Fast and sensitive screening of transparent composite materials using polarized light image processing

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Abstract

Composite materials of natural origin have remarkable material properties. In order to mimic the various functions of such materials for technical applications, it is necessary to understand the structure and the mechanisms of their formation, a scientific field which is called structural biology. A milestone in the era of structural biology was the application of the polarized light microscope for investigating biological specimens by W. J. Schmidt in 1924. A recent development in polarization technology is the LC-PolScope (Abrio IM™ Imaging System) that has been developed by R. Oldenbourg and his colleagues during the past 20 years. Like conventional polarization microscopy, it probes the local anisotropy of the specimen's optical properties, such as birefringence or dichroism. Since there is a close relationship between such properties and molecular order in bulk materials, polarization microscopy can be regarded as a submicroscopic technique. The LC-PolScope is especially suitable for investigating biological materials without the need for using contrasting agents such as fluorescent markers. We demonstrated the applicability of this technique for the fast and sensitive screening of biological composite materials. Implications for the characterization of biomineralization phenomena in a quantitative manner are discussed.

Introduction

In optically anisotropic materials, the refractive index $\mathbf{n}$ changes with the polarization of light passing through. With respect to the direction of molecular order, the refractive indices parallel and perpendicular differ by a certain value $\Delta\mathbf{n}$, which is called birefringence. One century ago, the invention of polarized light microscopy represented a milestone for the investigation of biological materials such as bone, teeth, and sea shells. It became possible to determine the orientation of inorganic crystals with respect to the body plan of biomineralizing organisms. As mentioned above one of the pioneers in the era of structural biology was W. J. Schmidt who applied the polarized light microscope to biological specimens [1]. A few years before, O. Wiener had presented a general theory of the dielectric constant of mixed systems based on expressions for the birefringence of lamellar sheets and suspensions of parallel cylinders [2]. While Schmidt continued to apply the polarized light microscope to all kinds of biological specimens in the macroscopic and microscopic regime of length scales during the following years [3-6], other scientists like Perutz [7] as well as Bragg and Pippard [8] employed Wiener's theory in order to characterize biological molecules and their interaction phenomena such as alignment and crystallization at the nanoscale. Biological macromolecules bear several analytical problems regarding the so-called form birefringence, a phenomenon that applies to objects substantially smaller than the wavelength of light. Thus, the controversies regarding intrinsic and form birefringence continued [9, 10]. The comeback of Wiener's theory happened in 1975, when Inoué and coworkers demon-

strated a direct relationship between the birefringence of the mitotic spindle and the alignment of tubulin proteins [11]. It was suggested that Wiener's theory is in good agreement with experimental observations of form-birefringence that were made with DNA and Tobacco Mosaic Virus assemblies. It turned out to be crucial to take solvent effects into account [12]. The past 20 years brought further development of polarized light microscopy by using liquid crystal compensators. Combined with efficient image processing techniques it is now possible to quantify birefringence at different levels of sensitivity [13-15]. The analogies of conventional polarized light microscopy and the LC-PolScope (Abrio IM™ Imaging System, CRi, Woburn, MA, U.S.A.) are summarized in Figure 1. With respect to biological materials it is especially interesting to note that the orientations of extracellular fibrils such as cellulose in wood or plant cell walls correlate with the alignment of certain fibrillar proteins such as microtubules inside of cells [16]. Intracellular cytoskeletal structures are usually visualized by immuno-fluorescence staining techniques with the drawback of fixation that is not compatible with recording dynamic rearrangements *in vivo*. Here we demonstrate the applicability of an imaging platform for the fast and sensitive screening of mineralized biological materials based on an inverted microscope equipped with an Abrio IM™ Imaging System that will allow us to study the synthesis of extracellular matrix polymers and their crystallization behaviour as a function of intracellular cytoskeletal fibril assembly. Based on this fast and sensitive screening method, the relationship between particular calcium oxalate crystals and plant cells turned out very efficient in order to demonstrate the suitability of this instrumentation for screening composite materials of biological origin with respect to quantification of birefringence and the orientation of crystals.

Quantification of birefringence

In principle, the LC-PolScope measures the same properties which can be measured by a traditional polarized light microscope, but with limited speed, sensitivity, and accuracy. The LC-PolScope achieves significant improvements by measuring the birefringence parameters for all resolved specimen points simul-

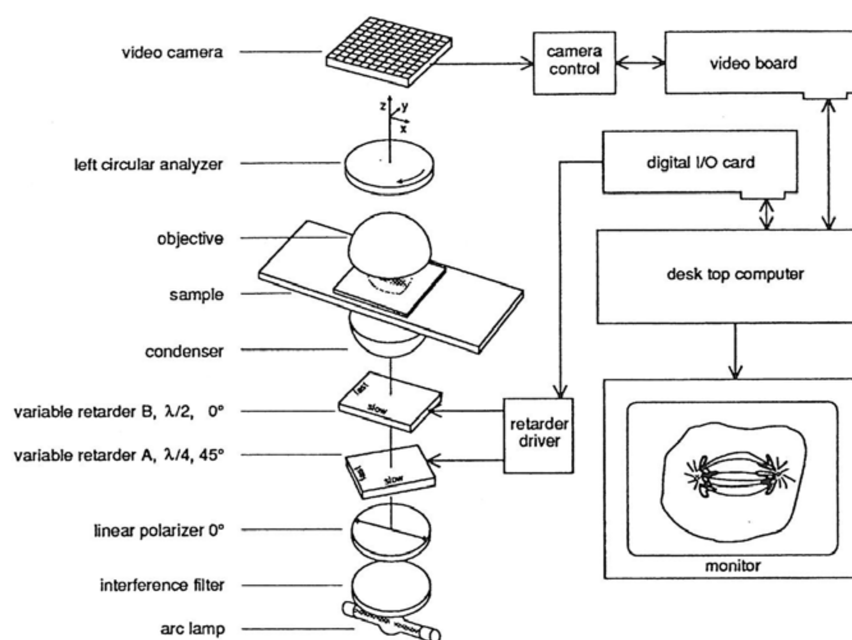

Figure 1: Principles of PolScope imaging technology as described in ref. [17]. The details of relevant imaging parameters are reviewed in ref. [14].

taneously. The heart of the instrument is an extremely sensitive high resolution ccd camera, in addition to an universal compensator which is built from two variable electro-optical retarder plates and a linear polarizer [17]. Depending on whether monochromatic light passes first through the retarder plates or first through the linear polarizer, this device functions as a "variable polarizer" or "polarization analyser", respectively. Any polarization state can be achieved: Linear polarization of any azimuth, or elliptical polarization of any handedness, ellipticity, or azimuth. The voltage for each retarder plate is specifically controlled by a software that synchronizes the image acquisition process and computes values for retardance $R = \Delta n \cdot t$ (relative optical path difference, or phase change, suffered by two orthogonal polarization components of light when interacting with an optically anisotropic material of a given thickness t) and principal axis orientation (e.g. slow axis azimuth corresponds to highest refractive index) at each resolved image point. A total of four images, which are in perfect register, are recorded with selected voltages applied to the liquid-crystal devices and processed. Since there is no mechanical change in orientation of the specimen required, inaccuracies due to a potential lack of reproducibility in the manipulation of the microsocope are eliminated. In such a way, high spatial resolution can be achieved. Since LC-PolScope image acquisition is based on circular polarized light, a monochromatic filter (e.g. 546 ±15 nm) is required. Although calibration and correction procedures are employed,

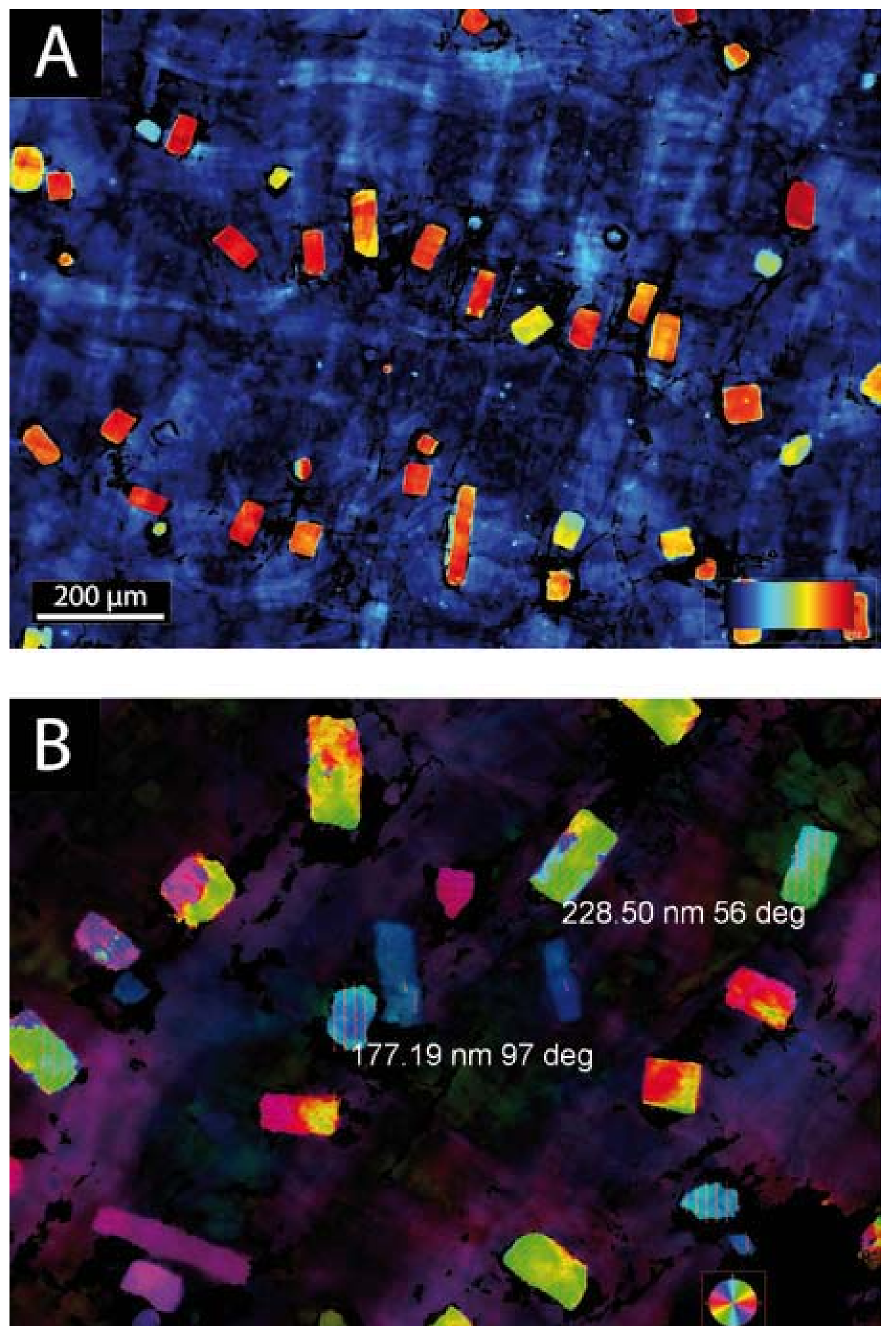

Figure 2: LC-PolScope images taken from *Allium spec.* in the retardance (A) and vector overlay mode (B). The range of retardance values in (A) is calculated from 0 nm - 272 nm (blue → red). The retardance in nm and azimuth of the slow axis in deg in (B) are calculated per pixel and colour encoded in the image. Depending on selected threshold values for the retardance, the azimuth vectors are displayed on regular grid points.

it is still crucial for measuring small retardance values in the range below 0.01 nm that extinction is optimized by the right choice of optics components as described [14]. The speed of image acquisition is to a large extent depending on liquid crystal settling time (e.g. 30 ms), among other parameters.

Investigation of birefringence in biological materials

The suitability of the LC-PolScope for investigating biological materials in an inverted microscope was tested using tissue samples from the onion (*Allium spec.*). It turned out that a large number of irregular cube-shaped objects of about 50 µm in length with high retardance values as compared to the surrounding tissue were observed in the outer epidermis of *Allium spec.*. As shown in Figure 2, the retardance in nm and azimuth in deg per pixel is quantitatively colour encoded in the image. The surrounding tissue gives significantly lower retardance values corresponding to a reduced brightness than obtained from the cube-shaped objects, which correspond well to calcium oxalate crystals that have been described in the literature to occur in *Allium spec.* [18-20]. As shown in Figure 2, it is possible to obtain information regarding the orientation of particular single crystals by vector overlay. As opposed to conventional polarized light microscopy, it was possible to visualize a whole population of crystals within the same image irrespective of their orientation. Furthermore, irregularities were observed within the crystals that would be difficult to detect by using conventional crossed polarizers. It remains, however, to be investigated whether the irregularities are due to crystal defects of the calcium oxalate, or due to any kind of form birefringence caused by surface roughness or liquid inclusions. In 2004, R. Oldenbourg wrote: "The art and science of relating measured retardance and azimuth values to structural information on the molecular level of the specimen is still in its infancy. The potential information in PolScope images and time-lapse movies is enormous" [14]. Our current investigation of *Allium spec.* demonstrates that Wiener's theory [2] will have to be consulted more extensively in order to see beyond the iceberg's tip of complex biological materials.

Outlook

We plan to transfer some concepts of mollusc shell formation [21-25] into suitable cellular model systems that allow us to study the formation of composite materials *in vivo* in a comparative manner. The main focus will be the biosynthesis of extracellular matrix polymers and the subsequent steps that are important to create mineralized composite materials. Since this is by no means going to be an easy task, it will be extremely important to apply a fast and sensitive screening technique. It is part of the strategy to obtain high-throughput information on the degree of mineralization and possibly the orientation of crystals with respect to intra- and extracellular polymer structures. Combined with standardized metallogra-

phic specimen preparation techniques [26-28], the LC-PolScope will thus be extremely useful for designing biological materials with respect to various applications.

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