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Dynamic Multiphoton Imaging: A Live View from Cells to Systems

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Leaps in scientific technology often occur at the interface of seemingly disparate disciplines. This holds true with the recent application of multiphoton microscopy to the biological sciences, leading to a new generation of imaging-based studies extending from the tracking of individual molecules within living cells to the observation of whole organisms.

The theoretical concept of multiphoton excitation was first proposed by physicist Maria Göppert-Mayer in 1931 (11) but was not experimentally proven until the invention of the laser 30 years later, and even then it required decades of further development in laser technology to become of practical utility. The fields of physical chemistry and physics were the first to apply this technique (12, 33), and biological research has only more recently embraced multiphoton excitation to image living cells (7, 8). Biological uses of multiphoton microscopy have been extensively reviewed (e.g., Refs. 9 and 29). Here, we provide an introduction to its operating principles, discuss its advantages and limitations, and illustrate some recent applications that bring to light the strengths of this technology.

Fluorescence imaging is widely used in biology for morphological and functional studies, but conventional (single-photon) fluorescence microscopy yields poor images, because in-focus structures are washed out by fluorescence outside the plane of focus. This can be mitigated by cutting thin sections (equal to or less than the width of a single cell), but that approach is clearly inapplicable for living tissue. The introduction of confocal microscopy thus represented an important advance, providing an "optical-sectioning" effect by using a pinhole aperture to reject out-of-focus fluorescence (41). Nevertheless, confocal microscopy still suffers from drawbacks, including limited (a few tens of micrometers) imaging depth into scattering biological tissues, photodamage owing to the use of short (high-energy) excitation wavelengths, and photobleaching. The subsequent development of multiphoton excitation greatly minimized these problems while sharing the optical-sectioning ability of confocal microscopy.

How Does Multiphoton Microscopy Work?

The principle of multiphoton excitation is elegantly simple. In the most commonly used case of two-photon microscopy, a fluorophore molecule is excited by the nearly simultaneous absorption (within 10^{-18} s) of two photons, each about twice the wavelength (half the energy) required for single-photon excitation. Likewise, three-photon excitation results from nearly simultaneous absorption of three photons, each having approximately one-third the energy needed for excitation of the fluorophore. The resulting fluorescent emission is then proportional to the square of the excitation intensity in two-photon absorption (or third power in the case of three-photon excitation). This nonlinear relationship provides the optical-sectioning ability of multiphoton imaging. Laser light focused on the specimen through a microscope objective has a high photon density in the focal point, but photon density, and therefore fluorescence excitation, falls rapidly away from this point. Fluorescence is generated only at the focal spot, and, unlike in confocal microscopy, there is essentially no out-of-focus light to reject (FIGURE 1A). A remaining problem is that multiphoton excitation requires enormously high light intensities that, if continuous, would almost instantly vaporize the specimen. The trick is to use lasers that produce amazingly brief (~10^{-13} s) pulses at a high repetition rate (typically ~80 MHz), thus generating very high instantaneous energy but low average energy (FIGURE 1B). Indeed, the adoption of multiphoton microscopy by biologists has largely followed progressive improvements in reliability and user friendliness of such "femtosecond" lasers.

In terms of optical-sectioning ability and image resolution, multiphoton microscopy has little advantage over confocal microscopy (6). However, several major advantages accrue secondarily from the use of long excitation wavelengths:

1) Light scattering declines steeply with increasing wavelength, so that infrared light (700–1,000 nm) used for two-photon imaging penetrates much deeper into tissue (~500 μm) than the equivalent blue light (350–500 nm) used for one-photon excitation (8, 41).

2) Infrared light causes negligible photodamage or phototoxicity to cells. This is particularly advantageous for fluorophores and endogenous proteins that would normally require excitation at short (<300 nM) and highly damaging ultraviolet wavelengths but can be made to fluoresce by three-photon excitation at infrared wavelengths three times longer (18, 44).

3) Photobleaching is restricted to the plane of focus.

4) The two-photon excitation spectra of most fluorophores are broader than for one-photon excitation, so a single multiphoton excitation wavelength can be used to simultaneously excite multiple fluorophores with distinct emission wavelengths.

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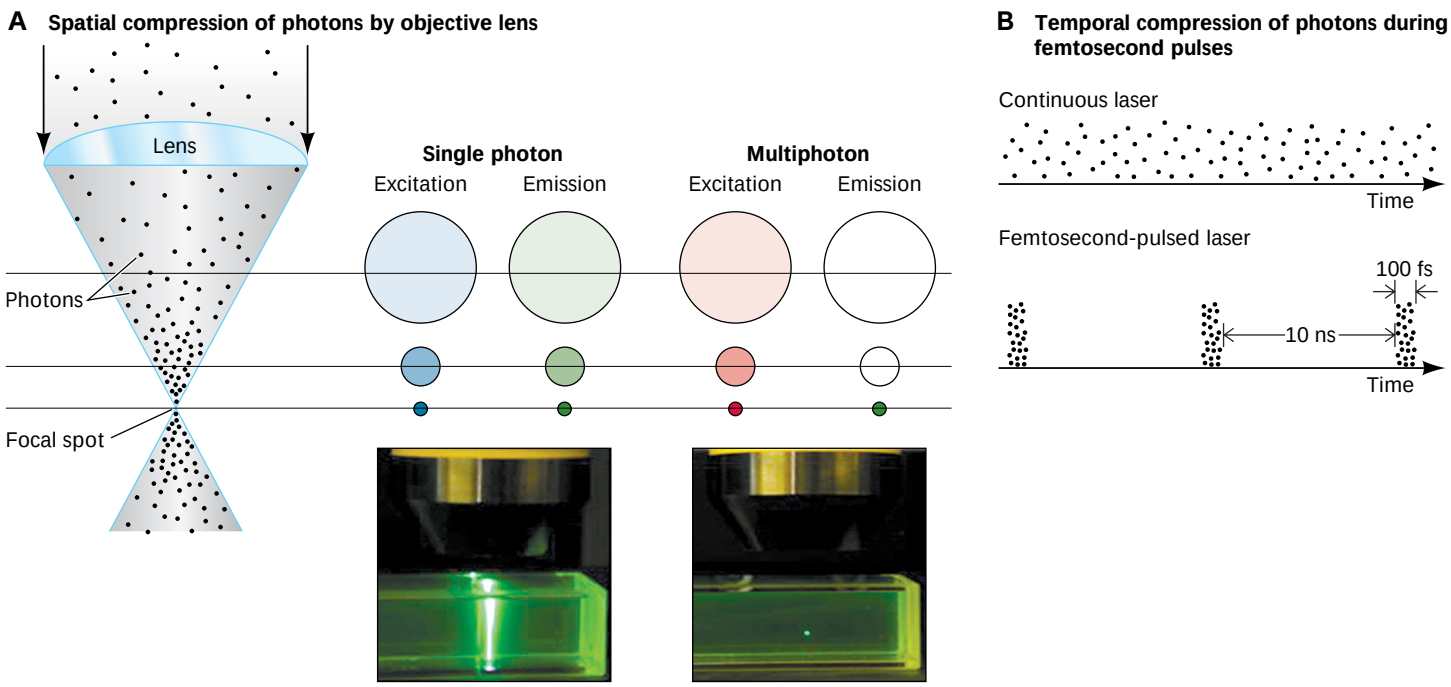

FIGURE 1. **Principles and imaging modalities of multiphoton microscopy**
A: demonstration of the differences between single-photon vs. multiphoton laser excitation. In both cases, the spatial compression of photons by an objective lens is greatest in the focal spot, and the photon density rapidly decreases away from this region. However, with conventional single-photon excitation, fluorophore excitation occurs throughout the beam path, resulting in fluorescence emission above and below the plane of focus. Multiphoton excitation restricts emission to the focal spot, owing to the quadratic relationship between excitation intensity and fluorescence emission. Photographs illustrate fluorescence in a cuvette of fluoresceine resulting from focused laser beams using single-photon excitation at 488 nm (*left*) and two-photon excitation with 780-nm, femtosecond pulses (*right*). Figure adapted from Ref. 4 with permission (see http://www.nature.com). *B*: temporal compression of photons into 100-fs packets achieves the high instantaneous power needed for multiphoton excitation, with an average power not much greater than a continuous laser beam as would be used for confocal imaging.

Novel Imaging Modalities

Multiphoton microscopy is most commonly used to acquire three-dimensional views of specimens labeled with a fluorescent probe but can also be used advantageously in conjunction with other microscopy techniques. One example is fluorescence correlation spectroscopy (FCS), in which fluctuations in fluorescence intensity of a small number of molecules within the focal spot are used to measure diffusion kinetics of individual molecules, protein-protein interactions, and molecular concentrations (15, 32). Applications of multiphoton FCS include analysis of aggregated β-amyloid peptides in human cerebrospinal fluid, multimerization of voltage-sensitive Ca^{2+} channels, and alterations in signaling proteins after changes in neuronal plasticity (24). FCS provides a quantitative, molecular assay in live cells and represents a marked advancement over existing quantitative diffusion-based approaches such as fluorescence recovery after photobleaching (FRAP; Ref. 31). Another example is fluorescence lifetime imaging (FLIM), which measures changes in the lifetime of a fluorophore probe that depend on its local environment (e.g., pH) but are independent of concentration. For example, although DNA and RNA molecules have a similar affinity for nucleic acid stains, they can readily be distinguished in living and apoptotic cells by virtue of differences in fluorescence lifetimes of certain nucleic acid dyes when bound to DNA compared with RNA (39).

FCS and FLIM are both possible using conventional (single-photon) microscopy but benefit from the increased depth penetration and minimal phototoxicity of multiphoton imaging. Beyond this, the femtosecond laser pulses required for multiphoton microscopy enable imaging techniques that are not possible by other means. Thus second harmonic generation (SHG) microscopy uses nonlinear optical properties that emerge at the very high energies during focused laser pulses. However, rather than being based on absorption of light energy by fluorescent molecules, SHG uses inherent properties of highly ordered structures that inherently function as frequency doublers of the excitation light. For example, infrared (900 nm) femtosecond pulses result in deep blue light at exactly one-half the wavelength (450 nm) traveling in the same direction as the incident light. SHG is well suited for imaging of ordered structures (e.g., collagen and myosin) deep within tissue without the need for any exogenous probes (5), provides information about molecular orientation and symmetries (27), and can be applied simultaneously with multiphoton fluorescence imaging to provide additional information about the specific molecular source of the SHG (27, 45). Recent developments of functional SHG probes, such as membrane voltage sensors with uniquely high sensitivity (30), hold further promise.

Finally, we note that multiphoton microscopy is not limited merely to imaging but can be applied to perturb biological tissues with very high spatial and temporal specificity. Examples include the localized "uncaging" of biologically active compounds such as neurotransmitters or intracellular messengers from photolabile precursors to reveal inter- and intracellular ionic communication (34); the three-photon induction of a red/green color change of a red fluorescent protein (DsRed) as a way of optically "highlighting" individual cells or subcellular regions (FIGURE 2A; Ref. 21); and the selective ablation of individual organelles, and even the use of the femtosecond laser beam as an optical microtome to construct three-dimensional images of entire fixed organs or organisms by sequentially imaging sections some tens of micrometers thick and then (by turning up the laser power) ablating that section to expose fresh tissue underneath (1, 38).

Biological Applications

Multiphoton imaging is not a panacea for all microscopy problems, and for many purposes confocal microscopy may be preferable (and less expensive), such as in protocols in which rapid exchange between excitation wavelengths is needed (e.g., ratiometric imaging). However, its inherent optical sectioning, deeper penetration in scattering tissue, and reduced phototoxicity are particularly applicable to dynamic imaging of living specimens. Here, we illustrate applications at levels from the subcellular to entire organisms that exemplify the advantages of multiphoton microscopy.

Subcellular/organelles

Neurobiologists were among the first to harness multiphoton microscopy for biological studies. A key functional component in the nervous system is the dendritic spine, a tiny structure

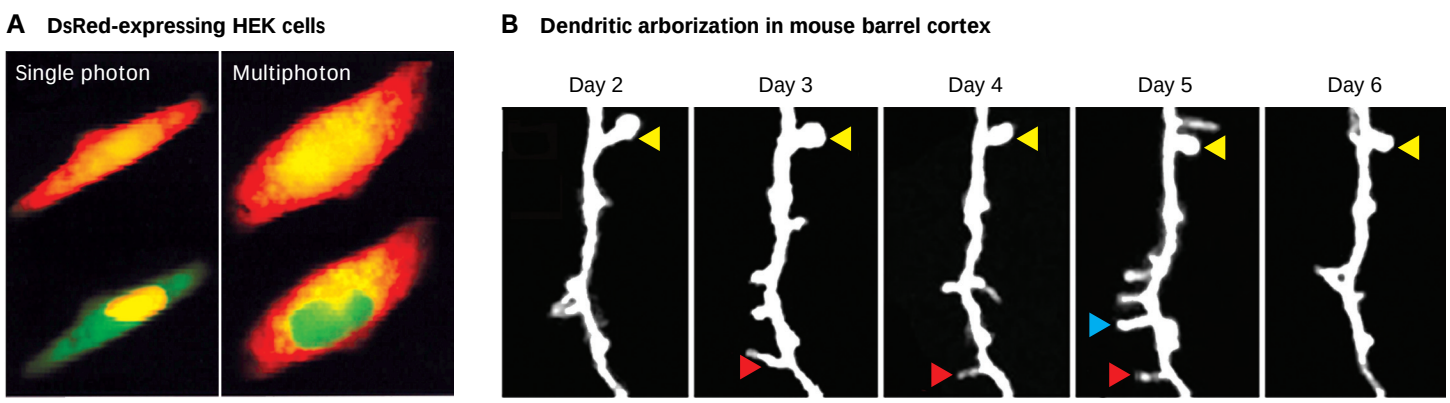

FIGURE 2. **Multiphoton applications for in vivo and in vitro cellular and subcellular imaging**

A: application of the multiphoton "greening technique" for labeling subcellular regions. Image pairs show DsRed-expressing human embryonic kidney (HEK) cells imaged by conventional (single-photon) epifluorescence microscopy, before (*upper*) and after (*lower*) greening the cytoplasm (*left*) or the nucleus (*right*) by local exposure to focused, 760-nm, femtosecond laser pulses. The color change arises from a selective three-photon bleaching of the mature, red form of DsRed, resulting in reduced red fluorescence and (via a reduction in Forster resonance energy transfer) enhanced fluorescence of the immature green species. Figure adapted from Ref. 21 with permission (see http://www.nature.com). *B*: Long-term two-photon imaging of the same dendritic arborization within the barrel cortex of a mouse. Arrows indicate examples of dendritic spines that changed morphology (yellow) or appeared and retracted (blue, red) throughout an 8-day period. Figure adapted from Ref. 37 with permission (see http://www.nature.com). *C*: subcellular distribution of Ca^{2+}-dependent fluorescence in a fura 2-loaded pyramidal neuron in an in vitro slice preparation imaged at different times following photorelease of the intracellular messenger inositol 1,4,5-trisphosphate. Figure adapted from Ref. 36 with permission. *D*: Ca^{2+} transients elicited by single action potentials along apical dendrites of a neuron visualized 400 μm into the brain of a living rat using two-photon microscopy. The image shows a side view of resting fluorescence in a neuron loaded with a fluorescent Ca^{2+} indicator, reconstructed from an image stack acquired at increasing focal distances. Traces show changes in Ca^{2+}-dependent fluorescence recorded at adjacent depths in image in response to single action potentials. Figure adapted from Ref. 40 with permission.

about a micrometer in size that forms the postsynaptic element mediating communication between neurons. Much interest focuses on the chemical and electrical signaling mechanisms in spines and on the plastic changes in their distribution, morphology, and function that may underlie learning and memory. However, it has been difficult to image these dynamic processes in live preparations. Neurons are sensitive to photodamage, brain tissue is highly scattering, regions of interest may lie hundreds of micrometers below the surface of the intact brain, and although it is possible to maintain viable slices of brain, these must be relatively thick to preserve functional connections. The introduction of multiphoton microscopy thus offered enormous advantages in terms of high-resolution, deep, and noninjurious imaging. Recent examples of multiphoton imaging in 300- to 400-μm-thick brain slices demonstrate the morphology, unique signaling dynamics, and rapid motility of dendritic spine head and neck regions (14, 19). Most spectacularly, it has been possible to resolve individual dendrites and spines in living animals by creating a viewing window into the brain by removing or thinning the skull of mice expressing a green fluorescent protein in a small subpopulation of cortical neurons (37). These could then be imaged repeatedly over several weeks, revealing that spines appear and disappear frequently in the adult cortex and suggesting that these changes might underlie adaptive remodeling of neural circuits in response to sensory experience (FIGURE 2B).

Dynamic cellular imaging

In addition to morphological studies, multiphoton imaging of cellular activity can be accomplished with the use of various fluorescent probes. For example, Ca^{2+}-indicator dyes can provide detailed spatial and temporal information about Ca^{2+} entry through voltage-gated channels in excitable cells or patterns of Ca^{2+} release from intracellular stores. Although visualizing cellular activities, such as ion flux or membrane voltage changes, can be accomplished by conventional fluorescence techniques, multiphoton excitation facilitates functional imaging of individual cells in thick tissue (FIGURE 2C; Ref. 34) and in intact animals (FIGURE 2D; Ref. 40). Yet a further advantage is nicely illustrated in recent studies using multiphoton excitation in retinal starburst amacrine cells that are involved in the processing of visual information yet are not activated by infrared wavelengths outside of the visible spectrum (10). Visible wavelengths were used to evoke patterned stimulation at the retinal photoreceptors, whereas multiphoton excitation (using infrared light) was used concurrently to excite a fluorescent Ca^{2+} indicator loaded into amacrine cells, revealing the functional responses to the patterned stimulation without complications due to photostimulation by the excitation light itself.

Imaging within intact organs

For many years biological research has pursued a reductionistic trend, with an increasing focus on genomics and proteomics. Multiphoton microscopy now offers an important tool toward a more integrative approach, because its ability to image function and morphology at cellular, and even molecular levels deep within intact organs and living animals provides a means to unify molecular, cellular and organismal studies. This approach has far-reaching applications for understanding how multiple cells and cell types interact to determine the function of an entire organ and can provide insight into disease mechanisms and aid development of therapeutic strategies. An example is the application of multiphoton imaging to open a new window onto the immune system (FIGURE 3A; see Ref. 4 for a review). Activation of the immune response depends on a complex choreography of interactions between different cell types, yet despite a wealth of molecular knowledge, little is known of the cell-cell interactions deep within native lymphoid tissue. Again, the depth penetration and minimal photodamage of multiphoton microscopy are crucial and, in conjunction with use of established fluorescent cell-tracker dyes to label adoptively transferred lymphocytes, permit six-dimensional imaging (*x*, *y*, *z*, time, intensity, and emission wavelength) into both excised lymph nodes and nodes in an anesthetized mouse. Time-lapse movies reveal strikingly dynamic movements and cellular interactions within the in vivo setting, in marked contrast to the static view of lymphocytes in culture, and are beginning to provide mechanistic insights into the processes by which T cells search for antigen and subsequently become activated by repeated contacts with antigen-presenting cells (2, 23, 26). The advantages of multiphoton imaging are further highlighted because individual naïve T cells can be followed within the lymph node for hours without loss of viability or motility (25, 26), whereas analogous studies using (single-photon) confocal imaging concluded that these cells were essentially immotile (35), a result that may reflect photodamage by the shorter-wavelength excitation.

Imaging whole organisms/embryos

At even larger scales, multiphoton microscopes can be "zoomed out" to capture an entire embryo during development while maintaining high (subcellular) spatial and temporal resolution over long periods (days to weeks). Transparent organisms such as *Caenorhabditis elegens*, zebrafish, and *Drosophila* are ideal candidates for such studies, and "in toto" imaging of entire embryos has revealed expression patterns at the cellular and genomic levels (FIGURE 3B; Ref. 22). Multiphoton imaging enables long-term observation of biological activity by providing the ability to repeatedly track and monitor cells deep within a growing embryo or organism while minimizing photodamage to cell structure and DNA.

Future Prospects

Multiphoton excitation is relatively new in the biological sciences, and future developments will likely improve its utility and overcome some existing shortcomings. These include optimization of optics for infrared wavelengths, improved imaging speed, and the introduction of fluorescent probes with high multiphoton cross-section.

Fast image acquisition is critical for capturing rapid biological events.However, almost all current multiphoton microscopes work by raster scanning a single laser spot, requiring a second or more to acquire each *x-y* image plane. One approach to improving on this involves the use of a resonant mirror to rapidly scan the laser in the *x* dimension, permitting acquisition speeds of 30 frames/s (28). An altogether different technology uses simultaneous scanning by multiple laser spots, thereby proportionately speeding the acquisition rate (3).

The optical requirements for microscope objectives are very different between conventional epifluorescence microscopy and multiphoton microscopy. With single-photon epifluorescence, aberrations with the short-wavelength excitation light are not important but the lens must be well corrected for the visible-wavelength emission used to form the image. On the other hand, multiphoton excitation requires good corrections in the infrared so as to focus the laser beam to a diffraction-limited spot, but on the emission side the objective needs only to collect as much light as possible, and aberrations are inconsequential. At present, multiphoton microscopists often make use of objectives originally designed for infrared differential interference contrast and that thus have good long-wavelength transmission, but development of new lenses specific for multiphoton applications may yield further benefits.

A major advantage of multiphoton microscopy is its ability to "see" deep within intact organs and organisms. However, that advantage is partly lost due to the large physical size of the objective lens. For example, it is difficult to insert a lens into the abdominal cavity of a mouse to image internal organs. Recent advances mitigate this problem, either by using a gradient index lens as a thin, rodlike probe to extend the working distance of a conventional objective (16) or by using fiber optics to a create multiphoton endoscopes. These developments have enormous potential for medical technology, such as minimally invasive endoscopic procedures, as well as scientific applications such as imaging

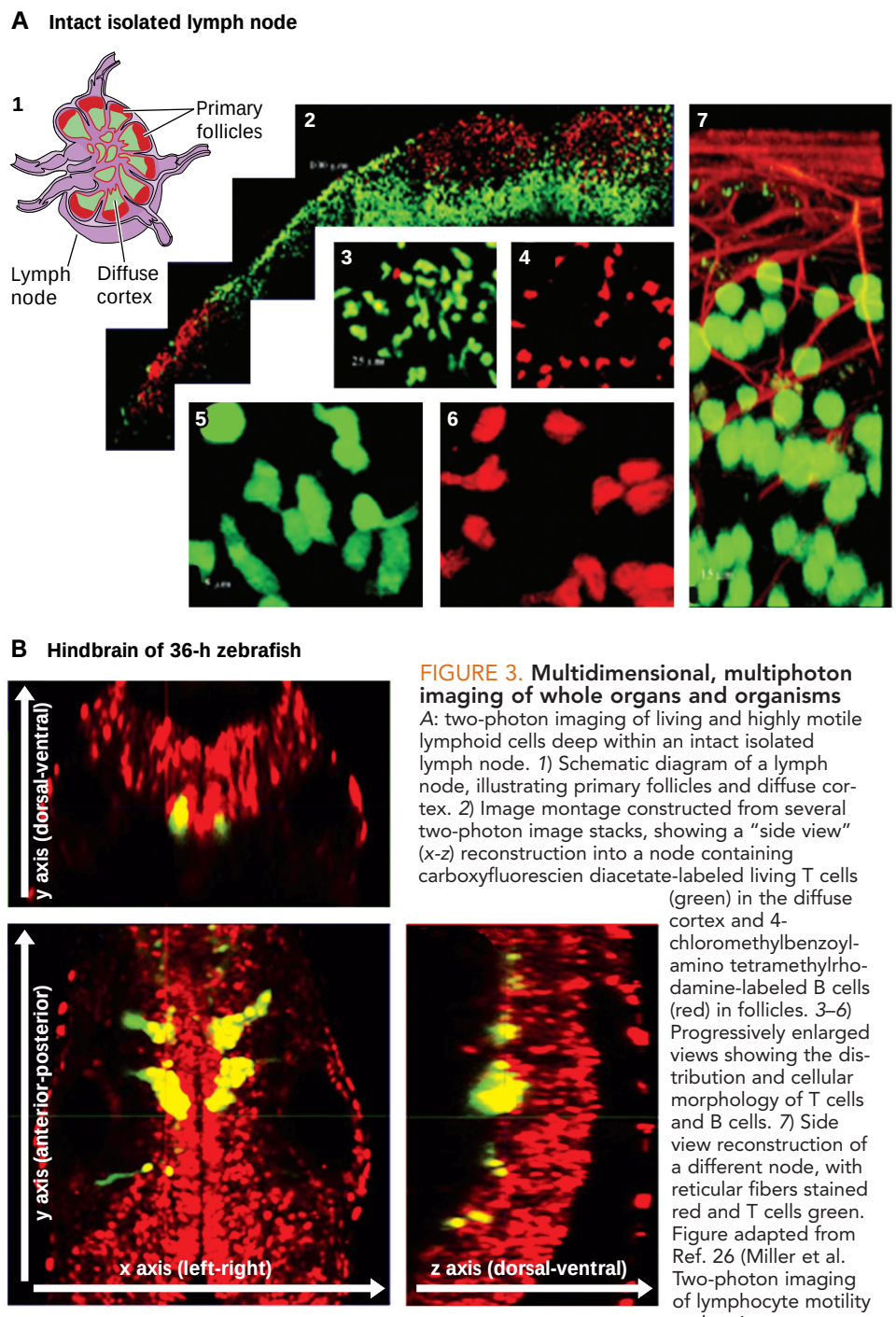

FIGURE 3. **Multidimensional, multiphoton imaging of whole organs and organisms** *A*: two-photon imaging of living and highly motile lymphoid cells deep within an intact isolated lymph node. *1*) Schematic diagram of a lymph node, illustrating primary follicles and diffuse cortex. *2*) Image montage constructed from several two-photon image stacks, showing a "side view" (*x-z*) reconstruction into a node containing carboxyfluorescien diacetate-labeled living T cells (green) in the diffuse cortex and 4-chloromethylbenzoylamino tetramethylrhodamine-labeled B cells (red) in follicles. *3–6*) Progressively enlarged views showing the distribution and cellular morphology of T cells and B cells. *7*) Side view reconstruction of a different node, with reticular fibers stained red and T cells green. Figure adapted from Ref. 26 (Miller et al. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 296: 1873–1876. Copyright 2002 AAAS). *B*: hindbrain of a live 36-h zebrafish embryo expressing Islet1:EGFP (yellow) and a Histone H2B-mRFP1 fusion protein (red). The *x-z, x-y, and y-z* sections into the embryo are shown. The ability to noninjuriously image fluorescently tagged proteins within a live embryo allows for the mapping of gene expression patterns throughout development. Figure adapted from Ref. 22 with permission from Elsevier.

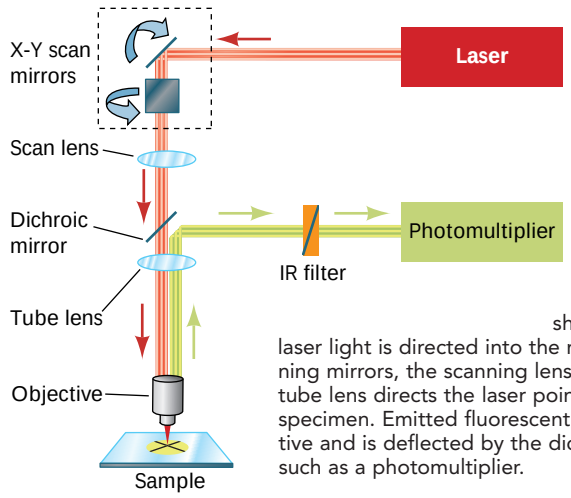

FIGURE 4. **Schematic layout of a multiphoton system setup**
The excitation laser beam path is shown in red, and the emission pathway and detection system are shown in green. The pulsed infrared laser light is directed into the microscope by orthogonal *x-y* scanning mirrors, the scanning lens serves as a beam expander, and the tube lens directs the laser point into the objective and onto the specimen. Emitted fluorescent light passes back through the objective and is deflected by the dichroic mirror into a detector system such as a photomultiplier.

cellular activity from awake, behaving animals (see Ref. 13 for further reading).

On the receiving end of multiphoton excitation, quantum dots have great promise as an extremely bright alternative to conventional fluorophores. These are nanocrystals of semiconductor that have a very high two-photon cross-section, show minimal photobleaching, and are available in a range of emission wavelengths. Initial problems with biocompatibility appear now to be largely solved, and quantum dots are available coated with various substrates, allowing them, for example, to be conjugated to specific antibodies. (43)

Applications

The key strengths of multiphoton microscopy lie in its applications for real-time imaging of dynamic processes occurring within the native environment of intact live tissues, organs, and organisms. Developmental biologists and neurobiologists were among the first to use this technique, and its more recent adoption by immunologists and molecular biologists indicates its potential within other disciplines. Future applications for multiphoton microscopy are broad and exciting, and they extend beyond basic research into the realm of noninvasive or endoscopic clinical and diagnostic work, such as detection of skin cancers, Alzheimer's disease, and metabolic disorders just by "looking" into the patient with a cellular level of resolution (45).

Appendix: Constructing Your Own Multiphoton System

Technological limitations of earlier generations of femtosecond lasers were originally a great deterrent to their use by biologists, but current models are compact and require no manual intervention by the user. However, the considerable expense of commercial multiphoton systems (~$700,000) remains a hurdle. Because of this, many multiphoton microscopes are purchased for multiuser facilities—a job for which they are often ill suited—and end up gathering dust! Instead, multiphoton microscopes are better employed within the laboratory of an individual investigator, where they can be dedicated for extended experiments and equipped as needed with ancillary systems for superfusion, environmental control, electrophysiological recording, etc. Several of the applications described in this review were made possible because the investigators constructed their own multiphoton microscopes at a considerable saving in cost (17, 20, 28) This task is not as daunting as might be feared and, beyond the intimate working knowledge and inherent satisfaction it provides, has further advantages, such as that the system can be customized for specific applications. FIGURE 4 outlines the basic working pathway and components in a multiphoton imaging system, and the references provide detailed instructions for constructing one's own system. ■

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