

Instant polarized light microscopy for real-time wide-field visualization of collagen architecture

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ABSTRACT

Collagen fiber architecture plays an important role in the mechanical properties of soft tissues. Conventional polarized light microscopy done using linear polarizers and, sometimes, quarter-wave plates is a label-free imaging technique for quantifying collagen fiber architecture, specifically distribution and orientation. However, this technique has several limitations. First, it requires acquiring multiple images with different polarization states, which precludes many time-sensitive applications. Second, post-processing, especially image registration, reduces the level of detail discernible. Third, the added optical elements may cause glare under coaxial illumination, thus complicating the use of reflected light microscopy. We have recently demonstrated instant polarized light microscopy (IPOL), that requires only one image and therefore no registration. IPOL utilizes wavelength-dependent polarization to modify the spectrum of the illumination, generating visible colors that depend on fiber orientation and density. Herein we present two further advances on IPOL: we extend it to work with coaxial illumination allowing transmitted and reflected light microscopy, and we integrate it in a dissecting microscope. This permits real-time imaging, limited only by the camera frame rate, making it possible to track dynamic events, such as fast-acting responses to external forces or moving objects. We demonstrate IPOL with a field of view of 11 mm and a long working distance of 65 mm, which simplifies testing of large samples. IPOL provides both fiber distribution and orientation information in a single true-color snapshot, and therefore, it is suitable for time-sensitive applications.

Keywords: Polarized light microscopy, coaxial illumination, transmitted illumination, collagen, biomechanics, dynamics, label-free

1. INTRODUCTION

Collagen fiber architecture is important in soft tissue dynamics and is therefore of great interest to biomechanics.¹⁻⁴ Collagen exhibits birefringence that affects light polarization, and therefore, polarized light microscopy (PLM) has been widely used to study collagenous tissues.⁵⁻⁹ Conventional PLM done using linear polarizers and, sometimes, quarter-wave plates is a label-free imaging technique for quantifying collagen fiber distribution and orientation.¹⁰⁻¹⁵ However, this technique has several limitations. First, it requires acquiring multiple images with different polarization states, which precludes many time-sensitive applications, such as fast-acting responses to external forces or moving objects. Second, post-processing, especially image registration, reduces the level of detail discernible. Third, the added optical elements may cause glare under coaxial illumination, which complicates the use of reflected light microscopy. In addition, when the distance between the objective and the stage is small, as is the case in many compound microscopes, it is difficult to use off-axis illumination.¹⁶ We have recently demonstrated instant polarized light microscopy (IPOL) implemented in a compound microscope, which can visualize the collagen fiber distribution and orientation with optical colors in a single snapshot.^{17,18} However, this setup only enables transmitted illumination, and the field of view is limited to a few millimeters, making it difficult to track the dynamic responses of large or fast-moving samples.

Our goal was to demonstrate an integrated imaging system that combined IPOL with a dissecting microscope, for imaging with either transmitted or coaxial illuminations. Our system benefits from the advantages of the dissecting microscope,

i.e., long working distance, wide field of view and large depth of field. We demonstrate that these advantages allow visualizing collagen fiber architecture of large moving samples in real time.

2. METHODS

2.1 Imaging setup

We implemented IPOL in a dissecting microscope (MVX10; Olympus, Tokyo, Japan) equipped with transmitted and coaxial illuminations (Figure 1). For each mode of illumination, a broadband light source, a set of polarization encoder and decoder, and a color camera were used. The light source was collimated to improve image contrast. Both the polarization encoder and decoder consisted of polarizers and polarization rotators. The light passing through the polarization encoder was linearly polarized with wavelength-dependent orientation, and then decoded by the polarization decoder. As the encoded polarized light passed through a birefringent material, such as collagen, the polarization state of each wavelength was modulated based on the relative orientations of the collagen and the wavelength. The polarized light passed through the polarization decoder, and was then captured by a color camera (acA1920-155uc, Basler AG, Ahrensburg, Germany). For IPOL images, the hue of the color represents the collagen fiber orientation and the brightness of the color represents the collagen fiber density. The implementation is similar to the one we described elsewhere^{17,18} and based on original ideas by Shribak.¹⁹

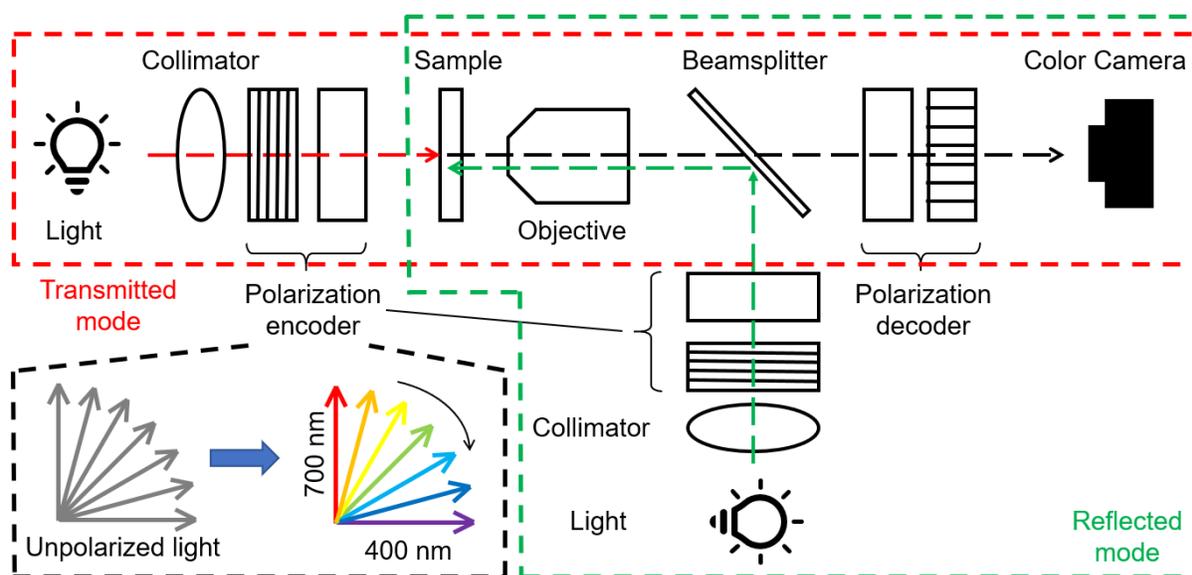


Figure 1. Schematic of instant polarized light microscopy (IPOL) using transmitted and coaxial illuminations in a dissecting microscope. For each mode of illumination, the broadband light source was collimated to improve image contrast. A polarization encoder, consisting of a polarizer and a polarization rotator, generated linearly polarized light with wavelength-dependent orientation. A polarization decoder then converted the polarized light into the colorful light, which was acquired by a color camera.

2.2 Sample preparation

We demonstrate the capabilities of our integrated system using cryosections of chicken Achilles tendon and sheep optic nerve head. The detailed procedure of sample preparation was described elsewhere.¹³ Briefly, chicken Achilles tendons were dissected from chicken legs purchased from a local grocery store. Following the dissection, the tendons were cryoprotected, embedded and cryosectioned at 30- μm thick along its longitudinal direction. The chicken tendon section was then washed with saline and mounted to a customized device for uniaxial stretching testing. No labels or stains or fixatives were applied. Sheep eyes were enucleated within 4 hours of death, followed by formalin fixation at an intraocular pressure of 5 mmHg. The optic nerve head region was isolated using a 11-mm-diameter trephine and cryosectioned coronally into 16- μm -thick sections. The sections were washed with saline before imaging.

3. RESULTS

Figure 2 shows IPOL images of a sheep optic nerve head section under transmitted illumination at two magnifications. The images illustrate the high-resolution imaging capability of the system, as well as its with a wide field of view. Collagen fibers are color-coded by local orientation, which significantly improves the visibility of fiber families and bundles, and reveals features of collagen microstructure and organization , such as crimp (red arrow) and collagen fiber interweaving (white arrow).

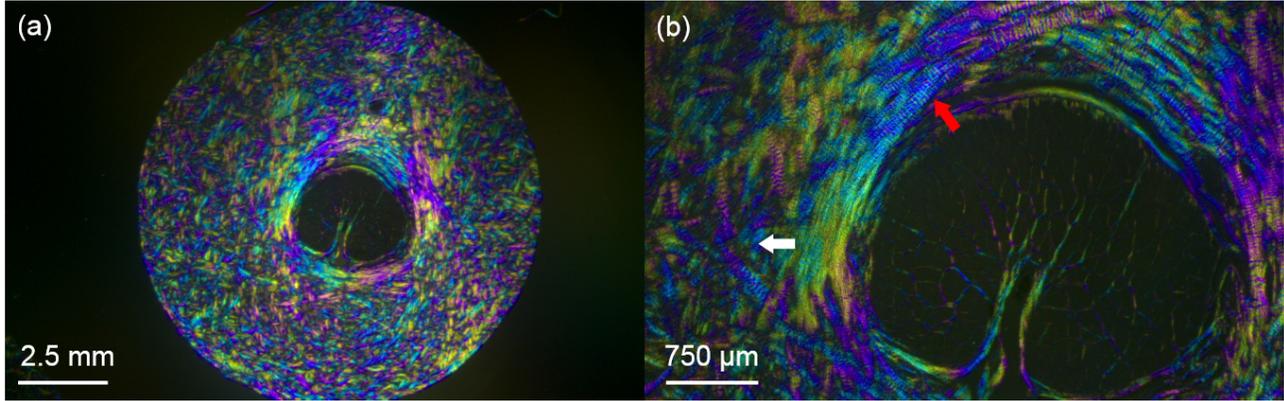


Figure 2. IPOL images of a section of sheep eye posterior pole under transmitted illumination. (a) Wide field view showing the whole cross-section with a field of view of 11 mm. (b) Close-up image of the optic nerve head region, showing the scleral canal and peripapillary sclera. Clearly discernible are collagen fiber crimp, i.e., undulations in color (red arrow), and bundle interweaving (white arrow). Brightness indicates local retardance and is, roughly, proportional to collagen density. The substantially lower density of the collagen tissues within the canal relative to the peripapillary sclera is easily discernible.

We evaluated the potential of our integrated system to visualize the stretch-induced deformation of a chicken tendon thin sample under transmitted illumination. Implemented in a dissecting microscope, IPOL has a working distance of 65 mm that is sufficient to place a custom stretching device between the objective and the stage (Figure 3a). Images show the stretch-induced collagen fiber uncrimping at different time steps (Figure 3b). Before stretch (time = 0 sec), the tendon tissue exhibits cyan/blue bands, indicating that collagen fibers were crimped. The color bands became less visible at later time points (time = 8 sec), suggesting that collagen fibers uncrimped due to stretch. With further stretching (time = 16 - 24 sec), the sample narrows, in part due to fiber failures, and the color became non-uniform.

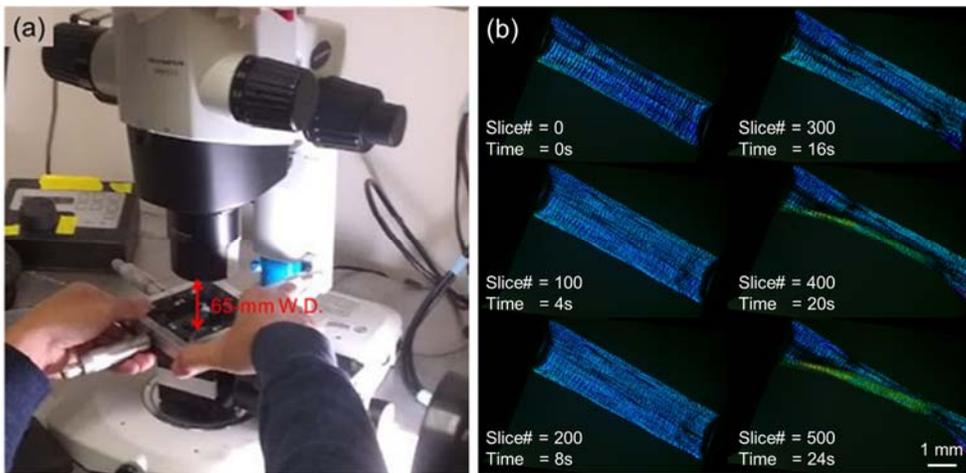


Figure 3. Visualizing the stretch-induced deformation of a chicken tendon section using IPOL under transmitted illumination. (a) IPOL was implemented in a dissecting microscope that has a long working distance of 65 mm, allowing to place the customized stretching device between the objective and the stage. (b) Time-lapse images of a chicken tendon section under stretching. The frame rate of the camera was set as 25 frames per second. W.D. – working distance.

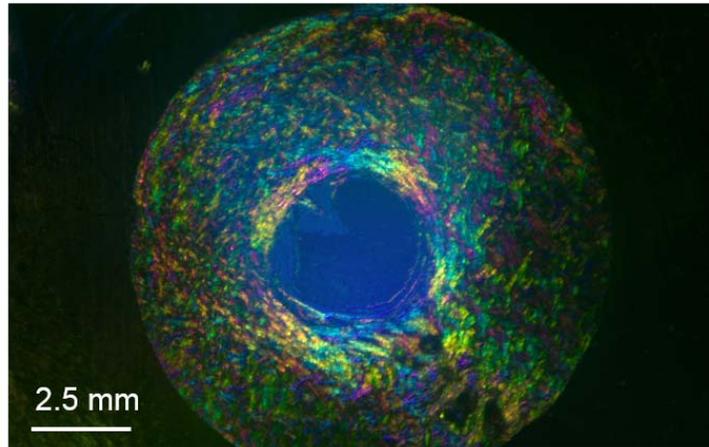


Figure 4. An IPOL image of a sheep optic nerve head section under coaxial illumination. The light reflected from optical elements and microscope slides was minimized by using crossed polarization between the polarization encoder and decoder, resulting in the dark background.

Figure 4 shows IPOL images of a sheep optic nerve head under coaxial illumination. The light reflected from the collagenous tissue encoded fiber orientation with color, and that from optical elements and microscope slides was minimized by crossed polarization in IPOL, resulting in a dark background.

4. DISCUSSION AND CONCLUSIONS

In this work, we demonstrated an integrated imaging system that combined IPOL with a dissecting microscope, equipped with both transmitted and coaxial illuminations. The system has two advantages. First, it allows IPOL with a long working distance of 65 mm, a field of view of 11 mm, and a large depth of field. This allows placing a custom mechanical testing device between the objective and the stage and to keep an entire large sample in focus in a single snapshot, eliminating the need for extended depth of field techniques.²⁰ Second, the system allows imaging thick and opaque tissues. In conventional PLM, the quarter-wave plate may cause glare under coaxial illumination. In our setup the crossed polarizers block avoid this problem. Although some of these benefits can be realized with off-axis illumination, this complicates quantitative imaging. Also, using coaxial illumination can capture more details of the sample surface. Integrating IPOL with structured illumination might further improve the ability to resolve surface details.¹⁶ In summary, our integrated imaging system can visualize collagen fiber architecture in real time, and therefore, it is suitable for time-sensitive applications, such as visualizing the stretch-induced deformation of collagenous tissues.

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