Two-Photon Microscopy

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Non-linear Optics 568 fall, 2008

Abstract
Two-photon microscopy is a nonlinear optical microscopy which allows imaging tissues through fluorescence. Here we review fundamental concepts of Two-photon microscopy and based microscope, and discuss some relevant issues.

1. Introduction
Two-photon excitation microscopy (2PEM) is a special variant of the multiphoton florescence microscopy (MPM). It may be in some cases be a viable alternative to a confocal microscopy due to its dipper tissue penetration and reduced phototoxity. 2PEM has overcome the limitation of light scattering in a tissue and provides large depth of penetration mainly because in this process even a multiply scattered signal photon can be assigned to their origins as the result of localized nonlinear signal generation.

In the process of two photon absorption\(^1\) an atom makes a transition from its ground state to an exited state by the simultaneous absorption of the two laser photons. The absorption cross section \(\sigma\) describing this process increases linearly with laser intensity according to the relation

\[ \sigma = \sigma^{(2)} I \]

Where \(\sigma^{(2)}\) is a two-photon absorption strength coefficient. Generally this idea is based on that, two photons of low energy can excite an atom in fluorophore in a quantum event, resulting in the emission of a fluorescence photon, at a higher energy than either of the two excitatory photons. The success of the event depends on the two photons both interacting nearly simultaneously (~\(10^{-16}\) sec), resulting in a quadratic dependence on the light intensity rather than the linear dependence of a conventional fluorescence. The process termed as ‘nonlinear’ because the rate at which they occur depends nonlinearly on the intensity. This squared intensity dependence is the basic of the localized nature of 2PE: Doubling the intensity produces four times the fluorescence. We will review this again in bit more detail in the oncoming section but first a little bit of historical development of the interest and field.

2. History of MPM; 2PME
2PE employs a concept first described by Maria Goppert-Mayer in her 1931 doctoral dissertation\(^2\). It was first experimentally observed in 1962 in cesium vapor using laser excitation by Isaac Abella. The application of mode-locked Ti:S crystal-based lasers to 2PEM, first demonstrated in 1992 was really the beginning of practical 2PEM. Before that, 2PEM involved femtosecond dye lasers, which were temperamental and required constant tweaking. Commercial Ti:S lasers from Spectra-Physics (Mountain View, CA, USA) and Coherent (Sunnyvale, CA), now with broadband optics permitting use of the full tunable range. Publication involving 2PEM have suggested as femtosecond laser sources became robust and commercially available, and as the first commercial multiphotone microscope (MPM; 2PME) were introduced in 1996 by BioRad Microscience (Hermal Hampstead, UK).

As the interest of researchers increased and number of publications increases exponentially (fig below) during the last 18 years: A survey of the instrumentation used in the
studies employing MPM for biological research (255 out of 560 total references) indicates that 66% of these studies made use of laboratory-built systems, usually based on modified confocal microscopes. The remaining one-third employed commercial systems-27% from BioRad and 3.5% each from Zeiss (Oberkochen, Germany) and Leica (Wetzel, Germany). Furthermore, the majority ~80% of the publication are from a small number of research groups (~12%) who have developed the required expertise to use the technique effectively. Taken together, these statistics indicate that MPM is still a specialized technology, used successfully by some, but apparently not yet at the level of routine use characteristic of conventional (single-photon) confocal microscopy.

Publications employing, developing or reviewing MPM; 2PEM

Bar height (white and black) indicates the total number of references for the given year; black bars represent publications focusing on instrumentation development, the remainder being work in which MPM was used to help clarify a specific biological research goal. Gray bar is the estimated number based on twice the 2003 half-year total; dotted line is an exponential fit of the data.

3. Two-Photon Microscopy

Florescent dyes are standard tools for tracking cell population in animals, for the microscopic imaging of individual cells. Important challenges arise when attempting to visualize fluorescently labeled cells deep in living tissues, such as lymph nodes. Optical resolution in that case is limited by three main problems. First: High-numerical aperture objective lenses, which are required for fluorescence microscopy, have a narrow depth of field. Objects appear in sharp focus over a depth of (<1 micro meter), but with a conventional epi-florescence microscopy, fluorescence label above and below this plane glows brightly, therefore obscuring the image. Second: Scattering done by most tissues, limits the image contrast as the microscope is focused to increasing depths. Third and final: Intensity of excitation light will bleach the dye and cause photodamage to the cells over time.

Limitation of confocal microscopy (CM):

CM provides a partial solution to the problem. This technique provides a sharp optical section at a given depth inside a specimen by rejecting out-of-focus fluorescence that arises from above and below the focal plane. The principal of CM is simple (fig1).
Fluorescence excitation light (from laser) is focused in the specimen by the objective lens, and the fluorescence emitted from that spot is collected through the same lens and refocused to a spot centered on a small aperture (pinhole) placed before the detector, whereas out-of-focused fluorescence from above or below (Fig 1) the imaging pane is largely rejected.

For number of applications, CM works extremely well, and, by serial acquisition of optical slices taken at increasing depths along z-axis, it allows subsequent 3-D (x-y-z) reconstruction of the sample. However, CM is limited in its ability to see deep into the biological tissues (typically to a depth of only few tens of microns), because of attenuation produced by the scattering at focal spot of laser light and the collection of light from resulting fluorescent spot. And fluorescent is imaged only from the focal plane, but the specimen is exposed to laser above and below that plane, so that regions suffer from photobleaching and phototoxic effects even when they are not being visualized. So these issues with CM pose serious limitations for the long-term imaging and usage of live cells.

**Principles of two-photon excitation (2PE):**

2PEM serves a solution to these problems. It provides a completely different principle, of the same ‘optical-sectioning’ capability, but has the advantages of greater imaging depth and minimal photobleaching and toxicity. Twophoton excitation is not a new concept and it was used first for biological research more than ten years ago. But only in the past few years, improvements in laser technology and the availability of commercial multi-photon microscopes (based on the principals of non-linear optics) made it a practical tool for biologists who lack specialized expertise in laser and nonlinear optics.
In conventional fluorescence excitation, a fluorophore molecule, such as fluorescein, absorbs energy from a photon and re-emits most but not all energy as a second photon in a few nanoseconds. As the energy of a photon is inversely proportional to its wavelength (\(\lambda\)), the emitted photon has a longer wavelength than the exciting photon (for example, green emission with blue excitation). But two-photon excitation involves the almost simultaneous absorption by a fluorophore of energy from two photons, each of which contributes one half of the total energy required to induce fluorescence (fig1). The emitted fluorescence light is, therefore, of shorter wavelength than the exciting light. For example, a molecule of fluorescein can be excited by two photons of near-infrared light (\(\lambda\approx800\) nm), each of which has approximately half the energy of a single blue photon (\(\lambda=400\) nm), and then emit a photon of green light, in the same manner as for standard (one-photon) excitation with blue light (fig1). Because excitation depends on the simultaneous absorption of two photons, the resulting fluorescence emission increases in proportion to the square of the excitation intensity. This quadratic relationship gives 2PEM its main advantages. The focusing of light by a microscope objective lens leads to a high density of photons at the focal point, whereas the density falls off rapidly above and below the focal point (fig2). So, the probability that a fluorophore might undergo two-photon excitation is highest at the focal point, and drops to nearly zero at distances of less than 1 micrometer above and below the focal point.

![Fluorescence at the focal point](a) Single-photon excitation  

![Fluorescence Above and below the focal point](b) Two-photon excitation  

![Fluorescence](Intensity)

![Fluorescence](Intensity)

**Fig 2**

To achieve a useful number of two-photon excitation events, the photon density at the focal spot must be high, because a fluorophore molecule has to absorb two photons within an unimaginably brief time (\(10^{-16}\) seconds) of one another. Under normal conditions, this does not occur; the light intensity of a laser scanning confocal microscope is about one million times too weak. Now the question is how a biological specimen can be imaged by 2PEM without
vaporizing it. And the answer is pulsed laser, for which the peak power is extremely high, but the average laser power is relatively low and not much greater than a conventional CM.

The most commonly used pulsed laser system is the mode-locked titanium-sapphire (Ti:sapphire) ‘femtosecond’ laser, which provides pulses of infrared light (λ = 700–1000 nm) with a duration of ~80 femtoseconds at a repetition rate of ~80 MHz. A visualized picture can be obtained that how brief the pulse can be, by considering the laser beam as a series of ‘packets’ of photons, each about 25 micrometer long, traveling at the speed of light and separated from one another by gaps of about 2 meters. A crucial measure is the ratio of the pulse duration to gap duration — in this case, about $10^{-5}$. Therefore, the peak power during each pulse is $10^5$ times greater than the mean laser power, so that the fluorescence emission from a fluorophore excited by two photons is $10^{10}$ times greater than would be achieved by a CW laser of the same mean power (related to this issue, serious readers should turn to the list of papers given in the end).

Now as we know that fluorescence at the focal spot is achieved by focusing the beam from a pulsed laser through a microscope objective. So, an image can be formed simply by measuring the fluorescence intensity while constructing an image by scanning the laser spot point-by-point and line-by-line, in the same manner that a picture is built up on a television screen. As fluorescence arises only from the focused laser spot, all emitted light carries useful information, even if it has been scattered in the specimen. Unlike for CM, there is no need to detect fluorescence through an aperture; instead, a ‘wide-field’ detector (usually a photomultiplier) should be positioned close to the objective lens to collect as much of the emitted fluorescence as possible. In the coming section we will review the basic setup for 2PE microscope and components.

**Two-photon excitation microscope:**

A 2PE microscope is very superficially described here (more serious readers should turn to the list of papers given in the end). Any commercial confocal microscope can be converted into two photon microscope (fig 3), but major modifications have to be made to achieve optimum performance. Custom made microscopes typically lack in a microscope corpus. Most microscopes essentially consist of a pulsed laser source, beam intensity controller, beam telescope and scanner and a detector (see fig 3).

A typical 2PE microscope requires a pulsed laser source. Most widely used is titanium-sapphire (Ti: sapphire) oscillator, with a repetition rate of 100MHz. The two-laser combination presently
has a broader tuning range than a single-box laser (as much as 690–1020 nm compared with 720–920 nm) and the ability to stop mode-locking, which is sometimes useful to verify that the signal is actually due to two-photon fluorescence. Laser power available varies depending on the size of the pump, with 5W pumped system providing up to 1 W at the Ti:S peak wavelengths (~800 nm) and a few hundred milliwatts near the edges of the tuning curve (700 nm and 1000 nm). System with a 10 W pump source produces ~50% more power across the wavelength range.

**Typical Laser System Used Specifications:**

- Average laser power at the specimen = 100 mW, focused on a diffraction-limited spot of 0.5 micrometer in diameter.
- Area of the spot = $2 \times 10^{-9}$ cm$^2$
- Average laser power in the spot = $0.1 \, \text{W} \times 1/(2 \times 10^{-9} \, \text{cm}^2) = 5 \times 10^7 \, \text{W cm}^{-2}$
- Laser pulse is on for 100 femtoseconds every 10 nanoseconds; therefore, the pulse duration to gap duration ratio = $10^{-5}$
- Instantaneous power when laser is on = $5 \times 10^{12} \, \text{W cm}^{-2}$
- Output of a typical nuclear reactor = $10^9 \, \text{W}$
- Therefore, the peak energy density at the specimen during each pulse is equivalent to the output of 5000 nuclear reactors converted to light and focused on a square centimeter.

But another problem arises due to the shortness of the pulses. When a pulse passes through a dispersive material such as glass, it becomes 'chirped'; the longer wavelength components travel faster so the pulsewidth lengthens, but the spectrum remains unchanged. In the visible and near-IR region, all materials have positive dispersion (red leads blue), so that femtosecond pulses passing through optics are always 'positively chirped' and thus longer than they were directly out of the laser. Positive dispersion can be offset by adding negative dispersion before the beam travels through the optics. This is known as dispersion compensation, or pre-chirping, and has been applied to 2PEM$^7$, but leads to an instrument that is more complicated to operate and out of the scope of this paper. For optimum performance of the microscope (fluorescence generation and detection can be optimized for deep imaging) some important issues; excitation wavelength, beam size, pulse width, repetition rate, scattering, inhomogeneity of refractive index in tissues, optimization and issues with resolution, objective lenses and other important relative issues are not discusses here because of time, scope and length constraints of writing this paper. For these issues a list of selected articles is given in the end.

**Beam intensity control:**

There are several choices for controlling the laser intensity: A collection of neutral-density (ND) filters a rotatable polarizer, an electro-optic modulator (EOM or Pockels cell) or an acousto-optic modulator (AOM). The two modulators have the ability to blank the beam during scanner turnaround and flyback; however, EOMs are typically less dispersive. For example, an EOM with a 50- to 80-mm-long KD$^\text{P}$ crystal has between 2,000 and 4,000 fs$^2$ of dispersion, whereas an AOM made of TeO$_2$ can be 4-fold more dispersive.

**Beam Telescope:**

A beam telescope can be used to adjust the size of the beam at the back aperture of the objective to be sure the lens is 'overfilled' for a diffraction-limited focus, or in some cases, underfilled for an axially extended focus. The position shown in fig 3, the range of useable beam diameters is ultimately limited by the size of the XY scanner mirrors, which may be only a few millimeters in diameter.
**Beam Scanner:**

Among the various XY scanner designs available, the most common is the nonresonant point scanner, which scans the focused beam across the specimen with an adjustable scan speed, permitting software ‘zooming’ (variable apparent magnification by scanning a smaller region, more slowly) and the important ability to rotate the scan axis.

**Detectors:**

Photomultiplier tubes (PMT) are the dominant detectors for both confocal and 2PEM because an imaging detector is not needed for point-scanning systems, and the high gain and absence of readout noise favors PMTs. A recent improvement in detectors for 2PEM are GaAsP photocathode PMTs (Hamamatsu H7422P), which offer high quantum efficiency (QE) values in the important 400- to 650-nm range compared with PMTs using conventional photocathode materials.

The overall technical detail and functionality of the microscope is rather tedious and out of the scope of this paper.

**Advantages and Applications:**

The main advantage of 2PEM is that excitation is confined exclusively to the focal plane, whereas other regions of the specimen are exposed only to the relatively harmless infrared light. Undesirable processes, such as photobleaching and photodamage, are not prevented entirely; however, unlike for confocal microscopy, these processes are restricted only to regions where imaging takes place. A secondary, but important, advantage arises from the long wavelengths that are used for excitation. The extent to which light is scattered varies as a steep, inverse power function of wavelength. Infrared light penetrates most biological tissues with minimal scattering or absorption, which allows two-photon images to be obtained at much greater depths than is possible with confocal microscopy. The two-photon technique has several advantages over conventional confocal fluorescence imaging, including reduced photobleaching, minimal phototoxicity and the ability to resolve detail at greater depths in living tissues.

Table below summarizes several applications with their probes, of 2PEM.

<table>
<thead>
<tr>
<th>Application</th>
<th>Probe</th>
<th>Ex. (nm)</th>
<th>Em. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term tracking of living cells</td>
<td>CellTracker™ Green CFDA (CFSE)</td>
<td>760</td>
<td>516</td>
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<tr>
<td></td>
<td>CellTracker™ Orange CMTPR</td>
<td>820</td>
<td>566</td>
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<tr>
<td></td>
<td>SNAP-1</td>
<td>700–510</td>
<td>539–540</td>
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<tr>
<td>Intracellular calcium measurements</td>
<td>Indo-1 free</td>
<td>700</td>
<td>490</td>
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<tr>
<td></td>
<td>Indo-1-with Co2+</td>
<td>700</td>
<td>436</td>
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<tr>
<td></td>
<td>Fura-2 free</td>
<td>720</td>
<td>512</td>
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<td></td>
<td>Fura-2-with Co2+</td>
<td>700</td>
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<td></td>
<td>Calcium Green-1 with Co2+</td>
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<tr>
<td></td>
<td>Calcium Orange with Co2+</td>
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<tr>
<td></td>
<td>Fluor-3 with Co2+</td>
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<td>526</td>
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<tr>
<td></td>
<td>Yellow cameline</td>
<td>760–820</td>
<td>535–480</td>
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<td>Fluorescent protein conjugates</td>
<td>FITC</td>
<td>780–820</td>
<td>510</td>
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<td></td>
<td>BODIPY</td>
<td>920</td>
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<td></td>
<td>TRITC</td>
<td>800–940</td>
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<td>Rhodamine B</td>
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<td>Texas red</td>
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<td>CY3</td>
<td>780</td>
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<td>Cy5</td>
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<td></td>
<td>Cascade Blue®</td>
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<tr>
<td>Genetically encoded protein tags</td>
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<td></td>
<td>eGFP</td>
<td>560–620</td>
<td>470–400</td>
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<td>eYFP</td>
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<td>DsRed</td>
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<td>DsRed-td</td>
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<td>Visualization of organelles</td>
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<td>Rhodamine 123 (mitochondria)</td>
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<td>DAPI (nucleus)</td>
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<td></td>
<td>Hoechst (nucleus)</td>
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<td>478</td>
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4. Conclusion and potential areas of development:

2PEM is quickly becoming a standard tool for determining the molecular mechanisms of cell-based processes in basic biological research, tissue engineering and transgenic mouse models of disease and development. The number of publications focused on nonlinear microscopy development indicates that 2PEM has created its own field of research. Areas of development that hold particular promise range from fabrication of minimally dispersive objective lenses designed to optimize collection of scattered emissions, to the application of adaptive optics\(^9\) to 2PEM for correcting aberrations of the point spread function.

As presently implemented, 2PEM can image hundreds of microns deep. But its believed that the ultimate depth limitation is not often a result of a lack of laser power but rather difficulty in collecting the generated fluorescence due to both absorption and scattering, which leads to collection losses, as well as reduced fluorescence due to degradation of the IPSF. A simple test for inadequate 2PE is to measure the power dependence of the fluorescence deep within the specimen to test for saturation. If it scales as less than the power squared, saturation is occurring, indicating that more than sufficient excitation exists in the focal plane. In practice, we often find blurriness and a significant reduction in contrast in many specimens when the imaging depth is increased past several hundred microns, well before all signals is lost. Aberrations of IPSF can be caused by either heterogeneity in the index of refraction in tissue or TPE focal volume saturation. It may be possible to overcome the former difficulty by adaptive optics, in which the spatial phase of the beam is modified to pre-compensate for the path the rays take through the tissue. Curing the latter problem requires lower power compensated by better detection.

Nonlinear optics, the 'magnificent' area of modern physics that helped spawn 2PEM, still has more to offer. Femtosecond lasers are being devised that can operate in the 1,000- to 1,300-nm range, just beyond that which a Ti:S laser can conveniently reach. This region is especially important for high-viability imaging of redder dyes and fluorescent proteins. Several groups are developing forms of multiphoton endoscopy, and new photonic crystal fibers now allow fiber delivery of 100-fs pulses through optical fibers with more than enough power for 2PEM and multiphoton endoscopy\(^10\). Finally, the trick of modifying nonlinear optical responses by changing the phase of the spectral components that make up a femtosecond pulse—a field known as 'coherent control'—may make it possible to fine-tune multiphoton excitation to increase multiphoton absorption, reduce two-photon photobleaching.

Surely the magic of nonlinear optics (2PEM) will continue to play an important role in bio and other sciences.

For serious readers: The list of some important selected papers (2PEM) is given below:-

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1 Boyd, Non-Linear Optics. Text Book 3\(^{rd}\) edition.