fixed rabbit corneal endothelium showing a detail of the intercellular contact of two epithelial cells and the micro-projections on their surface. 5-μm scan range; 2-Hz scan rate; scanning force <100 pN. **(B)** Height image of fixed rabbit corneal stroma after mechanical dissection. Collagen fibrils appear randomly arranged. In some of them the periodicity is apparent. 10-μm scan range; 2-Hz scan rate; scanning force <100 pN.


A
1
2
3
4
µm
B
1000.00 nm
2
4
6
8
µm

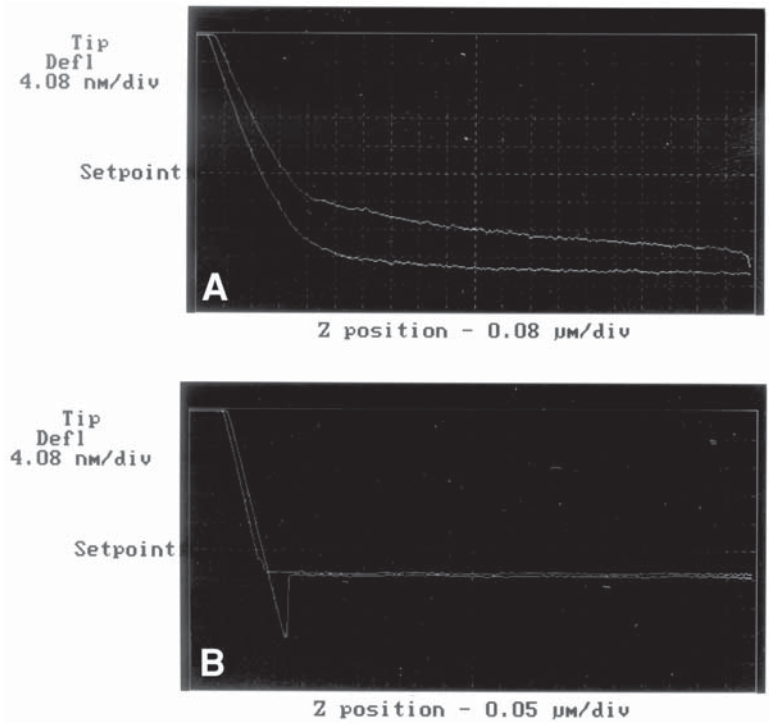


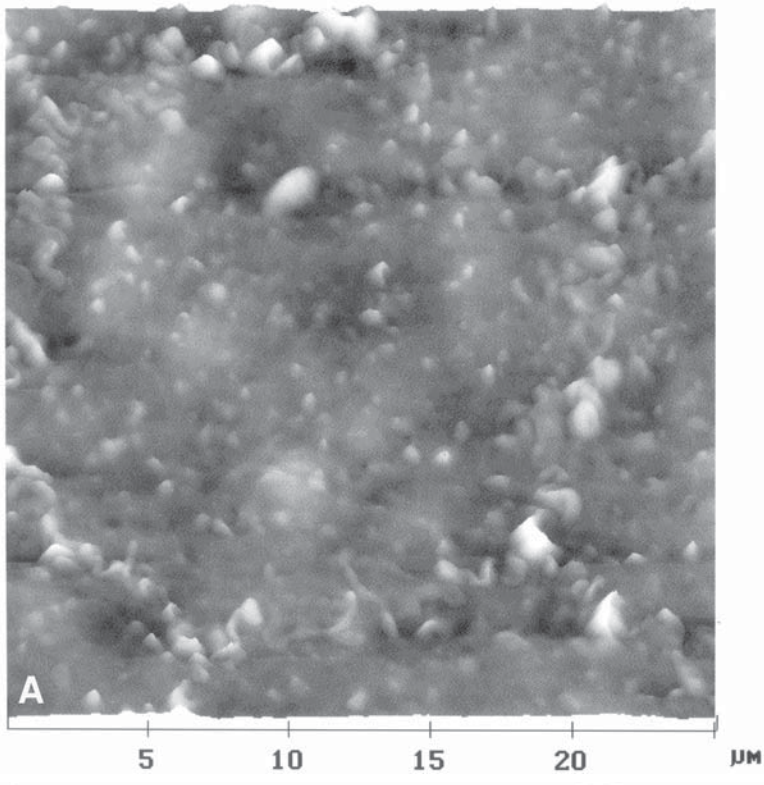
Fig. 3. **(A)** Force-vs-distance curve recorded on the fresh corneal surfaces. The cantilever's deflection in the vertical axis is converted into force using the relationship $F = k_{cl}$, where k_{cl} is the spring constant of the free cantilever. **(B)** Force-vs-distance curve recorded on the fixed corneal surfaces. Note the difference between fixed and fresh specimen curves.

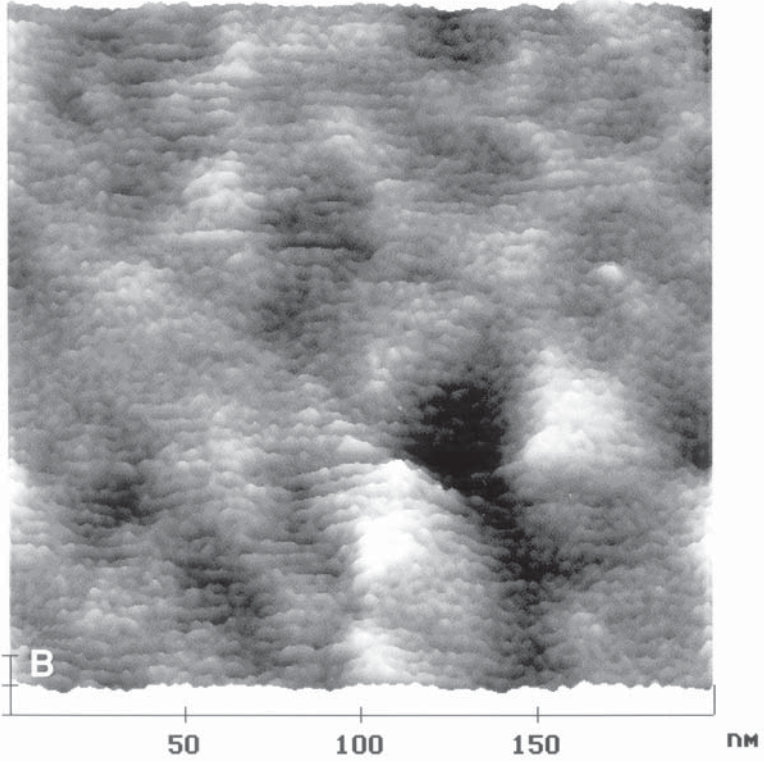
4. Roughness statistics are performed on height images 5 × 5 µm. Mean roughness (*Ra*) and root mean square (RMS), or *R(q),* are calculated. *R(q)* is the standard deviation of the *Z* values in a given area whereas*Ra* is the mean roughness value of the surface relative to the center plane.

4. Notes

To be able to extract information from AFM imaging it is important to minimize the risk of artifacts before or during the imaging.

Fig. 4. *(opposite)* Low-force contact-mode AFM images. **(A)** Height image of fixed rabbit cornea showing one endothelial cell. 25-µm scan range; 1.5-Hz scan rate; scanning force <100 pN. **(B)** Height image of fixed corneal endothelium showing a very fine structure of a few nanometers on the surface. 200-nm scan range; 10 Hz-scan rate; scanning force <100 pN.


A
5
10
15
20
μm


B
50
100
150
nm

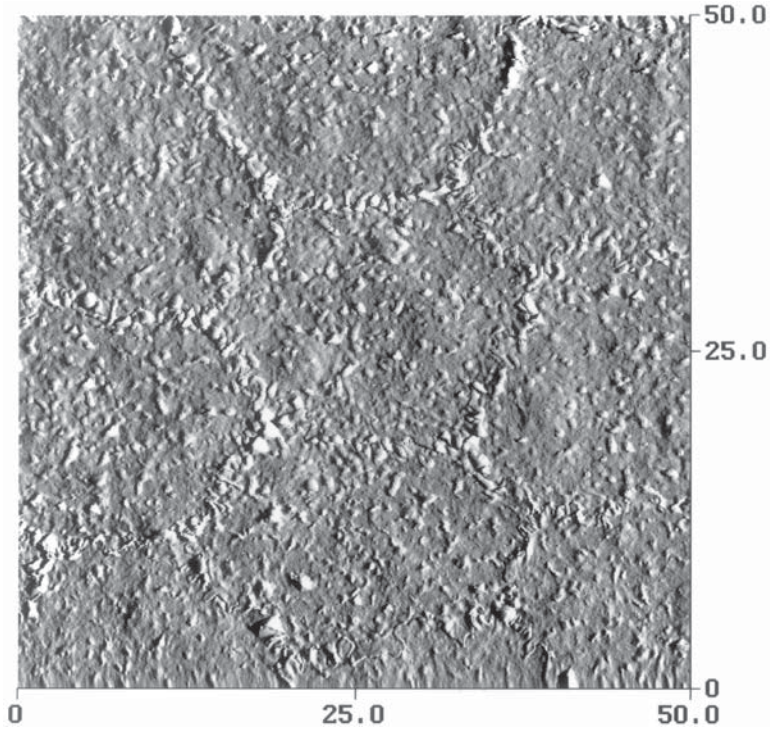


Fig. 5. Deflection AFM image. Fixed rabbit corneal endothelial surface. The contour of endothelial cells its easily detected. Because of their height contrast, these images are suitable for counting the features on the surface. 50-μm scan range; 0.5-Hz scan rate; scanning force <100 pN.

1. Before imaging, fresh tissue poses considerable imaging difficulties. The interaction of glycoaminoglycans chains of the glycocalyx layer with the microscope tip makes the imaging of the fresh tissue difficult (**Fig. 6**). When the imaging of a specimen is important not to fail, consider fixation. The removal of the glycocalyx that happens during fixation makes the imaging easier.
2. Enzymatic treatment represents another way to improve image acquisition from fresh tissue. The enzymatic process increases the unevenness of the sample surface thus increasing contrast. In addition, information concerning the sample's molecular composition can be revealed and help in the interpretation of the effect of the enzyme on the surface morphology (**Fig. 7**).
3. When imaging of the outer corneal surface is intended, it is important to handle the tissue very carefully during preparation. Contamination and distortion of the superficial corneal layers can happen very easily and will alter the surface morphology. When the tissue is going to be imaged fixed, the installation of a few fixative drops on the corneal surface while the animal is in deep anesthesia just

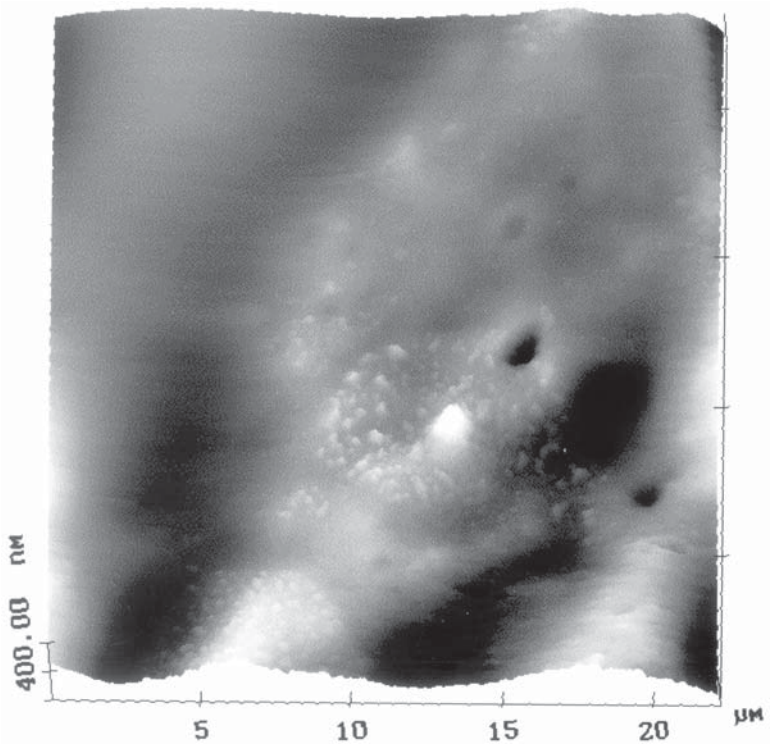


Fig. 6. Low-force contact-mode AFM image. Height image of fresh epithelium. 22-µm scan range; 1.5-Hz scan rate; scanning force <100 pN. A large part of the surface appears fuzzy.

prior to euthanasia ensures preservation of the superficial corneal layers in the best possible condition.

4. Gentle manipulation of the tissue in general is very important. Prepare all the instruments and materials in advance. It is essential to work under an operating microscope or a stereoscope especially when cutting the samples to be imaged. The working place, the instruments, and the solutions need to be very clean. Prior to imaging, inspect the sample's surface and ensure it is not defective or contaminated.
5. Time optimization: tissue preparation, cutting, and gluing on the pounches must be completed as quickly as possible to avoid tissue drying.
6. BSS represents our preferred medium for observation. This solution was selected because it contains all the essential ions necessary for maintenance of the rabbit and human corneal integrity ***(10,18,19)***.
7. Allow 15–30 min after the installation of the sample under the microscope for the system to reach a thermal equilibrium. This will elliminate thermal drifting.

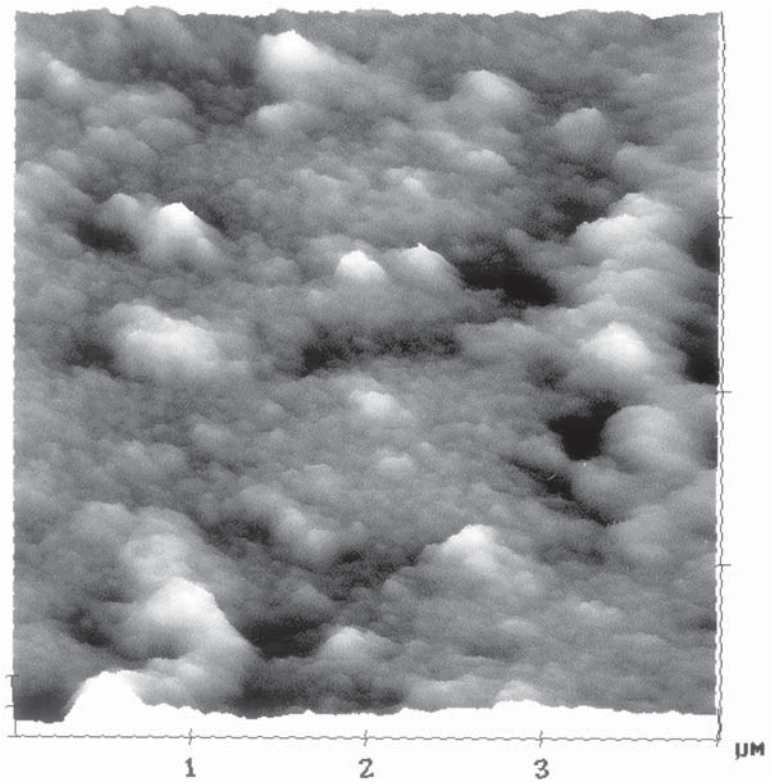


Fig. 7. Low-force contact-mode AFM image. Fresh rabbit corneal endothelium treated with neuraminidase. A microgranular structure can be seen on the surface together with elevated aggregates of different sizes. 4-μm scan range; 3-Hz scan rate; scanning force <100 pN.

8. Adjust the level of the set-point force by using the force-vs-distance curve (**Fig. 3**). This determines the force that the tip applies to the sample. A set–point level close to the jump-out point ensures an operation with minimal force.
9. When a soft cantilever is used, the applied force should be maintained in the sub-nano-newton level. Higher forces produce significant surface alterations. This effect is more pronounced in fresh tissue.
10. Duration of sample observation: in fixed tissue, the duration of observation of a specimen can be extended to 2.5 h without obvious morphological alterations. Observation of fresh tissue in BSS should not exceed 1–1.5 h. If the process is prolonged over this time the tissue hydration changes and edema occurs.
11. When there is any doubt concerning the tip's quality, check it and if necessary replace it.

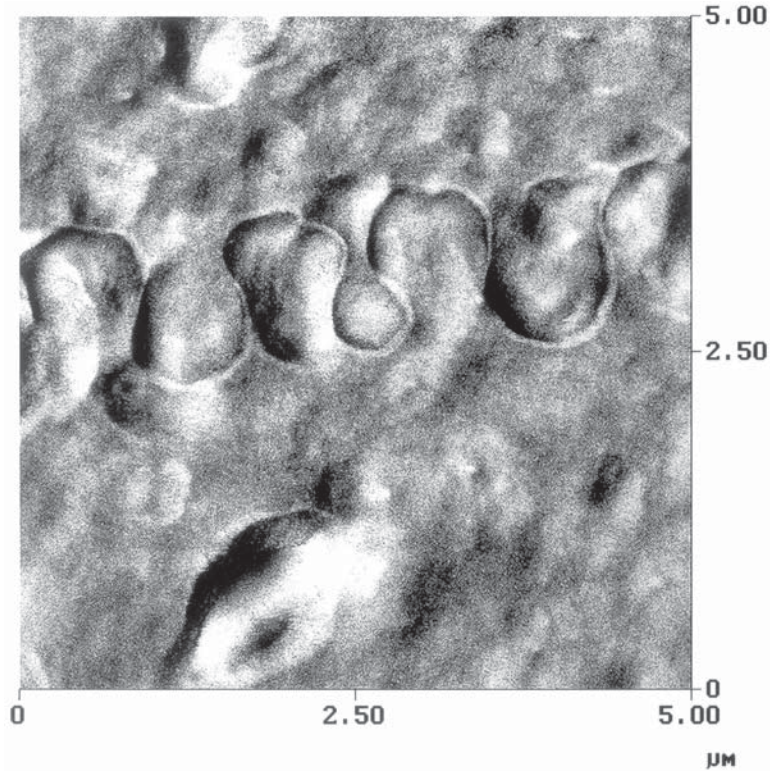


Fig. 8. Low-force tapping-mode AFM. Amplitude image of fixed rabbit cornea showing the corneal endothelial cell interdigitations. 5-µm scan range; scanning force <100 pN; 0.500-nm amplitude.

12. When the specimen's surface is rough, it may be necessary to alternate various imaging conditions (scan angle, scan frequency, etc.) to obtain an image of acceptable quality.
13. If the surface does not permit imaging using a large scanning area it is preferable to reduce the scanning area. This way you will save time and you will reduce the risk of damaging the sample and/or the tip.
14. Zooming to a smaller scanning area should be accompanied by an increase in the scanning speed.
15. Imaging is usually performed in contact mode. Taping mode gives height-images with a quality inferior to that of contact mode. The phase and amplitude images in tapping mode are usually of very good quality (**Fig. 8**). For the time being, however, the interpretation of these images is not easy.

References

1. Doughty, M. J. (1990) Morphometric analysis of the surface cells of rabbit corneal epithelium by scanning electron microscopy. *Am. J. Anat.* **189,** 316–328.
2. Doughty, M. J. (1990) On the evaluation of the corneal epithelial surface by scanning electron microscopy. *Optom. Vis. Sci.* **67,** 735–756.
3. Hazlett, L. D. (1993) Corneal and ocular surface histochemistry. *Prog. Histochem. Cytochem.* **25,** 1–60.
4. Hoffmann, F. (1972) The surface of epithelial cells of the cornea under the scanning electron microscope. *Ophthal. Res.* **3,** 207–214.
5. Hager, H., Hoffmann, F., and Dumitrescu, L. (1975) Scanning electron microscopy in ophthalmology. *Ann. Ophthalmol.* **7,** 1361–1371.
6. Doughty, M. J., Bergmanson, J. P., and Blocker, Y. (1997) Shrinkage and distortion of the rabbit corneal endothelial cell mosaic caused by a high osmolality glutaraldehyde-formaldehyde fixative compared to glutaraldehyde. *Tissue Cell* **29,** 533–547.
7. Doughty, M. J. (1994) The cornea and corneal endothelium in the aged rabbit. *Optom. Vis. Sci.* **71,** 809–818.
8. Hirsch, M., Renard, G., Faure, J.-P., and Pouliquen, Y. (1997) Study of the ultrastructure of the rabbit corneal endothelium by freeze-fracture technique: apical and lateral junctions. *Exp. Eye Res.* **25,** 277–288.
9. Lea, P. J., Hollenberg, M. J., Menon, I. A., Temkin, R. J., Persad, S. D., and Basu, P. K. (1989) High resolution scanning electron microscopy of rabbit corneal endothelium to show effects of UV-visible irradiation in the presence of chlorpromazine. *Lens Eye Toxic Res.* **6,** 119–133.
10. Edelhauser, H. F., Hanneken, A. M., Pederson, H. J., and Van Horn, D. L. (1981) Osmotic tolerance of rabbit and human corneal endothelium. *Arch. Ophthalmol.* **99,** 1281–1287.
11. Yamamoto, S., Hashiume, H., Hitomi, J., et al. (2000) The subfibrillar arrangement of corneal and scleral collagen fibrils as revealed by scanning electron and atomic force microscopy. *Arch. Histol. Cytol.* **63,** 127–135.
12. Bairaktaris, G., Lewis, D., Fullwood, N. J., et al. (1998) An ultrastructural investigation into proteoglycan distribution in human corneas. *Cornea* **17,** 396–402.
13. Borcherding, M. S., Blacik, L. J., Sittig, R. A., Bizzell, J. W., Breen, M., and Weinstein, H. G. (1975) Proteoglycans and collagen fibre organization in human corneoscleral tissue. *Exp. Eye Res.* **21,** 59–70.
14. Fullwood, N. J., Hammiche, A., Pollock, H. M., Hourston, D. J., and Song, A. M. (1995) Atomic force microscopy of the cornea and sclera. *Curr. Eye Res.* **14,** 529–535.
15. Tsilimbaris, M. K., Lesniewska, E., Lydataki, S., Le Grimellec, C., Goudonnet, J. P., and Pallikaris, I. G. (2000) The use of atomic force microscopy for the observation of corneal epithelium surface. *Invest. Ophthalmol. Vis. Sci.* **41,** 680–686.
16. Meller, D., Peters, K., and Meller, K. (1997) Human cornea and sclera studied by atomic force microscopy. *Cell Tissue Res.* **228,** 111–118.

17. Binning, G., Quate, C. F., and Gerber, C. (1986) Atomic force microscope. *Phys. Rev. Lett.* **56,** 930–933.
18. Doughty, M. J. (1992) Quantitative evaluation of the effects of a bicarbonate and glucose- free balanced salt solution on rabbit corneal endothelium in vitro. *Optom Vis. Sci.* **69,** 846–857.
19. Doughty, M. J., Newlander, K., and Olejnik, O. (1993) Effect of bicarbonate-free balanced salt solutions on fluid pump and endothelial morphology of rabbit corneas in-vitro. *J. Pharm. Pharmacol.* **45,** 102–109.