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Optical Filters for Multiphoton Microscopy

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Advances in filter technology enable improved multiphoton microscopy performance.

Multiphoton fluorescence microscopy is similar to conventional fluorescence microscopy in that it images fluorescence from molecules that tag a target of interest in a cell. However, in a two-photon microscope, each fluorescent photon results not from a single-excitation photon, but from two photons absorbed simultaneously — each with twice the wavelength and half the energy of the equivalent single-excitation photon (Figure 1).

The emitted fluorescence is essentially identical to that which would have resulted from excitation of a single photon at half the wavelength. Since the wavelength of the excitation source is now far removed from the wavelength of the emitted fluorescence, one can use a filter that collects a greater range of wavelengths over the emission band, yielding improved sensitivity. It also is possible to do three-photon fluorescence, where three photons, each with one-third the energy, are absorbed simultaneously to excite the molecule, replacing the original one-photon excitation.

A typical multiphoton imaging system comprises an excitation laser, scanning and imaging optics, a sensitive detector (usually a photomultiplier tube), and optical filters, such as a dichroic beamsplitter for separating the fluorescence from the laser and emission filters for blocking undesired laser light from reaching the detector (Figure 2). A longer-wavelength excitation source with sufficient peak intensity, such as a mode-locked, tunable Ti:sapphire laser, is used because the two photons must arrive at the fluorescent molecule at exactly the same instant to excite it.

A multiphoton imaging system offers many advantages. It provides true three-dimensional imaging, or optical sectioning, as does confocal microscopy. The technique can image deep inside live tissue (Figure 3), eliminates undesired fluorescence from outside the focal plane, and reduces photobleaching away from the focal plane, which increases sample longevity.

Multiphoton imaging allows imaging of fluorescent dyes with very short Stokes shifts and/or very low efficiencies, and even of inherently fluorescent molecules native to the sample or tissue. Its growing popularity is based on the availability of modern high-peak-power pulsed lasers, such as a widely tunable mode-locked Ti:sapphire. However, the technique has traditionally suffered from the lack of commensurately high-performance optical filters that provide excellent throughput across the whole emission range of interest and sufficient blocking across the laser tuning range.

Optical filters

Thanks to recent advances in optical filters, multiphoton users now can achieve ultrahigh transmission in the filter passbands, very steep transitions and deep blocking everywhere it is needed. These filters represent a simple and — relative to the rest of the system — inexpensive path to substantially boosting system performance.

The new breed of emission filters provides clear transmission from the near-UV to the near-IR (Figure 4). In fact, the filters look as clear as window glass, in contrast to the brownish tint of traditional filters. And the new high-damage-threshold dichroic beamsplitters are designed to reflect the precious fluorescence signal with exceptionally high efficiency. This throughput advantage is of critical importance, because the multiphoton fluorescence process is less efficient than conventional fluorescence; thus, it is desirable to collect every possible emission photon.

For best discrimination of the signal from scattered laser background noise, the emission filters also must provide deep blocking across the entire Ti:sapphire laser tuning range. Because of their sensitivity, photomultiplier tubes are the most common means of detecting multiphoton emission, but they also are sensitive to the excitation light, especially at the short end of the laser tuning range. Thus, high blocking is critical to achieve a high signal-to-noise ratio and measurement sensitivity. Without sufficient filter blocking, it is not uncommon for a substantial fraction of the measured signal (even tens of one percent) to consist of scattered laser light. This noise source can be almost completely eliminated with the new multiphoton filters.

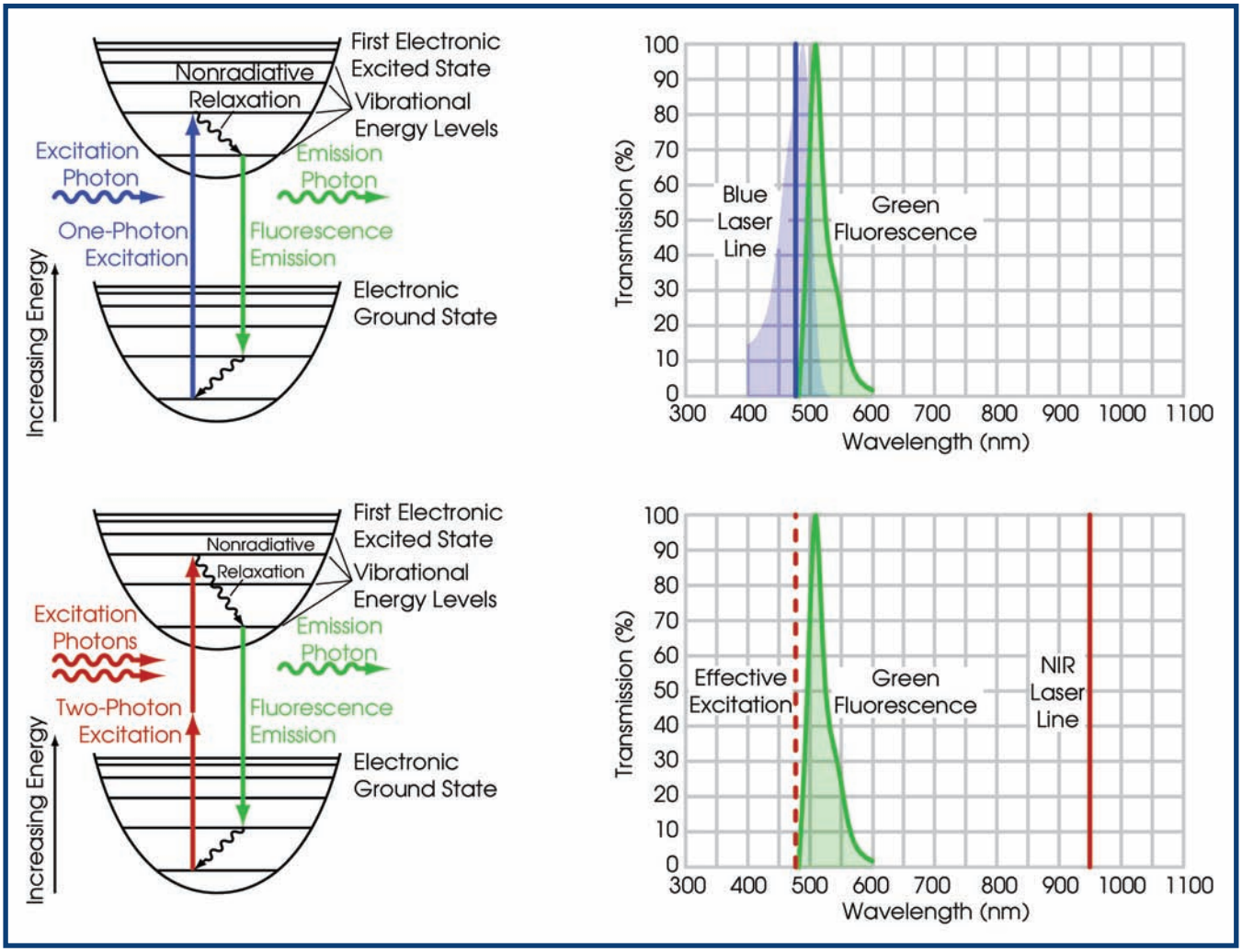


Figure 1. In standard single-photon fluorescence microscopy (upper pair of images) a laser or filtered light source excites fluorescent molecules from the ground state to the first excited state via absorption of a single photon for each emitted photon. The most efficient excitation happens when the wavelength of the excitation source matches the peak wavelength of the absorption curve, shown as the blue shaded region. Because this occurs very close to the emission band (green shaded region), one must sacrifice some of the desired fluorescence signal to minimize the excitation light noise. In multiphoton fluorescence microscopy (bottom two images) the same fluorescent molecule is excited with two photons (each twice the wavelength and thus half the energy of the original one-photon excitation) instead of one.

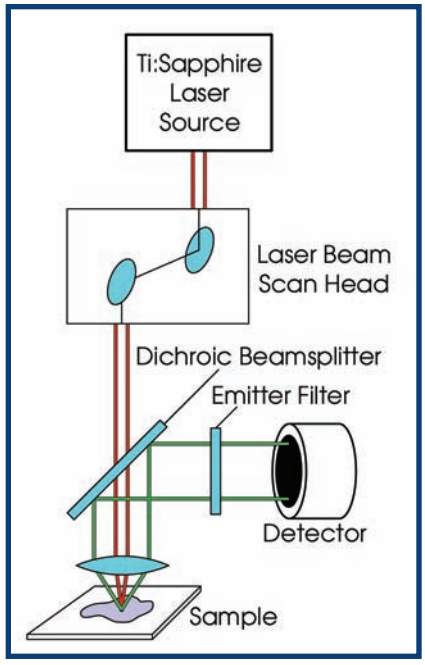
We have found that when using these filters in a typical experimental setup, the signal collection efficiency increased by 56 percent and at the same time the increased blocking of stray excitation light led to a 44 percent decrease in background noise — as a result, the signal-to-background ratio increased from about 7 to over 20. These numbers represent a significant improvement for this emerging imaging technology.

Sometimes it is desirable to restrict the spectral band of fluorescence emission that is detected, especially when multiple fluorophores are used to label different targets in a sample. Narrower bandpass emission filters are ideal for this purpose. Because standard bandpass fluorescence filters often do not provide sufficient blocking of a multiphoton excitation laser, researchers have typically had to combine multiple filters to achieve acceptable signal-to-noise performance. With the exceptional transmission of the new breed of multiphoton filters, researchers now can leave these filters fixed in their system to block the laser, and then exchange narrower bandpass emission filters when necessary to limit the fluorescence emission band for multi-fluorophore work.

Peering deeply

Dr. Karl A. Kasischke and his co-workers at the Center for Aging and Developmental Biology in the University of Rochester Medical Center's department of neurosurgery in Rochester, N.Y., use multiphoton fluorescence microscopy to measure the intrinsic fluorescence from nicotinamide adenine dinucleotide (NADH) as a functional imaging signal in the intact and living brain. NADH plays a crucial role in energy metabolism in the brain and is a fluorescent intracellular molecule, so it is possible to use multiphoton fluorescence microscopy to di-

Figure 2. In a typical configuration of a multiphoton fluorescence microscope, the high-peak-intensity (but moderate-average-intensity) pulsed laser source is focused on the sample and raster scanned, just as in confocal microscopy. The fluorescence is collected by a sensitive detector, typically a photomultiplier tube, using a dichroic beamsplitter to separate the fluorescence from the laser beam and an emitter to block the undesired laser light from the detector while transmitting as much fluorescence as possible.



rectly measure the concentration and location of this important coenzyme without introducing foreign dyes or tracers.

Importantly, the reduced co-enzyme (NADH) is fluorescent, while the oxidized co-enzyme (NAD+) is not. Therefore, intrinsic NADH fluorescence yields a direct measure of the cellular NADH/NAD+ ratio and has been used as an indicator for both oxidative (production of ATP) and glycolytic (anaerobic glucose metabolism) metabolism.

Kasischke and his colleagues have previously shown that two-photon imaging and spectroscopy of NADH provide quantitative and subcellular imaging of metabolic transitions in brain tissue. The in vivo application of this imaging technology offers great potential for the cellular localization of brain activation and for the investigation of the underlying biochemical changes.

In another example, Edward Brown III and his co-workers in the department of biomedical engineering at the University of Rochester Medical Center use multiphoton fluorescence microscopy to image deeply into internal regions of tissue and tumors. Intravital microscopy coupled with chronic animal window models has provided stunning insight into tumor pathophysiology, including: gene expression; angiogenesis; cell adhesion and migration; vascular, interstitial and lymphatic transport; metabolic microenvironment; and drug delivery. However, prior to the use of multiphoton fluorescence microscopy, analysis was limited to the tumor surface (<150 µm deep). Multiphoton microscopy can provide high-resolution, three-dimensional images of gene expression and function in deeper regions of tumors. These insights could be critical to the development of novel therapeutics that target not only the tumor surface, but also internal regions (Figure 5).

The researchers also are using a closely related imaging technique to study collagen in tumors. Called second-harmonic-generation microscopy, it involves a nonabsorptive conversion of two long-wavelength photons into one short-wavelength photon of the same total energy. The emitted photon results from a nonlinear optical interaction and is distinct from a fluorescent photon, which always has a lower energy than that of the excitation photon or photons.

In tumors, fibrillar collagen is a significant source of second-harmonic generation. The content and structure of collagen are essential for governing the delivery of therapeutic molecules to tumors. By using second-harmonic generation, Brown and his co-workers imaged fibrillar collagen within tumors growing in mice. Using this noninvasive technique, they quantified the dynamics of collagen modification after pharmacologic intervention and provided mechanistic insight into improved diffusive transport induced by the hormone relaxin.

This technology could enhance scientists' and clinicians' ability to estimate the relative penetrabilities of molecular therapeutics. And the new optical filters play a vital role in supplying second-harmonic-generation microscopy with the sensitivity needed for these applications.

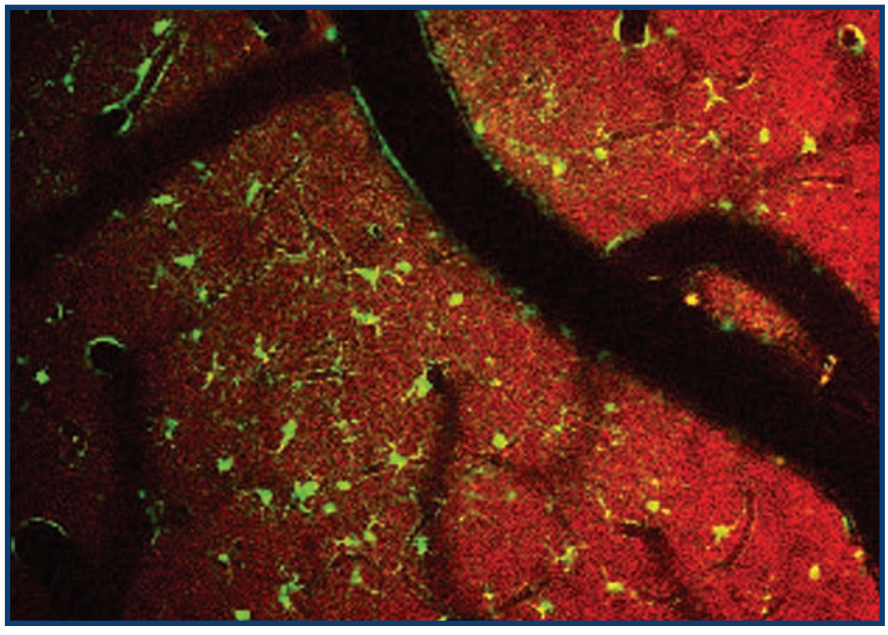


Figure 3. In this two-color in vivo two-photon image from an exposed mouse cortex, NADH fluorescence is shown as red and sulforhodamine-labeled astrocytes as green. Courtesy of Karl A. Kasischke and Nikhil Mutyal, University of Rochester Medical Center.

The technology

Optical filters are constructed by depositing alternating thin-film layers of high and low index of refraction. Typically, each layer has an optical thickness equal to roughly one-fourth of the wavelength of light, where optical thickness is the product of a layer's real thickness and its index of refraction. For example, green light has a wavelength of 0.5 µm (millionths of a meter), so the corresponding quarter-wave layer thickness is about 50 to 80 nm (billionths of a meter). Traditionally, thin-film filters have been constructed with tens of layers, and they have required many coatings for a single complex filter such as a multiphoton emission type. The new generation of high-performance filters is constructed with many hundreds of layers in each coating, so that each layer's thickness must be controlled with utmost precision. To achieve optimal spectral performance, sophisticated control of the thin-film deposition process is required to compensate for layer thickness errors.

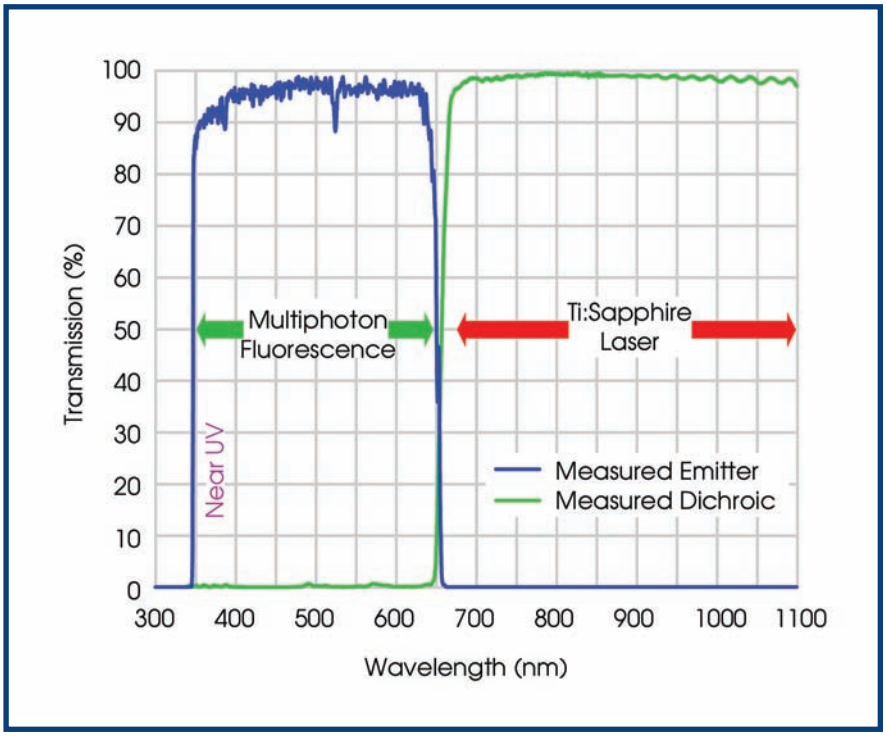


Figure 4. Typical measured spectra of the near-UV and visible emitter FF01-680/SP and dichroic beamsplitter FF665-Di01 are displayed. This filter combination provides high throughput from the near-UV (350 nm) through 650 nm, and very high OD >8 blocking along with high dichroic transmission across the entire commonly used Ti:sapphire laser tuning range, including the short-wavelength region down to 680 nm. It is ideal for near-UV fluorescence imaging.

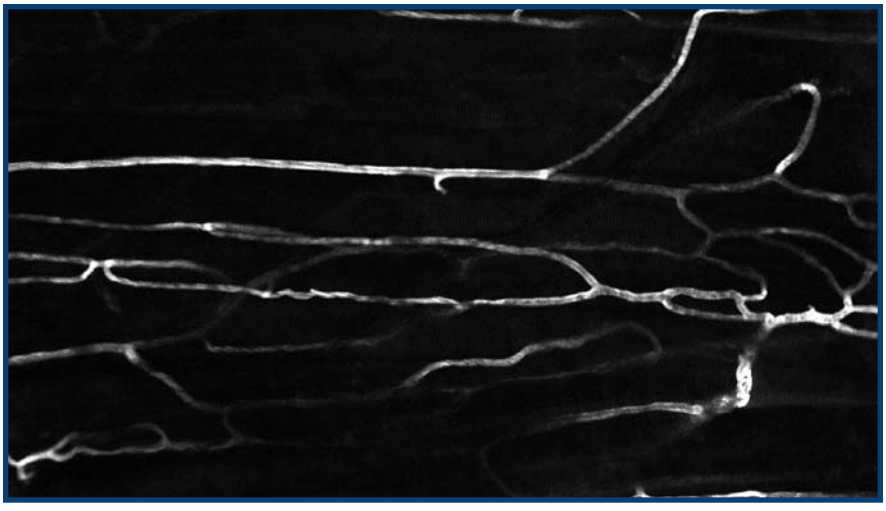


Figure 5. This in vivo two-photon image shows blood vessels in the dorsal skin of a live mouse labeled with intravenous injection of FITC-Dextran. Courtesy of Edward Brown, University of Rochester Medical Center.

The technology that meets these requirements is based on a combination of ion-beam-sputtering deposition systems (long renowned as the premier coating technology for low-loss, high-performance mirrors and filters) with an advanced deposition control technology. These filters are based on refractory metal-oxide materials that are as hard as the glass on which they are coated, with a single glass substrate design coated on one or both sides. There are no adhesives or epoxies in the optical path, eliminating the filter degradation, undesirable loss and autofluorescence that contribute to increased background.

The hard-coating technology and all-glass design of the new breed of filters provide exceptional reliability: The filters can be cleaned and handled like any standard glass optics, they have a laser-damage threshold higher than the laser power used in multiphoton microscopy, they do not photodarken or "burn out," and they are impervious to environmental factors such as temperature and humidity extremes.

Multiphoton microscopy is a vital biological imaging technique, and recent advances in optical filters are making it possible for researchers to significantly upgrade their systems' performance. Continued improvements in technology, especially in the excitation lasers, will further enhance the technique's efficiency and widescale accessibility. □

Meet the authors

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