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Seeing Detail Beneath the Surface

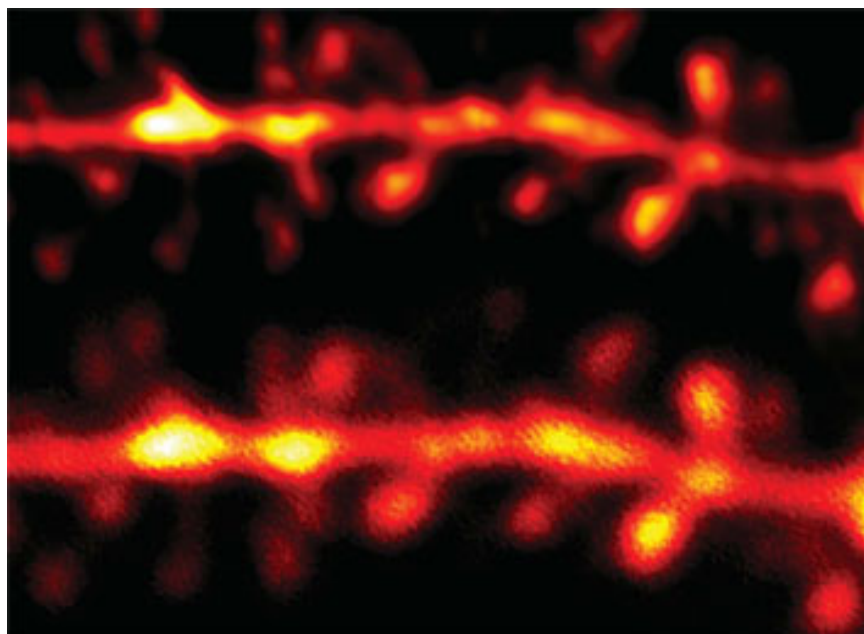


Image Title: A neuron's dendritic spines imaged with STED two-photon laser scanning microscopy (top), compared to traditional two-photon laser scanning microscopy (bottom). - Bernardo Sabatini

An adaptation of two-photon microscopy, the go-to method for seeing neurons below the surface of the brain, has improved its spatial resolution threefold. The new imaging technique will allow scientists to focus on tiny structures, such as those that mediate communication between neurons, within relatively intact samples of brain tissue.

Howard Hughes Medical Institute investigator Bernardo Sabatini led the development of the new method, which is described in the August 27, 2009, issue of the journal *Neuron*. Sabatini has built and used two-photon microscopes in his studies of the brain's plasticity for nearly a decade. Because the technique excels at revealing objects deeper with tissue than other kinds of light microscopy, he can examine slices of brain tissue that are thick enough to see how the connections between nerve cells are formed and

regulated. His group at the Harvard Medical School labels individual proteins with light-activated molecules, and watches them as neurons signal to one another across those connections, or synapses.

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- **Bernardo L. Sabatini**

But for studies examining the finer features of the synapse, Sabatini admits, "the images you get [using two-photon microscopy] are kind of blurry. What you know is supposed to be this tiny synapse that's 300 nanometers across appears larger because the image is so blurred," he says. And subcellular structures such as the slender projections called dendritic spines that extend from the edges of neurons can be particularly difficult to see.

Frustration at those blurry images convinced Sabatini that "we really needed to work on getting the resolution of this approach better," he says. To do that, his team took its cue from recent advances that have dramatically improved the resolution scientists can obtain with other types of light microscopes.

In the past few years, several different research groups have come up with ways to surpass a fundamental rule in physics called the diffraction limit. Light particles travel as waves, and because of the rule, scientists thought that microscopes wouldn't be able to see specimens — such as a protein within a cell — that are less than half the wavelength of light. In the 1990s, researchers developed technologies to see objects less than half the wavelength of light.

A technique called Stimulated Emission Depletion was the first to break the limit. It uses one laser light to excite or illuminate a small spot within the sample, and another laser to lower the excitation level in a donut-shaped area surrounding the spot. This focuses the portion of the sample visible to the microscope to such a small region that spatial resolution is greatly improved. But STED-- and other super-resolution techniques-- are unable to capture images at the same depth within tissue as two-photon microscopy.

About a year ago, Sabatini and his colleagues realized that they could combine the depth of two-photon imaging with the resolution of STED. All they needed was one piece of equipment: a vortex phase plate, which is a small piece of glass with a spiral-shaped coating. This piece would help them form the STED donut, by splitting the laser light so that one part would serve as the spot, and the other part as the donut.

Sabatini says many of his colleagues were skeptical at first. STED had so far been used to image individual cells dissociated from tissues, whereas he wanted to apply the approach to neurons

inside large chunks of tissue. Many scientists thought that the tissue blocks would bend and scatter light in multiple directions, destroying the effect of the depletion donut.

Indeed, when Sabatini's group first added the vortex phase plate to his microscope set-up in late 2008, it didn't work. The lenses and mirrors weren't lined up with the necessary precision to direct light from multiple lasers with to a spot in the tissue with nanometer precision. But in a few months, the group had rearranged and restructured many of the microscope's parts and was using it to see small artificial beads containing fluorescent molecules.

And soon they were able to see inside cells deep within brain tissue—at depths the equivalent of the width of 5 or 6 human hairs. At this depth, they were able to clearly see dendritic spines and smaller structures called filopodia.

Sabatini expects to obtain images with even higher resolution. To do this, "we need more power," he says. The current set-up uses brief pulses of light to illuminate the spots, while the depletion beam stays on constantly. To use the power more efficiently, Sabatini's group can pulse the depletion light in time with the excitation. "You've got to get the timing exactly right on these two beams," he says. "That should improve resolution by another factor of three."

They are also working to make the system foolproof for beginners, despite its requirement for fine-tuned assembly. To do this they've taken the whole microscope apart, and are writing computer programs that automate the process of aligning the laser beams. "What we really want it to do is have it align itself," he says.

Tweaks to the approach will help Sabatini achieve his larger goal of seeing how synapses work in healthy brain, as well as how they go awry in neuropsychiatric diseases such as autism and schizophrenia. The finer features of the synapses will give his group a chance to observe and form new hypotheses about how neurons signal to one another. "Oftentimes in the lab, we can take the approach that if we can look at something in a new way, we're going to discover completely new processes," he says.