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REVIEW

Practical intravital two-photon microscopy for immunological research: faster, brighter, deeper

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With its emphasis on minimally invasive high-speed imaging of intact tissues at depth, video-rate two-photon microscopy has revolutionized cell biology. This is particularly true in immunology, where the orchestration of cell migration, cell–cell interactions and intracellular signalling events in multiple distinct anatomical compartments within secondary lymphoid organs is fundamental for achieving an effective immune response. Until recently, access to this powerful tool has been limited to a handful of laboratories with the necessary skills and resources to either custom-build or purchase a commercial two-photon microscope. However, with the entry of more commercial vendors into the market and availability of turnkey solutions, two-photon microscopy is now becoming more accessible. Here, we discuss the practical aspects of establishing a basic intravital two-photon microscopy facility specifically for immunological research and how recent advances in ultrafast lasers, non-linear optics and localized photochemistry can be used to build more sophisticated instruments to support applications such as photoactivation and photobleaching, spectral fingerprinting and automated single-cell tracking. In addition, we discuss the next generation of fluorescent dyes and reporter mice and some of the microsurgical principles required to expose the relevant biology to interrogation by two-photon excitation.
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Time-lapse intra-vital microscopy has revolutionized *in vivo* cell biology. This is particularly true in immunology, where the orchestration of cell migration, cell–cell interactions and intra-cellular signalling events occurs in multiple distinct anatomical compartments over protracted periods of time. However, intra-vital microscopy is still limited by a number of fundamental problems that include (1) the disruption of image quality, signal strength and depth penetration by light scattering and autofluorescence; (2) limitations in physical access and field of view (FOV)because of the mechanical size and optical properties of commonly used objectives lenses; and (3) the issues of physiological maintenance, phototoxicity, anaesthesia and life support, which must be addressed during extended recording episodes. The incorporation of two-photon techniques specifically optimized for time-lapse imaging has helped overcome some of these issues. In particular, minimally invasive, video-rate two-photon microscopy has helped mitigate the effect of scattering and autofluorescence thereby maximizing image quality while at the same time maximizing temporal resolution and extending depth penetration.

Two-photon excitation was predicted by Göppert nearly 80 years ago.[1] However, it would take another 60 years before Denk *et al.*[2] at Cornell University combined two-photon excitation with a laser scanner to create the first two-photon microscope. Soon thereafter, the availability of tunable mode-locked Ti:Sapphire lasers made two-photon microscopy more practical as it no longer required a specialist laser laboratory.[3] The first commercial two-photon microscope was introduced by Bio-Rad (Hercules, CA, USA) in 1996. Since then there has been a rapid rise in use of two-photon microscopy,[4] which is now recognized as the gold standard for minimally invasive fluorescence microscopy of tissue explants and living animals.[5,6] Indeed, the parallel development of enabling technologies such as single-box turnkey ultrafast lasers, high-volume image data acquisition and analysis software, and fluorescent dyes and reporter mice has made two-photon microscopy much more accessible. Nevertheless, two-photon microscopy is still regarded as a specialized technology beyond the reach of most laboratories, unlike the almost universal adoption of confocal laser scanning (single-photon) microscopy.

Herein, we discuss the advantages of two-photon microscopy, which make it ideal for immunological research and describe the basic two-photon microscope and how recent innovations have made it more powerful and user friendly. The expiration of the patent for two-photon laser scanning microscopy has opened the market to competition from more commercial vendors, which may make it more affordable. Two-photon microscopy is now more accessible and it is timely to review the practical considerations that may help guide the prudent acquisition of a two-photon system.

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ADVANTAGES OF TWO-PHOTON EXCITATION

Two-photon fluorescence excitation is caused by two photons arriving simultaneously (within a femtosecond of each other) at a fluorophore. When compared with single-photon excitation, these photons need only possess half the energy for this excitation to occur and therefore light with twice the wavelength can be used. Hence, what are the advantages of two-photon over single-photon excitation? As excitation is localized to the focal plane (or optical section) where the photon density is sufficiently high for two photons to interact simultaneously with a fluorescent molecule, there is no out-of-focus excitation and negligible phototoxicity and photobleaching compared with single-photon excitation. Furthermore, the absorbance of light by haeme-containing proteins, water and lipids is minimal and the amount of light scattering is much lower at the longer infra-red (IR) wavelengths used for two-photon excitation[7] (Figure 1). Consequently, biological samples are more optically transparent to IR light and two-photon microscopes can penetrate much deeper into tissues (typically 400 µm in lymphoid tissue)[8] than confocal laser scanning microscopes (which can only penetrate 50 µm deep). This makes it possible to optically section an intact organ in an anaesthetized mouse without destroying the stromal cell networks, lymphatic vessels, blood supply and innervation that is critical for supporting tissue homeostasis and physiological responses to injury.

Although a confocal laser scanning microscope uses an emission pinhole to reject out-of-focus light and reduce image blurring this feature is not required in two-photon microscopy. Instead, non-descanned detectors (NDDs) can be used because the entire signal arises from the focal plane of interest. One advantage of using NDD is that it can significantly simplify the emission path of two-photon microscopes and facilitate multi-colour imaging.

It is also possible to achieve three-photon excitation with current ultrafast lasers. However, though *z*-axis resolution is improved with three-photon excitation,[9] optical sectioning, signal strength and signal-to-noise properties of three-photon excitation are not significantly improved compared with two-photon excitation,[10] and excitation wavelengths are typically moved away from the ideal 'optical window'.

Finally, another advantage of two-photon microscopy is the intrinsic fluorescence from second harmonic generation by collagen, and other non-centrosymmetric molecules[11] in biological tissues can provide important structural information and define anatomical landmarks such as the lymph node capsule,[8] subcapsular sinus,[12] blood vessels,[8] stromal cell networks[13] and follicular conduits.[14]

THE BASIC TWO-PHOTON MICROSCOPE SYSTEM

Two-photon microscopes represent a small niche market and most commercial systems are actually confocal laser scanning microscopes that have been modified to accommodate an added light path for two-photon excitation. This is not an ideal situation because it complicates the light path (often increasing temporal dispersion and reducing transmission efficiency) and for many years contributed to the superiority of dedicated custom-built two-photon microscopes such as the ones pioneered by Ian Parker at the University of California Irvine and Mike Sanderson at the University of Massachusetts Medical School.[15,16] An alternative is to 'strip down' a commercially available two-photon microscope by removing the unnecessary confocal elements such as confocal pinholes, continuous wave lasers (for single-photon excitation), epifluorescence filters, transmitted light illuminators and detectors, DIC and Dodt gradient contrast sliders, and so on. This will not only streamline the performance of the microscope but also substantially reduce its cost. More commercial vendors have entered the market and some now also offer dedicated two-photon systems. The modules that make up the basic two-photon instrument are described below and can be seen in Figure 2.

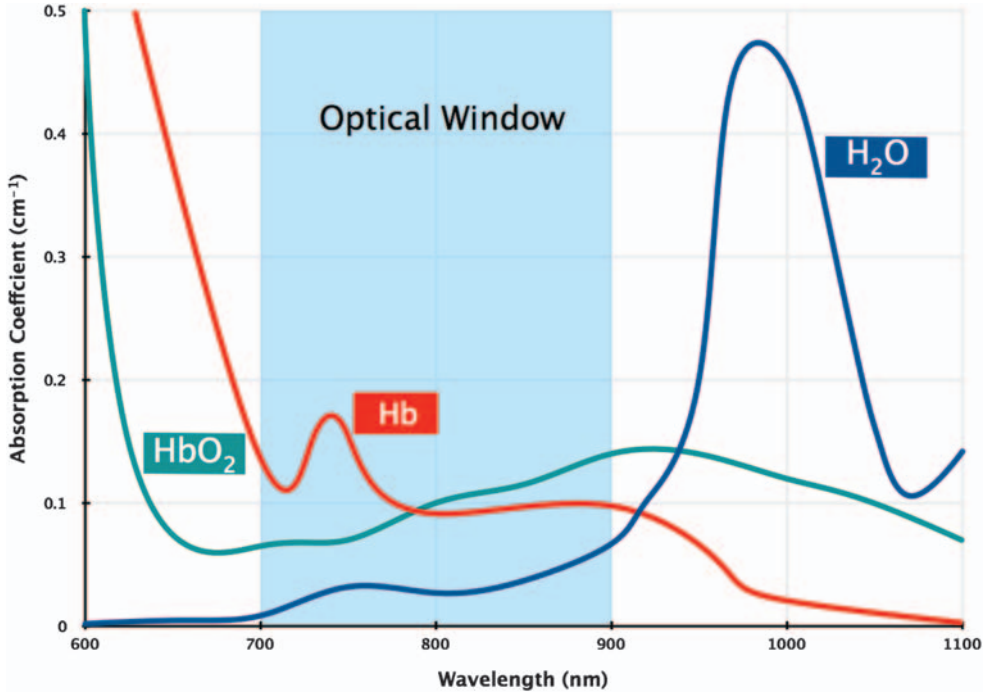

Figure 1 The absorption spectra of major tissue light absorbers haemoglobin and water. In the 'optical window' between 700 and 900 nm there is little single-photon absorption (except by melanin that has a high absorption coefficient and may be present in lymph nodes and spleens of non-albino mice). Biological tissues are considered optically transparent in this commonly used imaging window.

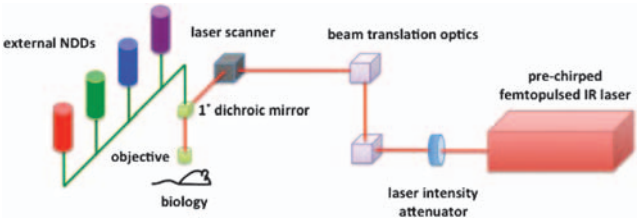

Figure 2 The basic two-photon microscope system mounted on a vibration isolation table comprises a single-box pre-chirped femtopulsed IR laser to provide two-photon excitation; beam translation optics to deliver the laser beam to the back aperture of the objective; a laser intensity controller to attenuate the laser power; a laser scanner to scan the specimen; a low magnification high numerical aperture water immersion objective; and a bank of external non-descanned detectors (NDDs) to simultaneously capture multiple fluorescent channels.

Ultrafast femtopulsed laser

Single-box turnkey solutions now available from Newport Spectra-Physics (Mountain View, CA, USA) and Coherent (Santa Clara, CA, USA) house the pump laser and Ti:S oscillator (required to mode-lock the output from the pump laser to produce a train of ultrashort pulses typically 100 fs wide at a frequency of 80 MHz) in the same factory-sealed box. Two-photon excitation with a pulsed laser depends on the average intensity squared and therefore the high frequency and peak power of these ultrashort pulses are critical for achieving efficient two-photon excitation. Although these Ti:S lasers have a wide tuning range (680–1080 nm), they work best at ~800 nm and peak pulse power drops off significantly at the limits of their tuning range. Another recent innovation is the incorporation of automatic dispersion compensation ('pre-chirping') by means of a grating, chirped mirror or pair of prisms to negate the pulse-broadening effect of refractive media in the light path such as lenses and mirrors. Separate pulse conditioners are therefore no longer required with the current generation of ultrafast lasers.

Laser power intensity control

Most microscopes use mechanical control device such as a neutral-density filter or a polarizer, or an electrical control device such as an electro-optic modulator (or Pockel cell) or acousto-optic modulators to attenuate the laser intensity. Both electro-optic modulators and acousto-optic modulators have no moving parts and can therefore provide much faster and more precise control over the laser power than the mechanical control devices. These devices can be coupled to a varying drive voltage determined by the z-focus position to enable automatic laser power adjustments to compensate for loss of signal at depth (adaptive illumination).[17] By reducing the amount of laser power attenuation as the objective scans deeper into the tissues, the image brightness and signal-to-noise ratio can be significantly improved.

Beam translation optics

The translation optics is used to steer the laser beam between various devices between the Ti:S laser and back aperture of the objective lens. These mirrors are optimized to operate over a wide spectral range at the power levels of Ti:S lasers with maximum transmission efficiency and minimal pulse dispersion. Nevertheless, efficient two-photon excitation is best achieved by having the most direct light path with the fewest optical devices in the way.

Objective lens

High numerical aperture and low magnification water immersion objectives such as the Olympus 20×0.95NA IR water immersion lens (Olympus, Tokyo, Japan) are ideal for two-photon microscopy as they provide a large FOV (to track displacement by highly motile cells across microanatomical compartments), high spatial resolution in *x, y* and *z* planes (300–350 nm and 1.3–1.5 µm, respectively, depending on the wavelength),[18] good transmission for both excitation and emission wavelengths and a couple of millimetres of working distance (for micro-manipulation and deep tissue penetration). In addition, water immersion lenses are ideal for intravital imaging as biology is best perfused with serum-free cell tissue culture media heated at 37 °C and bubbled with 5% CO_2/95% O_2 to maintain optimal tissue oxygenation and pH.

Laser scanner

For immunological research, a high-speed scanner is critical to capture rare short-lived events (such as calcium fluxes) as well as obtain accurate measurements of highly motile cells. Galvanometer-driven scan mirrors are limited by mechanical inertia and can only scan at a maximum line scan speed of 1 ms so the maximum frame rate for a 512×512 pixel full FOV scan is <2 frames per second (fps). This can be doubled by bidirectional scanning, which requires some image distortion correction. The advantage with point scanners is that they allow digital zoom, image rotation and targeting of region of interest with complex morphologies for photolysis. However, a common practice in commercial marketing brochures is to quote scan rates for the smaller region of interest scans rather than a 512×512 pixel full FOV scan, which misrepresents the true speed of the microscope. One attempt to overcome the mechanical limits imposed by a galvanometer is to multiplex the excitation laser beam with parallel beam splitters. This may work for confocal laser scanning microscopy but is not practical for two-photon microscopy as the probability of two-photon excitation depends on the average squared intensity and the splitting of the laser power exponentially reduces the two-photon signal achieved. The more practical solution is to use a resonant scan mirror as pioneered in the original custom-built microscopes.[15] The typical configuration uses a fast 8 kHz resonant scanner that undergoes fixed rotational vibration in the *x* direction and a slow galvanometer-driven scanner in the *y* direction. Bidirectional scanning using this configuration can achieve up to 30 fps for a full 400×480 pixel FOV scan.[19] However, unlike the saw-tooth waveforms that drive point scanners, resonant mirrors have a sinusoidal profile and require more complex correction of image distortion. In addition, there is no control over the pixel dwell time and depending on the signal-to-noise ratio it may be necessary to average the signal over multiple frames to improve image quality. For example, averaging five frames at 30 fps reduces the effective scan rate to 6 fps. Unfortunately, high-speed resonant scanners have a fixed path and do not support region of interest scans or targeted photolysis.

Detectors

The most efficient detection configuration is to use external NDDs placed as close as possible to the back aperture of the objective. The use of a pinhole to reject scattered light in descanned detectors in confocal laser scanning microscopes is unnecessary and actually degrades the image brightness because two-photon fluorescence originates from a small focal volume or 'point source'. NDDs also permit the simultaneous detection of multiple fluorescence channels by use of dichroic mirrors and bandpass filters.[19,20] Photomultiplier tubes (PMTs) offer high-signal amplification and low noise with a wide dynamic range making them ideal for use in two-photon microscopy. A more expensive alternative is to use charge-coupled device (CCD) cameras but these have narrow dynamic range and poor signal-to-noise discrimination despite their higher quantum efficiency.

In addition, CCD cameras are more susceptible to noise from scattered emitted light than PMTs making them less suitable for two-photon microscopy. A more useful innovation may be to couple the *z*-stage position to the PMT voltage to increase the signal gain as the microscope scans deeper into the tissue (adaptive sensitivity).

Vibration isolation table

Several manufacturers offer optical tables with breadboards to isolate the microscope optics from any vibration that can put the laser out of alignment. It is a good idea to choose the size of the table to not only accommodate the current microscope configuration but also allow for the addition of add-ons such as a second laser in the future.

MICROSCOPE ADD-ONS

Several optional extras are currently available that may enhance the performance of the basic two-photon system. However, some of the improvements are small and may come at a high cost. Moreover, it is important to future proof the basic configuration so that new developments can be added on in-the-field. For example, it may be prudent to customize the microscope frame with an extra port for a second laser before it leaves the factory floor.

Optical parametric oscillator

The first generation single-box ultrafast lasers (Spectra-Physics Mai Tai) had a tuning range limited to 720–920 nm and could not efficiently excite red fluorescent dyes and proteins. However, the current generation of lasers have a much wider tuning range (up to 1080 nm) that can efficiently excite red proteins such as dsRed and are more than adequate for practical purposes. There has been interest in optical parametric oscillators (OPOs) that can be used to extend the tuning range of ultrafast femtosecond pulsed lasers to 1000–1600 nm. OPOs may be useful for the IR fluorescent proteins that will become available in the future but the IR fluorescent proteins reported so far have a low quantum yield[21] and further work is required to optimize their spectral properties. Another potential application for an OPO is to use the longer wavelengths to generate third harmonic signals for label-free imaging of cellular and subcellular interfaces.[22] This may be particularly useful for *in vivo* human experiments in which biologically inert fluorescent reagents to label cells and structures are not yet available. However, one drawback with the use of the OPO is the potential toxicity from single-photon absorption of light by water, which has two minor absorption peaks at 1000 and 1200 nm and is opaque above 1300 nm.[7]

Second laser

The addition of a second ultrafast laser to the two-photon system opens up two opportunities. The first is that it allows the detection of two probes with similar emission spectra but distinct excitation peaks (for example, CMAC and CFP) to be used concurrently.[20] By use of an electrical control device such as an electro-optic modulator for fast switching between the two lasers it is possible to alternately scan at two different wavelengths (for example, 810 and 920 nm) to generate more detection channels than the number of equipped NDDs. In addition to alternate scanning, this also allows for simultaneous photoactivation at one wavelength (for example, 750 nm to photoconvert Kaede from green to red by two-photon excitation) and image acquisition at a second wavelength (for example, 920 nm). However, it is important to note that with the fast tuning capabilities of some modern lasers it is also possible to first photoconvert and then image after a few seconds delay without the need for a second laser.

Piezoelectric focus

The piezoelectric focus uses a piezoelectric actuator to move the objective lens to acquire a *z*-stack rather than moving the microscope stage. Therefore, it has much higher precision and greater speed than the stage *z*-motor. However, as the current high numerical aperture objective lenses cannot resolve focal spots $<1.5\,\mu m$ apart in the *z* plane,[18] it is superfluous to have such fine control to enable *z*-steps of as little as 50 nm. In addition, the major limiting factor in determining imaging speed is the laser scanner and benefits of the piezoelectric focus are not appreciable at the low scan rates of most point scanners.

Gallium arsenide phosphide detectors and electron multiplying CCD cameras

Ultrasensitive gallium arsenide phosphide PMTs with relatively high quantum efficiency are now routinely offered in commercial two-photon systems. However, current versions of these detectors are rapidly saturated at routine signal levels and are easily damaged making them difficult to use for day-to-day experiments. Another option is to use electron multiplying CCD cameras that offer high quantum efficiency and negligible noise but these are even more expensive than conventional CCD cameras and only allow modest depth penetration.[18]

'Hybrid' scanners

Several commercial vendors now offer a 'hybrid' two-in-one scanner for their confocal laser scanning microscopes. These systems typically include dual scanning paths (that is, fast resonant scanning path and a slower galvanometric scanner). Such systems combine the advantages of high-speed imaging with flexibility and higher spatial resolution commonly seen in regular confocal microscopes. These systems are also better suited for region of interest scanning and photoactivation applications. However, one concern with 'hybrid' scanners is that they require a more complicated light path and this may affect optical throughput and image quality. They are also more expensive.

Spectral detectors

Another detector option is to include a 32-PMT spectral detector for spectral unmixing to resolve fluorescent signals from different sources with overlapping spectra. However, many commercial microscopes now also offer spectral unmixing by component analysis of signals from the 4-PMT channel NDDs. An emerging application that will require a spectral detector is the use of photoactivatable fluorescent proteins to label multiple cells with distinct spectral signatures for large-scale real-time tracking by two-photon spectral microscopy.

FLUORESCENT DYES AND REPORTER MICE

It is worth remembering that two-photon excitation is non-linear and excitation cross-sections are not always predictable based on single-photon excitation spectra.[23] In general, the excitation spectra is shifted to shorter wavelengths than expected by just doubling the wavelength of the single-photon excitation spectra and is often much broader.[4] This applies to non-symmetric fluorescent proteins such as eGFP (which can be excited at 920–990 nm) but breaks down for symmetric molecules such as Alexa Fluor 488 (which can be excited at 800–830 nm). Therefore, it is critical that investigators obtain 'excitation fingerprints' by performing wavelength scans (λ scans)[24] for each fluorescent dye or protein to determine the optimal two-photon excitation wavelength for their experimental models.

Fluorescent labelling of cells and anatomical structures as fiduciary markers are critical to the success of intra-vital two-photon microscopy. Cells can be isolated and fluorescently labelled with

ready-to-use vital dyes such as CMAC (blue), CFSE (green), CMTMR (orange), CMTPX (red) and SNARF-1 (far red) before adoptive transfer into recipient mice for two-photon microscopy. Intra-cellular labelling is preferred to membrane labelling as the whole cell is labelled to provide brighter images with clear centre of mass that can be more easily tracked by image analysis software. In addition to these probes, a new generation of IR fluorescent dyes have been developed, which will enable even deeper tissue imaging.[25] Fluorescently labelled B and T cells can be used to define anatomical compartments such as the follicle and paracortical T cell zone in the lymph node. In addition, natural ligands such as lectins and specific monoclonal antibodies can be labelled with a range of fluorescent dyes that are now available in user-friendly formats such as amine-reactives succinimidyl esters. These reagents have been used for *in vivo* labelling of anatomical structures such as the subcapsular sinus macrophages,[26] lymphatic sinuses,[27,28] follicular stroma,[12] fibroblastic reticular cells[13] and follicular dendritic cells in germinal centres.[29–31] In addition, fluorescently labelled high molecular weight dextrans[8] and other impermeable compounds such as fluorescent nanoparticles[32] can be used to define the vascular anatomy and assess vascular permeability after perturbations.

Reporter mice ubiquitously expressing fluorescent proteins derived from GFP (eGFP, eCFP and eYFP) and dsRed (tdTomato and mCherry) can also be used as a source of fluorescent cells with the advantage that they are less susceptible to phototoxicity at high laser power intensities. Cells from these mice can be purified and adoptively transferred or mosaic mice can be made by mixed radiation bone marrow chimaeras. Mice with lineage-specific expression of fluorescent proteins have also been developed and these have the advantage of eliminating the need for isolation, cell labelling and adoptive transfer. These mouse strains are therefore particularly useful for imaging tissue resident, non-haemopoietic and radiation-resistant cells that are difficult to isolate and adoptively transfer. For example, the CD11c promoter has been used to drive eYFP expression in dendritic cells to visualize dendritic cell networks and their interactions with T cell *in vivo*.[33] Alternatively, inducible reporter gene expression using the *Cre*/LoxP system has been used to restrict fluorescent protein expression to cell lineages of interest.[34,35] These *Cre* reporter mice strains carry a targeted insertion of eGFP, eCFP or eYFP in the ubiquitous ROSA26 locus and take advantage of the large number of mice strains carrying *Cre* recombinase under the control of lineage-specific promoters available from the Jackson Laboratory (Bar Harbour, ME, USA). In addition to reporting cell lineages, mouse strains are available that can also report cellular function. For example, mice carrying bicistronic reporters for cytokines such as IL-4[36] and γ-interferon[37] have been made. Such mice can be used to image functional responses and cell fate decisions in real time.

Recently, IR fluorescent proteins derived from bacterial phytochromes were described.[21] These proteins are potentially used at wavelengths that are less affected by light scattering and tissue absorption making it possible to penetrate even deeper. However, the long wavelengths required for two-photon excitation of these proteins will require an OPO. Another exciting development is the recent discovery of Kaede[38] and subsequent development of other photoactivatable fluorescent proteins such as eosFP,[39] Dendra[40] and kikume Green-Red (kikGR).[41] These photoactivatable fluorescent proteins typically shift from green to red fluorescence on exposure to UV light making them ideal 'optical highlighters' for single-cell marking and tracking over prolonged periods of time and across large distances.[42] Kaede-transgenic mice have already been used to monitor the kinetics of immune cell trafficking.[43] KikGR is several fold brighter and undergoes more rapid and complete photoconversion than Kaede, making kikGR-expressing mice more suitable for two-photon microscopy.[44] Photoconversion is a relatively common phenomenon in orange and red fluorescent proteins[45] and it is expected that more mice expressing photoconvertible fluorescent proteins with a range of spectral properties will become widely available in the near future. This would enable optical marking of multiple cells with distinct spectral 'fingerprints' that can be tracked automatically at the single-cell level.

ANAESTHESIA AND MICROSURGICAL CONSIDERATIONS

A challenge with two-photon microscopy is to maintain the 'biology' under conditions as close as possible to the physiological state. Surgical-plane anaesthesia is ideally supported by cardiorespiratory monitoring using transcutaneous pulse oximeters that provide non-invasive recordings of oxygen saturation, respiratory rate and heart rate. Unfortunately, commercial pulse oximeters use red and infrared LEDs, which are absorbed by melanin in the skin of C57BL/6 mice. As most genetic mouse models are on the C57BL/6 background, albino C57BL/6 mice with spontaneous mutation in the tyrosinase gene[46] required for melanin synthesis are ideal for intra-vital microscopy. Moreover, melanin is transported to the lymph node and spleen where it is captured and retained by medullary sinus and red pulp macrophages, respectively. Albino C57BL/6 mice are therefore also ideal for two-photon microscopy as the absence of melanin may significantly improve depth penetration. Pulse oximetry may also allow synchronization of image scanning with respiration (physiological triggering) to minimize noise from chest and abdominal wall movements.

Microsurgery is often needed to expose the relevant biology for two-photon microscopy and this should be achieved with minimal trauma and tissue injury by blunt dissection along anatomical planes where possible. Intra-abdominal organs such as the pancreas and spleen may need to be mobilized and exteriorized for access and to minimize respiratory movement artefacts. Another option is to use 'stick' objectives that allow access to internal organs by minimally invasive 'keyhole' surgery.[47] Emptying the bladder with a small silastic catheter may help reduce abdominal wall movements and insensible water loss may need to be replaced by intravenous saline. Anaesthetized mice cannot maintain their core body temperature and heat lamps or a heated stage is needed as lymphocyte motility is very susceptible to changes in temperature.[48] Therefore, it is advisable to warm the cell tissue culture media or saline solution in a water bath and perfuse it through an in-line solution heater controlled by a heater probe and temperature controller to maintain the temperature under the meniscus at 35–38 °C. Some investigators use phenol-red-free media but we find this is unnecessary.

The use of explanted tissue has received some criticism as the loss of blood supply, lymphatic flow and innervation may affect cellular behaviour.[49] In optically dense tissues where sectioning by vibratome slices is required to expose the biology, explants may be less than ideal. For example, the marginal zone of the spleen is loosely attached to the white pulp and lymphocytes leak out of it in vibratome slices.[50] However, in intact lymph nodes the behaviour of cells in explant and intra-vital preparations are remarkably consistent and comparable providing optimal levels of oxygenation, pH and temperature are maintained.[5,20] Explants require less skill and can be prepared more rapidly than intra-vital preparations and therefore allow for higher throughput. Tissue explants are the only option currently available for fluorescent labelling and two-photon microscopy of human organs such as the liver.[51]

Table 1 Milestones in the development of two-photon microscopy

Year	*Advance*
1929	Maria Göppert predicts two-photon excitation[1]
1962	Osamu Shimomura discovers aquoerin[55]
1978	Thomas Cremer and Christoph Cremer develop laser scanning confocal microscope[56]
1990	Denk *et al.* develop two-photon microscope[2]
1991	Single-box Ti:Sapphire laser introduced for two-photon excitation[3]
1994	Martin Chalfie uses GFP as a genetic marker[57]
1995	Roger Tsien mutates wild-type GFP to enhanced GFP (eGFP)[58]
1996	Bio-Rad introduces first commercial two-photon microscope
1996	Tsien laboratory produces GFP variants: eCFP and eYFP[59]
1999	Lukyanov laboratory clones dsRed[60]
2002	First two-photon microscopic studies of lymphocytes from laboratories of Ian Parker and Michael Cahalan [48] and Richard Lewis and Ellen Robey[61]
2002	Miyawaki laboratory clones Kaede protein, which is photoconverted from green to red fluorescence on exposure to UV light[38]
2002	Tsien laboratory produces monomeric mRFP1 variant of dsRed[62]
2004	Tsien laboratory produces improved monomeric (mCherry) and tandem (tdTomato) variants of dsRed[63]
2008	Min Gu describes first hand-held two-photon microendoscope[54]
2009	Tsien laboratory develops infrared fluorescent dyes derived from bacterial phytochromes[21]

EXPERIMENTAL CONSIDERATIONS

The potential pitfalls of two-photon microscopy have been described in detail in several reviews.[5,52,53] These include observations of how cell motility and behaviour are highly susceptible to changes in tissue oxygenation, pH and temperature. These variables can be difficult to control from one experiment to the next and the use of internal controls is recommended. In addition, if fluorescent cell labelling with vital dyes is used it is a good idea to perform dye-swapping experiments to exclude phototoxicity as an explanation for observed differences in behaviour between the experimental and control cells.[28]

Fine resolution of biological processes in time and space requires frequent sampling of large imaging volumes.[19] The instantaneous velocity for a cell is calculated as the displacement divided by the elapsed time. As lymphocytes migrate along stromal cell networks,[13] they do not travel in a linear path but rather make frequent turns in a 'random walk'.[48] Frequent sampling at 30 s intervals, for example, would therefore more accurately measure the displacement and instantaneous velocities than less frequent sampling at 1 min intervals, which would underestimate it. More detailed discussions of image analysis artefacts and how to avoid them can be found in Beltman *et al.*[53]

Fluorescent imaging is dependent on the contrast provided by bright cells against a dark background and typical two-photon microscopy experiments aim to achieve 1–10% labelled adoptively transferred cells against a dark background of endogenous cells that have not been subject to isolation and *ex vivo* manipulation. This can vary depending on the goals of the experiment and the expected frequency of the event of interest. In addition, having a low frequency of labelled cells is critical for data analysis and automated cell tracking as it reduces pixel overlap between cells and allows distinction of cells based on their centre of mass (the centre of the pixel volume or voxel). However, this has lead to the important caveat that two-photon microscopy only illuminates a subpopulation of cells and only then if they occupy the imaging volume during data acquisition.[5,52] By its nature the data are visual and it is important to also consider the unmanipulated cells in the darkness when interpreting the data.

CONCLUDING REMARKS

As can be seen in Table 1, progress in two-photon microscopy and enabling technologies has accelerated in the past 10 years to the point where it is now an accessible multi-user tool that may one day become as commonplace as the flow cytometer and confocal laser scanning microscope. The key to successful implementation of a two-photon system is to keep the light path and therefore the instrument as direct and simple as possible—less really is more in the case of two-photon excitation. More innovations are anticipated in the near future involving pulse manipulations and alternative scanning techniques that will achieve the goal of faster, brighter and deeper image acquisition. Perhaps most exciting of all is the recent development of a fast handheld two-photon microendoscope using dispersion compensated photonic crystal fibres and piezoelectric actuator as a microscanner.[54] Such a probe can be used to examine the skin or surgically exposed tissue intra-operatively or adapted to an endoscope to examine the bronchial or gastrointestinal mucosa in human subjects. This coupled with the development of biologically inert affinity reagents for *in vivo* labelling of cells and structures in humans has the potential to transform immunological research. Watch this space!

CONFLICT OF INTEREST

TGP has received free flights from Nikon Instruments (Yokohama, Japan), Coherent Scientific (Adelaide, Australia) and Leica Microsystems (Sydney, Australia). AB declares no conflict of interest.

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