Axially resolved complete Mueller matrix confocal microscopy

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We introduce a technique that is capable of obtaining complete polarization-sensitive three-dimensional images that could reveal unknown anatomical conditions of living tissue that possess polarization-dependent signatures. Previously, the 16 Mueller coefficients were measured independently only by use of two-dimensional imaging techniques. We also present the experimental combination of a depth-resolved confocal imaging system with a complete Mueller matrix polarimeter. To calibrate the system, a double-pass method had to be implemented. We also indicate, experimentally, that the confocal sectioning of the system has a degrading effect on axially resolved Mueller matrix measurements. © 2006 Optical Society of America

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1. Introduction
Most of the conventional imaging systems used in a wide variety of applications such as biomedical imaging and material analysis can record only the intensity or phase of light that has been scattered or emitted from the sample under observation. Hence, some biological tissues and materials appear to be homogenous even when they may possess some kind of internal structure. Polarization-sensitive imaging is a form of optical inspection that can reveal features in a sample that appear invisible to intensity or phase detection systems. Its combination with a three-dimensional imaging technique results in a powerful high-dimensional detection system for material characterization or biological inspection and diagnosis.

Recent developments in polarization-sensitive imaging have given rise to polarization-sensitive optical coherence tomography (PS-OCT) systems.1–8 One of the advantages of optical coherence tomography is the rejection of the multiply scattered light that is no longer coherent after it returns from the sample. However, this also prevents current PS-OCT systems from measuring the depolarized part of the light that returns from the sample and thus from measuring the complete Mueller matrix of the sample. The results, however, have been commonly stated in terms of Stokes vectors and Mueller matrices, but to date, with PS-OCT only a subset of the 16 Mueller matrix coefficients has been measured. The definition of the Stokes vector in terms of irradiances makes the Mueller calculus most suitable for describing irradiance-measuring instruments.9 Incomplete interpretations of polarization-dependent features may arise if the limitations of the measuring instrument are not taken into account.

A Mueller matrix is a $4 \times 4$ matrix that represents the effect of a sample on the state of polarization of light as a linear operator that acts on a Stokes vector. The Mueller calculus can account for partially polarized incident beams and can handle the propagation through depolarizing optical systems. The 16 elements of a Mueller matrix are, in general, linearly independent. Thus, if no assumptions are made about the effect of a sample on the state of polarization of light, and if a complete characterization of the specimen is required, the 16 Mueller matrix elements need to be measured individually. In this case the polarimetry measurements are said to be complete (and incomplete otherwise).

In this paper we introduce a technique that can measure the complete Mueller matrix of a sample at different depths within the specimen. Previously the 16 Mueller coefficients were measured only with two-dimensional imaging techniques. The novelty of our work is the combination of a complete Mueller matrix polarimeter with a confocal microscope. We devel-
developed an instrument that we expect will be capable of measuring the complete Mueller matrix of contiguous planes of a biological sample. Of our particular interest is the identification of nonhealthy conditions of the human eye in vivo that led to specific decisions in the design of our experimental system. However, the technique is not limited to that particular application.

The confocal microscope used in the research reported in this paper was built in the reflection configuration, which is a requirement for obtaining Mueller matrix depth-resolved measurements in the paraxial approximation regime. In a transmission confocal microscope, the light must pass through the whole thickness of the sample (along the optical axis) before being detected, and the cumulative effect of the sample on the state of polarization of light would be the same for all axial positions. In microscopes with sufficiently high N.A. this restriction might not apply, but as a first approximation the reflection configuration is necessary to achieve complete polarization-sensitive depth sectioning. This need imposed a new requirement for the calibration that had not been addressed before. For the calibration of the double-pass polarimeter, we implemented a modified version of the eigenvalue calibration method (ECM) described previously by Compain et al. The method is highly robust and applicable to any polarimeter configuration.

The cumulative effect of the sample on the state of polarization and the double-pass nature of the measurements gave place to a new inverse problem concerning the disentanglement of the complete Mueller matrices measured from contiguous axial positions. We venture here on a first attempt to outline some difficulties that might arise in future investigations to solve this problem.

2. Experimental Setup: Mueller Matrix Polarimeter

The Mueller matrix polarimeter that was built was similar to one implemented by Delplancke in 1997. We also used two Pockels cells as linear variable retarders in the polarization state generator (PSG) and a division-of-amplitude polarimeter (DOAP) with nonpolarizing beam splitters for the polarization state analyzer (PSA). Significant modifications were introduced in our design, and they are explained below. This type of instrument can obtain measurements at high temporal frequencies. The typical rise time of a Pockels cell is of the order of 1 ns, and the DOAP is limited only by the speed at which the signal on each photodetector can be recorded and by the photon flux. There are no moving parts in this type of design, which simplifies its assembly and helps to make the calibration robust. The possibility of obtaining Mueller matrices at high acquisition rates made this type of polarimeter our choice of design because one of our ultimate motivations is to inspect biological samples in vivo.

A. Polarization State Generator

A schematic diagram of the experimental system is shown in Fig. 1. Light was generated with a 532 nm frequency-doubled diode-pumped solid-state laser (Melles Griot 58 GCS), and a Glan–Taylor prism (Melles Griot 03PTA401), oriented at 90°, ensured an initial high degree of polarization purity. Linear vertically polarized light that emerged from the Glan–Taylor prism passed through two electro-optical modulators (transverse Linos LM0202 Pockels cells) that acted as linear variable retarders. The two Pockels cells were combined successively to modulate the state of polarization of light. The fast axes of the first and the second Pockels cells were aligned at 45°.
Eq. (2), the generated states of polarization were re-
modulated set of states of polarization. Clearly, from
the retardances of the two Pockels cells
the PSG resulted in a Stokes vector that depended on
defining the transmittance of each modulator as
by azimuth angle
where

\[ \mathbf{R}(\theta) = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & \cos \Delta(t) & \sin \Delta(t) \\ 0 & 0 & -\sin \Delta(t) & \cos \Delta(t) \end{bmatrix} \times \mathbf{R}(-\theta), \tag{1} \]

where \( \mathbf{R}(\theta) \) is the matrix that rotates a Stokes vector by azimuth angle \( \theta \) about the \( z \) axis. Using Eq. (1) and
defining the transmittance of each modulator as unity, we found that the light that passed through
the PSG resulted in a Stokes vector that depended on
the retardances of the two Pockels cells \([\Delta_1(t) \text{ and } \Delta_2(t)]\) as

\[ \mathbf{S}_{\text{PSG}}(t) = \begin{bmatrix} 1 \\ -\cos \Delta_1(t) \\ -\sin \Delta_1(t) \sin \Delta_2(t) \\ -\sin \Delta_1(t) \cos \Delta_2(t) \end{bmatrix}. \tag{2} \]

To generate a complete set of incident states of
polarization with this PSG we set the values of the
retardances to

\[ \Delta_1(t) = 2o_0 t - 3\pi/2, \tag{3} \]
\[ \Delta_2(t) = o_0 t - 3\pi/2, \tag{4} \]

where slope \( o_0 \) defines the angular frequency of the
modulated set of states of polarization. Clearly, from
Eq. (2), the generated states of polarization were repeated
every \( T_0 = 2\pi/o_0 \) time units. The main advantage of this type of modulation is that, with the
PSA used in our setup, the elements of the Mueller
matrix to be measured were linear functions of a set of
only six Fourier coefficients of the detected intensity
signals. In Delplancke’s PSG\(^{11} \) the retardances produced with the Pockels cells were sinusoidal functions,
disadvantageously relating the Mueller matrix elements to an infinite set of harmonics to be detected
and therefore neglecting the high-frequency terms.

The periodicity of the sine and cosine functions in
Eq. (2) permitted emulation of the monotonically in-
creasing retardances by use of sawtooth functions. In
practice, the time-varying voltage signals applied to
the Pockels cells were sawtooth functions. A graph of
one cycle of the sawtooth signals used in the experi-
ment is shown in Fig. 2(a). A full cycle consisted of a
sequence of 256 different states of polarization, which
are represented by the small dots on the Poincaré
sphere in Fig. 2(b). The arrow indicates the direction of the modulation as time progressed, and the large
dot behind that arrow corresponds to an instantaneous (vertical) pair of retardances in (a). Retardances
at time \( t = 0 \) produced the large-dot on the sphere just behind the
arrow that indicates the direction of the modulation as time pro-
gressed.

**B. Polarization State Analyzer**

For every state of polarization that was incident
on the sample, a DOAP was used to simultaneously measure the complete Stokes vector of the light that
was scattered from the sample. The schematic diagram is also shown in Fig. 1. An important feature of the design that we chose is that a minimum amount of light is wasted when the Stokes vectors are measured. A nonpolarizing cube beam splitter (Newport 10BC16NP.3), Bs2, divided the beam into two equal branches. Along the first branch, a polarizing cube beam-splitter (Newport 10BC16PC.3), PBs4, was used to direct horizontal linearly polarized light to photodetector D1 and linear vertically polarized light to photodetector D2. In the second branch, another nonpolarizing beam-splitter cube, Bs3, divided the light again, without changing the state of polarization of the initial beam. For the last two branches a polarizer with its axis at 45°, P45, was placed before photodetector D3; and the combination of the beam splitter, a quarter-wave plate with its fast axis at 45°, and a linear horizontal polarizer resulted in a right-circular polarization analyzer placed in front of photodetector D4. Photodetectors D1–D4 (Si/PIN New Focus Model 2001) that measured the four optical signals were connected to the same data-acquisition board (Iotech Daqboard/2000) that was used to generate the voltage signals that modulated the retardation of the two Pockels cells. The two analog outputs of the board were updated synchronously relative to the four scanned input signals.

Since the introduction of the DOAP by Azzam in 1982,12 a number of papers concerning the optimization, calibration, performance, and application of DOAPs have been published.11,13,16–21 Its principle of operation is well understood and rather simple: Each of the four polarization analyzers in front of photodetectors D1–D4 can be represented by a Mueller matrix $\mathbf{D_i}$ ($i = 1, 2, 3, 4$). As it is only the intensity of light that can be measured by each detector, it is only the first Stokes component of the Stokes vector that measured Mueller matrix of all the possible possible passes through Bs1. After some algebraic manipulation, intensity vector $I(t)$ in Eq. (7) can be found to be a combination of only six Fourier harmonics:

$$I(t) = \mathbf{D}_{\text{PSA}} \mathbf{B}_{\text{out}} \mathbf{M}_{\text{sample}} \mathbf{B}_{\text{in}} \mathbf{S}_{\text{PSG}}(t),$$

where $\mathbf{B}_{\text{in}}$ and $\mathbf{B}_{\text{out}}$ are the $\mathbf{B}_1$ beam-splitter Mueller matrices that were either the $4 \times 4$ identity matrix or the Mueller matrix of a reflection. Envisaging that a future version of this instrument may be used in a clinical diagnosis environment in which ease of operation is more critical than in a research laboratory, we could dedicate the second branch that resulted after the first passage through $\mathbf{B}_1$ to calibration purposes. An additional mirror on this second branch could return the light into the PSA, and the system could be calibrated by use of the method explained in Section 3 below and the experimentally measured Mueller matrices of all the possible possible passes through $\mathbf{B}_1$.
This set of linear equations included the 16 unknown Mueller matrix coefficients of the sample, and the harmonics that constituted the modulated intensities recorded by each of the 4 detectors were a well-defined finite set. All the information needed to calculate the Mueller matrix of a sample was concentrated in the Fourier amplitudes of frequencies 0, \(\omega_0\), and 3\(\omega_0\) of the cosine terms and 2\(\omega_0\), and 3\(\omega_0\) of the sine terms. One advantage of the combination of the PSG and PSA described here is that the remaining Fourier coefficients were always zero, independently of the sample measured or of any linear errors in the system, which were compensated for by the calibration matrices discussed in Section 3 below.

The most widely used parameter in the evaluation and optimization of complete polarimeters is the condition number\(^{22}\) of the PSG and the PSA matrices.\(^{23–26}\) In the absence of systematic errors, the signal-to-noise ratio is maximum when the condition number is minimized.\(^{25,26}\) Nevertheless, it does not provide information about the overdetermination of the system or the number of times that each state of polarization is used or measured. The condition number can be used to compare PSAs restricted to four measurements to determine a Stokes vector, or PSGs that probe the sample with only four different states of polarization. The comparison of polarimeters that use different numbers of states of polarization should be done carefully; ultimately, operational restrictions and ease of implementation should also be considered.

The condition number for matrix \(D_{\text{PSA}}\) was 3.61. The PSG built during this study produced 256 states of polarization per modulation cycle of the Pockels cells. Nevertheless when it was combined with the PSA, all the information of the Mueller matrix of a sample was contained in 24 Fourier coefficients, 6 for each detector. Substituting the explicit time-varying retardances, \(\Delta_1\) and \(\Delta_2\) [Eqs. (3) and (4)], into Eq. (2) and using some basic trigonometric identities, we can use matrix \(Q\), which is defined by

\[
S_{\text{PSG}}(t) = \begin{bmatrix}
1 \\
\sin 2\omega_0 t \\
-\frac{1}{2}(\cos \omega_0 t + \cos 3\omega_0 t) \\
\frac{1}{2}(-\sin \omega_0 t + \sin 3\omega_0 t)
\end{bmatrix} = Q \begin{bmatrix}
1 \\
\cos \omega_0 t \\
\cos 3\omega_0 t \\
\sin \omega_0 t \\
\sin 2\omega_0 t \\
\sin 3\omega_0 t
\end{bmatrix},
\]

(10)

to evaluate the sensitivity of the PSG to errors in the calculated Fourier amplitudes. For our polarimeter, the condition number of \(Q\) was equal to \(\sqrt{2}\).

D. Modulation Parameters and Data Acquisition

The analog-to-digital signal-acquisition board (Daqboard/2000) could operate at a maximum sampling rate of 200 kHz distributed among the number of input channels used. Every time the modulation of the Pockels cells was started, a time delay of 5 s was introduced in the acquisition routine before the first measurement of the four photodetectors was recorded. This was done to let the voltage signals supplied by the Pockels cell amplifiers stabilize. For this reason, a fifth analog input channel of the board was used to monitor the beginning of every retardance modulation cycle. The signal-acquisition speed was limited by a maximum sampling rate of 40 kHz for each of the five input channels. Using the maximum detection sampling rate of 40 kHz and arbitrarily choosing 28 data points for every retardance modulation cycle, we calculated the discrete Fourier transform vector of the recorded signal at frequency intervals of 156.25 Hz. The result determined the experimental value for modulation angular frequency \(\omega_0\) that was defined in Eqs. (3) and (4):

\[\omega_0 = 2\pi(156.25 \text{ Hz})/\text{rad/cycle}.
\]

To reduce the influence of random experimental errors, every measurement consisted of a sequence of eight modulation cycles. The acquisition time for the data used to calculate a complete Mueller matrix was 51.2 \(\mu s\). For every recorded intensity signal, were computed the Fourier amplitude coefficients at integer multiples of the frequency 40/2048 kHz, using the Fastest Fourier Transform in the West algorithm built in Matlab 6.5. In accordance with Eq. (8) the cosine amplitudes of angular frequencies 0, \(\omega_0\), and 3\(\omega_0\) and the sine amplitudes of \(\omega_0\), 2\(\omega_0\), and 3\(\omega_0\) from the 4 detected signals were used to build an overdetermined set of 24 simultaneous equations and 16 unknowns. We found the best approximate solution, in the least-squares sense, of the set of simultaneous equations to obtain the 16 noncalibrated Mueller matrix coefficients of the measured sample.

3. Double-Pass Eigenvalue Calibration Method

The calibration method played a key role in the validation of the experimental results obtained in this study. It corrected all linear systematic errors introduced by the optical components in the nonconfocal polarimetry measurements and, as the calibration of the system was made in the exact configuration in which the polarimeter was used to obtain Mueller matrix measurements, it did not require two independent calibration routines, one for the PSG and one for the PSA. This advantage ensured that, after calibration, no additional optical elements were introduced (or removed) that could modify the state of polarization of the incident or detected light. In addition, two different matrices were computed to account for the linear errors in the PSA and the PSG independently. The calibration routine was based on the ECM developed by Compain \textit{et al.} in 1999,\(^{10}\) also described by De Martino \textit{et al.} in 2003.\(^{27}\) A necessary modification of the original ECM was introduced in the research reported here to extend its applicability to double-pass measurements.

Any experimental noncalibrated \(4 \times 4\) matrix (\(B_{\text{sample}}\)) or any raw data \(n \times m\) measurement matrix can be represented, as in Eq. (1) of De Martino \textit{et al.},\(^{27}\) by the product

\[\text{APPLIED OPTICS} 1921\]

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The matrices $W$ and $A$ contain the system errors associated with the PSG and the PSA respectively, and they may also contain all the polarimetry information associated with the specific designs of the PSG and the PSA. In the latter case, these two matrices may not be $4 \times 4$ matrices.\textsuperscript{10} The determination of these two matrices was the paramount result of the calibration method. The double-pass eigenvalue calculation method (DP-ECM) consisted of the same three main steps required in the original single-pass version\textsuperscript{10,27}:

1. The calibration samples were measured. In our work the samples were air, a linear horizontal polarizer, a linear vertical polarizer, and a linear retarder with the fast axis at $30^\circ$ ($B_{ih}$, $B_{iv}$, $B_{ih}$, and $B_{iv}$), as first proposed by De Martino et al.\textsuperscript{27}

2. The Mueller matrices that represent the samples were calculated by use of the eigenvalues of matrices $C_i = B_{ip}^i B_{ip}$ ($i = 1, 2, 3$).

3. The calibration matrices, $W$ and $A$, were found from the solution of the set of simultaneous linear equations in Eq. (18) of Ref. 10.

The double-pass measurements, however, originated one further mathematical constraint in the calculation of the Mueller matrices in step 2. The commutativity of the eigenvalues of a matrix product with respect to the order of the factors was no longer sufficient to allow the parameters that characterized the Mueller matrices of the measured calibration samples to be determined. The details are explained in the remainder of this section.

In the double-pass configuration of the polarimeter, the light passed twice across the measured sample, propagating in opposite directions. For these measurements, an additional mirror was introduced. Hence, instead of Eq. (11), the equation that represented a general experimental double-pass measurement was

$$B_{sample}^{dp} = AM_{sample}EM_{sample}^*W.$$

(12)

The plus and minus indicate the directions of light as it passes through the sample, and the superscript $dp$ stands for double pass. The plus corresponds to the first pass, when light propagated toward the mirror; the minus, to the second pass, when light had been reflected and traveled away from the mirror, toward the PSA. Mirror matrix $E$ in Eq. (12) represents a dielectric mirror with a nominal reflectivity greater than 99.9\%, on which the angle of incidence was 0\%. This mirror was assumed to be ideal.

As mentioned above, four measurements were also taken in the calibration routine (step 1) air, $B_0^i$; two polymer film linear polarizers (Newport 10LP-VIS), $B_1^i$ (horizontal) and $B_2^i$ (vertical); and a 633 nm third-order quarter-wave plate that in double pass became a seventh-order $-0.26\lambda$ retardation plate for the 532 nm light source that we used, $B_3^i$. It was assumed that the samples had the same behavior in forward and backward propagation. The 633 nm third-order quarter-wave plate was aligned at $30^\circ$ with respect to the first-pass coordinate system. The fast axis of the retarder was therefore aligned at $30^\circ$ during the first pass and at $-30^\circ$ during the second pass.

The characterization of the calibration samples, step 2, required the computation of the products

$$C^{dp}_i = (B^{dp}_i)^{-1}B^{dp} = (AEW)^{-1}(AM_iEM_i^*W)$$

(13)

and the commutativity of the eigenvalues with respect to the order of the factors was sufficient to ensure that

$$\text{eig}(C^{dp}_i) = \text{eig}(EM_iEM_i^*),$$

(14)

where eig($C$) refers to the eigenvalues of matrix $C$. Nevertheless, Eq. (14) is not sufficient to associate the calculated eigenvalues with the eigenvalues of the Mueller matrices of the calibration samples. A more detailed inspection of the matrix product on the right-hand side of Eq. (14) allowed us to overcome this difficulty.

Choosing the coordinate system of the first pass of light across a calibration sample ($i$), we can write the Mueller matrices for each pass through the sample as

$$M_i^+ = R(\theta_i)M_{vp,i}^+R(-\theta_i),$$

(15)

$$M_i^- = R(-\theta_i)M_{vp,i}^-R(\theta_i),$$

(16)

where $M_{vp,i}$ is the single-pass Mueller matrix of the calibration sample aligned at $0^\circ$. The double-pass Mueller matrix of a calibration sample then becomes

$$M_iEM_i^+ = R(-\theta_i)M_{vp,i}^+EM_{vp,i}^-R(-\theta_i).$$

(17)

The single-pass Mueller matrix of the calibration samples ($i = 1, 2, 3$) was assumed to be of the same kind as in the single-pass ECM, given by special cases of the matrix

$$P(\tau_p, \Psi, \Delta) = \tau_p [\begin{array}{cccc}
1 & -\cos 2\Psi & 0 & 0 \\
-\cos 2\Psi & 1 & 0 & 0 \\
0 & 0 & \sin 2\Psi \cos \Delta & \sin 2\Psi \sin \Delta \\
0 & 0 & -\sin 2\Psi \sin \Delta & -\sin 2\Psi \cos \Delta \\
\end{array}] .$$

(18)
At 0° the two polarizers were assumed to be ideally represented by $\mathbf{P}(1/2, \pi/2, 0)$, and the retarder by $\mathbf{P}(\tau_3, \Psi_3, \Delta_3)$. Matrix $\mathbf{P}$ always commutes with mirror matrix $\mathbf{E}$ because the two $2 \times 2$ matrices contained in its top-right and bottom-left corners are zero. One last rearrangement of Eq. (17) leads to

$$
\mathbf{M}_i \mathbf{E} \mathbf{M}_i^+ = \mathbf{E} \mathbf{R}(\theta_i) \mathbf{M}_0^+ \mathbf{M}_0 \mathbf{R}(-\theta_i) .
$$

(19)

Finally, Eq. (19) yielded

$$
eig(\mathbf{C}_{dp}) = eig(\mathbf{M}_i \mathbf{M}_i^+) = eig(\mathbf{M}_{dp}) .
$$

(20)

The double-pass Mueller matrices ($\mathbf{M}_{dp}$) of the calibration samples and calibration matrix $\mathbf{W}$ were then calculated exactly as in the original ECM$^{10}$: the eigenvalues of $\mathbf{C}_{dp}$ were used to characterize the calibration samples, and $\mathbf{W}$ was found as the unique solution of the set of equations

$$
\mathbf{M}_{dp} \mathbf{X} - \mathbf{X} \mathbf{C}_{dp} = 0 \quad (i = 1, 2, 3, 4). \quad (21)
$$

We also calculated the exact azimuth orientation of the samples by minimizing the ratio of the smallest to the second-smallest eigenvalue of the mapping $\mathbf{3\mathbf{F}}$ that was built, as by Compain et al.$^{10}$ to find the solution of Eq. (21).

Once $\mathbf{W}$ was determined, the other calibration matrix, $\mathbf{A}$, was calculated from the measurement $\mathbf{B}_{\text{dp}}$:

$$
\mathbf{A} = \mathbf{B}_{\text{dp}} \mathbf{W}^{-1} \mathbf{E}. \quad (22)
$$

At this point, the choice of coordinate system for the representation of the double-pass measurements was made. The orientation of a calibrated Mueller matrix was defined by use of the coordinate system of the first pass. The azimuth angle of a linear polarizer set at 45° in the first pass, for example, became $-45°$ in the second pass. An experimental Mueller matrix, however, contained information on the two passes together. According to Eq. (19), the choice of the first pass as a coordinate system required that, instead of calibration matrix $\mathbf{A}$, a double-pass calibration matrix,

$$
\mathbf{A}_{\text{dp}} = \mathbf{A} \mathbf{E} = \mathbf{B}_{\text{dp}} \mathbf{W}^{-1}, \quad (23)
$$

be used. With this matrix, the calibration of a measurement like $\mathbf{B}_{\text{sample}}^\text{dp}$ in Eq. (12) resulted in

$$
(\mathbf{A}_{\text{dp}})^{-1} \mathbf{B}_{\text{sample}}^\text{dp}(\mathbf{W}^{-1}) = \mathbf{E} \mathbf{M}_{\text{sample}}^+ \mathbf{E} \mathbf{M}_{\text{sample}}^+. \quad (24)
$$

If the measured sample commuted with reflection matrix $\mathbf{E}$, when it was rotated to 0°, the right-hand side of Eq. (24) became $\mathbf{M}_{\text{sample}}^\text{dp}$ [see Eq. (19)], with the azimuth angle defined as in the first pass. If the sample did not commute with matrix $\mathbf{E}$, the use of $\mathbf{A}_{\text{dp}}$ instead of $\mathbf{A}$ had no further consequences in the calibration because matrix $\mathbf{E}$ is obviously nonsingular.

We compared the results of 10 calibration routines to assess the accuracy and repeatability of the double-pass measurements. Objective lens Obj1 of the confocal optics was not included in this evaluation. The samples were inserted into the optical system instead of Obj1 in Fig. 1, and a dielectric flat mirror was placed at the sample plane. The working distance of the objective lens was not sufficiently large to allow the calibration samples to be introduced between the lens and the dielectric mirror used in the double-pass calibration, and the experimental performance of the Mueller matrix polarimeter had to be evaluated before it was used with the confocal microscope. The lens that focused the light toward the pinhole (Obj2), and the lens that collected the light after the pinhole and focused it on the detectors (Obj3), were always used.

During each calibration routine we measured the four calibration samples 13 times and used the averages of the first 10 noncalibrated matrices to compute matrices $\mathbf{W}_i$ and $\mathbf{A}_{\text{dp}}^i (i = 1, \ldots, 10)$, using the DP-ECM. The remaining three measurements were then calibrated and averaged. Additionally, two other samples were measured five times following each calibration: a linear polarizer placed at $-47° \pm 1°$ and a 532 nm zero-order quarter-wave plate with its fast axis at $0° \pm 1°$. These five measurements were also calibrated from the corresponding pair of calibration matrices, and then averaged.

The root-mean-square (rms) of the standard deviation of the 16 Mueller coefficients for six different samples that were measured several times (repeatability), and rms of the error in the 16 Mueller coefficients when they were compared with analytical matrices (accuracy). The values are shown as a percentage of the corresponding $m_{11}$ coefficient. We also show the maximum standard deviation and maximum error observed on a coefficient.

<table>
<thead>
<tr>
<th>Matrix $^a$</th>
<th>Repeatability (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mathbf{M}_{w1}$</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>$\mathbf{M}_{w2}$</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>$\mathbf{M}_{w3}$</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>$\mathbf{M}_{w4}$</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Polarizer</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Wave Plate</td>
<td>1.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$^a$Evaluation of the calibration: rms of the standard deviation of the 16 Mueller coefficients for six different samples that were measured several times (repeatability), and rms of the error in the 16 Mueller coefficients when they were compared with analytical matrices (accuracy). The values are shown as a percentage of the corresponding $m_{11}$ coefficient. We also show the maximum standard deviation and maximum error observed on a coefficient.
light of the experimental Mueller matrices. As the exact azimuth angle of the samples was not known, the polarizers and waveplates were also fitted to the experimental azimuth angle. The angles for the calibration samples, \( M_{B}^{dp} \), \( M_{B}^{dp} \), and \( M_{B}^{dp} \), were the mean of the ten sets of corrected angles calculated during the DP-ECM; for the two additional samples, polarizer and quarter-wave plate, the mean of 10 azimuth angles was found by use of the polar decomposition published by Lu and Chipman\(^{28} \) on each of the ten averaged calibrated Mueller matrices. Two extra parameters were used to fit the theoretical matrix of calibration sample \( M_{B}^{dp} \); retardance \( \Delta_{p} = -1.54 \) and diattenuation angle \( \Psi_{p} = 0.766 \), which were found within the DP-ECM routine. The rms of the errors in the 16 coefficients of each sample were calculated and are given in Table 1, together with the maximum difference obtained for each sample. The values were also normalized to coefficient \( m_{11} \) and are shown as percentages. The maximum observed error was 7.2\% and occurred in coefficient \( m_{24} \) of the polarizer at \(-47^\circ\); but the maximum rms error, which was measured on the calibration retarder, was equal to only 2.6\%. The magnitude of the errors reported here is comparable to or better than values previously reported in the literature: 0.5\%,\(^{10,27} \) 4\%,\(^{29} \) 5\%,\(^{11} \) and 10\%.\(^{30} \)

The confocal axial scans in this work (see Section 5 below) were performed manually. The samples were moved, in increments of 2.5 to 10 \( \mu \)m and by using a micrometer screw, through an axial distance of approximately 2 mm, a process that took nearly 1 h. We tested the time stability of the measurements, taking 1331 measurements of the 4 calibration samples every 5 s during a period of almost 2 h. The standard deviation was never larger than 1.3\% of the value of coefficient \( m_{11} \). No temperature control was implemented on the Pockels cells but, judging from this stability test, the errors induced in the Mueller matrix coefficients were considered small.

4. Experimental Results: Depth-Resolved Confocal Mueller Matrix Polarimeter

The confocal microscope used in this work was built in the reflection configuration (epi-illuminated).\(^{31} \) A schematic diagram of the confocal microscope is also included in Fig. 1. The collimated light that propagated away from the PSG entered beam splitter Bs1 and was reflected toward objective lens Obj1. This lens focused the light on the sample, and the light that was reflected or scattered from the sample was collected again by triplet Obj1. The objective lens and the second triplet (Obj2) formed an image of the sampled point onto the confocal pinhole plane. The last triplet (Obj3) collected the light passing through the pinhole and focused it on the four detectors of the PSA. The confocal polarimeter was built with three 30 mm focal-length triplet lenses (Linos HALO 03 8903). Given that the ultimate motivation for our study was to acquire in vivo depth-resolved complete polarization-sensitive measurements of the human retina, the N.A. of the system was similar to the N.A. of a human eye: 0.14 (30 mm focal-length objective and 8.5 mm pupil). The effective N.A. of the eye is \(-0.16 \) (22 mm focal length and 7 mm pupil)\(^{32} \).

According to a Zemax model of the experimental setup that included the curvature of the refracting surfaces, the spacing between them, and the type of glass of each lens (as specified by the manufacturer of each optical element), we found that the spot of light produced on the sample plane was diffraction limited for the 8.5 mm aperture used in the experiments. The numerical Strehl ratio was 0.97, and the Airy radius was 2.2 \( \mu \)m. On the pinhole plane, the Strehl ratio was calculated to be 0.83. The experimental lateral resolution was not tested in this work; we were interested only on the axial response of the confocal polarimeter, which we tested by scanning a flat mirror through the focal region of the illumination spot. We moved the mirror by manually turning a micrometer screw, which had a minimum division of 1 \( \mu \)m, in steps of 2.5 and 5 \( \mu \)m. While the mirror was scanned, the Mueller matrix at each axial position was measured and calibrated. Two different confocal pinhole sizes were used: 5 \( \mu \)m diameter (8 optical-unit radius) and 20 \( \mu \)m diameter (33 optical-unit radius). Optical units represent the real distance relative to the dimensions of the Airy disk, and they were calculated from \( v_{p} = (2\pi/\lambda) \text{ N.A.} r_{p} \), where \( r_{p} \) is the real pinhole radius. We used coefficient \( m_{11} \), which is equal to the intensity reflectivity for nonpolarized light (the typical axial response of a conventional confocal microscope), to calculate the axial resolution of the system, and the axial profile is shown in Fig. 3 for both pinhole sizes. The solid curves in Fig. 3 were calculated with the diffraction encircled energy function built in Zemax-EE Version January 1, 2003. We
made different Zemax models by increasing the distance between the objective lens (Obj1) and a planar mirror and then calculating the energy encircled by an aperture equal to the corresponding pinhole diameter. According to Török and Wilson, the asymmetry of the curves is typical of axial confocal measurements, mainly because of spherical aberration introduced by defocus. The agreement with Zemax models indicated that the alignment of the microscope was acceptable.

The complete Mueller matrices of the mirror axial scans for the 5 μm pinhole are shown in Figs. 4 and 5. To the best of our knowledge, this is the first time that axially resolved complete polarization-sensitive measurements have been reported. The data were calibrated without a pinhole for Fig. 4 and with a pinhole for Fig. 5. This means that the calibration samples used in the DP-ECM were measured twice, once without the pinhole and once with the pinhole in exactly the same axial position as when the scans were made. The Mueller matrices of the axially scanned mirror in both cases were slightly different from the identity matrix, which represents a mirror in the double-pass coordinate system chosen previously (see Section 3). Similar graphs were obtained for the 20 μm pinhole. Two different types of departure from the ideal identity matrix can be observed on the Mueller coefficients in Fig. 5: one that looks like an even function with respect to the highest signal (e.g., coefficient $\text{Mirror}_{42}$), and one that looks like an odd function (e.g., coefficient $\text{Mirror}_{43}$). The only lens that was not included in the calibration was the objective lens, and this could have contributed to the departure of the Mueller matrix from the identity. The introduction of the objective lens that focuses the light onto a sample can generate a significant axial component of the electric vector. It is shown below, however, that for our experimental system this error was small.

The N.A. used in this work was small compared with those in studies of the polarization changes produced by high-aperture lenses, and, as a first approximation, the axial component was neglected. The compromise between accuracy of the Mueller matrix polarimetry measurements and resolution of the microscope now becomes an interesting field of study. Polarization inhomogeneities across the pupil may introduce errors that the calibration method cannot remove. Only the average effect across the area used on the lens can be calibrated. In the presence of spherical aberration, for instance, the light that can pass through the confocal pinhole may be focused by...
use of different radial portions of the lens for different axial positions of the pinhole. This means that a spatially resolved calibration may also be necessary if the technique is to be extended to high N.A. systems.

The total retardance at three axial positions of the mirror scans was calculated from the Lu–Chipman polar decomposition. The three positions were the maximum and the two edges of the FWHM of coefficient \( \text{Mirror}_{11} \). The three parameters were calculated from matrices that were calibrated with and without the corresponding confocal pinhole. These results are compared in Fig. 6. It is noticeable that the effect of introducing the 5 \( \mu \)m pinhole into the system was significant, but when the larger, 20 \( \mu \)m, pinhole was introduced the calibration with and without the pinhole yielded the same retardance values. This is by no means an exhaustive analysis of the effect of the pinhole size on the axially resolved complete Mueller matrices, but it is evidence that further research is necessary for a full understanding of these artifacts.

5. Axial Scan of the Confocal Pinhole

As was mentioned above, objective lens Obj1 was not used during the calibration of the polarimeter; hence any polarization changes introduced by the lens were not removed from the Mueller matrix axial scans presented previously. In those axial scans, any polarization changes introduced by the optical sectioning of the confocal pinhole were combined with the non-calibrated artifacts of the lens. To isolate the pinhole effects, we made experiments in which the pinhole was moved axially through the confocal region instead of moving the mirror through the focal region of the illumination spot. Figure 7 shows a schematic diagram of the configuration of the system used to scan the pinhole without objective lens Obj1. A scan of the 5 \( \mu \)m confocal pinhole was also made with the objective lens (Obj1) inserted and the light focused onto the surface of mirror M2 that appears in Fig. 7. The results of the two pinhole axial scans are presented in Fig. 8. As an attempt to eliminate any bias towards a particular axial position of the pinhole, we calibrated both sets of Mueller matrices by using measurements that were taken without the confocal pinhole in the system.

The results obtained with and without the objective lens were similar to those previously obtained when the mirror was scanned. The FWHM of the scans was 71.1 \( \mu \)m without Obj1 and 87.0 \( \mu \)m with the objective lens. The value of 87.0 \( \mu \)m is approximately twice the FWHM of the mirror scans: 43.8 \( \mu \)m.
The off-diagonal Mueller coefficients of these scans showed shapes similar to those obtained when the mirror was scanned. The Mueller matrices at the intensity peak and at the edges of the FWHM were compared with the ideal identity matrix, and the residual rms errors obtained are listed in Table 2. The data in Table 2 is evidence that the pinhole can alter the Mueller matrix polarimetry measurements. The rms values and the consistent shape of the off-diagonal Mueller coefficients indicate that the pinhole effect appeared to be systematic. Figure 8 and Table 2 also indicate that the effect of the objective lens was small, almost reduced to only the broadening of the axial response of the microscope. In our system the polarization changes observed on the off-diagonal elements of the pinhole scans were smaller than 5% and 8% for the measurements without the lens and with the lens, respectively. These values were calculated with respect to the maximum value obtained: in both cases, the peak of coefficient $P_{33}$ (Fig. 8).

The results presented here indicate that the removal of the objective lens (Obj1) during the calibration measurements was not the only origin of the polarization artifacts observed during the axial scans of the mirror. Additionally, the magnitude of this effect was comparable to the magnitude of the polarization changes introduced by the optical sectioning of the confocal pinhole. Therefore we state here that the calibration of the system without the objective lens can produce a balanced compromise between accuracy and simplicity of the system.

6. Description of the Inverse Problem
The Mueller matrix obtained from two different axial depths within a sample contain the information of the cumulative double-pass effect of two layers in the sample. Previous to the acquisition of the Mueller matrix from the second (deeper) layer, the Mueller matrix from the first layer had to be measured; in the first measurement, only the effect of the first layer was contained, also in double pass. The solution of the inverse problem, for this case, should consist of identifying what the four Mueller matrices are that represent the effect of each of the four passes of light through the two layers in forward and backward propagation: the first and the second pass. In the most rigorous sense, the solution of this problem is underdetermined; there are four unknown matrices, and only two can be measured. And if a number $N$ of layers were measured, the solution would require $2N$ matrices. Clearly, some assumptions will need to be made when one is interpreting the double-pass data.

Previously, this inverse problem had not been addressed in the literature. Some studies, however, have analyzed the propagation of completely polarized light through layered birefringent media and layered birefringent turbid media. The $N$ matrices, which refer to the effect, on the polarization of light, of an infinitesimal path length within an optical element, were introduced in 1948 by Jones in light of the Jones calculus: For completely polarized light. Jones's work was extended by Azzam in 1978. Azzam developed a differential $4 \times 4$ matrix calculus to describe the continuous propagation of partially polarized light through linear anisotropic media that may exhibit depolarization. If the depth-resolved Mueller matrix measurements were made sufficiently close to each other, Azzam's work could be a good starting point with which to deal with this inverse problem. Despite not having rigorously covered that point in our work, in the following paragraphs we present some general observations concerning double-pass measurements that may be of help for future investigations. These observations do not only apply to complete Mueller matrix measurements but to previously reported incomplete polarization-sensitive instruments, four which the number of unknown parameters is, in general, larger.

A. Forward and Backward Propagation
The propagation through an optical element or a sample should not be assumed, in general, to be the
same in the first as in the second pass. If the depth resolution of the system used to obtain the Mueller matrices of a sample is not sufficiently high, layers with different polarization properties may be measured as a single layer. Mueller matrices are not commutative and, evidently, the combination of two layers that introduce different effects on polarization may not be the same when the order of the matrices (the layers in this case) is interchanged.

B. Ideal Polarizer

If the front layer of a sample were an ideal linear polarizer, much of the information about the polarization properties of the posterior (deeper) layers would be lost when a reflection configuration was used. Light returning from the sample would always be linearly polarized, and the polarization effect of the posterior layers would be projected as a mere intensity fluctuation of the returning linearly polarized light, i.e., as an effect on the first component of the Stokes vector. Nevertheless, a polarization-sensitive device would still be advantageous compared with a conventional imaging system (intensity, phase, or both) in this case, because the front layer would be identified as a polarizer, and any intensity fluctuations would not necessarily be associated with reflectivity fluctuations. Moreover, encountering an ideal linear polarizer in biological samples would perhaps constitute a more important scientific achievement than the study of what remained deeper in the sample.

C. Circular Retarder

An ideal circular retarder that is not a Faraday rotator would appear as a homogeneous medium if it were measured alone in double pass. The effect of such a rotator should be the same in forward as in backward propagation, but, for light that propagates in double pass, the effect of the circular retarder in the second pass would cancel the rotation of the first pass because of the change in the coordinate system that the reflection between the first and the second passes produces. During the measurement of a

<table>
<thead>
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<th>Condition of</th>
<th>–FWHM/2 (%)</th>
<th>Peak (%)</th>
<th>FWHM/2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Obj1</td>
<td>4.8</td>
<td>2.1</td>
<td>2.9</td>
</tr>
<tr>
<td>With Obj1</td>
<td>5.3</td>
<td>3.6</td>
<td>3.8</td>
</tr>
</tbody>
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*Error in the Mueller matrix of the pinhole scans, with and without objective lens Obj1, when compared to the identity matrix. We show the rms of the error (difference) in the 16 Mueller coefficients as a percentage of the intensity peak and the two edges of the FWHM.
deeper layer, however, the role of the rotator would have an effect on the polarimetry measurement because in this case the two optical rotations would not be separated only by a reflection, and the effect of the deeper layer would not necessarily commute with either rotation.

D. Depolarization Produced by Scattering

In a reflection confocal microscope, the light that returns to the system from a layer within a scattering sample (e.g., some type of biological tissue) and is detected can be depolarized by the scattering process itself. But when a deeper layer is measured it will be blocked (or almost blocked) by the confocal aperture, although some light will still be scattered at the front layer. Hence the depolarization produced by scattering that will be measured from the deeper layer can be independent (or almost independent) of the depolarization measured from the front layer. That is, the depolarization produced by scattering may not have a linear cumulative effect throughout the depth of the sample.

Not every $4 \times 4$ real-valued matrix is a Mueller matrix that can operate on a Stokes vector as a real optical element that can be built in the laboratory or found in nature. A good number of publications have dealt with finding valid criteria to test whether a matrix is a Mueller matrix. This subject falls beyond the scope of this paper, but it is important and must not be overlooked, especially during the analysis and interpretation of experimental results. We believe that the results obtained during this study are the first of their kind, and more work will be necessary for the solution of the inverse problem derived from the cumulative effect of the polarization properties of a sample $m$. The validity criteria of Mueller matrices may play an important role within the research that may result from this study.

7. Summary and Conclusions

For the first time to our knowledge, the combination of a depth-resolved imaging technique with a complete Mueller matrix polarimeter was introduced. A confocal microscope within a complete Mueller matrix polarimeter was designed and built. The system was used to measure the complete Mueller matrices at several depths of a mirror that was being moved axially. This study has shown that it is possible to measure the complete Mueller matrix of a sample at different axial positions and, therefore, in the three spatial dimensions.

As the elements of the Mueller matrix that represent a sample are in general linearly independent, polarization-sensitive imaging is a 16-dimensional imaging technique that includes intensity as one dimension. We are in the process of acquiring three-dimensional polarization-sensitive images from biological samples, which is in fact equivalent to developing a $3 \times 16$ dimensional imaging device. The technique that we present here can be implemented with any kind of complete polarimeter. The short rise time of the Pockels cells and the simultaneous operation of the division-of-amplitude polarimeter may allow our choice of implementation to be incorporated into fast clinical imaging devices such as confocal laser scanning ophthalmoscopes.

The double-pass eigenvalue calibration method was developed as a modification of the original eigenvalue calibration method previously published by Compain et al. Its accuracy and repeatability were evaluated for the polarimeter built. In our experiments the rms of the standard deviation of the Mueller matrix coefficients was smaller than 1.5%, and the residual rms error between the experimental and the analytical Mueller matrices of six samples was smaller than 2.6%. The time stability of the system was tested on four samples over a period of 2 h for each of them, and the standard deviation found in a single Mueller matrix coefficient was smaller than 1.3%.

The examples presented in Section 6 are ideas of some of the difficulties that future investigations may encounter in attempts to solve this inverse problem or to establish the circumstances under which a solution might exist. It may be important to note that, even if such a solution does not exist in a practical sense, some polarization parameters may still provide valuable information about the measured sample. If the depolarization produced by scattering is indeed independent at different depths, for example, one additional imaging dimension will be gained with this technique that no other existing three-dimensional imaging technique has yet reported. Additionally, the elaboration of statistical models of combinations of the depth-resolved polarization properties of a sample can lead to a better understanding of its composition and to a new way to detect anomalies in the specimen, such as diseases of biological samples, that have not been understood.

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References and Notes

22. The condition number that we use is the one that corresponds to the mathematical definition of the condition number in the $l_2$ form: the ratio of the largest to the smallest singular value in the singular-value decomposition of a matrix.