THREE PHOTONS ARE BETTER THAN TWO

Three-photon microscopy was suggested in the 1990s, but laser technology at the time was just not up to the challenge. Lauren Ware explores how recent technology advances are bringing three-photon microscopy back into focus.

When Cornell University physicist Chris Xu started working with multiphoton fluorescence microscopy in the 1990s as a graduate student at Cornell, the technique was brand-new—having been recently developed by scientists Winfried Denk, Watt Webb, and Jim Strickler. Although the potential for using three photons was known and explored early on, in practice it was inefficient owing to the laser technology available at the time. As a result, two-photon fluorescence microscopy became the standard.

But Xu believes the time is right for three-photon fluorescence microscopy to re-emerge. In a paper published in *Nature Photonics* in 2013, Xu and his team revealed their three-photon microscope, which is efficient, doesn't damage tissue, and can image up to 3.5 millimeters deep into tissue, deep enough to see below the white matter of the brain—a feature beyond the limits of two-photon microscopy (1).

Strong Enough

Multiphoton fluorescence microscopy (MFM) creates high-resolution images with incredible selectivity, imaging only what is labeled with a fluorescent dye. MFM has the advantage of being able to image more deeply into tissue with less photobleaching than confocal fluorescence microscopy. It's a technique especially popular with neurobiologists because it allows imaging of individual synapses and can be performed on brain slices or in live, intact animals.

"If you really want to see what the brain does, you have to be at cell-level resolution," says Kevin Eliceiri, an advanced light microscopy expert and director of the Laboratory for Optical and Computational Instrumentation at the University of Wisconsin, Madison. "Multiphoton microscopy is the only technique where you can get some depth with resolution and see cells actually moving."

To perform MFM, researchers begin by introducing fluorophores, chemical compounds that emit light upon excitation, into the specimen as a dye. Then, a focused laser beam scanned in a raster pattern emits long-wavelength photons which can excite an electron in the fluorophore to a higher energy state once multiple photons are simultaneously absorbed: two photons for two-photon microscopy or three photons for three-photon microscopy. As the electron decays, it emits a fluorescence signal that is captured by the microscope. In MFM, the excitation wavelength is longer than the emission wavelength—the opposite of traditional fluorescence microscopy.

As exciting as the technique can be, it does have imitations—specifically, the depth at which MFM can image due to scattering and the refractive index. If researchers used longer wavelengths of photons, it would be possible to image more deeply since the photons would be less susceptible to scattering. But when wavelengths approach the 1300-nm range, water in the specimen begins to absorb the photons, rendering them useless. So the question of whether one could image more deeply using three photons instead of two had gone largely unanswered since the 1990s.

But Xu's work showed that even though there is this water absorption window, three photons of fluorescence is strong enough so that in many cases you can still see clearly. What's more, longer wavelengths don't seem to damage cells as previously thought.

Laser Light

To make three-photon microscopy a reality, developments in laser technology have been key, rendering the method efficient and workable. And for this, Xu was particularly well-positioned to move the approach forward. After studying multiphoton imaging as a graduate student, he worked on fiber optics in the telecom industry, providing him with valuable experience in laser technology. "We created our own laser system," says Xu. "With this system we don't need to use a lot of power, only 20 milliwatts, to get superb image quality. This is a lot lower power than the two-photon laser."

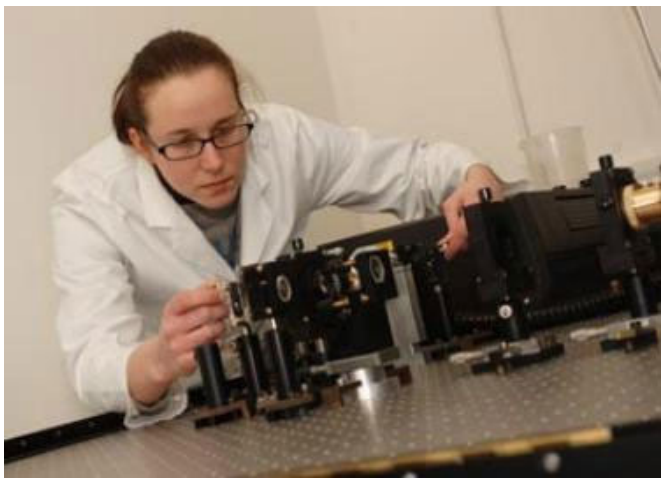
The laser Xu created has a high pulse energy and a low pulse repetition rate, making it more efficient for three photons than the standard two-photon titanium sapphire laser. He started with a fiber laser that generated a wavelength of 1550 nm, a standard wavelength used in the telecom industry. Then he used a photonic crystal rod to shift the laser's wavelength to 1700 nm, while scaling up the energy about 100,000 times by forming a wave called a soliton, which keeps its shape when it travels at a constant rate.

The rod is glass with a special structure around it so that when the laser beams into it, the spatial profile is maintained. "I knew that the bigger the rod, the more fiber, the more energy. So I wondered, how big can we make it? How far can we push in this direction?" recalls Xu. He discussed his idea with coauthor Frank Wise, also a physicist at Cornell University. "'I have this wonderful idea,'" Xu told Wise. "'I need a big, long rod to form a soliton, so I can make this energy very useful.' [Wise] said, 'Yeah, I have a rod like that,' and literally the next day we just got it, put out a pulse, and yes, the energy was up."

Other developers have also revisited three-photon fluorescence microscopy. In a paper also published in 2012 in the *Journal of Microscopy,* Gail McConnell, a physicist at the University of Strathclyde in Glasgow, UK, and colleagues reported that their three-photon microscope can produce images safely and effectively as well (2).

Like Xu, McConnell developed a laser for her three-photon microscope—in this case, a bidirectional pumped optical parametric oscillator. While it is a fiber laser that emits at a wavelength of 1500 nm like Wu's laser, McConnell's has an additional feature: it's tunable—she can adjust the wavelength, giving greater flexibility.

Although her group has done some unpublished research looking at mammalian cells, their published paper imaged plant cells, showing that these cells were not damaged by the increased power. "We could image for hours," she says, "compared to seeing damage after about five minutes with a titanium sapphire laser." The next step for McConnell is to improve the resolution even further—something her team is actively working toward.



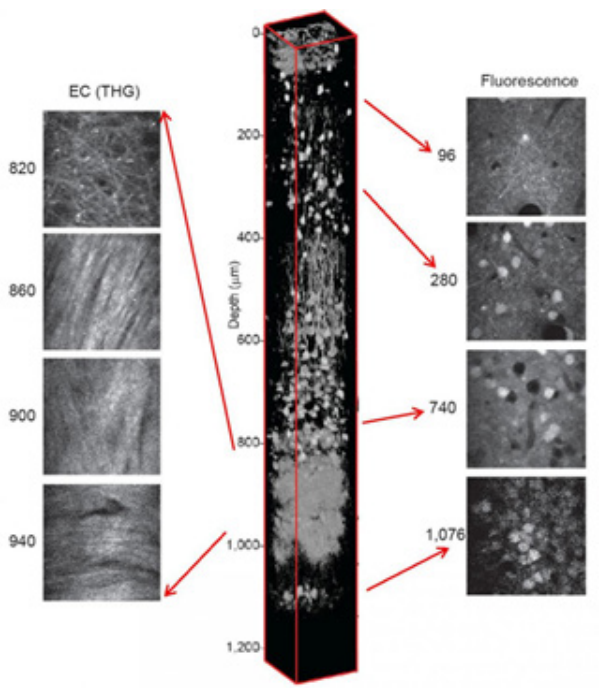
Gail McConnell and colleagues reported that their three-photon microscope can produce images safely and effectively. Source: University of Strathclyde

Three, Four, or More

If three photons works, why not four? The trouble with four photons is that you need more photons and therefore brighter lasers, which results in a greater potential for cell damage. Considering that the technique is typically used to image live animals, this becomes problematic. "There may be circumstances that allow you to do that," Xu says. "You would have to redo the optimization. And we haven't reached the depth limit with three photons yet."

Overall, a lot of potential has yet to be realized in the development and optimization of three-photon microscopy, according to Xu. "We can develop better contrast agents, better optics, and perhaps use a little less power to get the same signal, as well as optimize the system to collect the photons. We may be able to do an order of magnitude better on each of these things, which is a factor of 1000 when added together—not a trivial amount." For now, three photons seems to strike the best balance between absorption and scattering.

Excitement over Xu and McConnell's work continues to grow. "Both have really shown the great potential of three-photon microscopy," says Eliceiri. "This work points out the importance of looking at other wavelengths and doing a survey of what's there. But this shows the real promise of going very, very far red." With this new tool able to produce high-resolution, three-dimensional images of individual neurons deep in the brain, scientists will hopefully be able to learn much more about how the brain works.



Xu and his team reveal their new three-photon microscope, which is efficient, doesn't damage tissue, and can image up to 3.5 millimeters deep into tissue, deep enough to see below the white matter of the brain. Source: Chris Xu

References

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Written by Lauren Arcuri Ware

BioTechniques 57:237-239 (November 2014) doi 10.2144/000114225