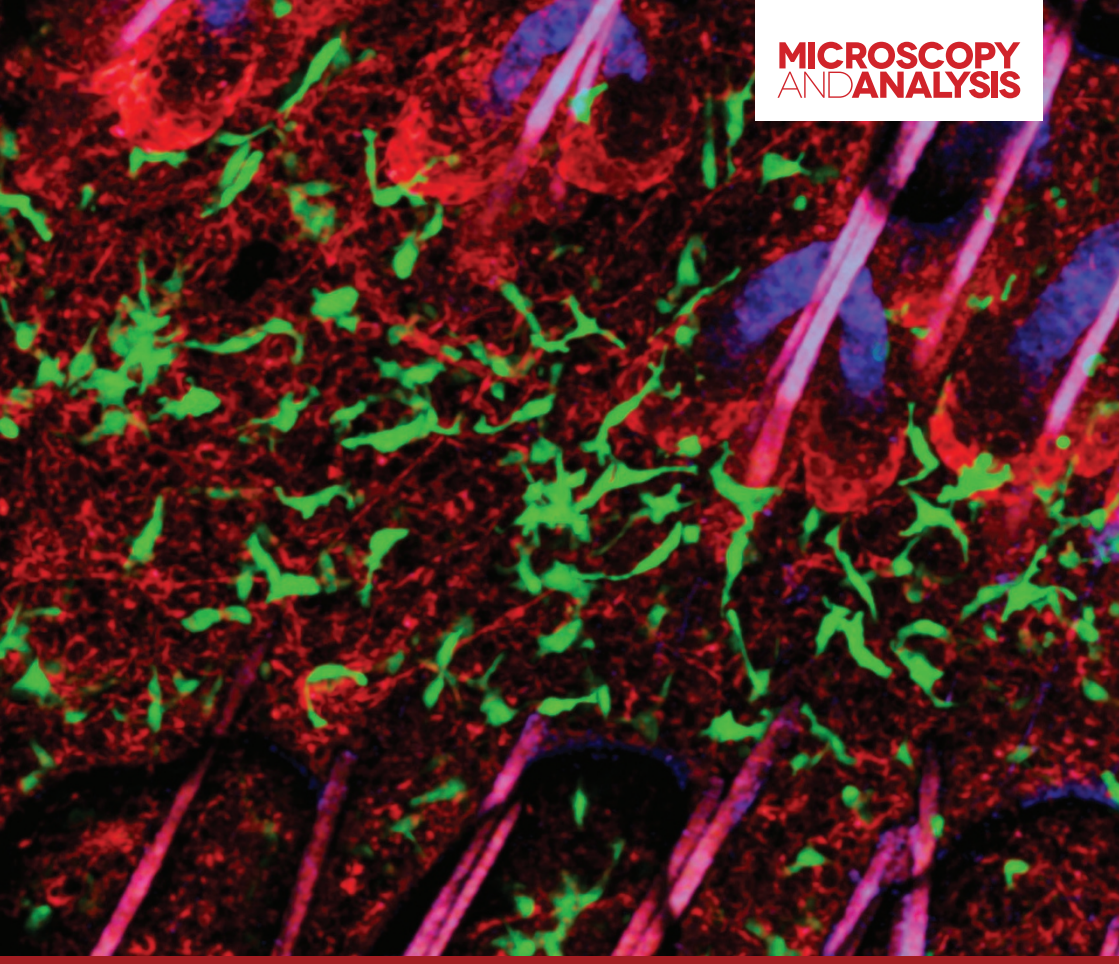
MICROSCOPY AND ANALYSIS



Multiphoton Microscopy

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Front cover: enhanced green fluorescent protein (eGFP)-expressing dendritic epidermal T cells (green) in the skin of a reporter mouse expressing GFP in T cells. Intravenously injected Rhodamin 6G demarcates the dermal–epidermal border (red). Hair shafts (purple) are rooted in hair follicles revealed by their sebaceous glands (blue autofluorescence). Image courtesy of Thorsten Mempel, Massachusetts General Hospital, USA

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CONTENTS

4 INTRODUCTION
6 HISTORY AND BACKGROUND
12 IN PRACTICE
22 PROBLEMS AND SOLUTIONS
29 WHAT'S NEXT?

About Essential Knowledge Briefings

Essential Knowledge Briefings, published by John Wiley & Sons, comprise a series of short guides to the latest techniques, applications and equipment used in analytical science. Revised and updated annually, EKBs are an essential resource for scientists working in both academia and industry looking to update their understanding of key developments within each specialty. Free to download in a range of electronic formats, the EKB range is available at www.essentialknowledgebriefings.com

INTRODUCTION

Modern light microscopy was born with the invention of the fluorescence microscope in the early 1900s. By labeling specimens with fluorescent tags, or fluorophores, that emit visible light when excited with light at other wavelengths, fluorescence microscopy was able to image cells and subcellular compartments for the first time. But early fluorescence microscopes suffered from such problems as out-of-focus flare and limited sensitivity, which led to the widespread adoption of confocal microscopy in the late 1980s. By focusing a laser beam at one depth level at a time and using a pinhole to block the out-of-focus flare, confocal microscopy is able to generate thin optical sections, and has remained the gold standard technique for imaging both fixed and living samples for many years.

Nevertheless, confocal microscopy still suffers from certain limitations, especially in terms of how deep it can image inside a sample. These limitations have now spurred the development of multiphoton microscopy (MPM), a more powerful fluorescent microscopy technique that can produce three-dimensional (3D) images of biological structures and processes at unprecedented depth scales.

By combining two photons with half the energy to excite fluorophores (both synthetic and natural) in a specimen, MPM can reveal dynamic biological processes occurring deep within living tissue. As well as providing 3D images at unprecedented depths, MPM can reduce the damage caused by extended illumination with the excitation wavelengths, known as photobleaching. It also provides more

efficient light detection and superior sectioning compared to confocal microscopy.

There are a number of multiphoton imaging techniques, ranging from two-photon microscopy of synthetic fluorophores to second-harmonic imaging of biological structures, as well as newer developments using three-photon absorption.

This Essential Knowledge Briefing (EKB) introduces these techniques in the context of life sciences research. It begins with an outline of the development of MPM from earlier, one-photon techniques before looking in detail at the workings of a multiphoton microscope, including the different imaging modes and sources of image contrast.

Aimed at new users, the EKB provides a detailed explanation of the operation of a typical multiphoton instrument, with particular focus on the importance of the laser source. It goes on to detail the specific practical requirements for different fields of research and to offer solutions to the universal challenges of imaging live samples and dynamic biological processes. There are also several case studies demonstrating how MPM is currently being used by researchers working in diverse fields such as neuroscience, cancer and immunology.

Finally, the EKB looks to the future of MPM. This future promises new and brighter fluorophores, longer wavelengths for deeper imaging, exciting new applications in neuroscience including simultaneously activating and imaging specific neuronal populations, and lasers powerful enough to permit the simultaneous absorption of three or more photons.

HISTORY AND BACKGROUND

The most widely used form of MPM is two-photon microscopy. This is the method of choice for imaging living biological tissue and even intact animals at high resolution and in real time, providing unparalleled insights into dynamic biological processes.

Two-photon microscopy evolved from confocal microscopy, which also generates optical sections and uses focused laser beams scanned in a similar pattern, allowing 3D images of a sample to be built up. In confocal microscopy, one photon is absorbed by a fluorophore (in a linear interaction), causing it to emit a single fluorescent photon. In two-photon microscopy, by contrast, two photons are absorbed by the same fluorophore at the same time (in a non-linear interaction).

Although the concept of two-photon absorption was first described by physicist Maria Goeppert-Mayer in the 1930s, it wasn't until 1990 that the first two-photon microscope was developed, when researchers at Cornell University in the USA combined two-photon excitation with established laser scanning microscopy. Since then, the technique has become increasingly popular thanks to further technological developments and cost reductions.

Because the energy absorbed by the fluorophore is equal to the sum of the two photons, a fluorophore may be excited by two photons with approximately half the energy (or double the wavelength) of the photon required for conventional, single-photon microscopy. For example, a fluorophore normally excited by a single photon at a wavelength of 500nm may be excited by two photons at a wavelength of 1000nm.

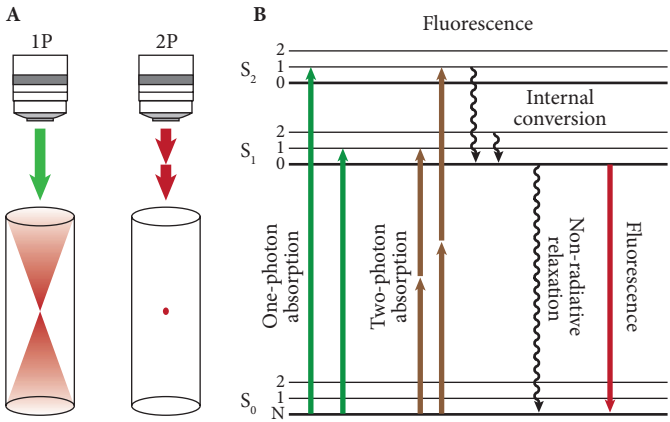


Figure 1. Principle of two-photon excitation. (A) The excitation volume of two-photon laser scanning microscopy is confined within the focal plane, while single-photon excitation shows strong out-of-focus contribution. (B) Fluorescence represented in a simplified Jablonski diagram. When a fluorophore absorbs two photons, each with half the energy necessary for single photon excitation within 10^{-18}s, two-photon excitation is elicited and fluorescent light will be emitted a few nanoseconds later. Figure from Wu Z, *et al.* Multi-photon microscopy in cardiovascular research. *Methods* 2017;130: 79–89. Reproduced with permission from Elsevier, Copyright 2017 Elsevier

Crucially, this means that excitation with infrared wavelengths will produce fluorescence at visible wavelengths (Figure 1).

As might be expected, two-photon absorption is ordinarily a very rare occurrence, and so making sure it happens sufficiently frequently requires concentrating the excitation photons in both time and space. The photon density needed to generate two-photon excitation is approximately one million times that required for one-photon excitation.

The simultaneous absorption of three photons – each with a third of the energy or three times the wavelength of the single photon – has also been demonstrated and requires only 10-fold greater photon density than two-photon absorption.

Because three-photon microscopy uses even longer excitation wavelengths and higher-order non-linear excitation, it offers even deeper imaging capabilities. Although at a much earlier stage of development than two-photon microscopy, three-photon microscopy has already been used to visualize neurons at greater than 1mm depth in a mouse brain, allowing non-invasive recordings of neuronal activity at high resolution.

Producing a high enough photon density for two-photon microscopy requires higher-powered lasers than used for confocal microscopy. To avoid damaging the specimen, these high-powered lasers emit ultrashort pulses of photons, which can be tuned to occur at different frequencies and to last from femtoseconds to picoseconds: typical Ti:Sapph lasers used in two-photon excitation have pulse widths from 70fsec to 140fsec (with a period of 12.5nsec). The pulse widths for three-photon excitation are around 40fsec. By ensuring the laser is off for much longer than it is on, ultrashort pulses provide photons at sufficient densities to stimulate two-photon absorption while avoiding damage to the specimen. The goal is to increase the individual pulse energy without increasing the average power.

The high-power, ultrashort pulsed laser beam is focused to a single point within the sample by the optical components of the two-photon microscope, ensuring the highest spatial concentration of photons is achieved at the focus of the beam. It is only at this focal point that the photons are sufficiently concentrated to produce two-photon absorption.

This confinement of the absorption volume is a primary advantage of MPM. In confocal microscopy, the excitation light

interacts with fluorophores all along its path through the specimen, generating fluorescence as it goes. This requires placing a pinhole in front of the detector to block the out-of-focus flare. In two-photon microscopy, however, the excitation light only excites molecules at the focal spot of the microscope. In other parts of the specimen, where photons are present at much lower densities, two-photon absorption generally does not occur, minimizing unwanted fluorescence and out-of-focus glare. This means MPM can distinguish weaker signals (typically from deep inside the specimen) than confocal microscopy.

MPM also benefits from reduced light scattering. Scattering of light by tissues is highly complex, but, broadly speaking, longer wavelengths of light are scattered less than shorter wavelengths. Thus the infrared light used in two-photon microscopy undergoes less scattering than the shorter wavelengths of light used in confocal microscopy. As a consequence, these shorter wavelengths can penetrate much further into the tissue sample, up to 1mm for some applications.

Some scattering of the resultant fluorescence is inevitable, but this is less of a problem for two-photon microscopy than for confocal microscopy. Whether a fluorophore is excited by one photon or two, the photons it subsequently emits are identical and will be identically scattered. However, scattered emission photons have a greater chance of reaching the detector in two-photon microscopy than confocal microscopy, as there is no pinhole to obstruct them.

Because of its ability to build up 3D images of samples in real time without causing damage, MPM has proved adept at studying both biological samples and living organisms. As

such, it is now commonly used for basic science studies in a wide range of life-science fields, including cell biology, embryology, cancer and immunology, as it is able to track cells as they migrate and interact with each other and the surrounding environment. More recently, it has begun to be used for clinical applications such as diagnosing skin cancer and screening cancer biopsies for drug resistance.

Some of the most exciting uses of two-photon micros copy are, however, in neurophysiology, where it is being used to image both extracted samples of brain tissue and neurons in the brains of living animals. It is also being combined with various other techniques to extend its capabilities. These techniques include calcium imaging, which uses fluorescent molecules that respond to the binding of calcium ions to monitor neuronal firing, and optogenetics, which uses light to activate individual neurons genetically engineered to express light-sensitive ion channels. Activating specific subsets of neurons with light and simultaneously imaging them with two-photon

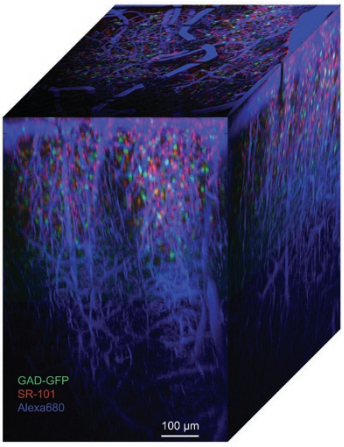


Figure 2. *In vivo* two-photon imaging through an optically transparent electrode array. Three-dimensional projection of a two-photon image stack. The vasculature is visualized by intravascular injection of Alexa 680-labeled dextran. Inhibitory neurons are labeled with eGFP. Astrocytes are labeled with SR101. Image courtesy of Anna Devor and Shadi Dayeh, University of California San Diego, USA

microscopy can reveal network connectivity in the brain with unprecedented temporal and spatial precision.

Two-photon microscopy can also be used to release a specific molecule into a sample in a temporally and spatially controlled manner, known as 'two-photon uncaging'. Releasing neurotransmitters in this way can mimic synaptic activity in the brain, allowing the stimulation of highly localized neuronal structures and an assessment of their structural and functional effects over time. This technique has been used to study synaptic signaling and to map complex neural circuits.

Although two-photon microscopy has almost become synonymous with MPM, there are other microscopy methods based on multiphoton interactions. The most important of these is second-harmonic imaging microscopy (SHIM), which is based on another non-linear optical effect known as second-harmonic generation (SHG). This is a scattering process whereby two photons of the same wavelength interact simultaneously with certain biological structures, leading to the emission of a single photon with twice the energy (and half the wavelength).

SHIM is particularly useful for biological imaging, as the technique is non-absorbing and thus does not cause photobleaching. It is most often used for imaging cell and tissue structure and function, and is particularly useful for providing label-free imaging of ordered crystal-like molecules in the body, such as collagen and elastin.

IN PRACTICE

All multiphoton microscopes have several core components, although the precise specification may vary depending on the application. These core components include: a femtosecond laser to produce a high-power photon beam; a scanning system to scan the focal point of the beam and create the image pixel-by-pixel; an objective lens, normally with a high numerical aperture; and a detector (Figure 3).

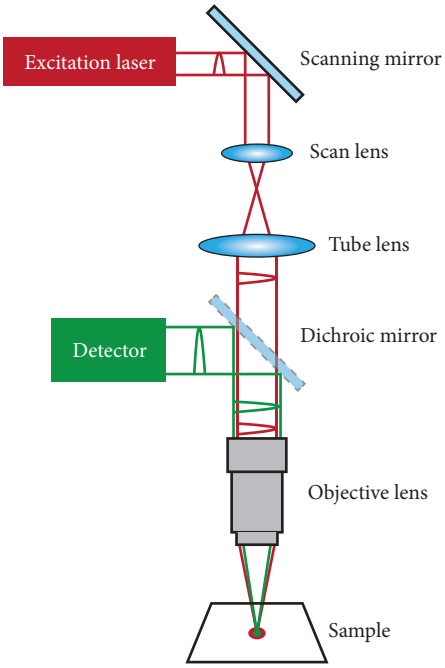


Figure 3. A schematic of a scanning two-photon microscope, showing excitation by infrared laser pulses and collection of the resulting visible fluorescence or phosphorescence. Reprinted from the February 2015 edition of *Laser Focus World*. Copyright 2018 by PennWell

Laser

The advent of ultra-fast pulsed (mode-locked) lasers was the main impetus behind the widespread adoption of multiphoton microscopes for life-science applications. The laser source must be high power and mode-locked to create the photon concentration needed for two-photon absorption at the focal point. The most widely used ultra-fast mode-locked lasers are the Ti:sapphire laser and the Nd:YLF laser. Both of these are able to produce femtosecond pulses, with the Ti:sapphire laser having the advantage of being able to be tuned from wavelengths of 700nm to 1100nm, while the Nd:YLF laser only emits light at one wavelength (1047nm).

Scanning system

Two-photon microscopy relies on exciting fluorophores within a single focal point at a time. So to build an entire 3D image, this focal point must be scanned over the sample, with each focal point creating a single image pixel. Electrically controlled, moveable mirrors known as galvanometric (galvo) mirrors are typically used to scan the beam.

With standard scanning galvo mirrors, the mirror angle is varied by motors. By having two mirrors for the x and y plane, it becomes possible to create any arbitrary beam path. This is useful for various applications, such as tracing the outline of a cell membrane. The disadvantage of scanning galvo mirrors, however, is the limited speed at which they can move, due to heating of the motors caused by repeated acceleration and deceleration.

A more recent development is the incorporation of a resonant galvo mirror into multiphoton systems. Resonant mirrors

rotate or oscillate at a fixed frequency controlled by an electrical input; the beam is scanned across the sample at this resonant frequency, allowing resonant galvo mirrors to create an image far faster than scanning galvo mirrors. Resonant galvo mirrors do not illuminate the sample as evenly, however, because the resonant mirror moves at different speeds during the scanning process. Some MPM systems offer both sets of scanners, providing the option of slower but more even scanning with standard scanning galvos or faster scanning with resonant galvos.

Lenses

The objective and relay lenses focus the laser beam down to the focal point required for imaging, and also focus the resultant emitted photons towards the detector. The numerical aperture of a lens and the wavelength of light used to image a sample impose the theoretical resolution limit of the microscope. The objective lens also has a defined field of view, which determines the maximum angle at which scattered photons from the sample can still be detected, and hence the area the lens can image.

Lenses are continually being developed to offer a higher numerical aperture with an increased field of view, as well as to correct for various aberrations that can cause the focal point to become larger than its theoretical limit. High numerical aperture oil and water immersion lenses are often used with multiphoton microscopes.

Detectors

The detectors most commonly used in MPM are photomultiplier tubes, which are highly sensitive detectors of light. The

geometry of the detectors is a particular advantage of MPM over confocal microscopy. In confocal microscopy, it is essential that the excitation photons and the photons emitted from the sample follow identical paths. If the paths were different, it would not be possible to assign the photons to a specific pixel, as they could have come from other parts of the sample. This type of light path, where the excitation and emission light path is identical, is known as descanned. The disadvantage of a descanned geometry is that photons are lost each time they pass through mirrors or other optical components, and any photons that came from the focal point but are scattered will be lost.

With MPM, as all the emitted photons can only come from the single focal point, they do not need to travel along the same optic path to the detector to determine which pixel they belong to. This means one or more detectors can be placed anywhere after the objective lens. Known as a non-descanned configuration, this allows for more efficient photon collection because the detectors can now be placed much closer to the objective lens. This means highly scattered photons can be collected and photon loss reduced, as the emitted photons now pass through fewer optical components before reaching the detector.

CASE STUDY 1. Memory circuits

Atilla Losonczy and his group at Columbia University, USA, are using two-photon microscopy to reveal the formation of memory within the living brain. A small region in the hippocampus called the dentate gyrus is a major focus of this research. The hippocampus is the brain's center for learning and memory, and the dentate gyrus houses a neuronal population of particular interest, as it is one of very few regions in the brain that continues to create new neurons during adulthood.

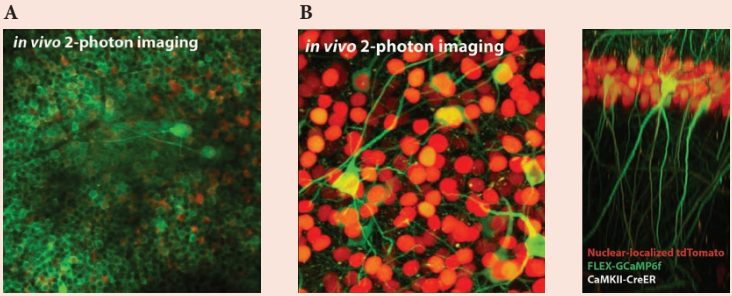
This area is believed to be important in helping the brain to distinguish similar but slightly different stimuli, an ability known as pattern separation: for example, the ability to distinguish two faces with very similar features. As the dentate gyrus is buried deep within the brain surrounded by tightly packed neurons that are rarely active, scientists have previously had difficulty studying it.

'We have used two-photon microscopy to image populations of cells in the dentate gyrus while mice are moving along a treadmill with different visual, audio, taste and odor stimuli,' explains Losonczy. He and his team incorporated a fluorescent calcium indicator into these neurons so that when firing they fluoresce for a short period of time.

By monitoring this firing, Losonczy and his team were able to show how networks of neurons within the dentate gyrus respond to these stimuli. They also showed that there are several neuron types within the dentate gyrus, each with a specific role in pattern separation and memory formation.

'This work is key not only to understanding the basis of memory formation but also psychological pathologies such as

schizophrenia, where changes in the cells in the dentate gyrus can be seen histologically,' Losonczy says.



(A) *In vivo* two-photon image of hippocampal dentate gyrus. Green: GCaMP; red: tdTomato in young adult-born granule cells. (B) Left: *in vivo* two-photon image of hippocampal area CA1. Red: nuclear localized tdTomato in CA1 pyramidal cell bodies; green: Cre-dependent sparse expression of GCaMP in some CA1 pyramidal cells labeling cell bodies and dendrites. Right: z-stack of the imaged volume. Reproduced with permission from Attila Losonczy

Danielson N, Turi G, Ladow M, *et al*. In vivo imaging of dentate gyrus mossy cells in behaving mice. *Neuron* 2017;93:552–9.e4. (https://doi.org/10.1016/j.neuron.2016.12.019)

Danielson N, Kaifosh P, Zaremba J, *et al*. Distinct contribution of adult-born hippocampal granule cells to context encoding. *Neuron* 2016;90:101–12. (https://doi.org/10.1016/j.neuron.2016.02.019)

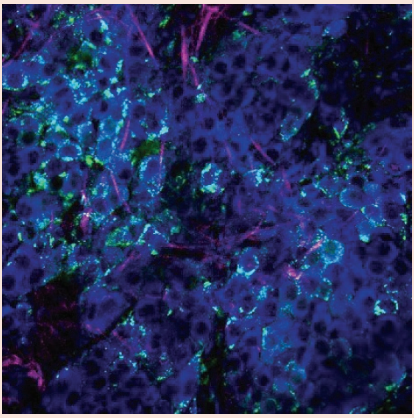
CASE STUDY 2. Personalized cancer treatment

Melissa Skala (an Associate Professor of biomedical engineering) and her group at the University of Wisconsin–Madison, USA, are investigating whether MPM can be used as a platform for personalized medicine and drug discovery for numerous types of cancer. 'Our group uses multiphoton microscopes to probe the metabolic state of cancer cells in response to treatment, both in 3D in vitro *and* in vivo *cancer models,' explains Skala.*

Her group assesses cellular metabolism in tumor cells, which can indicate whether or not a cancer treatment is working within a matter of days. 'We assess the cellular metabolic response by imaging two endogenous, fluorescent co-enzymes, NAD(P)H and FAD, which are involved in multiple metabolic processes,' says Skala. Looking at the relative abundance of these co-enzymes and measuring their fluorescent lifetime (the time it takes for an electron of an excited molecule to decay back to its ground state and emit a photon) provides a quantitative readout of cellular metabolism. This is powerful information when used in combination with MPM.

MPM is able to provide single-cell resolution, which is critical when assessing a tumor's response to treatment. Small subpopulations of tumor cells can still be resistant to a given treatment, even if the majority of cells respond. These subpopulations can then grow into a new tumor and cause a recurrence of the cancer in patients.

'Multiphoton microscopy allows us to screen for drugs that would kill all subpopulations of cancer cells to prevent recurrence,' Skala says. 'It also offers intrinsic optical sectioning, and



Multiphoton image of a melanoma. NADH fluorescence in blue, FAD fluorescence in green, SHG from collagen in pink. Image courtesy of Tiffany Heaster, University of Wisconsin–Madison, USA

the use of near-infrared excitation wavelengths allows us to image deeper into tissue and causes less photodamage than the visible wavelengths used in other common fluorescent microscopes.'

This application of MPM is still in preclinical studies but, if proven accurate, could be used for matching cancer patients with the most effective drugs for treating their tumors and for screening experimental drugs to determine if they would be good candidates for clinical trials.

Sharick J, Favreau P, Gillette A, *et al*. Protein-bound NAD(P)H lifetime is sensitive to multiple fates of glucose carbon. *Sci Rep* 2018; 8:5456. (https://doi.org/10.1038/s41598-018-23691-x)

Heaster T, Walsh A, Zhao Y, *et al*. Autofluorescence imaging identifies tumor cell-cycle status on a single-cell level. *J Biophotonics* 2017;11:e201600276. (https://doi.org/10.1002/jbio.201600276)

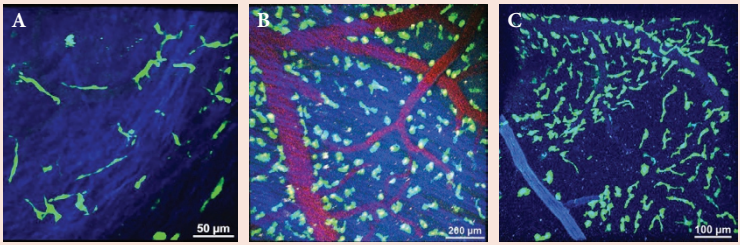
Cannon T, Shah A, Skala M. Autofluorescence imaging captures heterogeneous drug response differences between 2D and 3D breast cancer cultures. *Biomed Opt Express* 2017;8:1911–25. (https://doi.org/10.1364/BOE.8.001911)

CASE STUDY 3. A new look at the eye

Pedram Hamrah and his team at Tufts University, USA, are developing MPM techniques to study inflammatory and infectious diseases of the eye in living mice.

Immune cells in the eye are critical for maintaining its clarity and preserving vision, but the mechanisms underlying their entry and exit from the cornea remain poorly understood. By fluorescently tagging these cells and imaging them in the corneas of living animals, two-photon microscopy could help to unravel this issue.

Hamrah and his team are applying two-photon microscopy to the eyes of anesthetized animals to track immune cells moving to and from the cornea, creating 3D time-lapse movies at subcellular resolution. These movies allow them to visualize the movement and interaction of these immune cells and to investigate the signals that might guide the cells to both healthy and inflamed corneas.



(A) Multiphoton micrograph of bulbar conjunctiva of a normal transgenic mouse with GFP-tagged dendritic cells showing GFP+ dendritic cells in green and collagen (SHG) in blue. Scale bar: 50µm. (B) Multiphoton micrograph of the brain of a normal transgenic mouse with GFP-tagged dendritic cells injected intravenously with quantum dots shows dendritic cells (green), blood vessels (red), and collagen (SHG; blue). Scale bar: 200µm. (C) Multiphoton micrograph of the brain of a normal transgenic mouse with GFP-tagged dendritic cells shows dendritic cells (green) and collagen (condensed in the media of the vessels; SHG; blue). Scale bar: 100µm

This work could provide new drug targets for inflammatory, infectious and autoimmune diseases of the eye. In addition, it could lead to new and more targeted strategies for immunotherapy that involve modulating the movement of immune cells, which could also be used to prevent rejection during corneal transplants.

Lopez MJ, Yamaguchi T, Harris DL, *et al.* Multi-photon intravital microscopy of corneal draining lymph nodes demonstrates immune cell alterations and kinetics after corneal allotransplantation. *Invest Ophthalmol Vis Sci* 2014;55:5044. (https://iovs.arvojournals.org/article.aspx?articleid=2270613)

PROBLEMS AND SOLUTIONS

Live imaging of dynamic biological processes

One of the greatest challenges for MPM is imaging dynamic biological events in real time. The dynamics of many biological events, such as immune cell interactions or neuron firing, occur over extremely short timescales. For any microscopy technique, capturing these dynamics requires imaging at high speeds without causing photodamage to the sample.

MPM already has an advantage over other fluorescent microscopy methods for imaging dynamic biological processes. The longer wavelengths of light used in MPM inherently cause less photodamage and, as only fluorophores at the focal point of the beam are excited, photodamage to the sample surrounding the focal point is also reduced.

Imaging at rapid speeds requires delivering sufficient photons to the focal point in a short space of time and the ability to move the beam across the sample faster than the biological process being studied. An obvious way to deliver more photons to the focal point is with higher-power lasers, but another way is to use existing power more efficiently. This can be done by beam shaping, in which special lenses alter the

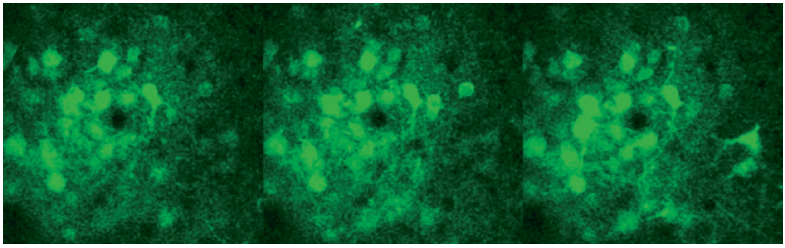


Figure 4. Resonant scanner imaging of neuronal signaling, indicated by changes in calcium level of layer 2/3 cells in somatosensory cortex of an awake mouse during behavioral trial. Changes in calcium level measured using GCaMP

shape of the laser beam in both space and time to provide more efficient delivery of photons to the focal point.

Scanning the focal point across the sample quickly is currently achieved through the use of resonant scanners. When operating a galvo mirror at a resonant frequency, images can be obtained at up to 40 frames per second, whereas scanning galvo mirrors are limited to 1.5 frames per second. Nevertheless, further speed increases are always being sought after and one avenue of research is the use of electrically tunable lenses, which allow the focal point to be scanned in the axial plane. These could replace the slower piezoelectric motors that are currently used to move the sample in the axial direction.

Image processing

Image processing in MPM comes in as many forms as there are applications. In the case of live *in vivo* imaging, the most common image processing need is to correct for motion artefacts produced by movement of the area being imaged between frames. Motion artefacts can be caused by animal movements, breathing or even heartbeats.

Good experimental design is the first step in correcting for motion artefacts. Many specialist experimental setups exist, such as head frames or kidney cups, to keep organs and organisms still during imaging. However, there will inevitably be some motion artefact, particularly in awake animals or when imaging the visceral organs or heart. One of the most common methods to process this data is with a reference image. One image is chosen from all those acquired during the experiment, either by the researcher or a software package, so

that other frames can be compared to this reference image and rejected if they are too dissimilar.

Another strategy is to gate the two-photon recording: measuring the two-photon signal only at specific points in the breathing or cardiac cycle. To do this, the multiphoton image capture software has to be linked to other physiological recording devices, such as an electrocardiogram device or respiratory pads. This is more commonly used in cardiac MPM, as the heart cannot be completely immobilized.

Image processing is also important for imaging extracted organs that have been rendered transparent by a chemical process (cleared), as this can generate single-cell resolution data sets for whole organs that are several terabytes in size. Here, specific image processing pipelines are often developed depending on the type of analysis.

Multicolor multiphoton

As with all fluorescent imaging techniques, the ability to image multiple fluorophores allows more sophisticated experiments to be performed. The fact that different fluorophores are excited by and emit slightly different wavelength photons can be used to distinguish multiple fluorophores within one sample, allowing selective excitation or labeling of specific structures or processes.

In theory, different fluorophores can be distinguished by exciting only one fluorophore at a time, through a tunable laser source, or by exciting all fluorophores and filtering the emitted fluorescence for the wavelength of choice. In practice, fluorophores normally have overlapping spectra, particularly

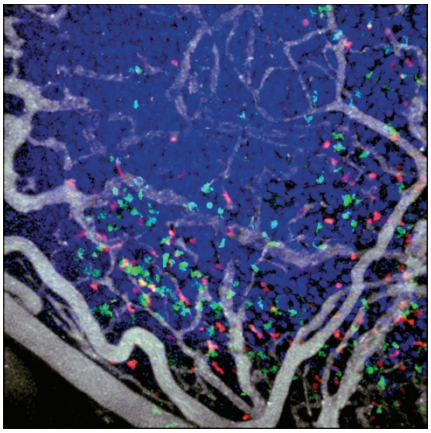


Figure 5. GFP-expressing cytotoxic T lymphocytes (CTL, green) and TdTomato-expressing regulatory T cells (Treg, red) infiltrating a mouse colon carcinoma implanted into a dorsal skinfol chamber. Tumor cell nuclei (blue) are tagged through expression of a Cerulean-histone H2B fusion protein. Blood vessels (white) are highlighted through intravenous injection of QTracker 655 quantum dots. The question here was whether CTL and Treg interact with each other directly, which turned out not to be the case. Image courtesy of Thorsten Mempel, Massachusetts General Hospital, USA

for two-photon excitation, as these peaks are generally very broad. Therefore, multicolor MPM tends to employ a combination of different laser excitation wavelengths, emission filtering and an analysis technique known as spectral demixing to separate the signals from individual fluorophores.

To maintain the imaging speed, multiple emission filters and a small number of excitation wavelengths are generally used. For example, two laser pulses can be used at wavelengths of 800nm and 940nm, with the emissions then detected by numerous detectors, each with a different filter. Non-descanned configurations with multiple detectors and filters make such a setup possible and maximize the photons collected at each detector. Using this kind of measure, a research group recently reported the simultaneous detection of seven fluorophores by MPM in different cell and tissue compartments.

Fluorophore considerations

An important requirement when conducting MPM experiments is to understand the spectral properties of the fluorophores

being used. The two-photon cross-section of a fluorophore is a measure of two-photon absorption at different wavelengths and needs to be determined before a fluorophore can be used in two-photon microscopy. This is a technically challenging procedure, requiring tunable lasers that must be calibrated alongside the detectors for the various wavelengths and laser powers.

For two-photon microscopy, the proximity of the maximum cross-section of the fluorophore to the absorption peaks of water is an important consideration. Water makes up a high proportion of all tissues and has a broad absorption peak centered around 1500nm and beyond 1800nm. To prevent heating of the specimen, these wavelengths are generally avoided, and so two-photon cross-sections centered at 1300nm and 1700nm are often preferred for deep tissue penetration.

Simultaneous stimulation and imaging

Simultaneous optical stimulation and imaging offers huge potential for studying neural circuits. In model animals such as mice, MPM offers the possibility of imaging active neural circuits with single-cell precision though the depth of the neocortex.

Monitoring neuronal activity is possible using fluorescent microscopy alongside various synthetic or genetically encoded proteins that fluoresce in response to the binding of calcium ions. A particularly specific and reliable marker is the genetically encoded calcium indicator GCaMP, a GFP-containing fusion protein.

In addition to monitoring neural activity, however, light can also be used to manipulate it. Optogenetics was first conceived in the late 1990s and took off rapidly after its first

demonstration by a team at Stanford University, USA, in 2005. Optogenetics relies on opsins, a family of naturally light-sensitive ion channels, which are found in the cell membrane of many microbes and allow the passage of different chemical species into or out of the cell in response to light. By genetically engineering neurons to express these proteins, they can be made to fire when illuminated with a particular wavelength of light.

Neurons in the mouse brain can be manipulated to express both opsins and GCaMP. When stimulated at one wavelength, the opsin channels open, causing the illuminated neuron to fire. The GCaMP calcium reporter can then be imaged via a second laser system, allowing neural activity to be monitored in response to the specific activation.

Multiphoton microscopes that combine neuronal photostimulation and calcium imaging are now commercially available. Other recent developments include the ability to selectively excite ensembles of neurons in 3D within the brain whilst monitoring the response of all the neurons in the field

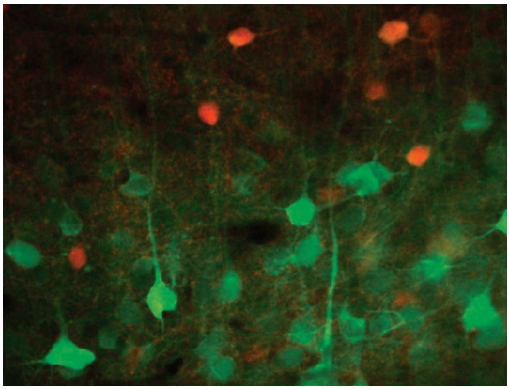


Figure 6. Chronic imaging through microprism in a live mouse, with interneurons labeled in red (td Tomato) and principal cells labeled in green (GCaMP6). Image courtesy of Michael Goard, University of California Santa Barbara, USA

of view. This is made possible by the use of spatial light modulators (SLMs), which split a single laser beam into a number of spots in an arbitrary pattern. These single spots can then be scanned over a set of neurons to stimulate them. This technique has so far been used to stimulate tens of neurons simultaneously, while monitoring the response of all the neurons in the field of view.

WHAT'S NEXT?

Since the first demonstration of two-photon microscopy in 1990, the applications of this technique have expanded rapidly and MPM has proven to be a powerful tool for life sciences researchers across the board. The ongoing development of new and more advanced component parts should continue this into the future, ensuring that MPM becomes an ever more valuable tool for biologists.

As well as efforts to increase the speed, resolution and field of view of MPM, longer excitation wavelengths are also on the horizon. Longer wavelengths are beneficial because they are less susceptible to scattering and so can penetrate further into biological tissue for deeper imaging. Alternative approaches for increasing penetration depth include combining two-photon microscopy with micro-optics such as gradient-index (GRIN) lenses. GRIN lenses are less prone to the optical aberrations of conventional spherical lenses and so can provide access to deeper structures, allowing imaging up to several millimeters into the mouse brain.

MPM can also be combined with tissue clearing, which renders a sample translucent, to reduce light scattering and allow deeper imaging of a sample. This is particularly useful for anatomical imaging of entire organs. Another approach is to try to collect more scattered photons by, for instance, adding a ring of optical fibers around the objective.

Three-photon microscopy also offers the potential to image deeper into samples, without the need for any additional modifications. As longer wavelengths are scattered less, three excitation photons can penetrate deep into tissue – up

to 3.5mm. In addition, three-photon microscopy benefits from an enhanced signal-to-noise ratio because the focal point of light at which three-photon excitation occurs is more tightly focused. Three-photon microscopy is fast becoming a research reality, as developments in more powerful laser technology render the method efficient and workable.

As well as obtaining images from deeper within the sample, efforts are ongoing to increase the speed of imaging. Fluorophore signal-to-noise ratio improvements will be essential here, as faster scanning generally results in a reduced signal.

Researchers are particularly interested in longer wavelength fluorophores, as they suffer from less scattering and can reduce background interference, such as that caused by autofluorescence from the sample. Researchers have recently developed red fluorophores (which have a longer emission wavelength than the conventionally used green fluorophores) for imaging live cells with two-photon microscopy. Developing fluorophores with more diverse kinetic profiles is also important, as those with a fast response can be stimulated with a higher frequency.

MPM can also image samples without any fluorescent labels at all by taking advantage of cellular molecules that are naturally fluorescent, known as endogenous fluorophores, such as the co-enzymes detailed in Case Study 2. Researchers have recently described the multicolor imaging of several endogenous fluorophores with two-photon microscopy, using a technique called wavelength mixing. This involves using two synchronized laser pulse trains to generate an additional two-photon excitation wavelength,

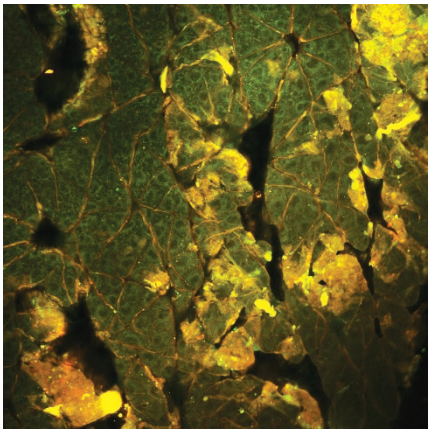


Figure 7. High-resolution two-photon excited fluorescence intensity image of mouse epithelium. Intrinsic NADH fluorescence is in green, while autofluorescence from keratin is in red/yellow. Cell morphology can be inferred from NADH signal, which primarily emanates from mitochondria. Image courtesy of Kyle Quinn, University of Arkansas, USA

allowing the simultaneous excitation of blue, green and red fluorophores in a sample.

Other, related imaging techniques such as coherent anti-Stokes Raman spectroscopy (CARS) and SHIM require no exogenous tags. CARS microscopy uses signals from inherent molecular vibrations, while SHIM relies on SHG signals produced naturally by some biological structures.

As already mentioned, some of the most interesting future applications for MPM are in neuroscience. The unprecedented depth power of MPM offers exciting new possibilities for investigating the body's most complex and least understood organ.

Imaging of live, active brain circuits is an area that is only starting to be explored. Thanks to the advent of photostimulating and calcium-sensing proteins, there is huge potential for imaging neural circuits deeper in the brain. Two-photon microscopy and optogenetics have already been used to stimulate and image specific neuronal populations, creating a hugely powerful technique for linking neural activity to

behavior. Combining these techniques with multicolor labels and other label-free techniques, such as SHIM, will only serve to create more opportunities for highly sophisticated neural investigations.

Efforts already underway to improve the use of MPM for neuroscience include using SLMs to shape the laser beam in order to rapidly excite large numbers of cells in parallel, allowing for simultaneous imaging of neurons in different locations. Other exciting work just emerging involves combining two-photon microscopy with functional magnetic resonance imaging – the gold standard for assessing brain activity – to connect individual neuron dynamics to whole brain activity.

Moving beyond basic science, there are also clinical applications in the pipeline for MPM. The ability of MPM to provide high-resolution images of unfixed and unstained tissue in real time has high clinical potential. Miniaturized multiphoton endoscopes could, for example, be used to provide real-time diagnoses of cancer.

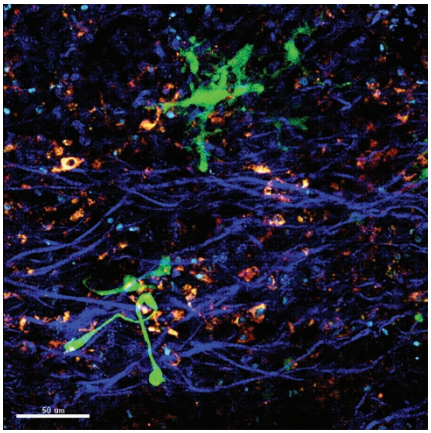


Figure 8. A humanized mouse (a mouse with a human immune system) was injected with a human immunodeficiency virus that expresses GFP in infected cells. The image shows two infected T cells (green) amidst collagen fibers (blue, second harmonic signals) and autofluorescent cells (white/orange hues) in a lymph node. The movie that this image is from was recorded to assess the migratory behavior and other characteristics of HIV-infected T cells. Image courtesy of Thorsten Mempel, Massachusetts General Hospital, USA

Currently, cancer diagnoses generally require an invasive biopsy, which is both painful for the patient and delays diagnosis. Multiphoton endoscopy combines the high resolution of MPM with the ability to penetrate into the body – necessary for real-time tissue diagnosis.

Researchers at Cornell University, USA, have recently developed a compact and portable multiphoton endoscope that can generate images of unstained tissue at submicron resolution. This could be used to guide tissue biopsy and even as a standalone tool for diagnosing cancerous lesions.

In the years since its development, MPM has become the tool of choice for imaging biological processes in living cells, tissues and animals. Offering unmatched penetration depth and low phototoxicity, a wealth of applications for MPM continue to emerge. And as the development of MPM continues to accelerate, many more applications are undoubtedly still to come.

FURTHER INFORMATION

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http://www.spectra.arizona.edu

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