Atomic Force Imaging of Ocular Tissues: morphological study of healthy and cataract lenses

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This study focuses the recent application of Atomic Force Microscopy (AFM) to determine structural details of crystalline lenses. We are interested in the application of AFM to distinguish structural and physical properties changes in ocular tissues and associated diseases. Crystalline lenses have its well-defined structure, however, details about pathological stages are still to be determined. In this investigation we evaluated semithin sections and rudimentary incise of crystalline canine lens. AFM showed to be a high-resolved imaging tool for the scanning of both healthy and diseased lenses. Three-dimensional images of tissue sections with resolution on a nanometer scale were obtained. In addiction, some results were compared between histological optical microscopy analysis and AFM. The promising of AFM applications for characterizing healthy and diseased ocular tissues is discussed.

Keywords canine lenses; cataract; morphology ; AFM

1. Introduction

In the two last decades, atomic force microscopy (AFM) has been established as a technique for high resolution providing original information about cellular and biological structure and to obtain time-dependent dynamic information about biological systems in their natural environment [1-4]. The great advantage of AFM compared with conventional scanning electron microscopy (SEM) is its ability to obtain topographic particulars from the specimen surface in aqueous, nonaqueous, or dry environments. To this, a scanning AFM tip over the sample is performed and recording the z axis displacement required maintaining a constant contact force. The atomic force microscope (AFM) proposed [5] produces image contrast by a device entirely dissimilar from other types of microscopy. The main disadvantages to use of electron microscopy (SEM) and transmission electron microscopy (TEM) is that these techniques provide limited ability to in-situ investigation of the physical and mechanical properties in a variety of environments. The literature shows a multiplicity of applications involving biological tissues. Some cells are intrinsically difficult to image because of poor adhesion, while others reveal a modest structure. Surfaces of hard samples are routinely imaged with atomic resolution. Soft images, however, remain challenging [6]. Atomic force microscopy (AFM) is a technique particularly suited to supply detailed information on structure, elasticity and interactions at high resolution and, in addition, it allows to image biological samples in their native state. A whole range of techniques for AFM were developed during the last years. They are aimed to analyze the morphology of biological surfaces at high spectral resolution and for probing interaction forces. The direct interaction of the AFM tip with the sample firstly prompted concern that soft biological material might be damaged during imaging [7]. Functional properties of biological systems are determined by a complex set of mechanical and electromechanical interactions on the length scales of several orders of magnitude: from macro to nano. Thus, it has been proposed that the

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highly ordered arrangement of lens fibers contributes to lens transparency by transforming the individual fibers into a series of coaxial refractive surfaces [8, 10]. Collagen is the most abundant protein in mammalian organisms and structures the extracellular matrix. Its interaction with different proteins leads to diverse biological responses by many types of cells. Knowledge of mechanical properties of cells and other biological tissues at high resolution could be important to understanding how mechanical interactions affect function. The advantages of AFM are that it can be operated in many modes and environments depending on the need and the material being studied. The optimization of system is a disadvantage because some parameters can be adjusted such as including the sensitivity and calibration of the photodiode, and calibrating to determine the spring constant of the AFM cantilever.
In this paper, we describe a simple procedure that makes it potential to image, in the Mac mode and tapping mode. The utility of the AFM varies considerably depending on the tissue or cell type. Atomic Force Microscopy (AFM), originally developed for the study of surface sciences, has emerged as a practical tool for conducting studies on living biological materials [11, 12]. As a multifactorial disease, cataract is an unknown disease from the point of view of its causes and associated disorders. Numerous mechanism have been appointed as responsible for cataract formation, being among them the development of protein aggregates, oxidative stress and changes of protein, all involving factors such as aging factors, dietary habits, radiation exposure and metabolic disorders. The main physical alteration occurs at the transparency of the lens and consequently its hardness. The immature cataract is a diffuse cortical opacity. However, it does not block all light to the fundus and the animal still has visual functions. The mature cataract is a complete cortical opacity that blocks the fundus or tapetal reflection completely and the eye is blind. In the hypermature cataract the lens has shrunk and the lens capsule is wrinkled. Lens resorption can be partial or complete and vision can still occur.
Prior to the disease instauration, the aspect of the lens changes gives rise to an aggregate, precipitation and eventually a different coloration. Two problems associated with this disease are the excessive cost of the surgery and the large number of patients that need it. Presently, surgery is the main approach for cataract treatment, except if they are in initial period of development. Applications of new drugs to retard and/or consequently prevent cataract can be used. The main objective of this paper is to explore the opportunity to use AFM in differentiate stages of maturation of cataract, on the basis of their surface morphology in the first step, and interactions and elasticity subsequently. Results obtained by topography AFM on different canine cataract clinical cases are showed. New studies are required because there are only a few published papers related to AFM for the characterization of ocular tissues [9].

2. Materials and Methods

2.1 Tissue preparation

The lens consists on a mass of closely packed fiber cells delimited at the anterior part by an epithelial monolayer, and enveloped by the lens capsule. The canine lens samples were obtained directly after surgical procedures. The lenses were classified according to the disease progression. The stage of cataracts was based on the degree of opacity, tapetal reflection, clinical vision, and visibility of the ocular fundus by indirect ophthalmoscopy. All exams were performed by experts. All surgery was performed using a standard procedure to extracapsular extraction at Faculty Veterinary Medicine- University of Sao Paulo, consisting on an incision of capsule and removal of the lens. For histological examination, the lens were fixed in 10% formaldehyde, embedded in paraffin, and histological slides of four micrometers thick were stained with haematoxylin and eosin (HE) and examined in an optical microscope. The fixation of lens is a routine procedure to avoid deterioration. No other preparation was performed. Thick sections of 1mm were obtained for AFM analysis.

Samples of mature and immature cataract corresponding respectively to cases of total and partial opacity were selected considering clinical cases covering a distinct canine breeds and ages. Some clinical cases were selected in this study to morphological evaluation using AFM and histological analysis. The

study was carried out in accordance with Bioethical Commission of the Faculty of Veterinary Medicine and Zootechny of University of Sao Paulo (Protocol n°644/2005).

2.2 AFM Imaging

AFM imaging was performed with large area scanners (>160 μm x 160 μm). Imaging parameters were adjusted and optimized to produce clear images with minimal distortion or damage to the tissues. Typically, scan rates were 0,3 lines/sec, resulting in image acquisition average time of 15 min. AFM measurements were performed using PicoSPM I (Molecular Imaging, Tempe, AZ, USA) with PicoScan 2100 controller coupled with MACmode controller. The system was used to operate on MAC Mode SFM employing type II 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 Multi-purpose Scanner was used with scan range of 80 μm in the X–Y direction and 7 μm 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. Others studies published in the literature involve complicated and time-consuming preparation methods.

3. Results and Discussion

The crystalline lens is a transparent biological material, and a complex inhomogeneous optical element in the vision system of mammals. Approximately 90% of the fiber cells composition is proteins responsible for the high refractive index of the lenses. Several studies suggest that cataractogeneses is associated with perturbation of lens membrane composition, structure, and function [5]. The comprehension of the structure of the lens proteins, its arrangement, and interactions within the lens are fundamental to investigate the mechanisms of cataract formation and consequently aspects relative to the preservation inhibiting the disease process [10, 11]. 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 consecutive topography measurements to great scan size indicated that the applied force did not have an effect significant on the topography. We have not detected the presence of artifacts in high resolution images obtained during this investigation.

3.1 Morphology of cataractous canine lens imaged by histology and AFM

Fibers presented distinct alterations due to their localization. Lens fibers from the cortex of the canine lens are around 7-13 mm in extent, and are extremely thin. Cortical fibers are very thin and are hexagonal in cross section. The fibers appear strongly associated by interconnections. Particularly, gap junctions are rich and frequently linked with processes. Using scanning electron microscopy (SEM), three types of fiber surface structures have been identified. The first type is the interlocking processes along the six edges of the lens fiber, which appear to join adjacent fibers [10]. Recently, AFM imaging of the ultrastructure of fibers in healthy rabbit lenses was determined [11].

Tongue and groove patterns were determined using AFM to clinical case of mature cataract. Some types of structures in cortical and nucleus region show this connection. Using scanning electron microscopy (SEM), three types of fiber in the deep cortex and nucleus, with all six surfaces displaying folds, sometimes called tongue and groove junctions, were determined [10].

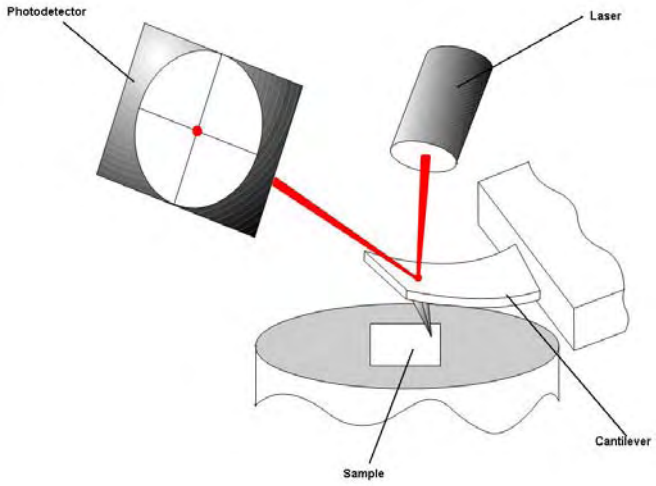

Fig. 1 Schematic drawing of an AFM and of the sample positioned. An AFM consists of a flexible shaft with a tip at its extremity. The deflection signal is monitored by an optical system consisting of a diode laser and a photodetector that is sensitive to variations in position. Deflections of the sample are plotted point to point while scanning on surface.

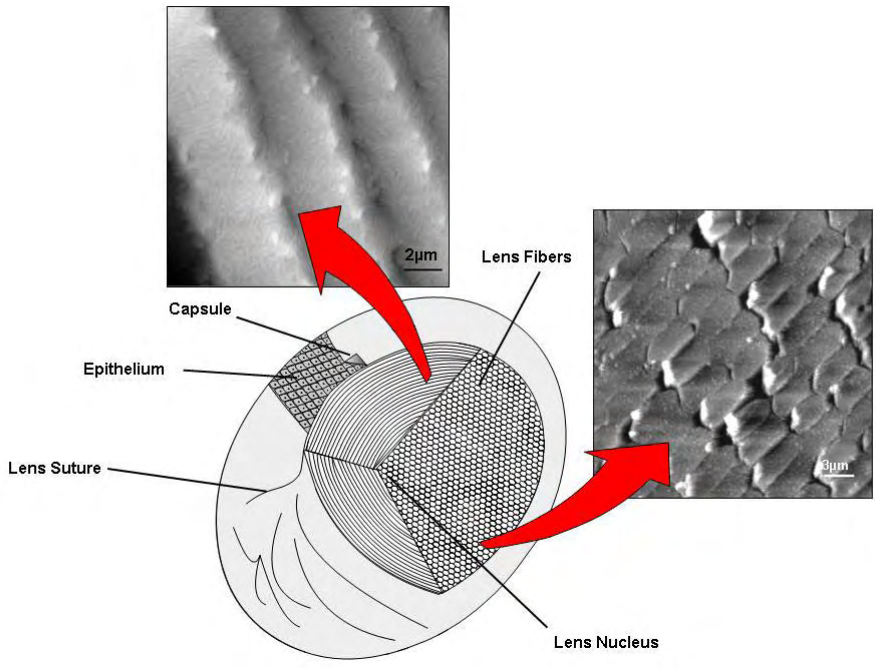

Fig. 2 The figure shows a diagram of the lens section and details of structural information obtained by AFM. The structures showed in the figures A and B were obtained to healthy lens [13]. Scale bars in Left= 2µm and Right = 3µm.

Morphological studies using SEM, TEM and Field Emission-SEM have appointed to the incidence of sphere-shaped bodies in animal and human lenses. The presence of these structures has been correlated

with mechanisms of degradation that occur in lens cytoplasm during cataract development [10]. Our study also indicated the same aspect in clinical cases of progression of cataract. We show some topography of immature cataract lens of distinct parts of the tissue, and histological images as a comparative illustration with an imaging mode that emphasizes the surface detail of tongue and groove pattern, such as 3D-imaging of lens fibers (Fig. 3).

Mature cataract represents an advanced stage of maturation and the structural information is lost (Fig 4).

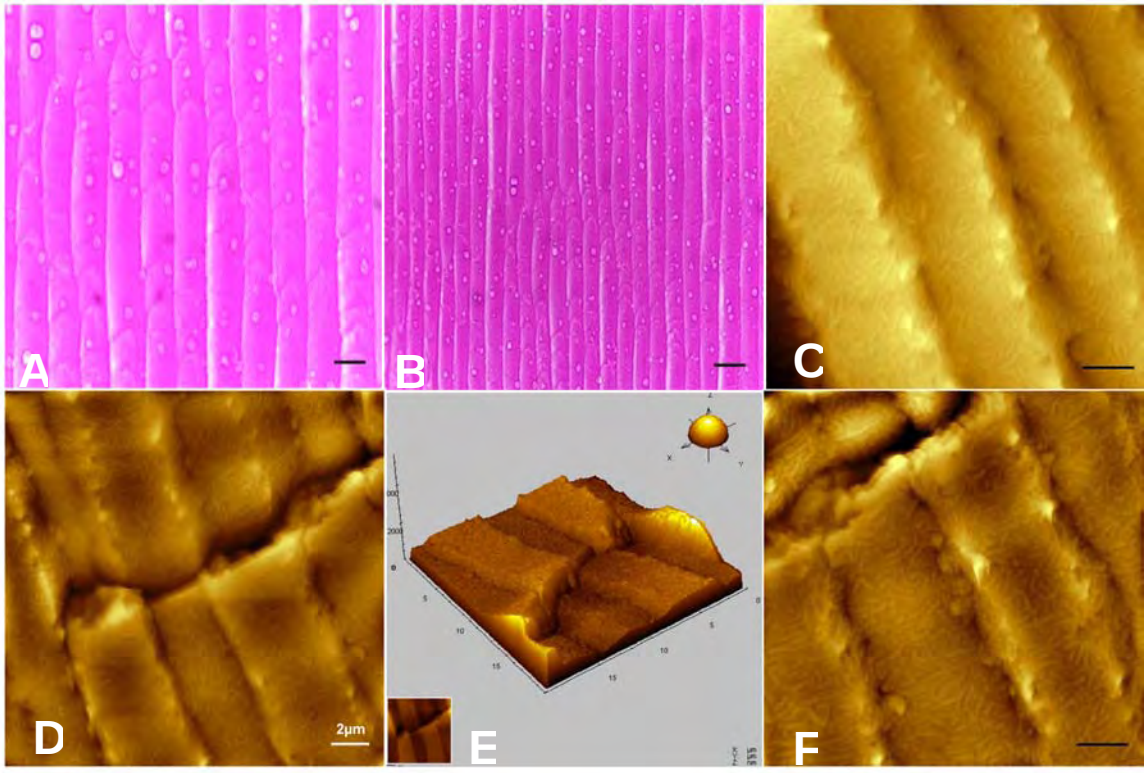

Fig. 3 For immature cataracts the level of opacity is partial and a few areas of the lens are involved, however, the opacity can reach very high levels in some points. High resolution images obtained by tapping Mode AFM of nucleus region are presented. (A) Histological image of cortex region showing the fibers organization. (B) Histological image of the nucleus region showing the fibers and the spherical bodies. Note the aspect of a healthy tissue due the fibers organization. (C) Small structures can be seen in the superior part of the image, the tongue and groove pattern is visible on the fiber surface. (D) Fiber dimensions to healthy portion of lens to clinical case of immature cataract. (E) An AFM image (topography) of a typical immature cataract fiber cells in 3D. (F) Topographic details of fiber cell and tongue and groove pattern. Scale bars in A= B= 10µm, C=D=E=F= 2µm).

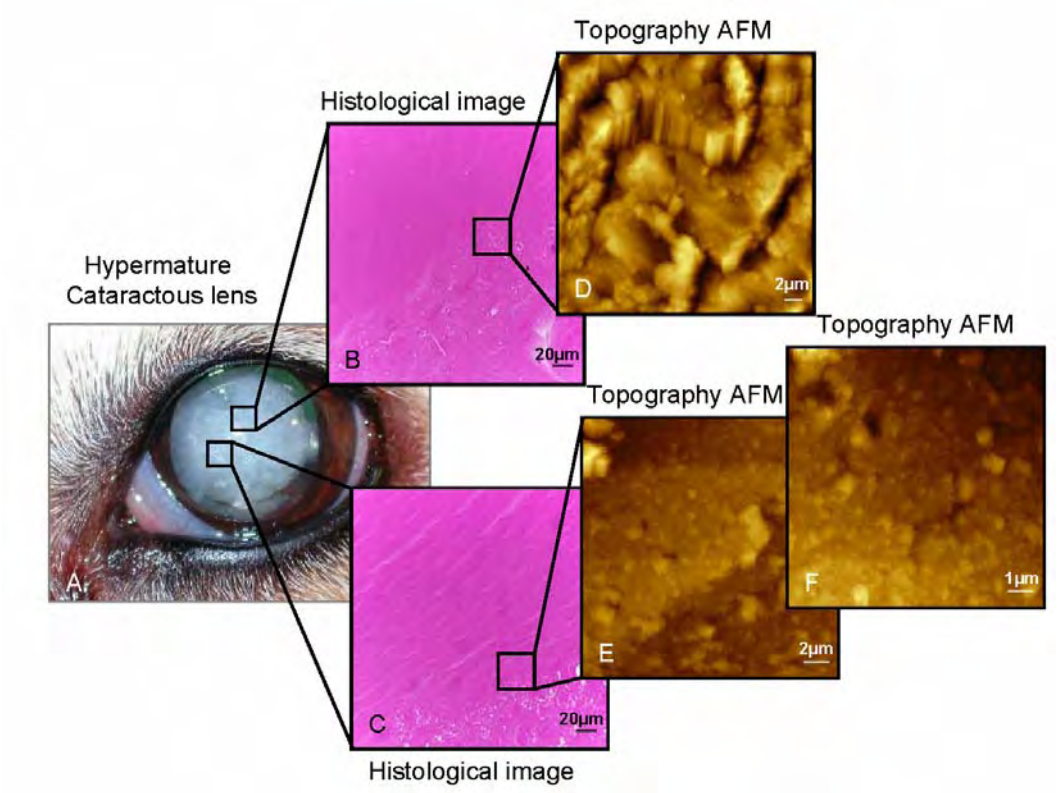

Fig. 4 The images show three distinct topography of hypermature cataract. MAC Mode AFM image of cortex region can be visualized in figures. (A) Macroscopic fragments of the mature lens; (B) Histological image of cataractous lens. There is absence of fiber structure indicating the total opacity of mature lens. (C) Histological image of the cortex region. AFM images are in figures (D), (E), (F) with the presence of small spherical bodies recovering on lens area.

In the Figure 3, we have fiber cells exhibiting elongated shape, indicating that some areas of the lens present fiber structures well defined that are related with transparency. Similar AFM images (Figs 3 and 4) are not found in the mature and hypermature cataract. In these clinical cases there is an accentuated alteration in the lens transparency, what represent a total opacity.

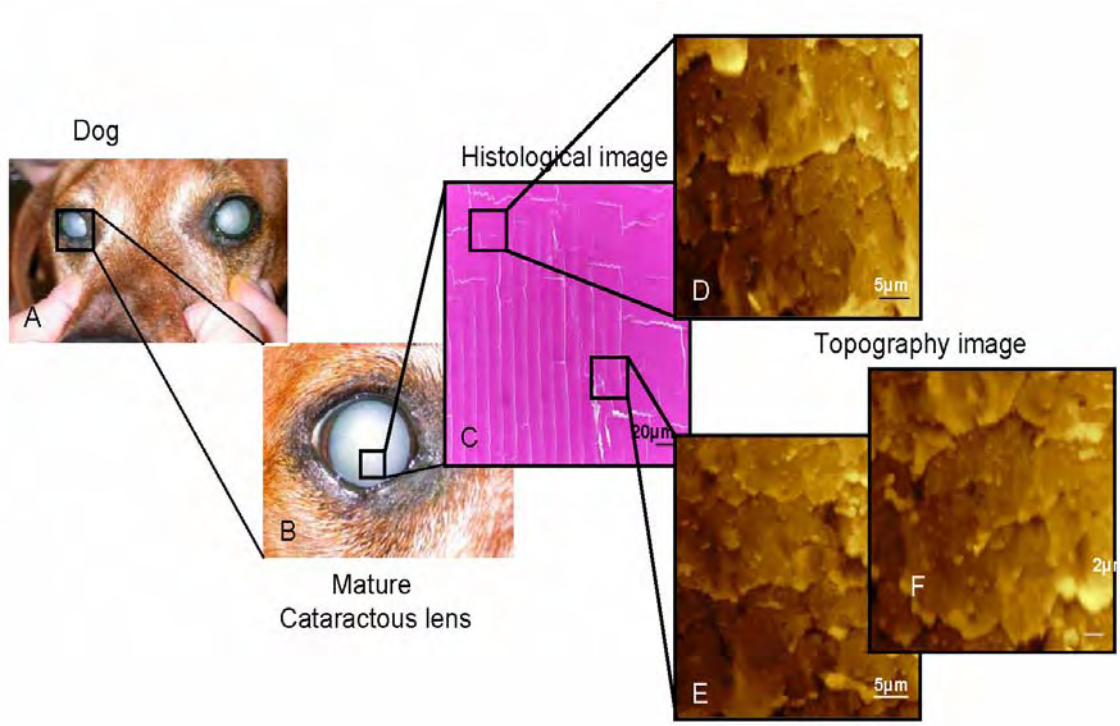

Fig. 5 The images show three distinct topography of mature cataract. (A) and (B): macroscopic views of a canine mature cataract; C. Histological images of mature cataractous lens. (D), (E) and (F) are AFM images of distinct parts of mature canine lens. There is an absence of fiber structure indicating the total opacity of mature lens.

Our results have appointed that the AFM also allow us to categorize tissue structures that are regularly arranged when observed with other microscopic techniques. Fiber in the cortex region of immature cataract (Figure 3) appeared organized what is not occurs to others cataract progression stages (Figs 4 and 5). This study recognized the morphology and organization of fiber during cataract progression.

4. Conclusions

AFM allows direct imaging of the topography of cataractous canine lenses and their images can be compared with morphological information obtained by histology technique. Thus, AFM presented an auxiliary tool in the characterization of cataract formation to distinct development stages. High resolution images of the fibers shells such as their intershell separations were accessed by AFM. We understand that MAC mode AFM methodology can be applied to the study of distinct ocular tissues and their diseases. In recent work, we have presented the relationship between fiber diameter and position in tissue of rabbit healthy lens [13]. New AFM images can be analysed to the determination of physical properties of fiber lens in different stages of cataract maturation. We expect that this article can contribute for further investigations in ocular tissues, and, more generally, for the applications of nanotechnology in Veterinary Ophthalmology.

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