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Abstract. We developed a new theory-based azimuthal phase retardation microscope to visualize distributions of actin filaments in biological cells without having them with exogenous dyes, fluorescence labels, or stains. The azimuthal phase retardation microscope visualizes distributions of actin filaments by measuring the intensity variations of each pixel of a charge coupled device camera while rotating a single linear polarizer. Azimuthal phase retardation δ between two fixed principal axes was obtained by calculating the rotation angles of the polarizer at the intensity minima from the acquired intensity data. We have acquired azimuthal phase retardation distributions of human breast cancer cell, MDA MB 231 by our microscope and compared the azimuthal phase retardation distributions with the fluorescence image of actin filaments by the commercial fluorescence microscope. Also, we have observed movement of human umbilical cord blood derived mesenchymal stem cells by measuring azimuthal phase retardation distributions. [DOI: 10.1117/1.3615665]

Keywords: imaging systems; microscopes; microscopy.

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1 Introduction

The ability of eukaryotic cells to adopt a variety of shapes, organize the many components in their interior, interact with the environment, and fulfill coordinated movements depends on the cytoskeleton, an intricate network of protein filaments that extends throughout the cytoplasm. The cytoskeleton in a cell that helps to support the large volume of cytoplasm and is reorganized as a cell changes shape, divides, and responds to its environment.[1] The cytoskeleton is composed of three principal types of protein filaments: actin filaments, intermediate filaments, and microtubules, which are held together and linked to organelles and the plasma membrane by a variety of accessory proteins. The major cytoskeletal protein of most cells is actin, which polymerizes to form actin filaments. Within the cell, actin filaments are organized into higher-order structures, forming bundles or three-dimensional networks.[2] Recently, visualization of cytoskeletal filaments was mainly limited to immunofluorescent staining of fixed cells or fluorescent analog cytochemistry for living cells.[3,4] Especially, the introduction of green fluorescent protein (GFP) technology has made it possible to visualize cytoskeletal filaments in living cells through the expression of a chimeric fusion protein formed from GFP and the cytoskeletal protein of interest.[3,5–7] Although this technology is a great advance, it suffers from the drawback that some cells are difficult to transfect and the background fluorescence of transfected cells was obscure even when it was successful to transfect cells with GFP.[3]

Recently, polarization microscopy has been introduced to visualize anisotropically ordered fibrous structures in biological materials without having to stain or label them. This technique can visualize fibrous structures of biological materials from acquiring their phase retardations and orientations of the principal axes which come from birefringent characteristics of biological materials.[8–14] Two different types of polarization microscopes have been reported. One type uses two liquid crystal variable phase retarders for generating four discrete input polarization states, and the other type uses two Faraday rotators for continuous rotation of linearly polarized light with respect to a sample by controlling an external magnetic field. The first type of polarization microscope was proposed by Oldenbourg et al. and has been used for cell imaging applications. Four intensity images of a biological sample are measured through an analyzer while the polarization states of the incoming light to a sample are changed into four predetermined states: left circular, right circular, crossed, and parallel polarization states. From these intensity images, four Stokes polarization parameters can be calculated on a pixel-by-pixel basis. The second polarization microscope, with two Faraday rotators, proposed by Kuhn et al., is called a "modulated polarization microscope." The two Faraday rotators, one positioned before a specimen and the other after the specimen, are used to modulate polarization angles. The polarization microscope uses the single-frequency Fourier filtering algorithm to calculate phase retardation.[8,14]

Although the phase retardation and the orientation of the principal axis have been used as important concepts in polarization microscopes, the phase retardation and the orientation of the principal axis in biological materials may not have important meaning in the polarization microscopes with the type of

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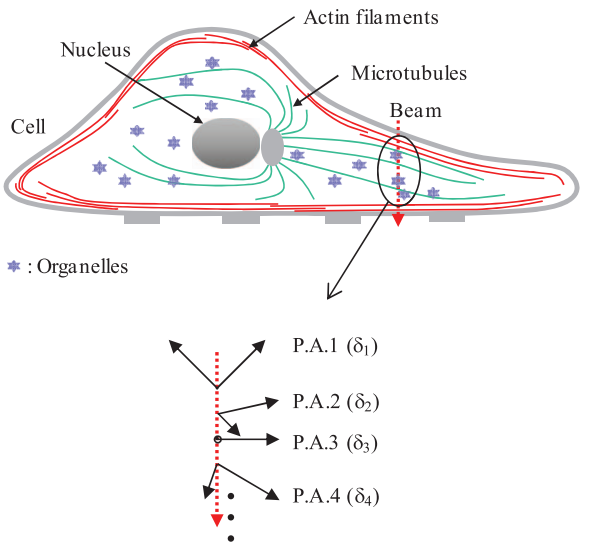


Fig. 1 Schematic diagram of the phase retardations (δ) and orientation of the principal axes along a beam path in a cell.

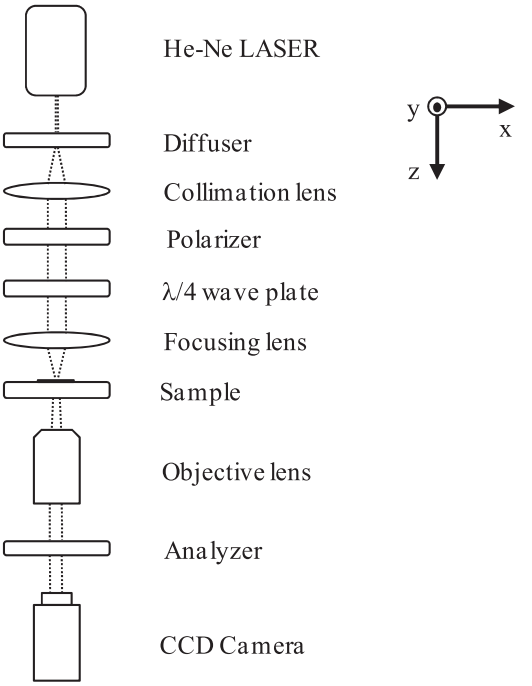


Fig. 2 A schematic diagram of proposed azimuthal phase microscope system.

light transmission because biological cells have microtubules, actin filaments, nucleus, cell membranes, and organelles such as lysosome, endoplasmic reticulum, Golgi apparatus, peroxisome, and so on, and these components in biological cells have unique orientations of principal axes and phase retardations about the principal axes along a beam path. Figure 1 shows the schematic diagram of the phase retardations and orientations of the principal axes along a beam path in a cell with typical thickness. Actually, the orientation of the principal axis acquired by the conventional polarization microscopes is a vector summation about the orientations of the principal axes along a beam path. Also, the phase retardation acquired by the conventional polarization microscopes is a scalar summation about the orientations of the principal axes along a beam path.

In this paper, we introduce a new definition of the azimuthal phase retardation that is the phase retardation difference, δ , between two fixed axes (y- and x-axes in Fig. 2) because finally acquired orientation of the principal axis in the conventional polarization microscope is a vector summation and the orientation of the principal axis and phase retardation about the principal axis do not have important meaning any more. We also describe a new azimuthal phase retardation microscope and experimental results of the azimuthal phase retardation as the birefringence characteristics in biological materials. Biological materials generally exhibit birefringence, although the birefringence is weak because they have anisotropic protein structures. Therefore, it is possible to visualize cytoskeletal filaments in biological materials with the azimuthal phase retardation microscope. Major advantages of the azimuthal phase retardation microscope for biological cells and tissues are that the structural order in biological cells and tissues can be revealed and analyzed without having to treat them with exogenous dyes, fluorescence labels, and stains like the conventional polarization microscopes,[3,8–15] and that the azimuthal phase retardation microscope can visualize biological cells by just one factor, an azimuthal phase retardation unlike the polarization microscope.

To acquire and visualize the azimuthal phase retardation distribution of the biological materials, the developed azimuthal phase retardation microscope uses a polarizer, a quarter wave plate, and an analyzer. The polarizer rotates to modulate polarization state in the measurement system. There are several methods to modulate polarization state such as the polarization modulation by the liquid crystal phase retarder and the polarization modulation by Faraday rotator. Especially, Faraday rotator gives fast measurement speed in the polarization microscope. However, since the high current in the solenoid of Faraday rotators generates a lot of heat, cooling systems should be added to the Faraday rotators of the microscope. This makes the system expensive, and the whole assembly becomes bulky, which makes it difficult to fit this system into existing laboratory microscopes.[8] So, we used a polarization modulation method by a stepping motor. The major advantage of our method is that more accurate azimuthal phase retardation can be obtained compared to modulated polarization microscopy (MPM) by having 30 or even more phase scanning data points and fitting them with a squared-sinusoidal function.[8,14] A charge coupled device (CCD) camera is used for significant improvements in measurement time and accuracy instead of a scanning photodiode. Diffraction and interference effects are effectively eliminated by employing an incoherent imaging system in this setup.[16]

2 Theory and Experimental Setup

Figure 2 shows the schematic diagram of a newly developed azimuthal phase retardation microscope. In this setup, fixed analyzer and fixed quarter wave plate are adjusted to 45° with respect to the x-axis, respectively. Also, the polarizer is initially adjusted to 45 deg with respect to the x-axis. Image acquisition is synchronized to the rotation angle of the polarizer such that successive frames captured as the polarization angle is ramped linearly. Jones matrix representations for polarization states of

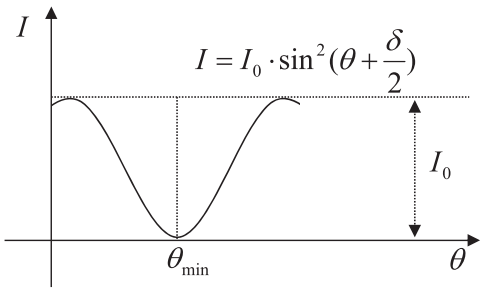


Fig. 3 Intensity variation of light emerging at one point on a sample.

the light just after each polarization component can be written as Eq. (1).

$$\begin{bmatrix} E_x \\ E_y \end{bmatrix} = \begin{bmatrix} \frac{1}{\sqrt{2}} & 0 \\ 0 & \frac{1}{\sqrt{2}} \end{bmatrix} \begin{bmatrix} e^{i(\delta/2)} & 0 \\ 0 & e^{-i(\delta/2)} \end{bmatrix} \times \begin{bmatrix} \cos\frac{\pi}{4} & -\sin\frac{\pi}{4} \\ \sin\frac{\pi}{4} & \cos\frac{\pi}{4} \end{bmatrix} \begin{bmatrix} e^{i(\pi/4)} & 0 \\ 0 & e^{-i(\pi/4)} \end{bmatrix} \times \begin{bmatrix} \cos\frac{\pi}{4} & \sin\frac{\pi}{4} \\ -\sin\frac{\pi}{4} & \cos\frac{\pi}{4} \end{bmatrix} \begin{bmatrix} \cos(-\frac{\pi}{4}+\theta) \\ \sin(-\frac{\pi}{4}+\theta) \end{bmatrix} E_0^{i\omega t}. \quad (1)$$

Here, E_0 and $\bar{\omega}$ are the amplitude and the angular frequency of the light vector, respectively.

Then, the intensity equation of the light detected at a point on a sample (as seen by the CCD camera) $I(\theta)$ can be written as

$$I(\theta) \equiv |E_x|^2 + |E_y|^2 = I_0 \sin^2\left(\theta + \frac{\delta}{2}\right), \quad (2)$$

where $I_0 = E_0 E_0^*$ is the amplitude of the intensity.

As seen in Eq. (2), the intensity will vary squared-sinusoidally with the changes in modulation angle of the rotating polarizer, θ. This intensity equation can be illustrated like Fig. 3, and the azimuthal phase retardation, δ can be calculated from Eq. (2) when the intensity distribution is zero as

$$I(\theta) = I_0 \sin^2\left(\theta + \frac{\delta}{2}\right) = 0, \quad (3)$$

$$\delta = -2\theta_{\min}, \quad (4)$$

where $\theta_{\min}$ is the modulation angle of the rotating polarizer with minimum intensity.

Generally, a laser which has narrow spectral width is an effective optical source in the polarization microscope system because polarization states of polarization components such as polarizer, wave plate, and liquid crystal depend on wavelengths of optical source.[14] So, a randomly polarized He–Ne laser with 7 mW average power (JDS Uniphase Corp.) has been used as a light source in the azimuthal polarization microscope system. As a coherent imaging system exhibits a ringing effect at a sharp edge of an object due to diffraction, a rotating diffuser (Korea Electro-Optics Corp.) is used to make the setup an incoherent imaging system. Unwanted speckle pattern on the image is effectively eliminated by rotating the diffuser during measurement.[14,16]

The scattered light from the rotating diffuser is collected by a collimating lens. A rotating polarizer (linear, $\lambda = 632.8$ nm, Thorlab) aligned to 45 deg with respect to the x-axis is placed after the collimating lens and controlled by a motion controller. A quarter wave plate (Zero order, $\lambda = 632.8$ nm, Thorlab) aligned to 45 deg with respect to the x-axis are placed after the rotating polarizer and fixed analyzer (linear, $\lambda = 632.8$ nm, Thorlab) aligned to 45 deg with respect to the x-axis are placed after the objective lens (Edmund Optics). The magnified images of a sample are detected by a CCD camera (640×480 pixels, COHU) through the analyzer and are digitized by a frame grabber with 8 bit resolution. In order to remove the interference effect between the CCD sensor and its protecting cover glass, the cover just in front of the CCD camera is removed.[14,16] The acquired intensity data of each pixel is fitted with a squared-sinusoidal function of the input polarization angle, θ to calculate the azimuthal phase retardation, δ of each pixel according to Eqs. (3) and (4).

3 Preparation and Imaging of Biological Cells

Azimuthal phase retardation distributions in human breast cancer cells and human stem cells as the biological materials, were measured with a newly developed azimuthal phase retardation microscope because movement speeds of the cells are below 10 nm/s, which is appropriate for measurement by the azimuthal phase retardation microscope. The human breast cancer cells, MDA MB 231s, were used in this measurement. The cells were grown on 18 mm square glass coverslip in 60 mm culture dish filled with growth mediator for 2 days after planting. For live cell observation under the azimuthal phase retardation microscope, the glass coverslip was attached with silicon vacuum grease at its edge to glass slide with a thin space. The space between coverslip and slide was filled with Tyrode's solution (Sigma). After sealing, the prepared cells can be observed for several hours without visible loss of cell architecture. Figures 4(a) and 4(b) show the photographic image and the azimuthal phase retardation distribution of breast cancer cells, MDA MB 231s, which were measured with the azimuthal phase retardation microscope by rotation of a single linear polarizer. In Fig. 4(b), the scale bar indicates the azimuthal phase retardation value (radian) of each breast cancer cell in Fig. 4(a). After acquisition of the azimuthal phase retardation image, a fluorescent dyeing process has been accomplished in each breast cancer cell in Fig. 4(a). The glass coverslip with the cells was fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) immediately after measuring azimuthal phase retardation. Also, the glass coverslip was incubated for 30 min at room temperature in Anti-Alpha Tublin Alexa Fluor 488 (Invitrogen) diluted 1:200 and Alexa Fluor 594 phallodin (Invitrogen) diluted 1:500 in PBS. The specimen was mounted on a glass slide and observed under the fluorescence microscope (IX71 and DP71, Olympus). In Figs. 4(c) and 4(d), red and green fluorescent images indicate actin filaments and microtubules of the MDA MB 231s in Fig. 4(a), respectively. By definition of the azimuthal phase retardation, plus and minus symbols of the azimuthal phase

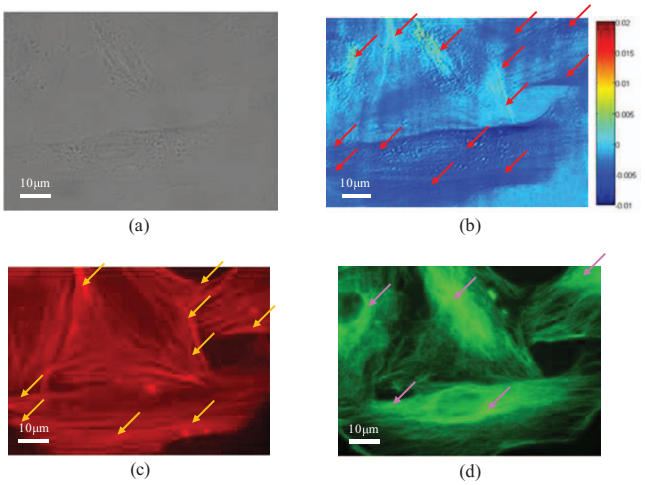


Fig. 4 (a) Optical image; (b) azimuthal phase distribution; (c) red fluorescent image of actin filaments; (d) green fluorescent image of microtubules of highly invasive breast cancer cells, MDA MB 231s.

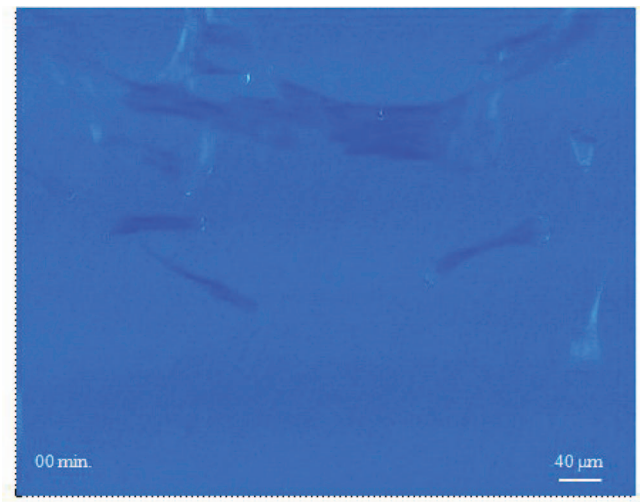
retardation in Fig. 4(b) mean vertically- and horizontally-formed cytoskeletons.

4 Azimuthal Phase Retardation Images of Biological Cells

By the definition of the azimuthal phase retardation, plus and minus symbols of the azimuthal phase retardation in Fig. 4(b) mean vertically- and horizontally-formed cytoskeletons. In this sense, Fig. 4(b) resembles Figs. 4(c) and 4(d). Especially, from the measured distribution of the azimuthal phase retardation of breast cancer cells, we can confirm that mapping of azimuthal phase retardation characteristics [see arrows in Figs. 4(b)–4(d)] can visualize the structure of actin filaments in breast cancer cells which generally does not appear in photographic image by light microscopes.

Also, we observed movement of human umbilical cord blood derived mesenchymal stem cells (hUCB–MSCs) and Video 1 shows the movement of hUCB–MSCs by measuring azimuthal phase retardation distributions. During the measurement, a humidified atmosphere with 5% CO_2 was provided in a confocal dish with the cells which were placed on a temperature (37 °C) controlled stage on a polarization microscope. The measurement was accomplished with a time interval of 1 min for 39 min.

From the measurement of azimuthal phase retardations of MDA MB 231s and hUCB–MSCs, we can demonstrate that newly developed azimuthal phase retardation can be a novel tool for visualization of actin filaments' structure in cells and for observation of biological cells' reaction to external conditions such as drugs, heat, etc., although its system is very simple.



Video 1 Movement visualization of hUCB–MSCs by measuring azimuthal phase retardation distributions. (WMV, 3.4 MB) [URL: http://dx.doi.org/10.1117/1.3615665.1]

5 Conclusions

In summary, we have developed a new and simple azimuthal phase retardation microscope by a single polarization rotation method which has special advantage to analyze the structural order of biological cells and tissues, without having to treat with exogenous dyes, fluorescence labels, and stains. The theory is simple in that a series of intensity of a pixel on a CCD, measured while rotating a polarizer, is fitted to a sine square curve to find rotation angles of the polarizer at the minima of the curve. From the intensity minima, the azimuthal phase retardation of the sample area corresponding to the pixel is obtained. To demonstrate the utility of the microscope, we visualized the azimuthal phase retardation image of human breast cancer cells, MDA MB 231s. Also, the azimuthal phase retardation image of MDA MB 231 cells have been compared with red and green fluorescent images for actin filaments and microtubules of the same cells. Also, we observed the movement of hUCB–MSCs by measuring azimuthal phase retardation distributions. From measured data, it has been verified that cytoskeletal structure in highly invasive breast cancer cells and stem cells, which generally does not appear in photographic image by light microscopes, can be visualized by a newly developed azimuthal phase retardation microscope. Especially, actin filaments of cells are seen clearly in the azimuthal phase retardation distribution of breast cancer cells. Hereby, it can be demonstrated that the azimuthal phase retardation microscope can make it possible to observe and visualize reactions of biological cells on drugs and variation mechanisms of actin filaments in movements of biological cells.

Acknowledgments

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