Mueller matrix polarimetry for characterizing microstructural variation of nude mouse skin during tissue optical clearing

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Abstract: We investigate the polarization features corresponding to changes in the microstructure of nude mouse skin during immersion in a glycerol solution. By comparing the Mueller matrix imaging experiments and Monte Carlo simulations, we examine in detail how the Mueller matrix elements vary with the immersion time. The results indicate that the polarization features represented by Mueller matrix elements m22&m33&m44 and the absolute values of m34&m43 are sensitive to the immersion time. To gain a deeper insight on how the microstructures of the skin vary during the tissue optical clearing (TOC), we set up a sphere-cylinder birefringence model (SCBM) of the skin and carry on simulations corresponding to different TOC mechanisms. The good agreement between the experimental and simulated results confirm that Mueller matrix imaging combined with Monte Carlo simulation is potentially a powerful tool for revealing microscopic features of biological tissues.

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1. Introduction

Polarization imaging methods are increasingly applied in the field of biomedicine [1]. Especially, through analyzing the Mueller matrix large amounts of information about the microstructures and optical properties of the tissues can be extracted [2]. It has been demonstrated that Mueller matrix imaging is capable of differentiating abnormal tissues and revealing the pathological microscopic origins [3–6]. In previous studies, we have developed a sphere-cylinder birefringence model (SCBM) for anisotropic tissues and the corresponding polarization-sensitive Monte Carlo simulation program, which have been used to investigate the polarization features and explore the microstructural origin of pathological abnormality in several tissues [7–9].

Tissue optical clearing (TOC) is a technique to improve the penetration depth, resolution and contrast of tissue images by applying high refractive index and/or hyperosmotic liquid, i.e. optical clearing agent (OCA), to highly turbid samples [10]. Although TOC has demonstrated its potential applications in diagnosis, its mechanism remains elusive [11]. Many groups have tried to explain how OCA affects the microstructure of the tissues during the optical clearing process [12–15]. For example, the transmittance and reflectance spectra have been used to extract the scattering properties of tissues during TOC, and to monitor the process of OCAs penetrating the tissue [13]. Transmission electron microscopy (TEM) is also a common technique for examining the microstructure, such as collagen fibril distribution [14]. Since polarization sensitive methods can extract much richer microstructural and optical information of tissues [2], they are potentially powerful tools for characterizing the microstructural changes of tissues during the optical clearing process [16,17].

In this paper, we take in vitro Mueller matrix images of nude mouse skin during TOC and carry out Monte Carlo simulations using scattering models corresponding to different TOC mechanisms. By comparing the polarization features in experiments and Monte Carlo simulations, we are able to acquire large amounts of information about the microstructures of the tissues.

2. Methods

2.1. Experimental setup and nude mouse skin sample

The backscattering Mueller matrices are measured using the dual rotating retarder configuration [18], as shown in Fig. 1(a). The 633 nm LED source (3W) is collimated by the lens L1 (Thorlabs, USA), then modulated by the polarization state generator (PSG), which consists of a fixed linear polarizer (extinction ratio>1000:1, Thorlabs, USA) and a rotatable quarter waveplate QW1 (Thorlabs, USA), before illuminating the sample. Part of the backscattered light passes through the polarization state analyzer (PSA), which consists of a rotatable quarter waveplate QW2 (Thorlabs, USA) and a fixed linear polarizer P2 (extinction ratio>1000:1, Thorlabs, USA), then collected by the lens L2 and recorded by the CCD camera (QImaging 32-0122A, 12 bit, Canada). There is an oblique angle $\theta$ of 20 deg between the illumination light and the detection direction to avoid the sample surface reflection.
Fig. 1. The backward detection Mueller matrix imaging configuration. Source (LED, 633nm, 3W), L1 and L2 are lens (Thorlabs, USA), P1 and P2 are linear polarizers (extinction ratio>1000:1, Thorlabs, USA), QW1 and QW2 are quarter waveplates (Thorlabs, USA), and CCD (QImaging 32-0122A, 12 bit, Canada) (a). The photograph of nude mouse skin samples before immersion (left) and after immersion for 20 min in 80% glycerol solution (right), respectively (b).

During the experiment, the polarizers P1 and P2 are both fixed in the horizontal direction, and the waveplates QW1 and QW2 rotate with a 5:1 ratio of rotation angle. The intensity is calculated as a Fourier series,

\[ I = \alpha_n + \sum_{n=1}^{12} (\alpha_n \cos 2n\theta + \beta_n \sin 2n\theta) \]

where \( \alpha_n \) and \( \beta_n \) are the Fourier coefficients from which the Mueller matrix elements can be calculated [19]. The system is calibrated by measuring the Mueller matrices of standard samples such as air or an waveplate in the transmission mode [20]. After the calibration, the PSA arm is rotated to the back-scattering configuration for measuring the tissue samples as shown in Fig. 6(a).

We cut the skin samples from newly sacrificed nude mice into slices of 1.5 × 1.0 cm² area, then remove the subcutaneous fat layers with tweezers. The sample thickness is about 0.5 mm. For the TOC operations, we immerse the skin sample in a 80% glycerol solution for 0 to 30 min, then take it out and wipe off the remaining OCA from the surface. The sample is put on a piece of glass with the dermal side touching the glass for backscattering Mueller matrix measurements. Figure 1(b) is the photograph of skin samples before immersion (left) and after immersion for 20 min (right), respectively. Note, due to the angle of 20 degrees between the incident and detecting arms, reflection from the sample surface will not be detected. All animal care and all experimental protocols were performed in accordance with the guidelines of the Animal Ethics Committee of Tsinghua University, and have been approved by the Committee.

2.2. FDHs of the Mueller matrix elements

Mueller matrix is a comprehensive representation on the polarization properties and encode rich information about micro-structures of samples. Since the tissue optical clearing process may involve different mechanisms corresponding to complex changes in the micro-structure of the sample, in this study we use all the Mueller matrix elements, instead of the few parameters derived from the decomposition method, to characterize such micro-structural variations. Although the detailed textures also encode qualitative microstructural features of the 2D images, we may regard the mouse skins as homogeneous samples and characterize quantitatively their microstructures by the frequency distribution histogram (FDH) of each...
MME [21]. The FDHs show the probabilities for pixels of an image falling into a particular intensity range. The peak, width, and shape of FDH curves may all contain information on the tissues. The average values and standard deviations of the Mueller matrix elements (MMEs) correspond to the peak positions and widths of the FDHs respectively. The MMEs encode rich information about the tissue. For example, $m_{22}$ & $m_{33}$ & $m_{44}$ values are related with the depolarizing ability [8], $m_{12}$ & $m_{13}$ & $m_{21}$ & $m_{31}$ values are connected to the anisotropy induced by aligned fibrous scatterers and $m_{24}$ & $m_{34}$ & $m_{42}$ & $m_{43}$ values are closely connected to the anisotropy induced by the birefringence [22]. We can determine the axis direction of aligned fibers through the signs and intensities of $m_{12}$ and $m_{13}$ [23], and the direction of optical axis through those of $m_{24}$ and $m_{34}$, similarly.

2.3. Monte Carlo simulation

Due to the complexity of the structures and properties of biological tissues, it is hard to solve the problem of light transport in turbid biological tissues with electromagnetic theory. Monte Carlo simulations offer a flexible, yet rigorous approach to examine in detail the behaviors of photons as they transport in turbid tissues and solve the positive and inverse problems of Vector Radiation Transport Equation (VRTE), unaffected by the morphology and boundary conditions of the sample [24,25]. By establishing appropriate scattering models to mimic the complex microstructure of the sample and the mathematics equations corresponding to each step of the stochastic photon transport process, we can trace the spatial and polarization features of the photons and systematically study the relationship between the polarization and structural parameters of tissues. The combination of polarization measurements and Monte Carlo simulations provides a powerful tool for probing the microscopic structures of tissues and revealing the physical origin of TOC.

In previous studies, we have established a series of scattering models and the corresponding Monte Carlo simulation programs to study the polarized photon scattering in complex anisotropic turbid media. The scattering models consist of spherical and infinitely-long cylindrical scatterers [7], which mimic the scatterings by particles such as the cells and intracellular organelles and by fibrous structures such as skeletal muscle fibers [26,27], and an interstitial medium, which takes into account all the none scattering effects during the propagation of the photons such as birefringence and optical activities [28,29]. The models are simple, but very versatile for adapting to different types of turbid media. The sizes, densities and optical properties of the scatterers, the spatial orientations and degree of alignment of the cylinders, the anisotropic optical properties of the interstitial media are all possible variables in the simulations. It has been demonstrated by simulations that apart from the birefringence of the interstitial media, the size, density [30] and birefringence [31] of well aligned cylindrical scatterers may also contribute to the anisotropic optical properties of the sample. Monte Carlo simulations using appropriate scattering models allow us to separate these different contributions and identify from the polarization images of tissue’s specific microstructures which correspond to pathological features of cancers [8,9]. Monte Carlo simulations will help us understand how the skin’s microstructure changes during the immersion.

According to the literature [32,33], the average scattering properties of the skin are defined by the scattering properties of the dermis, which mainly consists of fibrous structures where collagen fibrils are packed in collagen bundles and form lamellae structure. Both Rayleigh scattering and Mie scattering exist in the skin. The sphere-cylinder birefringence model (SCBM) is applicable to simulate such anisotropic fibrous tissues [22,28]. The SCBM model of skin consists of spherical and cylindrical scatterers which simulate the scatterings by collagen fibers and cell organelles, and an interstitial birefringent medium. The detailed simulation parameters are shown along with the simulation results. Using a GPU program [34], each sphere-cylinder scattering single-precision simulation for 10$^7$ photons for four groups based on single-kernel scheme only takes about 38.8 seconds.
3. Results and discussion

3.1. Two-dimensional images of Mueller matrix elements (MMEs) of the skin sample

Firstly, we obtain two-dimensional images of backscattering Mueller matrices of the skin tissues at different immersion times, as shown in Fig. 2. The MMEs are normalized at each pixel to m11. The Mueller matrix images immediately show some characteristic features, e.g. their diagonal elements m22 & m33 & m44 vary during the immersion, and the values of m34 & m43 are relatively large. These features are characteristic of the microstructure of the skin tissues and will be used in the following analysis.

![Fig. 2. Two-dimensional backscattering Mueller matrix images of the skin tissues at different immersion times: 0 min (a), 4 min (b), and 8 min (c) (the image size is 400 × 400 pixels; θ = 20 deg.).](image)

3.2. Frequency diagram histograms (FDHs) of the Mueller matrix elements and microstructures of the skin sample

To display more clearly the changes of MMEs, we choose an area of 100 × 100 pixels on the images to obtain the FDH curves at different immersion times. Figure 3 shows the Mueller matrices in FDH corresponding to 0 min, 4 min and 8 min immersion times respectively. The following features are becoming evident as the immersion time increases. (1) The FDH curves of m22 & m33 & m44, and m34 & m43 change significantly. (2) The values of the diagonal elements m22 & m33 & m44 increase indicating the decrease of depolarization as the results of TOC [8], and the values of m22 & m33 are greater than m44 reflecting that the scattering characteristics of the tissue are dominated by Rayleigh scattering [35]. (3) The absolute values of m34 & m43 and m12 & m21 increase indicating an increase in the tissue anisotropy during immersion, which can also be confirmed by the increasing difference between m22 and m33 [22] (the difference between the central values of the FDH curves of m22 & m33, which are marked by black solid lines showing their values in Fig. 3, increases from 0.01 (0 min) to 0.12 (8 min)). (4) The variations of m34 & m43 are much larger than those of m12 & m21, meaning that the tissue anisotropy and its change during immersion are mainly due to the birefringence rather than scattering by well aligned fibrous structures [22]. Compared with the 2D images of the MMEs, the semi quantitative FDH curves provide more characteristic information.
We further calculate the averages and standard deviations of the MMEs to examine quantitatively how the polarization properties of the skin vary with the immersion time. Figure 4 shows the kinetic changes in the FDHs as functions of the immersion time. The central value and the half width represent the average value and standard deviation (SD) of the FDH, respectively. The quantitative data in Fig. 4 confirm the qualitative conclusion obtained from the Mueller matrix images and FDH curves in Figs. 2 and 3 and demonstrate quantitatively how the immersion in the glycerol solution affect the polarization properties of the tissue samples. In addition, from the negative averages of $m_{12}$ & $m_{21}$ and the averages of $m_{13}$ & $m_{31}$ being close to zero, it can be determined that the fibrous scatterers are aligned around the y-axis direction [23], and from the negative average of $m_{34}$ and the much smaller absolute value of $m_{24}$, it is confirmed that the optical axis is also around the y-axis direction. Figure 4 clearly shows that the variations of the MMEs of the skin, or TOC, mainly happen within the first 10 min immersion time. The optical properties of the tissues stay stable after 10 min, and the small changes in $m_{24}$ and $m_{42}$ are due to variations in the direction of optical axis.
To be noted, diffuse reflection at the skin surface contributes very little to the experimental results [36], since the interaction time of optical clearing (mainly refractive index matching (RIM)) on the surface of the skin is only seconds and the time for optical clearing acting on the inner part of the skin usually lasts several minutes or more, which thus determines the experimental results. Also, as seen in Fig. 1(b), the surface of the nude mouse skin sample is relatively flat resulting in smaller diffuse reflection.

3.3. Monte Carlo simulations of the characteristic polarization features corresponding to different TOC mechanisms

To understand how the immersion in the glycerol solution changes the microstructure and hence the polarization characteristics of the skin sample, we simulate the backscattering Mueller matrices using the SCBM model which approximates the skin to a mixture of spherical and infinitely long cylindrical scatterers embedded in a birefringent interstitial medium. Following different TOC mechanisms proposed by various authors [12–14], we can set up the scattering models of characteristic microstructures and examine in detail how they affect the polarization properties of the sample. The simulations are compared with the experimental results shown in Fig. 4 to confirm their validities.

According to previously published data [32,33] and our own studies [22,28], the SCBM model parameters before the immersion are set as follows. The diameters of the scatterers are 0.2 \( \mu \)m for the spheres and 1.5 \( \mu \)m for the cylinders. The scattering coefficients for spherical and cylindrical scatterers are 20 cm\(^{-1}\) and 180 cm\(^{-1}\), respectively. The cylindrical scatterers are aligned along the y-axis direction. Considering that the collagen fibers are packed in bundles and arranged in a lamellae structure [32,33], the directions of the cylinders are allowed to fluctuate by 40 deg within the lamellae but by 5 deg perpendicular to the lamellae. The refractive index (RI) are 1.43 for both types of scatterers. For the interstitial medium, RI is 1.35, birefringence is \( 3.0 \times 10^{-5} \), and the optical axis is in the y-axis direction. The thickness of the sample is 0.5 mm. The incident wavelength is 633 nm and the number of photons for a simulation is \( 10^7 \). It should be noted that the initial birefringence value is chosen according to the experimental results shown in Fig. 3.
Since scattering in biological tissues is originated from the microscopic fluctuations in RI, primarily the differences in RIs between the scatterers and the surrounding medium, refractive index matching (RIM) is considered to be one of the main mechanisms of TOC [13]. When the nude mouse skin sample is immersed in the glycerol solution, water in the skin will transport into the glycerol solution because the glycerol solution is hyperosmotic and make the skin dehydrated. This causes the increases in the density of the tissue fluid and its RI. In the meantime, glycerol molecules will migrate into the interstitial fluid and increase its RI. These two factors will improve the degree of RIM between the scatterers and the interstitial fluid, resulting in reduced tissue scattering. We simulate the RIM process by increasing the RI of the medium from 1.35 to 1.40 at 0.01 intervals, corresponding to the RIM stage progress from the first to the sixth stage, as shown in Fig. 5. The behaviors revealed in the simulations and a comparison with the experimental results (as shown in Fig. 4) are as follows. As the degree of RIM increases, (1) the values of $m_{22} & m_{33} & m_{44}$ and $m_{34} & m_{43}$ change significantly, which are consistent with the experimental results, (2) $m_{22} & m_{33} & m_{44}$ increases, which reflects a decrease in the depolarizing ability of the medium due to the reduction of scattering by RIM, but the changes look slightly less than the experimental results, (3) the absolute values of $m_{34}$ and $m_{43}$ increase, indicating an increase in the birefringence-induced anisotropy or retardance which is caused by the increase of mean free path in weaker scattering [37], but the increase in the absolute values of $m_{34} & m_{43}$ is smaller than what was observed in the experiments, (4) the difference between $m_{22}$ and $m_{33}$ increases slightly, which is much smaller than the experimental result.

Fig. 5. The variation of MMEs with RIM between the scatterer and the surrounding medium by Monte Carlo simulations. The horizontal coordinates are the clearing stage numbers, corresponding to the RI of the medium increased from 1.35 to 1.40 at 0.01 intervals. The map in $i^{th}$ row and $j^{th}$ column corresponds to $m_{ij}$.

Therefore although the RIM process seems responsible for the increase of $m_{22} & m_{33} & m_{44}$ during the TOC period, it cannot explain quantitatively the variations of $m_{34} & m_{43}$, $m_{12} & m_{21}$, and the difference between $m_{22}$ and $m_{33}$ well. Since $m_{34} & m_{43}$ and $m_{12} & m_{21}$ are related mainly to the anisotropic properties induced by birefringence and scattering of the aligned-cylinders respectively [22], we also need to consider effects due to the variations of the interstitial birefringence and cylindrical scatterers during the immersion. It has been
reported [14] that the immersion by OCA will make the fibrils in the skin packed more densely, which should improve the alignment of cylindrical scatterers and increase the birefringence value. Therefore, we should take into account of these effects in the simulations.

Figure 6 shows the simulated Mueller matrices with all the three possible TOC mechanisms being considered, i.e. increasing RI of the medium from 1.35 to 1.40 at 0.01 intervals, increasing the interstitial birefringence from $3.0 \times 10^{-5}$ to $6.0 \times 10^{-5}$ at $0.5 \times 10^{-5}$ intervals and decreasing the orientation fluctuation of the cylindrical scatterers in the y-axis direction from 40 deg to 15 deg at 5 deg intervals. The experimental results are also plotted in the same graph for direct comparison. The results show that the simulations accurately reproduce some of the experimental features, such as the varying trends and amplitudes of the diagonal elements $m_{22}$ & $m_{33}$ & $m_{44}$, which reflect the depolarization, and off-diagonal elements $m_{34}$ & $m_{43}$, which reflect the birefringence-induced anisotropy. The relative values of $m_{12}$ & $m_{21}$ & $m_{34}$ & $m_{43}$ and $m_{22}$ & $m_{33}$ & $m_{44}$ by both simulations and experiments also agree qualitatively with each other, although there are discrepancies in the varying amplitudes of elements $m_{12}$ & $m_{21}$. Simulations including the three TOC mechanisms can explain the dominant polarization features of the skin during the first 10 min of immersion in the glycerol solution. During the TOC process, the increasing degree of RIM between the scatterer and the interstitial medium reduce the tissue scattering coefficient which results in a reduction in depolarization as well as increasing the mean free path of the photons. In the meantime, TOC also changes the tissue anisotropy. The increase of the interstitial birefringence enhanced by the increase in mean free path takes the major contribution in anisotropy while the improving alignment of fibrous scatterers takes a minor contribution. To be noted, considering some other possible microstructural and optical changes during clearing, we also investigate such factors as variations of the sample thickness, the diameter of cylindrical scatterers and the ratio between the spherical and cylindrical scatterers. It indicates that the impacts of these factors on the polarization features are rather small, which further verifies that the feasible polarization scattering explanation is the co-action of RIM, the increase of the interstitial birefringence and the alignment of the cylindrical scatterers.
4. Conclusion

We investigate the variations of polarization features of nude mouse skin immersed in a glycerol solution for different time periods by measuring the backscattering Mueller matrices, and quantitatively analyzing by Monte Carlo simulations the polarization features corresponding to different changes in the microstructures of the skin during the TOC process. The experimental results show that during immersion, the diagonal Mueller matrix elements $m_{22}$ & $m_{33}$ & $m_{44}$ increase indicating a decrease in the depolarizing ability of the sample, the absolute values of $m_{34}$ & $m_{43}$ and $m_{12}$ & $m_{21}$ increase, which reflect increases in the birefringence-induced and aligned fiber scattering induced anisotropies, respectively, and the increasing difference between $m_{22}$ and $m_{33}$ also indicates the increase of the anisotropy. The absolute values and variations of $m_{34}$ & $m_{43}$ are much larger than those of $m_{12}$ & $m_{21}$, which indicate that the skin anisotropy is dominated by the interstitial birefringence.

Monte Carlo simulations using properly designed sphere-cylinder birefringence models (SCBM) provide the insight on the relations between the polarization features and the microstructure of the skin during the immersion. Based on previously reported mechanisms for TOC, i.e. increasing RIM between the scatterer and the surrounding medium and more closely packed collagen fibrils in the skin, we simulate the behaviors in Mueller matrices corresponding to changes in RIM, the interstitial birefringence and the alignment of cylindrical scatterers. Comparisons between the experimental and the simulated Mueller matrices show that increasing RIM, birefringence of the interstitial medium and alignment of the fibrous structures may all contribute to the effect of TOC. RIM reduces the depolarizing ability of the tissue and increases the photons’ mean free path, which, in conjunction with the increased interstitial birefringence and alignment of the cylindrical scatterers, increases the transparency and anisotropy of the tissue. This study proves again that the Mueller matrix
imaging combined with Monte Carlo simulation, is potentially a powerful method to connect the microstructure of biological tissues with their polarization optical features, and shows a wide range of further clinical applications.

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**Competing financial interests**

The authors declare that there are no conflicts of interests related to this article.