Exploring multiphoton microscopy in depth

Over the last two decades, the use of multiphoton microscopy has spread to all major areas of biological research. **Marie Freebody** speaks to John Girkin about the remarkable potential of this powerful technique and the innovations that have aided its growth.

John Girkin is an associate director of the Institute of Photonics at the University of Strathclyde, UK. Previously, Girkin worked for Keeler, UK, producing instruments for the treatment and diagnosis of a range of ophthalmic diseases, including the first commercial diode laser ophthalmic photocoagulator. His interests primarily focus on the use of photonics in biotechnology, including the applications of femtosecond lasers for multiphoton imaging.


John Girkin, Institute of Photonics

John Girkin hopes that multiphoton microscopy can be used in clinical settings in the future.

What is multiphoton microscopy?

Multiphoton microscopy is a term that now covers a range of nonlinear optical microscopy methods with one key feature – they can all obtain optically sectioned images with high resolution. A common factor in all of these methods is that two or more photons combine to generate a contrast difference within the sample being imaged.

The recent growth in the field was prompted by a team at Cornell University led by Watt Webb in 1990. A femtosecond laser was used to excite fluorescence within a sample through the absorption of two photons. Since then the method has broadened beyond using simple fluorescence as the contrast method, with the addition of three-photon excitation, second and third harmonic imaging and CARS microscopy.

In all of these methods, two or more photons are used to excite either fluorescence or vibrational modes. Under normal circumstances such events do not take place with a high probability, but at the focus of a high numerical aperture objective lens the probability of such an event rises.

Typically, several watts of laser power is required for excitation but, by using femtosecond laser pulses, the peak power is high enough for an acceptable excitation rate while the average power (a few milliwatts) is low enough not to cause any thermal damage to the sample. As the excitation only takes place at the focus of the beam, you automatically have a method of taking optical sections through the sample.

Why is multiphoton microscopy an important technique?

The inherent optical sectioning provided by multiphoton microscopy enables three-dimensional images to be obtained from ever increasing depths into samples. The excitation light is generally in the near-infrared portion of the spectrum (say 700–1000 nm), which passes through the sample (in particular tissue) with minimal scattering and linear absorption.

The main application of the method is in life sciences as it provides a means to image deeply (between around 500 μm and 1 mm) into a sample with minimal damage. Key advantages of the method for biological applications include shorter sample preparation time, the ability to view the area of interest without dissection and, in particular, in-depth *in vivo* imaging.

What are the main applications and when do you expect them to occur?

The main application is in life-science research. However, it is crucial to point out that the growth of multiphoton imaging has been boosted by the ability to produce living samples via genetic manipulation, in which only specific and selected features fluoresce. Multiphoton microscopy is now being applied in all major areas of biological research ranging from stem cells to cancer and heart studies.

What is the most significant advance in recent years?

Initially, imaging was undertaken using a colliding pulse dye laser – not the type of instrument to be used lightly in a biology laboratory. The development of reliable femtosecond near-infrared sources has enabled life scientists to concentrate on the biology rather than having to become laser experts. The change from water-cooled argon lasers to compact diode-pumped, frequency-doubled solid-state pump lasers has also contributed to the revolution in imaging.

What key challenges remain?

The main challenge now is to image ever more deeply into the sample. As you image deeply, however, the optical properties of the tissue through which you image cause a loss in image resolution and contrast, thus setting a limit on the depth at which high-quality images can be captured. This problem is currently being tackled using adaptive optics originally developed for astronomy. By shaping the imaging laser beam to counteract the sample-induced aberrations, resolution and contrast can be returned to the image and even sample movement (an issue for *in vivo* imaging) can be overcome.

What do you think the next big breakthrough will be?

One of the aims of current research is to take the method from the life sciences and into the clinic for improved disease diagnosis. This requires the optical system to be made smaller, more reliable and even easier to use so that clinicians can be provided with images of organs with sub-cellular resolution. This is leading to the development of miniature MEMS-based systems linked to sources via optical fibres. Such methods, however, present significant physics challenges in a number of areas. □