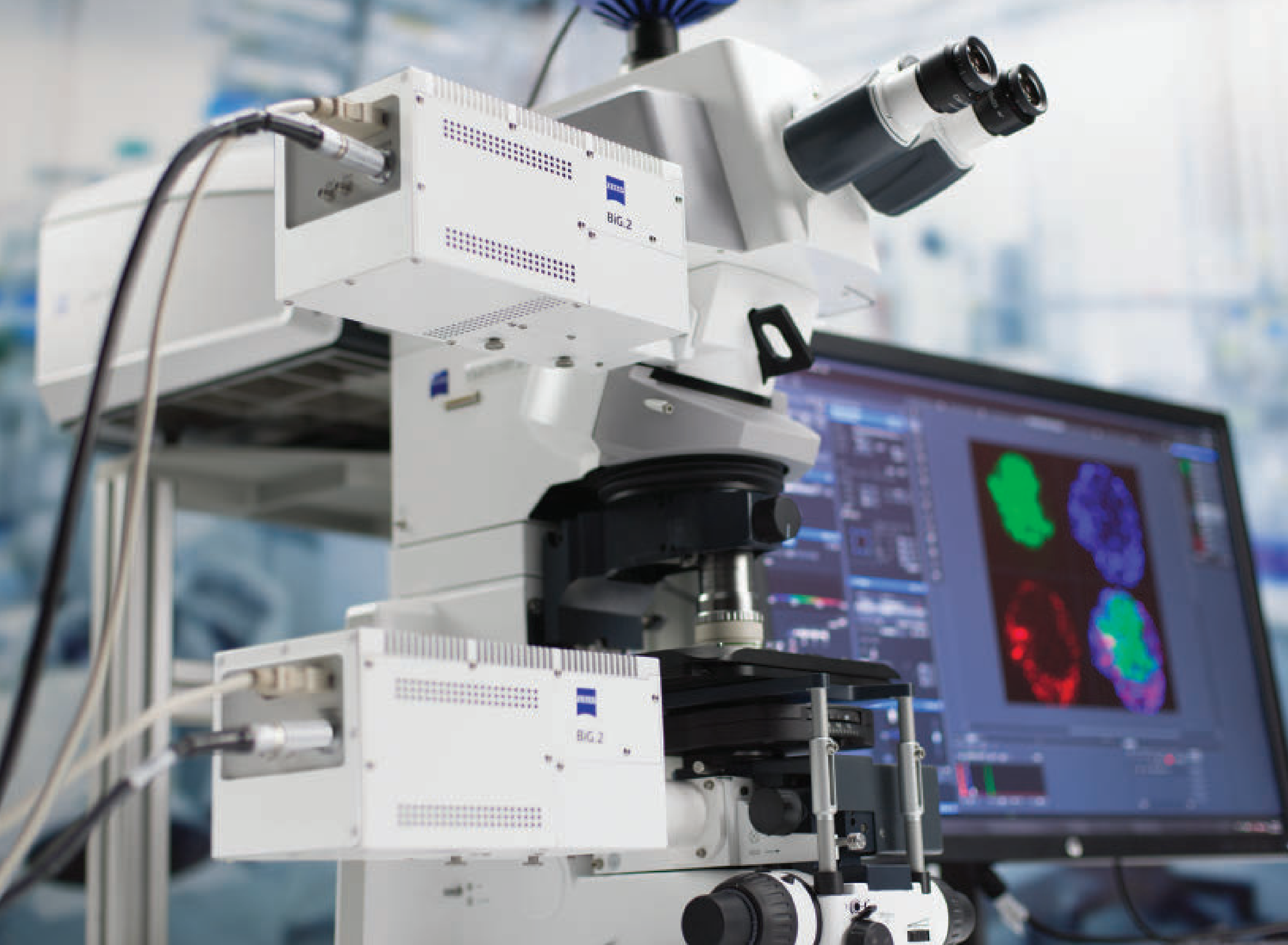


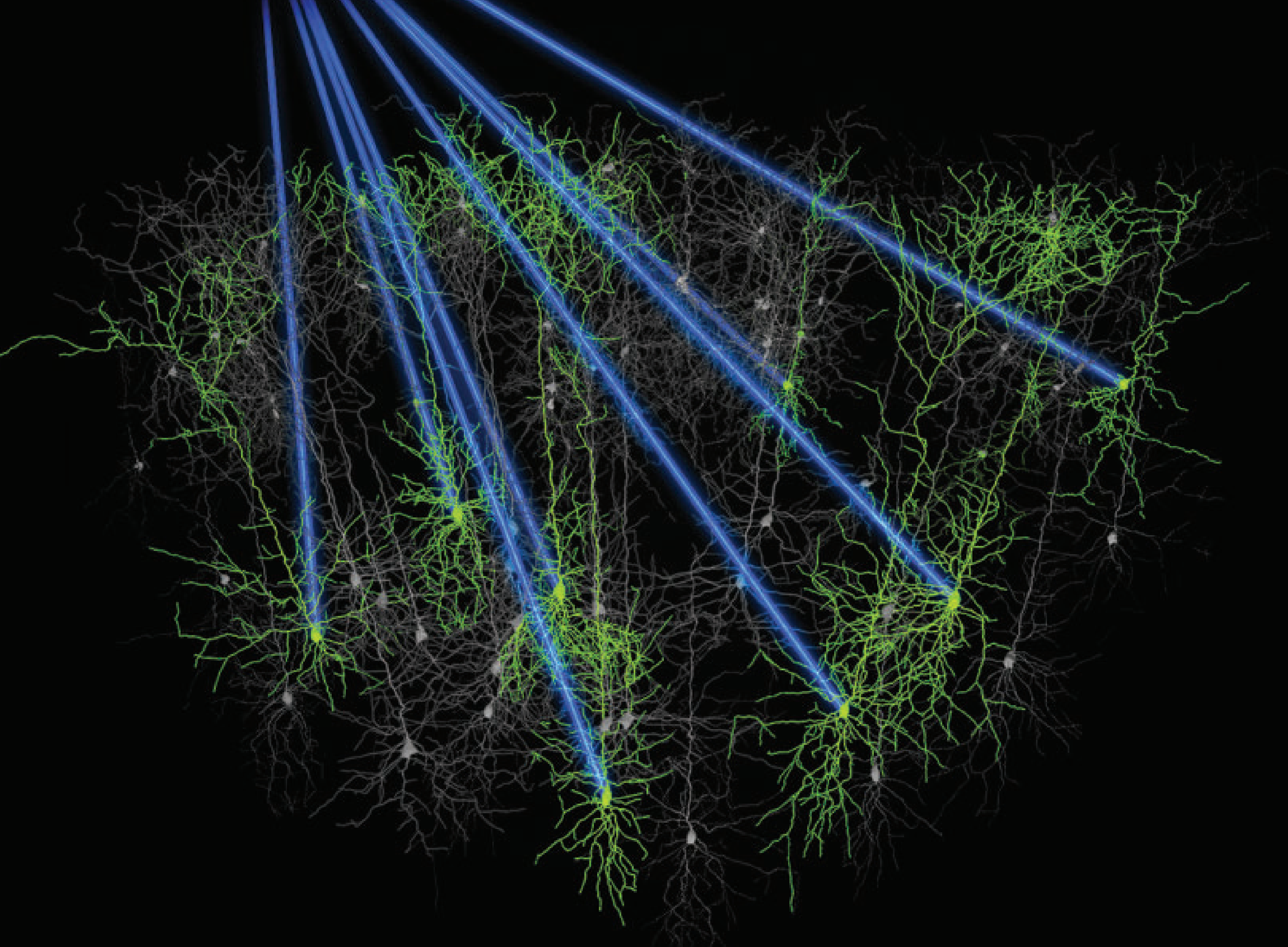
The Zeiss LSM 880 confocal microscope equipped with BiG.2 for multiphoton microscopy applications enables deep tissue imaging of biological tissue samples and living organisms. Courtesy of Zeiss.

Multiphoton excitation fluorescence (MPEF) microscopy, an optical imaging technology that's vital to the life sciences, is advancing as quickly as its applications. The technique is on track to transition to clinical uses within the next several years.

MPEF Microscopy
Shines Best on Living Samples

BY RODD M. PEDROTTI, EDITOR

MPEF microscopy, along with other nonlinear methods that include coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS) and second harmonic generation (SHG), is suitable for imaging whole cells — especially cells within intact tissue samples. While many fluorescence microscopy schemes use filtered lamps, LEDs and lasers as light sources that drive excitation-produced visualizations, MPEF methods require an ultrafast laser source that is focused onto the sample's plane of observation. Typically, it also uses a combination of dichroic mirrors and longpass filters to filter the emitted light to the wavelengths of interest.



Higher power from femtosecond laser sources allows for simultaneous activation of multiple neurons within a population. Courtesy of the Hausser Lab, University College London.

Marco Arrigoni, technical marketing director at Coherent Inc., spearheads the company's support of nonlinear microscopy using ultrafast lasers. He provided unique insight into how MPEF techniques are currently employed and where this technology is going. He also addressed its fundamental differences from confocal microscopy. In short, "The applications for multiphoton microscopy continue to grow at a very healthy pace, and I see no chance of this slowing anytime soon," Arrigoni said.

Ultrafast momentum

Sir Isaac Newton's first law of motion states in part that anything in motion will remain in motion unless acted upon by an outside force, as evidenced by gravity's influence on inertia. Technological advancements seem to play by similar rules; in this case, their momentum is pushed to an ever-increasing pace by innovative minds.

MPEF microscopy is very popular within the field of optogenetics, primarily due to its unique ability to produce high-resolution 3D images via ultrafast lasers (typically operating at 900 nm at up to 100 MHz). MPEF methods also confine undesirable sample damage to the laser's focal plane. Even though an MPEF technique may use an ultrafast laser that produces multiple watts of output, the low probability of two-photon absorption and other nonlinear imaging mechanisms means that only a very small amount of the near-infrared (NIR) laser power is absorbed by the sample.

Because this type of nonlinear method has a nonlinear (squared or cubed) dependence on laser intensity, the absorption and subsequent fluorescence excitation only occur at the beam waist of a tightly focused laser. This event is confined to a single, microscopic pinpoint of the specimen, allowing the researcher to analyze a very specific "dot" of the sample. By X-Y scanning the beam and stepping the focus of the laser, or stepping the sample, a 3D image can be acquired.

Arrigoni described three distinct benefits associated with MPEF-based applications, including those of other nonlinear techniques:

- MPEF methods enable deeper imaging into a tissue sample. As MPEF techniques use longer NIR wavelengths, the approach does not rely on conventional excitation methods dependent upon the absorption of a single, visible photon of light.
- MPEF microscopy yields very little tissue damage with regard to photobleaching, as well as photothermal damage, making it ideal for imaging live tissues. It should be noted, though, that because most MPEF methods rely on fluorescent proteins and dyes injected into the sample, many versions

of the technique are not suitable for imaging human tissue in vivo.

• MPEF techniques provide inherent 3D resolutions. In his explanation of the difference between 3D resolutions provided by MPEF imaging and those produced by single-photon confocal imaging, Arrigoni confirmed, "Whereas a confocal microscope relies on a pinhole (i.e., the 'confocal aperture') to block light generated away from the focal plane of the sample, a multiphoton microscope excites fluorescence only at that focal plane, where the laser intensity is the highest. The actual resolution is limited by diffraction (as in confocal microscopy), with a typical value of 0.3 to 0.4 µm in the X and Y axes, and two to three times worse along the Z axis."

The current state of MPEF microscopy and its growing relevance were anticipated more than a decade ago. In early 2007, Victor David and Philippe Feru of Spectra-Physics indicated that, since only a few years prior, the average power output provided by ultrafast pulsed Ti:sapphire lasers, which are commonly used in MPEF applications, had increased ("Seeing Deeper with Multiphoton Microscopy," www.photonics.com/A39645). "This capability has enabled deeper tissue imaging, but increases in the average power will damage most samples because of the heating effects of high laser power. A new approach, based on dispersion compensation, can allow imaging to occur deeper in the tissue," they stated.

The new approach they referenced was an evolutionary advance in the lasers used for MPEF microscopy, namely the ability to minimize the pulse duration (i.e., higher peak power) at the sample. Combined with longer excitation wavelengths, the approach enabled deeper imaging within a biological sample while minimizing cellular damage, such as photodamage.

According to Arrigoni, because the various multiphoton methods used are based on low-probability mechanisms, regardless of whether very tightly focused high powers are employed, the average power absorbed by the sample is quite small.

Clinical potential

In 2011, researchers at Cornell University were in the process of developing a multiphoton endoscope prototype designed for use in clinical settings. The work was spearheaded by Dr. Watt W. Webb, a professor of applied physics and the S.B. Eckert Professor in Engineering in the university's department of Applied and Engineering Physics ("Multiphoton Endoscopy May Equal Fewer Biopsies," www.photonics.com/A48933).

Webb has studied multiphoton microscopy for decades. With his colleagues' help, he invented the scientific field in the 1990s. His goal has been to make the technology readily available in clinical settings, enabling better imaging applications for use on patients.

Consequently, he and his team sought to develop a multiphoton technology — a two-photon technology, to be specific — housed within the end of a thin endoscope to directly image tissues or tumors. According to Webb, "The motivation all along was to look at human cells."

Arrigoni believes that such application-specific developments represent the bulk of MPEF microscopy's advancements over the next three to five years. He said that roughly 60 percent of all multiphoton microscopes are used for some type of neuroscience application, due in part to the increased funding for specific initiatives in several geographical areas. In the U.S., for example, the National Institutes of Health's Brain Research through Advancing Innovative Neurotechnologies (BRAIN) initiative is a prime example of such an endeavor (www.photonics.com/A57712).

Arrigoni says fulfilling the project's ultimate goal — to understand how local neuronal networks operate — will require utilization of MPEF microscopy resources. "The rest of the market is a combination of various fields including cancer research, stem cell studies and other aspects of immunology. While many of these applications involve laboratory research, there is an overarching although still elusive goal of moving toward clinical (i.e., human) applications."

His assessment mirrors Webb's approach: As the primary founder of the field of multiphoton microscopy, he sought to prototype a device with a very specific application about five years ago.

Another application-specific development was recently reported by David Leunberger, of Optotune AG, and the University of Zurich's Fabian F. Voigt ("Focus-Tunable Lenses Enable 3D Microscopy," www.photonics.com/A57323). They described the use of electrically tunable lens (ETL) technology in the promising field of in vivo two-photon microscopy. In another study, referenced by Leunberger and Voigt, and published by Benjamin Grewe et al., a two-photon microscope equipped with acousto-optical deflectors was modified with ETL and an offset lens. Using a mouse as a test subject, a femtosecond laser beam rapidly targeted a large quantity of neurons — in a random-access pattern at kilohertz rates — within the animal's brain.

Down the road, Arrigoni believes a key trend for research-based MPEF microscopy will be the increased development of genetically encoded fluorescent probes. "These are probes that can be expressed directly in model animals using viruses to introduce them into the cell DNA. These complex molecules (like green fluorescent proteins) have several advantages over conventional dyes: They are not as toxic, and one can create a colony of animals with the same fluorescent characteristics," he said, adding that existing companies supply model animals, such as fruit flies or mice, with a diversity of genetic expressions. Arrigoni also noted that progress has been made to develop functional probes for applications such as optogenetic stimulation and monitoring of metabolic activity.

Multiphoton vs. confocal microscopy

A common misconception regarding MPEF microscopy versus confocal microscopy, said Arrigoni, is that although damage at the focal plane actually is very similar for single- and multiphoton excitation, single-photon excitation damages the samples over a much larger volume, as damage occurs above and below the focal plane. Consequently, total damage to the sample is much higher when employing confocal methods.

"To put this in numerical perspective, in 1997, Schoenle and Hell showed that irradiation with 100 mW of ultrafast power at 800 nm on a diffraction-limited spot for 1 second resulted in only a 0.2-°C temperature rise, well-compatible with cell functionality," said Arrigoni. He indicated that although laser confocal microscopy is suitable for thin samples on a dish or slide, it's generally not suitable for prolonged in vivo imaging applications.

Also, confocal microscopy can't be used to construct the same type of 3D

image as multiphoton methods can. Much like raster scanning, confocal microscopy only allows the user to view a single point of the sample at any given instant — it's not possible to view a full 3D image of a specimen in real time. Instead, confocal microscopy relies on software algorithms to construct a point-by-point 3D image based upon the technique's 2D optical sections.

Regarding confocal microscopy's inherent limitations, Arrigoni said, "One of the major limitations on imaging depth is scatter loss — scattering of both the laser itself, and of the resultant fluorescence or other type of optical signal."

He added, "In an inhomogeneous sample like biological specimens, there are several scatter mechanisms involved, but Mie scattering [very simply defined as the scattering of light via particles ranging from about 5 nm to 50 µm in size] is probably the dominant one, and this has a fourth-order-inverse dependence on wavelength." In short, the more light scatter present, the lesser the possibility of increased imaging depths. Additionally, confocal microscopy is optically inefficient; its microscope/filter combination throws away approximately 95 percent of the laser's light.

Refining the method

As for the downsides of MPEF technology, Arrigoni indicated that the most obvious ones are its associated costs and the complexity of its execution. Speaking on neuroscience applications, Arrigoni noted, "The complexity of the experiments is unlikely to drive equipment price down, however progress and possible adoption of [MPEF] microscopy for clinical applications will definitely require less-expensive dedicated scopes and probably simpler lasers, with targeted functionality."

Additionally, only a few benign dyes are suitable and available for human applications (e.g., during surgery). The use of endogenous fluorescence or nonlinear techniques that do not need fluorochromes, such as SHG and third harmonic generation (THG), are being investigated for potential use in these areas. Since the vast majority of MPEF microscopy techniques' observable excitations rely on fluorescent dyes/substrates being injected into the sample, or for the sample to be genetically modified with green fluorescent proteins or one of the mFruits, they are not fit for human applications.

Laser technologies and their respective power outputs for use in MPEF applications are expected to rapidly advance. In fact, users are already requesting powers ranging from 5 to 10 W, and two different lasers currently exist at 18 W and 40 W. Speaking to the particular needs of optogenetics, Arrigoni indicated that higher power is needed to photoactivate and interrogate an ever-larger number of neurons, and to be able to do so at deeper cortex locations.

"Higher power also enables faster imaging which will benefit all of these applications. This includes simple speed accelerations by faster scanning and multipoint scanning, as well as novel high-speed imaging modalities," said Arrigoni.

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