Two-photon microscopy enables sub-diffraction limit characterizations of millimeter-depth features in living specimens

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Two-photon microscopy allows for the imaging of both fixed and live tissues orders of magnitude deeper than what is possible with standard widefield microscopy techniques and does so at higher resolutions than those conventional techniques. There are numerous structures that were previously unable to be imaged in live specimens until the development of two-photon microscopy due to their depth in the tissue. While confocal microscopy can also image deeper into tissues than conventional widefield techniques, the penetration depth is still insufficient for many biological structures of interest. Before the development of two-photon microscopy, the primary methods for imaging such structures were tissue sectioning and scaling, i.e. making the tissues transparent via soaking in chemical solvents. Both of these methods are incompatible with living samples, and while they can also achieve two-photon-like resolutions, they cannot visualize any dynamics. Two-photon microscopy exploits the nonlinearity of its namesake effect, and by only exciting near the waist of the beam in the imaging plane, is able to achieve millimeter-depth imaging in tissues at higher resolutions.

Keywords: Two-photon microscopy, two-photon excitation, confocal microscopy, fluorescence, nonlinear optics, biological imaging

I. INTRODUCTION

There are numerous cases where the spatiotemporal organization of deep tissues and the biological processes within them were previously not able to be studied in live specimens. Two-photon microscopy (and later, multi-photon derivatives) enabled the study of such structures where no previously existing techniques could, such as its original intended target, the neuron [1, 4], along with more recent applications to systems like skin cells [2] and cancer metastasis [3]. In addition, is has been applied to the live imaging of numerous organisms such as fruit flies [5], zebrafish [6], mice [7], and humans [2].

Many of two-photon microscopy's benefits become apparent when compared to alternative techniques. Conventional widefield fluorescence microscopy techniques are not very well suited for deep tissue imaging. While these techniques can generally illuminate up to around 700 μm, they cannot clearly resolve anything at that depth, and while total internal reflection fluorescence microscopy (TIRF microscopy) is gaining popularity for its sensitivity, it is generally limited to penetration depths of one to two hundred nanometers [8]. Confocal microscopy is often used for three-dimensional imaging deeper in tissues, but it is limited to imaging structures up to a few microns deep [9, 10]. Scientists interested in deeper structures like the brain were initially able to circumvent the limit on imaging depth by euthanizing the animals, then taking brain slices [11]. Later, scaling, a chemical method of making a mouse embryo optically transparent enough to directly image the brain was developed [12]. However, both of these techniques relied on the termination of the specimen before imaging. In order to probe the dynamics of neuronal activity, scientists needed a way to image deep into animal tissue without the need for euthenasia. Two-photon microscopy filled this gap, and is able to image over a millimeter deep into tissue. In addition, it does so at a resolution higher than conventional widefield techniques, and comparable to confocal microscopy: well below the diffraction limit. After introducing the principles and practical considerations behind two-photon microscopy, the underlying reason for both increased penetration depth and resolution will be explained.

Two-photon microscopy relies on two-photon excitations (or more fundamentally, absorptions), a phenomenon initially theorized by Dr. Maria Göppert-Mayer in her doctoral dissertation [13]. As shown in figure 1, a fluorophore that is typically is excited by a photon with energy $E_b = h\nu_b$ can also be excited by the near simultaneous arrival (within a femtosecond) of two photons with energies $E_r = h\nu_r = \frac{1}{2}E_b$. A large amount of energy (generally hundreds of thousands of watts) is needed to guarantee a sufficient number of two-photon excitations. Continuous wave lasers (CW lasers) cannot maintain sufficient powers, so pulsed lasers like Ti-Sapphire lasers which can produce pulses of around $150,000W$ at a frequencies around $80MHz$ are used in their stead. The benefits of replacing a more simple, statistically likely excitation scheme with a less accessible nonlinear one may not be immediately obvious. However, the nonlinear nature of two-photon excitations is actually responsible for both two-photon microscopy's ability to achieve millimeter-depth imaging and its ability to image with increased resolution.

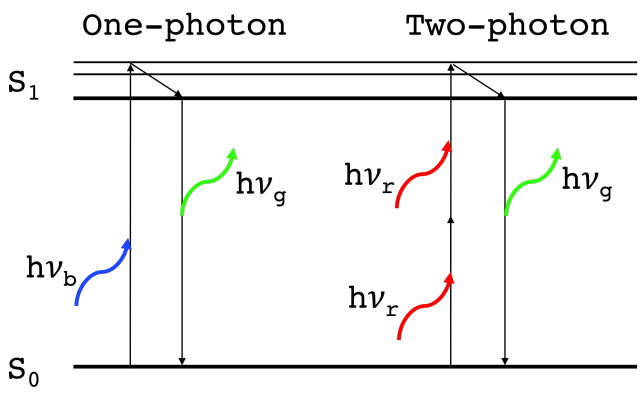

FIG. 1. Jabłoński diagram comparing one-photon and two-photon excitation events. For a two-photon excitation to occur, it is necessary that the combined energies of the red photons equal the energy of the blue photon, i.e. $h\nu_b = 2h\nu_r$, and that both red photons arrive within a femtosecond of one another. Of course, there is no requirement that the photons actually be red or blue, the colors are just for illustrative purposes.

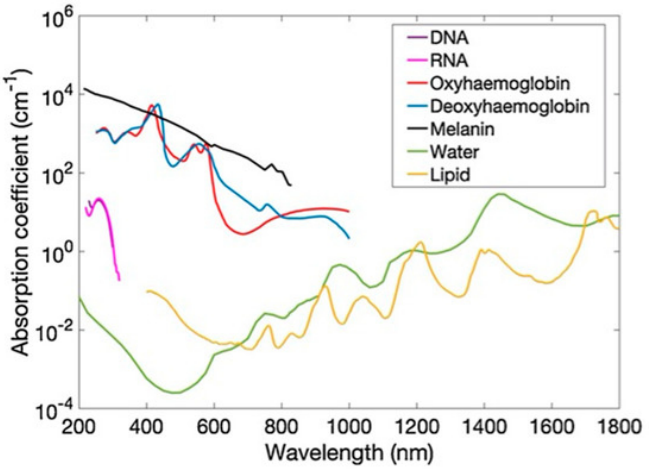

FIG. 2. The absorption coefficients of common biological materials. While the absorption coefficients of water and lipids increase with wavelengths in the red to infrared, the relevant materials in millimeter-depth biological imaging show decreased absorption coefficients under the same range of wavelengths. Reproduced from [14] under Attribution 4.0 International (CC BY 4.0).

II. TWO-PHOTON ENABLES MILLIMETER-DEPTH IMAGING

The primary optical effects that limit imaging depth in live tissues are scattering and absorption. Two-photon microscopy allows us to reduce the impacts of both of these interactions. It is often preferred that the fluorophores used in biological imaging have emission wavelengths in the visible. Consequently, when using a two-photon process to excite such fluorophores, an excitation beam with a wavelength in the red to infrared regime must be used. It is well established that the intensity of Rayleigh scattering scales with the inverse of the wavelength to the fourth power, i.e. $I \propto \frac{1}{\lambda^4}$. Then, it follows that increasing the wavelength of our excitation beam reduces the amount of scattering in the sample.

Using an excitation beam with a longer wavelength also reduces the amount of absorption in the tissue. As shown in figure 2, while the absorption coefficient of water and lipids is higher at higher wavelengths, the relevant biological mass in millimeter-depth imaging like blood and melanin feature dropoffs in their absorption coefficients at longer wavelengths (in particular in the red to infrared regime). Two-photon microscopy allows for the imaging of standard, biological fluorophores using excitation beam wavelengths that scatter and absorb less in living tissues.

Of course, the penetration of the excitation beam is not the sole factor in determining the maximum imaging depth of a given technique. In order to image deeper in the tissue while avoiding the detection of fluorescence from areas not of interest (and not in focus), millimeter-depth imaging techniques like two-photon microscopy need some way to isolate the signal from the imaging plane before detection. Because of both the rarity of simultaneous photon arrival and the fact that the emission intensity scales with the square of the excitation intensity, the only place in the beam where two-photon excitation occurs is near the waist, where the photon flux is maximized. In other words, the nonlinearity of two-photon excitations guarantees that the bulk of the detected signal originates from the imaging plane (as shown in the bottom pane of figure 3). The size and position of the excitation volume are also responsible for two-photon microscopy's ability to image at a higher resolution than conventional widefield techniques.

III. TWO-PHOTON ENABLES SUB-DIFFRACTION LIMIT IMAGING

The resolution of light microscopes was traditionally thought to be fundamentally limited by the diffraction limit, given as

$$d = \frac{\lambda}{2 * \mathrm{NA}}, \tag{1}$$

where NA represents the numerical aperture, or the range of angles over which a lens, in this case the objective lens, can accept light. This means that in general, fluorescence microscopes used for biological imaging are limited to resolutions of a few hundred nanometers. Numerous techniques have been pioneered that circumvent this limit, broadly referred to as super-resolution microscopies. While there are numerous types of super-

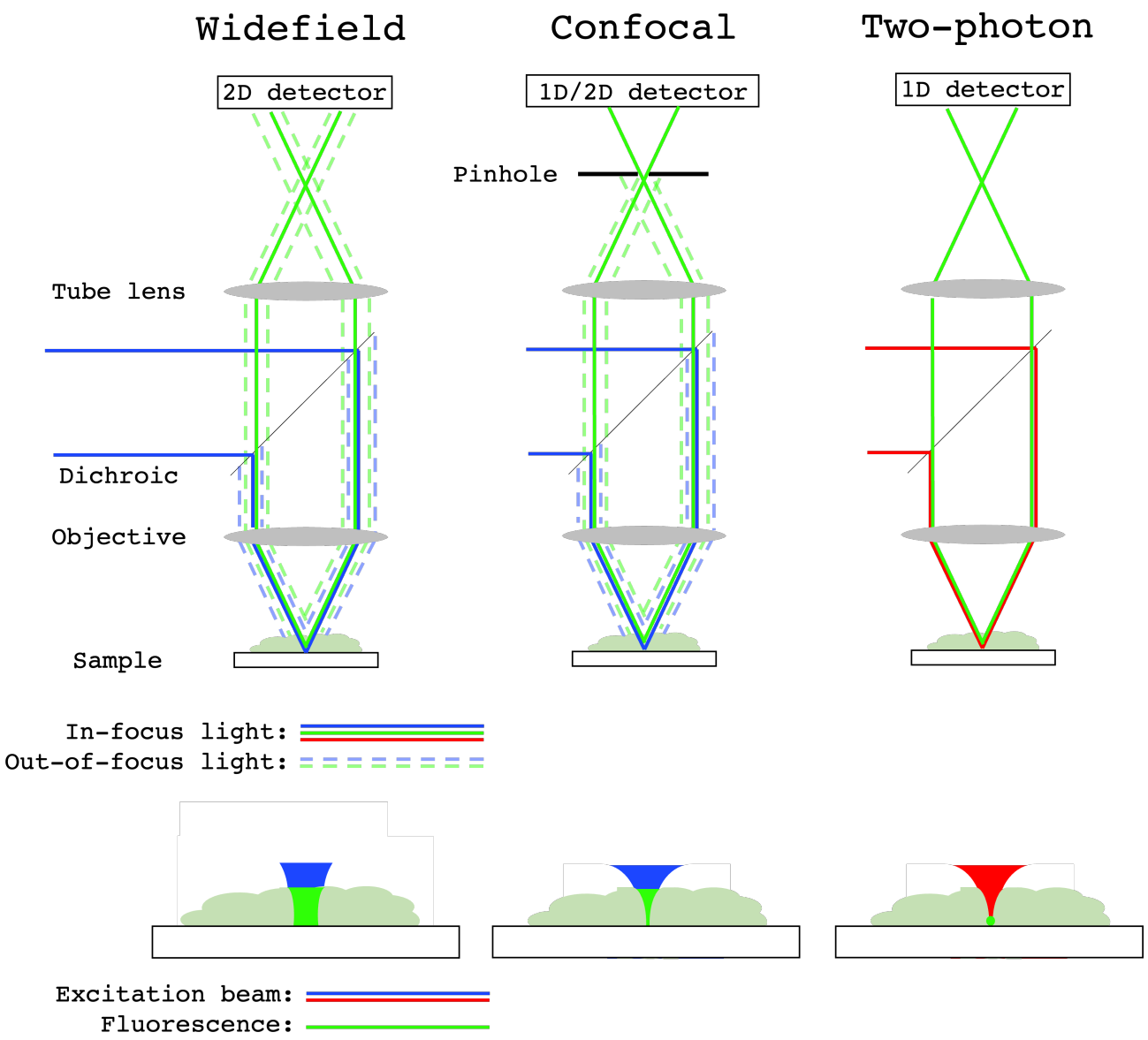

FIG. 3. Scheme comparing the optical excitation and detection paths of widefield, confocal, and two-photon microscopy. Notably, confocal microscopy acheives higher resolution both laterally and axially by using a pinhole in the excitation beampath (non shown here) to narrow the focal volume and another pinhole between the tube lens and the detector in order to block out of focus light. Two-photon microscopy has no need for these pinholes because the nonlinear nature of two-photon excitations constrains the excited region to only near the waist of the beam where the photon flux is maximized. The focal and excitation volumes are depicted in the bottom pane.

resolution techniques, such as localization-based techniques like STORM, PALM, and fPALM ([15–18]) and correlation-based techniques like SOFI and variants ([19–21]), they all require global single-molecule resolution, a feat generally only achievable at or near interfaces. While these techniques can achieve near-molecular resolutions, they are not yet suitable for the investigation of millimeter-depth features. However, there are other ways to get more modest increases in resolution, one of which is by preventing out of focus (both lateral and axial) light from reaching the detector, an effect most easily understood by comparing conventional widefield and confocal microscopes.

Traditional widefield microscopies have their resolutions limited partially by the presence of out of focus light in both the lateral and axial directions. Generally, confocal microscopy filters out the out of focus light in order to achieve higher resolutions at the cost of field of view.

This is done through the use of a set of pinholes. The first is used just before the beampath intersects with the dichroic (colloquially known as the illumination pinhole and not shown in figure 3 due to space) and is responsible for ensuring that the light source is as close to a point light source as possible. The second pinhole (colloquially known as the detection pinhole) is responsible for filtering out all the out of focus signal that results from excitation events outside of the imaging plane. As shown in the bottom pane of figure 3, the entire focal volume is excited in the confocal scheme. Much of the out of focus signal from the focal volume can be filtered out by the pinhole, but a combination of scattering and absorption still limits the imaging depth of confocal microscopy.

However, due to the nonlinearity of two-photon microscopy, nearly all of the signal from the sample is guaranteed to be from the imaging plane. As shown in figure 3, while the excitation beam still passes through much of the sample, only the fluorophores near the waist of the beam (incidentally in the imaging plane) are excited. This means that there is little to no out of focus light to be filtered out, and two-photon microscopy is able to achieve a finer resolution in a similar manner as confocal microscopy without a need for pinholes. In addition, fluorescent signal that is scattered by the sample is generally filtered out by the pinholes in confocal microscopy, but in two-photon microscopy, there is no need. Because the signal is nearly guaranteed to be from the focal volume at the imaging plane, scattered fluorescent signal can still be used by the one dimensional detector, often a photomultiplier tube (PMT), and actually serves to increase the sensitivity of detection in two-photon microscopy.

IV. CONCLUSION

The investigation of millimeter-depth tissues in live specimens was long impossible due an inability of linear fluorescence microscopy techniques to resolve features more than a few microns deep in biological tissue. These deeper features could be imaged by cutting slices of tissue and making specimens transparent by chemical means, but neither of these methods can be performed in live specimens. The nonlinearity of the two-photon excitations that underly two-photon microscopy enables the use of red to infrared wavelengths to specifically excite the small volume where the excitation beam and imaging plane intersect. This permits the imaging of live, millimeter-depth biological features (unachievable by confocal microscopy) at resolutions greater than achievable with conventional widefield techniques. It is for this reason that the development of two-photon microscopy has had such a major impact on biology as a whole.

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