Methods 128 (2017) 105–118

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Polarization resolved second harmonic microscopy

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ARTICLE INFO

Article history:
Received 21 February 2017
Received in revised form 23 May 2017
Accepted 13 June 2017
Available online 15 June 2017

Keywords:
Optical scanning microscopy
Second harmonic
Stokes vector
Polarization
Chirality
Fluorescence lifetime imaging (FLIM)

ABSTRACT

Second harmonic (SH) microscopy has proven to be a powerful imaging modality over the past years due to its intrinsic advantages as a multiphoton process with endogenous contrast specificity, which allows pinhole-less optical sectioning, non-invasive observation, deep tissue penetration, and the possibility of easier signal detection at visible wavelengths. Depending on the relative orientation between the polarization of the incoming light and the second-order susceptibility of non-centrosymmetric structures, SH microscopy provides the unique capacity to probe the absolute molecular structure of a broad variety of biological tissues without the necessity for additional labeling. In addition, SH microscopy, when working with polarimetry, provides clear and in-depth insights on the details of molecular orientation and structural symmetry.

In this review, the working principles of the polarization resolving techniques and the corresponding implements of SH microscopy are elucidated, with focus on Stokes vector based polarimetry. An overview of the advancements on SH anisotropy measurements are also presented. Specifically, the recent progresses on the following three topics in polarization resolved SH microscopy will be elucidated, which include Stokes vector resolving for imaging molecular structure and orientation, 3-D structural chirality by SH circular dichroism, and correlation with fluorescence lifetime imaging (FLIM) for *in vivo* wound healing diagnosis. The potentials and challenges for future researches in exploring complex biological tissues are also discussed.

Contents

1. Introduction

Optical microscopy, with contrasts including transmission, phase contrast, differential interference contrasts (DIC), and fluorescence, has long been an essential and routine tool in biomedical researches [1]. The unique capacity of optical microscope depends on minimal sample invasiveness, high resolution, a broad variety of contrast and high brightness that could enhance the visualization [1,2]. Among the vast modalities of optical microscopy, polarization light microscopy enhances contrast for optically anisotropic materials in live cell imaging [3–5]. The visualization of internal organization of spindles in living cells

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http://dx.doi.org/10.1016/j.ymeth.2017.06.012
1046-2023/

was revolutionized in 1940s and 1950s with Schmidt's and Inoué's polarization microscope [4,6]. The PolScope was developed accordingly for qualitative as well as quantitative analysis regarding the molecular alignment and microstructure, such as spindles in the large oocyte irrespective of the perpendicular orientation [7]. Multifocus PolScope employed multifocus optics of up to 25 focal planes simultaneously for 3D polarization imaging of living biological samples [8].

Inoué and Ernst et al. [4,5] illustrate the optical setup and critical aspects concerning the interaction between the polarized light, the optically anisotropic sample, and the analyzer. In order to accomplish this task, the conventional optical microscope must be equipped with a polarizer, positioned in the light path before the specimen, and an analyzer, placed in the optical pathway between the objective rear aperture and the observation tubes or camera port. Importantly the depolarization effects due to high numerical aperture objectives have to be taken into account [1,9]. Imaging contrast arises from the interaction of plane-polarized light with a birefringent (or doubly-refracting) specimen. The polarized light is usually decomposed and analyzed via two orthogonal wave components. The relative phase difference and intensity of these two components is used to determine the molecular structures and orientations of the specimen, including the molecular dynamics of macromolecules in membranes, microtubule and microfilament bundles in native environmental conditions [10–13]. The polarization microscope is further improved in axial resolution using confocal imaging system with Mueller matrix polarimetry [14]. In this way, the depolarized component can be quantitatively accounted. A complementary polarization characterization based on Stokes-Mueller formalism was implemented to study the scattering properties of turbid media and tissue using linear Lu-Chipman decomposition. This technique provides high contrast and rapid convenience for pathological study and is applied to assess the severity of cancer invasion [16].

As discussed above, the low extinction ratio due to large depolarization effects when working with high NA objectives is well addressed by Inoue and Odenbourg's PolScope [7] in wide field microscopy and later by Török's use of confocal microscopy in scanning one [9]. However, molecular specificity remains a challenge in linear polarization microscopy, since the birefringence effects due to molecular structures and layered interfaces are indistinguishable. For comparison, SH microscopy naturally encompasses all the above developments and advantages with specificity on molecules and structures of non-inversion symmetry. The development of high resolution SH microscopy has demonstrated immense capacity to reveal the biological structures with sub-millimeter depth penetration [18]. It reveals material and structural anisotropy and chirality via polarization resolving with molecular specificity. Due to their large SH responsivity, the interested targets include collagen in fibrosis [21], human dermis, keloid [22], cornea [15], microtubules, myosin of the skeletal muscle [23] as well as starch granules [24].

Recently, for both linear and non-linear optics, polarization resolved measurements are adopted for in-depth understanding of specific molecules [17–19]. Stokes vector formalism has also been implemented to SH microscopy to extract the critical polarization parameters, including the degree of polarization (DOP), the degree of linear polarization (DOLP), and the degree of circular polarization (DOCP) [17,24]. Additionally, Zhuo et al. [25] has developed precise polarization state determination without repetitive image acquisition, to improve imaging speed for better disease assessment.

This review elucidates the working principles of polarization analysis for linear and non-linear optical microscopy. Linear interaction for scattering light are analyzed by linear polarimetry with limited molecular contrast, which is extensively improved in the nonlinear tissue-light interaction centered on second harmonic generation, including SH anisotropy for wound healing, second harmonic generation circular dichroism (SHG-CD), and Stokes vector analysis. Importantly, the nonlinearity of SH microscopy not only allows pinhole-less optical sectioning, non-invasive observation, deep tissue penetration, and the possibility of easier signal detection at visible wavelengths, as commonly recognized for multiphoton microscopy, but also highly enhances the polarization sensitivity.

2. Characterizing the polarization state of second harmonic

Fundamentally, polarization analysis can be carried out with Jones calculus, in which polarized light is expressed by the two-element Jones vector and the polarization elements are represented by the 2×2 Jones matrix [26,27]. However, Jones calculus is limited only for perfectly polarized light and the complete polarization state cannot be revealed due to the lack of the phase difference between the two components of the measured polarization vector. For example, use of cross-polarized two-channel detection does not allow the relative ratio of the polarized and un-polarized components of the electric field to be determined [26,27]. For comparison, Stokes-Mueller formalism is a powerful approach that accommodates all polarization states, including incoherent, partially polarized, and unpolarized ones [26–29]. Any beam of light can be completely characterized in terms of its intensity and state of polarization by the 4×1 Stokes vector $S = [S_0\ S_1\ S_2\ S_3]^t$, where the superscript t denotes the transpose of the matrix. The components of the Stokes vector are defined as below:

$$S = \begin{bmatrix} S_0 \\ S_1 \\ S_2 \\ S_3 \end{bmatrix} = \begin{bmatrix} < E_{\parallel}E_{\parallel}^{*} + E_{\perp}E_{\perp}^{*} > \\ < E_{\parallel}E_{\parallel}^{*} - E_{\perp}E_{\perp}^{*} > \\ < E_{\parallel}E_{\perp}^{*} + E_{\perp}E_{\parallel}^{*} > \\ < i(E_{\parallel}E_{\perp}^{*} - E_{\perp}E_{\parallel}^{*}) > \end{bmatrix} \propto \begin{bmatrix} I_0 + I_{90} \\ I_0 - I_{90} \\ I_{45} - I_{135} \\ I_R - I_L \end{bmatrix}, \tag{1}$$

where $E_{\parallel}$ and $E_{\perp}$ are horizontal and vertical electric field components with respect to the direction of propagation of light, '<>' denotes the time average over an interval much larger than the wave period and the asterisk denotes the complex conjugate of a complex variable.

Importantly, these Stokes vectors are based on measurable quantities relative to the six intensities (I) [28,29] as described in above Eq. (1), where S_0 is the total intensity that corresponds to sum of the two orthogonal component intensities I_0 and I_{90}, S_1 is the difference between the 0^0 (I_0) and 90^0 (I_{90}) polarization intensities, S_2 is the difference between the $+45^0$ (I_{45}) and -45^0 (I_{135}) polarization intensities, and S_3 is the difference between the left (I_L) and right (I_R) circular polarization intensities. These elements are generally normalized to the value of S_0 so that they range between +1 and −1. A typical Stokes polarimeter consists of a light source, polarization state analyzer (PSA) and intensity based detection (Fig. 1(a)). Specifically, a Stokes polarimeter measures the polarization states of the detected light as parameterized by the associated Stokes vectors. Accordingly, the sample and the complete polarization states of light can be characterized by Mueller polarimeter.

When light propagates into an optically thick turbid media such as biological cells and tissues, the polarization state of the incident light is altered as a result of light matter interaction, which is depicted by the Stokes vectors, S_{in} in and S_{out} out. Notably, the input (S_{in}) and the output (S_{out}) Stokes vectors can be linearly related by a 4×4 transformation matrix known as the Mueller matrix (M_{ij}) under linear optics setting, as given below

$$S_{out} = \frac{1}{k^2 r^2} M_{ij} S_{in}, \tag{2}$$