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Published in:
Proceedings

DOI:
10.1117/12.2573210

Publication date:
2020

Document Version
Peer reviewed version

Towards single-photon deep-tissue microscopy

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Abstract

Fluorescence light-sheet microscopy is gaining rapid adoption in developmental biology. With irradiation levels well below that of confocal and multi-photon microscopy, it enables the study of intact organs and organisms for prolonged time periods during development. Minimal sample exposure is achieved by selectively illuminating the focal plane with a second objective orthogonal to the detection axis. The light-sheet microscope's ability to study intact biological samples as and when they grow highlights the importance of imaging deeper into biological samples. Yet, deep-tissue microscopy is hampered by autofluorescence and the scattering of light. Direct observations are therefore limited to highly transparent and thin samples. Here, we show how autofluorescence can be eliminated effectively by relying on reversible photo-switching fluorescence while we propose a way forward to study and control light propagation in optically-thick tissues.

Keywords: Fluorescence Microscopy, Optical Scattering, Reversible Photoswitching, Auto-fluorescence

I Introduction

Fluorescence light-sheet microscopy is increasingly adopted by developmental biologists to study how cells divide and differentiate to form organs and even entire organisms [7]. A light-sheet microscope differs from the conventional microscope in that the sample is illuminated by a plane of light orthogonal to the detection axis, thus keeping out-of-focus areas dark while limiting potentially detrimental exposure of the sample. Light-sheet microscopy has become a key technology for long-term and non-invasive studies of intact, and therefore three-dimensional, fluorescent specimen.

While the light-sheet microscope is able to rapidly image larger samples such as the developing zebrafish, this comes with a trade-off between resolution and field-of-view. Diffraction prevents the conventional Gaussian light-sheet to remain focused to a thin plane throughout the entire width of the sample. High axial resolution can only be obtained near the beam waist, while other regions suffer from reduced axial resolution and increased out-of-focus blur. Propagation-invariant Bessel beams can form extended light-sheets; however, their extended transverse profile is detrimental to contrast without relying on two-photon excitation [8,11], or blocking of the out-of-focus light using a confocal slit [3]. Both solutions do lead to a significant increase in sample exposure when the same number of fluorescence photons is collected. It is unfortunate that this diminishes the low sample exposure advantage of light-sheet microscopy, a primary reason for using the technique. We demonstrate how the asymmetric profile of the Airy beam can overcome the trade-off between axial resolution and field-of-view without sacrificing the scarce fluorescence signal and without relying on the high—often photo-bleaching and damaging—peak-powers associated with two-photon excitation. The Airy light-sheet enables sub-cellular resolution throughout a 10× larger volume [5].

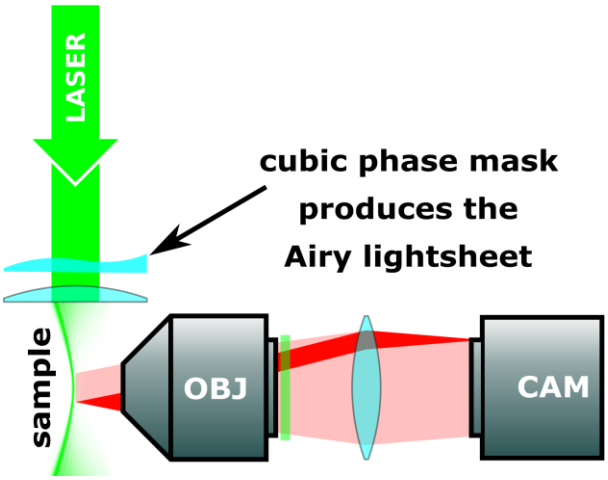

Figure 1 The Airy light-sheet fluorescence microscope. A collimated laser beam (green) is modulated by a cubic-polynomial phase mask and focused into the sample to produce a propagation-invariant Airy light sheet. The emitted fluorescence (red) is collected by the objective and reimaged onto the camera (CAM). The asymmetry of its profile enables high-resolution image reconstruction over an order of magnitude larger field-of-view.

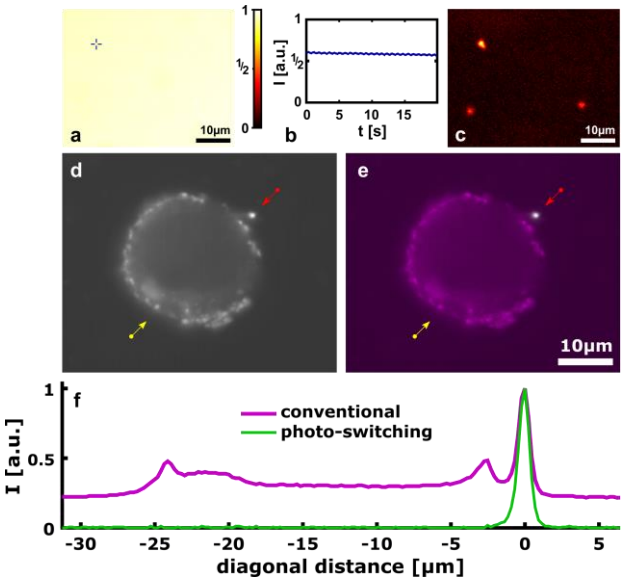

Figure 2 Reversible photo-switching contrast light-sheet microscopy. Microspheres labeled with the reversible photo-switchable protein rsEGFP within a highly fluorescent medium. (a) Conventional light-sheet fluorescence image. (b) The fluorescence intensity fluctuations, in lock-step with the activation laser, at the pixel indicated by the cross-hair in panel (a). (c) Photoswitching-enabled contrast enhancement of the light-sheet image. (d-f) Photo-switching contrast used to identify E-coli bacteria. (d) Conventional fluorescence image of a green-fluorescent HaCaT (aneuploid immortal keratinocyte) cell. (e) Photo-switching contrast overlaid on conventional image in magenta. (f) Diagonal cross-section through the cell shows that the small dot on the top right is the photo-switching E-coli bacterium. Adapted with permission from [12]. Copyright 2017 American Chemical Society.

The larger imaging volume also comes with new challenges. Optical aberrations are a reflection of the natural inhomogeneity of biological specimen, and scattering quickly become significant when imaging through multiple layers of cells. Conventional light-sheet microscopy is therefore restricted to thin or quasi-transparent specimen such as the zebrafish (*Danio rerio)*. Nevertheless, most biological samples tend to be turbid or opaque to light, in particular after the later stages of development. Moreover, the indigenous auto-fluorescence of biological tissues can severely reduce contrast and hamper the correct interpretation of the light-sheet fluorescence microscopy images. We demonstrated that reversibly photo-switching fluorescent proteins such as rsEGFP can be successfully used to boost weakly fluorescent features by two orders of magnitude (Figure 2). This enables high contrast and resolution, even under the adverse conditions of turbid, highly auto-fluorescent, samples.

Auto-fluorescence is not the only obstacle to deep-tissue microscopy. Light scattering becomes progressively more important as one attempts to image deeper into biological specimen. The issue is two-fold in light-sheet microscopy. Since the illumination and detection light paths traverse different areas of the specimen, adaptive optics are required in both independent light paths. While early forms of light-sheet microscopy were relatively forgiving when the light-sheet deteriorates; novel forms of light-sheet microscopy increasingly rely on precisely patterned illumination to form a higher resolution image through computational methods [1,5].

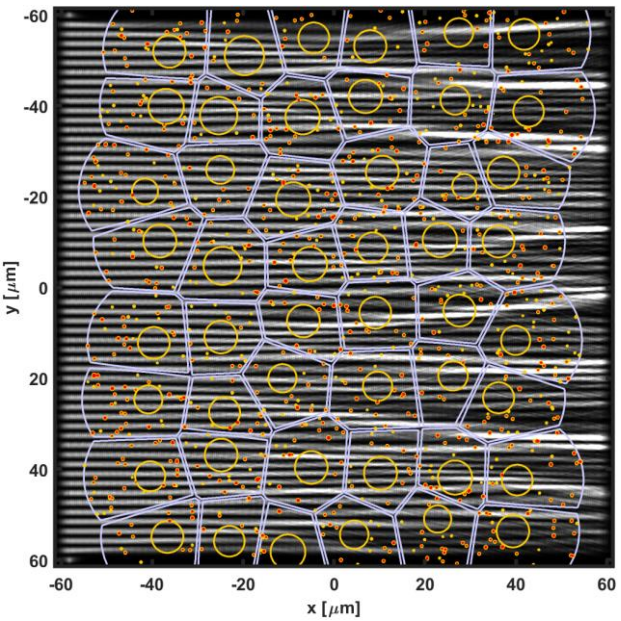

Figure 3 Structured illumination light-wave propagation through biological tissue from left to right. Cell membranes are indicated with blue lines, nucleus membranes with orange lines, dense regions are indicated in red. The sample is illuminated from the left (along the *x*-axis) with a periodic pattern. The periodic pattern is distorted after propagation through six layers of cells as it exits the sample on the right-hand side.

Understanding the effects of heterogeneity on light propagation requires adequate models of the sample as well as novel algorithms that enable accurate and

efficient calculation of the light field in optical materials as complex as biological tissue. Conventional methods such as Finite-difference time-domain (FDTD) and finite-element methods do not scale well to the volumes of interest to biological microscopy. A novel class of modified Born-series methods was recently discovered and put forward to bridge this gap [9]. We show that this type of method can be extended to Maxwell's electro-magnetic vector fields [6], and applied to very general materials, including biological tissues and metamaterials [13]. Figure 3 shows how this can be used to study how structured illumination is refracted and scattered by the heterogeneities within a model specimen. The highly-accurate map of the internal electro-magnetic field can complement experiments that only have access to the field as and when it exits the sample.

Accurate information of the light propagation can drive adaptive optics techniques and aid in the study of different optical memory effects [4,10]. Commonly, adaptive optics are introduced to correct the detection light path. We demonstrate how also light-sheet illumination can be controlled in turbid samples to achieve high axial resolution even in turbid samples [2].

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