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# Analysis of the healthy rabbit lens surface using MAC Mode atomic force microscopy

A. Antunes [a,*], F.V. Gozzo [c], M. Nakamura [b], A.M.V. Safatle [a], S.L. Morelhão [c], H.E. Toma [b], P.S.M. Barros [a]

[a] *Laboratório Experimental de Oftalmologia Comparada, Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil*
[b] *Laboratório Supramolecular e Nanotecnologia, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil*
[c] *Departamento de Fisica Aplicada, Instituto de Física, Universidade de São Paulo, São Paulo, SP, Brazil*

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**Abstract**

In this investigation healthy rabbit crystalline lenses were characterized by atomic force microscopy (AFM). The lenses were cut in slices with thickness with 1 mm and thus, put after cortex distinct regions of nucleus and cortex for AFM examination. AFM analysis were carried out using a PicoSPM I operating in Mac Mode. We obtained topographic images of rabbit lenses and a quantitative analysis of the width and height of fibers for nucleus and cortex regions. The longitudinal section analysis of fibers in the nucleus region indicated structures with an average width of 200 nm and average height of 200 nm. The intershells distance was determined as 4 μm. Fiber cell cross-section dimensions, longitudinal and transverse widths, could be estimated in these regions from the AFM images. Structures with average widths as small as 1.0 μm are observed in the nucleus; the intershell distance is 4.0 μm. In cortical regions, hexagonal structures with average longitudinal and transverse widths of 5.0 μm and 3.0 μm, respectively, were identified. Three-dimensional images of tissue sections with resolution on a nanometer scale were obtained. The potential of AFM analysis for characterizing healthy and pathologic lens tissues is discussed.


*Keywords:* Atomic force microscopy; Mac Mode SPM; Morphology; Crystalline lens; Fibers; Rabbit

## 1. Introduction

The crystalline lens is an avascular cellular structure composed of six-sided prismatic cell, consisting of a large number of fibers that are distributed and organized in the tissue for preserving the transparency condition which is necessary for executing their function in the vision system. The lens is a naturally elastic structure. As is the case for others tissues of the body, these cells are complex structures with specific architecture and composition. The main disorders reported about the lenses are associated cataract formation. Several techniques has been used to characterize ocular structures such as corneal surface, membrane, cloned lens among others. It is well known that light microscopy (LM) and electron microscopy (EM) have become essential tools in biology over the past decades (Jongebloed et al., 1998). Atomic force microscopy (AFM) is a technique by which surface topography can be measured to a precision which, in some cases, reaches sub-angstrom levels (Bining et al., 1986). The main advantage in using AFM is that it involves minimal sample preparation and tissue perturbation. It is also a useful tool for analyzing biological systems at and below the ultrastructural level (Hansma and Hoh, 1994). Nowadays, a brief description about application of *in vitro* and *in vivo* AFM studies in Life Sciences includes a wide range of applications. AFM has been applied to the imaging of various fibrous systems, including collagen and keratin (Gale et al., 1995; Paige et al., 1998; Parbhu et al., 1999). Among other investigations, a technique suitable for the study of collagen fibrils produced by *in vitro* assembly has been successfully utilized for the examination of healthy collagen (Baselt et al., 1993; Revenko et al., 1994) and of its *in vitro* assembly (Gale et al., 1995; Goh et al., 1997). Studies in the field

* Corresponding author at: Faculdade de Medicina Veterinária e Zootecnia da Universidade de São PauloAv. Prof. Dr. Orlando Marques de Paiva, 87CEP 05508-900São Paulo/SP Brazil; Universidade de Lisboa, Centro de Física Atómica da Faculdade de Ciências, Av.Prof. Gama Pinto, 2, 1649-003, Lisbon, Portugal.
*E-mail addresses:* antunes@cii.fc.ul.pt, antunes_ap@yahoo.com.br (A. Antunes).

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of structural biology by AFM have also produced significative results (Shao and Yang, 1995; Shao et al., 1995) and accordingly, several reviews have recently been published (Shao and Zhang, 1996; Engel et al., 1999; Heinz and Hoh, 1999). Other relevant aspects of AFM include its nondestructive character and the possibility of working in nearly physiological conditions and without any need for staining or coating (Tiribili et al., 2005; Morris et al., 2000). With respect to ocular tissues, studies of the ultrastructure of corneal and scleral collagen fibrils using distinct microscopic techniques have been reported in the literature. These studies reveal that these distinct parts of the ocular system have different diameters and arrangement (Yamamoto et al., 2000). Other important results indicate that D-periodicity of the fibrils differs between corneal and scleral fibrils (Yamamoto et al., 2000). The detailed architecture of the lens fibers still needs to be determined. In this investigation we utilize AFM imaging for an examination of the ultrastructure of fibers in healthy rabbit lenses. We compare the ultrastructure in the cortex and nucleus region aiming to determine variations in diameter and D-periodicity for each region. Topographic images of fibers *in vitro* have been obtained showing that the fibers have peculiar arrangement in the cortex and nucleus, respectively, according to the examination performed using field emission scanning electron microscopy (FE-SEM) and light microscopy (LM). In order to obtain these images, however, a number of fixation procedures are required, together with the use of a microtome. In the present study, the characterization of the healthy lenses by AFM has been reported for the first time. These results suggest that, for example, AFM could be used to examine changes in the fiber cell during the test of new medicaments to prevent or inhibit lens diseases. The focus of this investigation is a general application of AFM for examining hexagonal cells and consequently indicating a novel tool for the characterization of alterations and initial stages of formation of lesions in the lens.

## 2. Materials and methods

### 2.1. Sample preparation

Five New Zealand white adults rabbits (all weighing from about 2.5–3.0 kg) were used. The rabbits were anesthetized and sacrificed after procedures of tissue removal. Lens materials was removed using an extracapsular surgery procedure. This removal procedure is performed frequently at the College Veterinary Medicine- University of Sao Paulo. It consists of an incision of the capsule and removal of the lens. The lenses evaluated were completely transparent. Normal rabbit lenses were immersed in 10% formalin solution. The fixation of lenses is a routine procedure for avoiding deterioration. No other preparation was performed. Thick sections of 1 mm were produced for topographic analysis. In Fig. 1 we show a diagram of a lens section. Another important reason for performing the sample fixation is the duration of the examination process. In this experiment a minimal 2 hours per specimen was required. Therefore, we have produced a limited number of high resolution images.

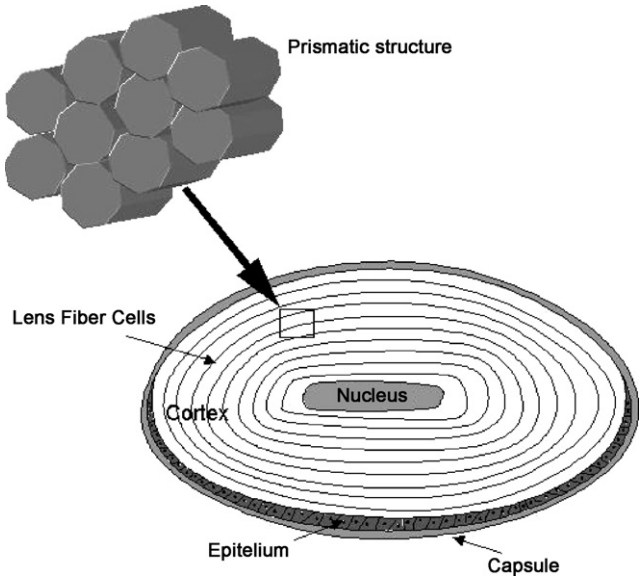


Fig. 1. Diagram of the lens section.

### 2.2. AFM analysis

AFM measurements were performed using PicoSPM I (Molecular Imaging, Tempe, AZ, USA) with PicoScan 2100 controller acopled with MAC Mode controller. The system was used to operate on MAC Mode SFM employing type I MACLevers under ambient conditions. Type I Mac Levers are silicon cantilevers with length ranging from 90 to 110 μm and typical tip radius of curvature less than 10 nm. Typical values of spring constant and resonant frequency in air are 0.6 to 1.75 N/m and 75 to 155 kHz, respectively. In all AFM measurements a used Multi-purpose Scanner was used with scan range of 80 mm in the $X$–$Y$ direction and 7 mm in the $Z$ direction. Several papers have been dedicated to detailed descriptions of the technical aspects of the atomic force microscope, including enhanced aspects such as its sensibility

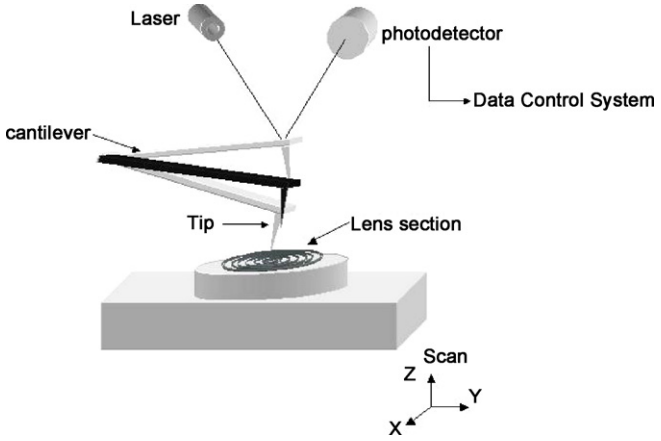


Fig. 2. Schematic drawing of an AFM and of the sample positioned. An AFM consists of a flexible shaft with a tip at its extremity. The deflection signal is monitored by an optical system consisting of a diode laser and a photodetector that is sensitive to variations in position. Deflections of the sample are plotted point to point while scanning on surface.

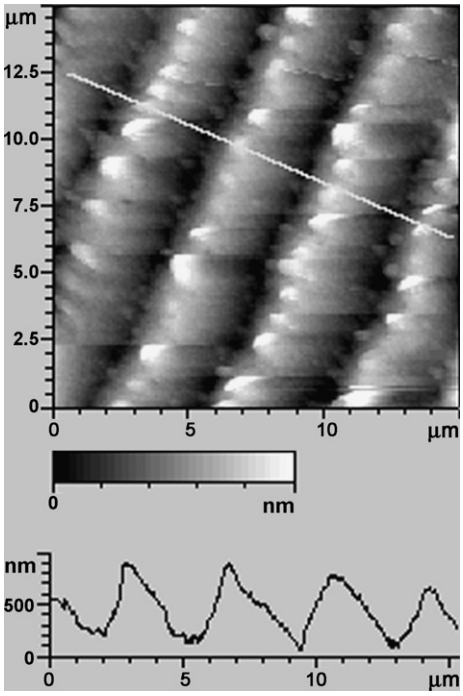


Fig. 3. (a) AFM image of the healthy rabbit lens fibers to longitudinal section in the nucleus area, 15 µm × 15 µm. (b) Quantitative analysis of the D-band spatial period and the gap depth between fibers of healthy rabbit lens for which the distance between fibers is 4 µm.

and resolution (Grant and McDonnell, 2003). A scheme of an AFM is presented in Fig. 2.

Images were recorded at a scan rate of 1 Hz, over a maximum area of 80 × 80 $mm^2$ with a pixel size of 256 × 256 operating with amplitude setpoint around 60% of free amplitude oscillation. Images were processed by flattening in order to remove the background slope, and the contrast and brightness were adjusted.

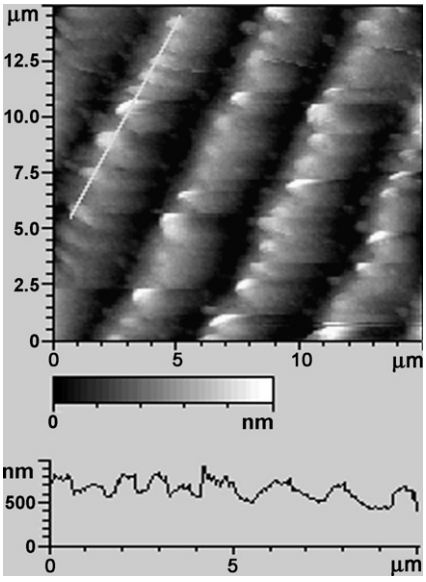


Fig. 4. (a) AFM image of the healthy rabbit lens for a longitudinal section in the nucleus area, 15 µm × 15 µm. (b) AFM images of healthy lens and sectional analysis of fibers showing the periodicity among fibers.

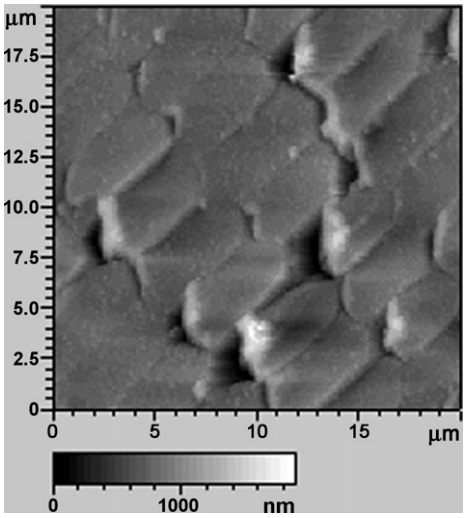


Fig. 5. AFM of lens fibers in the cross-section in cortex region (20 µm × 20 µm).

## 3. Results and discussion

AFM images revealed fibers cells in healthy rabbit lenses for areas from both nucleus and cortex. The AFM identified individual lens fibers with a mean diameter of 200 nm according to Figs. 3 and 4. The resolution is in nanometer range and quantitative three-dimensional images are obtained, allowing the visualization of details of the fiber structure in the nucleus and cortex regions. The borders in the hexagonal cell appear well defined as shown in Fig. 5. Comparing with images produced by SEM available in the literature, no changes associated with sample fixation are apparent. Cortical fibers are very thin and are hexagonal in cross section. Fibers presented distinct alterations due to their localization.

The relationship between fiber diameter and position in tissue can be evaluated by examining Table 1.

Here, we can visualize these structures in Figs. 5 and 6. Others studies published in the literature involve complicated and time-consuming preparation methods. Limitations related to the technique or tissue type conducting AFM as a complementary technique to traditional methods (Shao, 1999). We have not detected the presence of artifacts in our high resolution images. An advantage to AFM examination is the ability to perform 3D scanning (*XYZ* planes). In Fig. 7 we can visualize a 3D-view of the nucleus fiber cells. The reproducibility of the high resolution images was evaluated throught duplicate measurements.

This information is relevant for investigating changes due to the action of different pathologies that alter the fiber. In particular, our results are consistent with the proposition that in

Table 1
Dimensions of lens fibers to each region analyzed by AFM

| Region | Fiber diameter |
|---|---|
| Nucleus | 200 nm |
| Cortex | 5 µm |

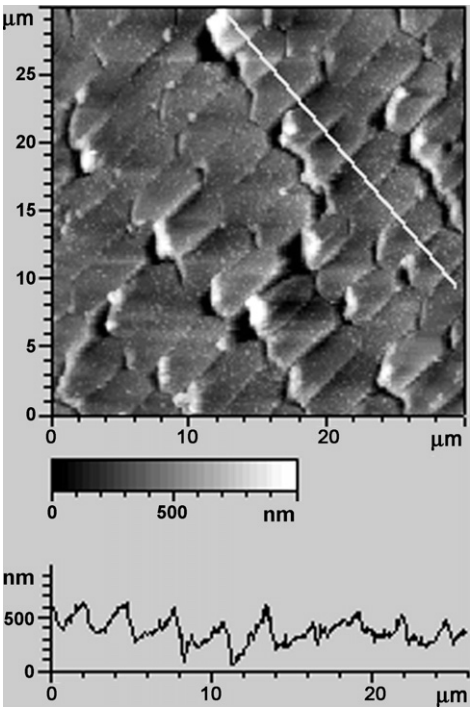


Fig. 6. AFM of lens fibers in the cross section in cortex region (30 µm × 30 µm). Sectional analysis showing the periodicity of the fibers.

the nucleus the fibers are closer together than in the cortex. In this peculiar soft tissue, a degradation process occurs due to diseases or aging. It is useful to compare the conclusions of AFM analysis with the histological examination due to different specific preparation procedures for each technique. In the thin section of the lens without any preparation several morphological details can be viewed. Detailed morphology revealed by SEM can be found in published literature (Jongebloed et al., 1998; Freel et al., 2003). The potential of AFM as an important tool in life science has been demonstrated in several investigations during the last decade. In this same period, AFM has been improved in its current limits however, it still requires improvements to determination physical properties as elasticity of soft tissues, and in the analysis and interpretation.

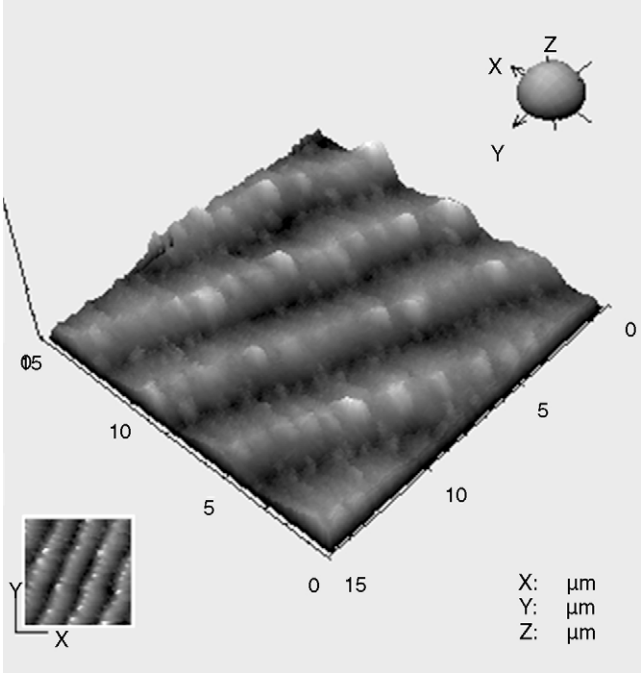


Fig. 7. 3D view of fiber cells.

## 4. Conclusions

AFM allows direct imaging of the morphology and is an important auxiliary tool in the characterization of healthy lenses. We believe that it is the first time crystalline lenses have been evaluated by Mac Mode AFM indicating the D-periodicity of the fibers shells such as their intershell separations. MAC mode AFM methodology can be applied to the study of ocular diseases. As a next step, AFM could be used as a powerful tool for evaluating the disorganization of lens fiber cells during the stages of progression of cataracts.

The observations on lens suggest that fixative agent has not altered its structure. In situation where the lenses are exposed to drugs, for instance, their structure can be evaluated using Mac mode AFM. However, other fixative agents can be used to analyze fiber cells. The fact that the lens has a specific shape and arrangement allows a morphological evaluation in fragments. This can be used to inspect changes in their structure such as those due to the formation of a spherical body during cataractogeneses as mentioned in the literature. This investigation has demonstrated that AFM can be an important technique for analyzing ocular tissues and therefore for studying related diseases. It is shown here that fiber cell dimensions, periodicity and intershell distances can be evaluated by Mac mode AFM.

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