Shedding Light On Deep Tissue: Multiphoton Microscopy

Multiphoton microscopy and other approaches for more precise imaging of tissue samples—such as lightsheet imaging and tissue-clearing methods—are growing in popularity as they turn more mainstream. As more scientists use these deep-imaging methods, new applications are continually arising, such as watching some of the cellular processes that control brain development, testing bones for lead levels, and studying a wide range of disease processes. By Mike May

Throughout the history of biological imaging, scientists and engineers have worked together to see more. Sometimes their advances enabled us to see things with higher resolution; other times they helped us to see deeper into a sample. Yet biologists want to do both things at once, which has led to new methods such as multiphoton microscopy.

In confocal microscopy, a single photon excites a fluorescent label in a sample. In two-photon microscopy—the most common form of multiphoton microscopy—two photons are absorbed by the label at virtually the same instant. Multiphoton microscopy also uses longer-wavelength photons, which are lower energy and penetrate more deeply, creating less tissue damage while imaging farther into a sample.

The ability to resolve structures deeper in biological samples keeps evolving with improvements in technology and modified or new methods—while still requiring considerable expertise in preparation, operation, and analysis. In many ways, these two key elements of scientific progress—technology and techniques—are moving in tandem to help imaging reveal more of the biological world.

Putting the tech together

To expand the use of multiphoton imaging, scientists need more and better commercial platforms to consider. One of them is the A1R HD multiphoton confocal microscope from Nikon in Melville, New York. A key feature of this microscope is speed—capturing data from the entire field-of-view at 30 frames per second. “With the A1R HD, scientists can capture great quality images at a high rate,” says Adam White, a biosystems product manager for Nikon.

The LSM 880 with Airyscan from ZEISS, headquartered in Oberkochen, Germany, also provides multiphoton imaging. “Adding the Airyscan detector improves the signal-to-noise ratio by four to eight times,” says Joseph Huff, solution manager and application development engineer at ZEISS. “Using this setup on mouse brain, we can acquire data at depths up to 500 microns.”

However, there are drawbacks that come with deeper focus. “As you go deeper in tissue, the amount of spherical aberration increases,” says Carlo Alonzo, product manager at Olympus in Waltham, Massachusetts. “This degrades the focus and results not just in poorer resolution, but also dimmer images because of less efficient multiphoton excitation.”

For that reason, Olympus developed TruResolution Objectives. “A motorized optical correction collar dynamically ad-..."
justs with the z-focus of the microscope,” Alonzo explains. By automating this process, these objectives “deliver a volume image that better maintains brightness and resolution from the top to the bottom of the z-stack,” he says. These objectives work with the Olympus FLUOVIEVIEW FVMP-E-RS multiphoton microscope, and it’s an easy update to install. With this device, a researcher can more easily capture small features deep within tissue, like submicron-sized dendritic spines on neurons.

**Professor of neuroscience**

Sebastian Jessberger uses multiphoton microscopy to study living mouse brains, to understand how neurons are generated in the mammalian hippocampus throughout the life of an organism.

Getting some clarity

It’s not just hardware that improves deep-tissue imaging. Tissue clearing, a technique that makes a sample virtually transparent, also makes it easier to image at deeper levels. Tom Villani, chief scientific officer at Visikol in Whitehouse Station, New Jersey, points out that in addition to letting photons go deeper in tissue, “a lot of clearing agents stabilize fluorophores, which is a useful side effect.” Plus, the Visikol HISTO clearing agent is easy to use. “I have high school interns clearing tissue!” Villani says.

The type of tissue involved impacts the process. “Brain, skin, and lung are easier,” Villani says, “but tissue with lots of pigment, like kidney and liver, is harder.” The microscope also matters. With just about any confocal scope, images can be obtained 1–2 mm into tissue that has been cleared with Visikol HISTO. “You need two-photon or lightsheet fluorescence microscopy and water immersion objectives to go deeper than 4 millimeters,” he says.

To get particularly sharp images from cleared samples, Nikon developed a new 20x glycerol immersion objective. It provides a high numerical aperture, 1.0, and a good working distance, 8.2 mm. To make that clarity possible, the objective needs to be big: It’s 90 mm long and 48 mm in diameter—both dimensions being about 50% bigger than an average 20x objective.

The Lightsheet Z.1 microscope from ZEISS works with cleared samples. “For bulk tissue imaging,” says Huff, “a clearing agent and lightsheet is a good choice.” This imaging platform includes illumination paths from the right and left sides. As a result, “you can see twice the depth of confocal for live imaging,” says Scott Olenych, North American product marketing group manager for light microscopy at ZEISS. Despite the added depth, the real benefit of using lightsheet technology is speed. “By comparison, imaging on confocal is fairly slow,” Olenych explains. “Lightsheet can plow through multiple millimeters and even deeper, because it uses a camera instead of photomultiplier tubes.”

**Picturing pathogenesis**

At the University of Texas Medical Branch in Galveston, Joan Nichols, professor of internal medicine, and her colleagues use multiphoton microscopy to study the pathogenesis of human disease. Disease processes often involve changes in both the cells and the extracellular matrix, the scaffolding that supports cells. To understand these processes, Nichols likes to image changes in the matrix and in the live cells that produce and modify it. “[In the matrix], multiphoton is amazing,” she says. “It lets you do some deep imaging—at 100 microns or better.” From that depth, she gets a better assessment of the disease-driven changes that take place in the tissues, cells, and supporting extracellular matrix. “We can do a control and experimental condition side by side and image it over and over during a chosen period of time,” she says.

The key to this work is looking in the same place over the course of a disease progression. With traditional light microscopy, the samples could be fixed and imaged slice by slice. “But seeing the control and experimental tissue over time without having to fix the tissues lets you watch live changes—going to the same point over and over to find the change in that region,” Nichols explains.

One health condition that Nichols and her colleagues study is pulmonary fibrosis. To do this, they developed bioengineered lung tissue by making a 3D group of cultured cells called an organoid, which mimics the features of an organ. This organoid can be imaged with multiphoton microscopy to compare and analyze healthy and fibrotic or diseased tissue.

“A ‘window’ into the brain”

But sometimes organoids are not enough. At the University of Zurich in Switzerland, professor of neuroscience Sebastian Jessberger uses multiphoton microscopy to study living mouse brains, to understand how neurons are generated in the mammalian hippocampus throughout the life of an organism, specifically in a structure called the dentate gyrus. Previously, Jessberger looked at fixed cells, but he wanted a better way to study neuron creation. So, through careful surgery, Jessberger has exposed the hippocampus and made a “window” into the brain that can be used for live imaging over months and even years as the mice continue to perform their normal range of behaviors.

Jessberger labels stem cells in these mice and follows those cells over time with multiphoton microscopy. “We look at neurons born in the adult hippocampus and those in embryonic development,” he explains. “From that, we’re trying to understand what happens at the cellular level.” So far, the technique allows imaging to a depth of about 1 mm.

Using three-photon microscopy—developed by Chris Xu and the Xu Research Group of Cornell University in Ithaca, New York—Jessberger hopes to see much deeper, maybe 2 mm. “Eventually, we hope to reach the dentate gyrus or hippocampus without removing part of the cortex,” he says.

With these advances, Jessberger and his colleagues expect to explore even more aspects of brain development. For example,
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Xu Research Group, Cornell University  
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ZEISS  
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Nichols mentions yet another challenge for multiphoton microscopy users: availability. Some facilities will have a few instruments, but that’s not always the case.

Just wanting to use multiphoton imaging, then, is not enough. A scientist needs to purchase or schedule an instrument to use, develop the needed skills for this type of imaging, and learn to prepare samples for the best results.

Creating combinations

Some of the biggest advances could come from combining advanced imaging with other technologies. For example, Jessberger describes the approach his group performed in imaging a clone or cluster of the same cells and then looking at its RNA. “You can use the knowledge of the RNA content in those cells and then follow the exact history of those cells for a certain period of time,” he says, adding that these types of studies would start to connect molecular mechanisms with a range of anatomical changes.

Nichols also combines technologies in studying lung function. She has used microcomputed tomography to see the respiratory tree in an entire lung, and multiphoton microscopy to see structural changes at a cellular level.

Biologists have only begun to probe the possible combinations of multiphoton microscopy, deep-tissue imaging with clearing techniques, and other technologies. Certainly, many more uses will emerge for these imaging techniques—and others yet to be devised—as more scientists explore the opportunities they present.

Making it work

Although multiphoton microscopy is much easier than it used to be, it differs significantly from conventional light imaging. “It’s not like a regular confocal that you buy and put on the bench and it works,” Jessberger says. “You need people who understand the physics who can be at least partially available.” He adds, “Adjustments have to be done from time to time.”

Nichols agrees on the need for expert assistance. “We work closely with an [outside] team that helps us set up the imaging and evaluate it,” she says. “Most scientists need to rely on a microscopy expert to work with them.” Most big universities, in fact, employ someone in-house dedicated to working with multiphoton microscopy users.

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