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Deep tissue imaging with multiphoton fluorescence microscopy

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Abstract

We present a review of imaging deep-tissue structures with multiphoton microscopy. We examine the effects of light scattering and absorption due to the optical properties of biological sample and identify 1300 nm and 1700 nm as ideal excitation wavelengths. We summarize the availability of fluorophores for multiphoton microscopy as well as ultrafast laser sources to excite available fluorophores. Lastly, we discuss the applications of multiphoton microscopy for neuroscience.

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Introduction

The quest for new techniques capable of rapid, three-dimensional imaging with cellular resolution in intact tissues has fueled rapid advances in optical microscopy. Two-photon fluorescence microscopy (2PM), developed in the 1990's [1], is the most widely adopted method for minimally invasive *in vivo* brain imaging due to its ability to image in three dimensions. Recent *in vivo* imaging of neuronal circuits [2–4] and vascular networks [5,6] in the brain with micron-scale resolution has revealed significant new insight into cortical function and organization. Three-dimensional multiphoton imaging is possible because of a nonlinear dependence on excitation intensity, which confines two-photon fluorescence generation to the focal volume [7]. The resulting imaging depth is significantly greater than in confocal fluorescence microscopy due to the longer excitation wavelength [8] and the ability to detect multiply scattered emission photons. However, the imaging depth for most 2PM imaging studies is around 500 μm, which is sufficient to image superficial cortical layers in rodent experiments. This limitation on penetration depth arises from light scattering and absorption of tissue, both of which are wavelength dependent.

Traditional 2PM relies on Ti:Sapphire (Ti:S) lasers because of their ability to output mode-locked ultrafast pulses with high average powers, and their ability to excite a variety of fluorophores with one-photon absorption peaks in the visible range. Due to their reliability and robustness, Ti:S lasers have served as the workhorse of 2PM for the last 25 years and are commercially available from multiple laser companies as turn-key systems. While 2PM with Ti:S lasers substantially increases imaging depth relative to other optical microscopy approaches like confocal microscopy, Ti:S lasers are not optimized for deep imaging. To push the imaging depth further and visualize the entire mouse cortex (which is about 1 mm thick) and beyond, many new tools have been developed.

To appreciate the recent development of tools that extend the imaging depth of 2PM, it is important to understand that the optical properties of the biological sample serve as the fundamental limiting factors for imaging depth. Both excitation light and emission light are attenuated by absorption and scattering in biological tissue. Scattering and absorption effects vary by wavelength; thus, approaches to extend imaging depth focus on optimizing the excitation wavelengths, emission wavelengths, or a combination of both. The development of longer wavelength laser sources aims to reduce the scattering of excitation light. The advent of brighter fluorophores aims to both increase the amount of excitation light absorbed by the fluorophore as well as increase the likelihood that the fluorophore emits a fluorescent photon after absorption, and red-shifted fluorophores reduce the scattering effects of the emission light.

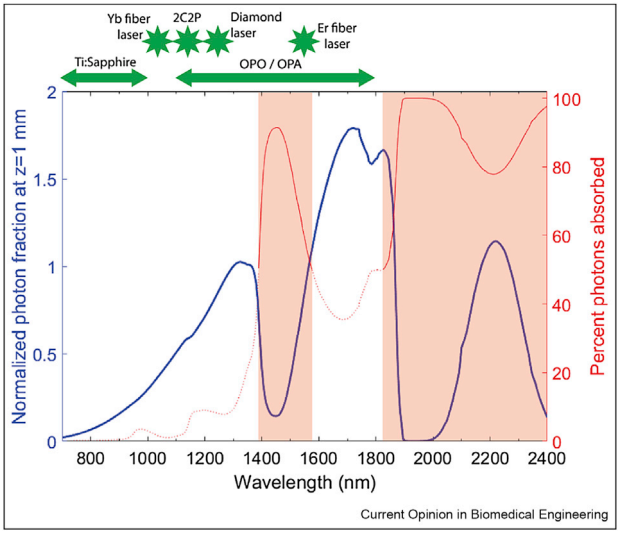
Role of tissue optical properties in imaging depth

Scattering and absorption, characterized by the scattering coefficient (μ_s) and the absorption coefficient

(μ_a), respectively, impact imaging depth by attenuating both the excitation laser light and emitted fluorescence, ultimately reducing the number of signal photons that reach the detector. The amount of scattered light decreases with increasing wavelength; however, absorption due to water increases significantly near 1500 nm and beyond 1800 nm. Figure 1 illustrates this wavelength-dependent effect by showing the normalized fraction of photons (blue line) that reach a depth of 1 mm for wavelengths from 700 to 2400 nm. Any scattered excitation light does not contribute to the multiphoton absorption process, so the fraction of photons of wavelength, λ, reaching a depth, z, can be modeled as $\exp[-(\mu_a(\lambda) + \mu_s(\lambda))z]$. The wavelength dependence of absorption is governed by water absorption, while the wavelength dependence of scattering is significantly more complex and difficult to measure, but is commonly approximated as $\mu_s(\lambda) = a/(1-g)(\lambda/500)^{-b}$, where g is the scattering anisotropy and is usually assumed to be approximately 0.9 for biological tissues [9]. Under this approximation, the scattering coefficient is determined by the values of a and b, which can vary considerably, even for brain tissue [9]. The scaling factor a and scattering power b are parameters used to characterize the scattering coefficient.

Figure 1 illustrates the contributions of scattering and absorption in biological tissue. The solid blue line is calculated using average values for brain optical properties (g = 0.9, a = 1.1 mm^{-1}, b = 1.37, water content = 75%) [9]. The plot demonstrates that 15 times more photons reach a depth of 1 mm using a wavelength of 1300 nm versus 800 nm, and 1.8 times more photons at 1700 nm versus 1300 nm. There is an additional peak near 2200 nm; however, it is in a red-shaded region indicating that more than 50% of the photons are absorbed by tissue. Although scattering and absorption attenuate photons with equal magnitude from a physics perspective, they are not equal from a physiological perspective. Excessive absorption is harmful to biological samples because it causes tissue heating. Wavelength regions in which >50% of photons are absorbed may not be viable for deep imaging without significant cause for concern due to excessive heating of tissue; thus, the peak at 2200 nm is not feasible for deep imaging. However, the peaks near 1300 nm and 1700 nm are viable and are the optimal wavelengths for deep imaging. At 1300 nm, conventional fluorophores may undergo two- or three-photon excitation depending on the fluorophore absorption characteristics, whereas most fluorophores undergo three-photon excitation at 1700 nm. We will use the term multiphoton microscopy (MPM) to collectively refer to fluorescence microscopy using two- or three-photon excitation, or any higher order excitation.

Figure 1



Effects of scattering and absorption. Photon fraction at a depth of 1 mm for average brain tissue optical properties (g = 0.9, a = 1.1 mm^{-1}, b = 1.37, water content = 75%) [9] is demarcated by the blue line. Regions shaded in red indicate areas in which 50% or more of photons are absorbed, as calculated by the red line (dashed red indicates below 50%; solid red indicates over 50%). Available laser options are outlined for their respective wavelength range (Yb = ytterbium, 2C2P = two-photon two-color of Yb fiber laser and diamond laser, Er = erbium, OPO = optical parametric oscillator, OPA = optical parametric amplifier).

Laser sources for deep imaging

Ti:S lasers have dominated the 2PM field because they reliably produce wavelength-tunable ultrafast pulses with high average power. Modern Ti:S lasers are robust, turn-key systems that enable routine 2PM imaging; however, they are not the optimal source for deep imaging within biological tissue. There are several approaches to increase imaging depth by reducing the effects of scattering in brain tissue while avoiding deleterious water absorption regions. A recent approach, adopted from astronomy, is the use of adaptive optics to shape the wavefront of excitation light to overcome tissue scattering [10]. An imaging depth of 700 µm was reached in mouse cortex using adaptive optics [11]. Another approach is to increase the pulse energy of a laser source such that a greater number of photons per pulse penetrate to deeper tissue. Ti:S lasers typically produce pulse energies on the order of 10 nJ for repetition rates around 80 MHz. Using a Ti:S laser as a seed for a regenerative amplifier, pulse energies on the order of microjoules can be attained for repetition rates between 100 and 500 kHz. Using a regeneratively amplified Ti:S laser at a wavelength of 925 nm and repetition rate of 200 kHz, Theer *et al.* (2003) achieved an imaging depth of 1 mm [12]. A third approach to deep *in vivo* imaging is to use longer excitation wavelengths for which scattering effects are not as significant. Ti:S lasers typically have a wavelength maximum near 1000 nm. An optical parametric