LARGER, SHARPER & DEEPER

Microscopy continues to expand our knowledge of brain structure and function. Sarah Webb looks at some of the latest tools and techniques leading the charge.

Credit: P. Keller

Over the last 20 years, neuroscientists have discovered that many neurons in the body spontaneously fire in groups. “We’re pretty certain that these neuronal ensembles exist in the brain, but we still don’t know what they do,” says Rafael Yuste of Columbia University. While traditional optical microscopy paired with tools such as calcium imaging can follow active neurons, what these neurons are actually doing within the brain is still a mystery.

Yuste is keen on solving this mystery. His group has focused recently on new techniques that can write neuronal activity, such as optogenetics and optochemistry, as well as complementary microscopy techniques to observe the experimental results. Recently, they used two-photon methods to write firing patterns into groups of neurons in live mice and then monitored the activity based on calcium signaling to connect those patterns with behavior. As a result, the team successfully imprinted new neuronal circuits in the brain of a mouse by simultaneously stimulating those neurons and testing whether the artificial patterns interfere with a specific behavior—licking for juice in response to a pattern of horizontal or vertical lines (1). Interestingly, the imprinted circuits persisted over time, and stimulating a single cell in the neural circuit could trigger the entire pattern.

“At least it’s very easy to build these ensembles in the cortex and also recall them later,” says Yuste, even if it remains unclear what specific functional role the neural ensembles play.

Yuste’s work is just one example of how a growing range of sophisticated optical imaging techniques are helping neuroscientists map signaling and correlate neural pathways with behavior in living organisms. From chemical agents to clarify opaque brain tissue samples to improvements in spatial and

temporal resolution through light-sheet and multiphoton microscopy, neuroscientists now have a toolkit for uncovering the brain’s specialized structures and functions.

A clearer picture

Imaging neurons within the brain is not a trivial matter. Like many other tissues, the brain is made up of a host of biomolecules, particularly lipids, that scatter light as it travels through them. Depending on when and where light scattering occurs in a sample, this can prevent deep penetration of light, minimizing the excitation of fluorophores and ultimately decreasing the resolution of the detected image. Though a German researcher had done some work to make heart tissue transparent in the early 20th century, Stanford University researchers capitalized on this idea with brain tissue in 2012.

The CLARITY method was developed in the laboratory of Karl Deisseroth at Stanford University. Today, it is one of the more widely used techniques for making opaque tissues transparent. The protocol requires researchers to infuse samples with a combination of formaldehyde, acrylamide, and a thermal initiator. Monomers crosslink with the amine moieties in biomolecules, and heating prompts the hydrogel polymerization reaction. The resulting soft hydrogel structure serves as a support, making it possible to actually remove lipids, the primary molecules responsible for light scattering. This passive clearing process takes roughly 2–3 weeks for a mouse brain, according to Raju Tomer of Columbia University, who worked as a postdoctoral fellow in Deisseroth’s lab. The resulting transparent sample is a hybrid of tissue and hydrogel. Since CLARITY first emerged, a variety of tissue clearing procedures such as Scale, SeeDB, CUBIC, and LUMOS have been developed, al-

lowing researchers to improve the optical properties of samples and acquire high-resolution images from ever-larger pieces of cortex, whole mouse brains, and even peripheral neural connections in whole mice.

With a cleared tissue sample, researchers can choose from an array of optical techniques to map tissue structure, including confocal, two-photon, or light-sheet microscopy. According to Tomer, light-sheet microscopy, which shines a plane of light into a sample and collects the emitted signal using an orthogonally arranged objective, is a natural pairing with cleared tissue. When he was in Deisseroth's group, Tomer developed a technique that brought these ideas together: CLARITY-optimized light-sheet microscopy (COLM) (2). Whereas confocal and two-photon microscopy are both point-based methods and require more time and photons for high-resolution imaging, light-sheet microscopy shines a plane of light into a sample, and scans can be orders of magnitude faster.

COLM is now routinely used to map neural connections in whole mouse brains. Northwestern University researcher Talia Lerner, who also worked with Deisseroth, uses the approach to explore the role of dopamine neurons. "It's a good technique for getting a broad overview of the brain-wide connections through various chemical labeling strategies," she says, and that unbiased overview can help in forming more specific

The limited brightness of fluorescent probes places an upper boundary on imaging speed according to Na Ji of HHMI's Janelia Research Campus. Credit: M. Stanley/HHMI.

research questions. For example, Lerner can go back and examine the areas of the brain that show the most interesting connection clusters, areas she might want to focus on for functional experiments or other types of assays.

Researchers have also used CLARITY to image whole model organisms with light-sheet microscopy. For example, another Deisseroth alumna, Viviana Gradinaru from Caltech, has developed PARS, a perfusion-based CLARITY method, to map peripheral neural connections in whole cleared mice. Her team has also employed the technique as part of a study that developed a virus to deliver genes across the whole brain (3). "Whole-body clearing was instru-

mental in providing detailed transduction maps of the novel gene delivery vectors," Gradinaru says.

Finding the best image

While tissue clearing methods have led to mapping of the structure of whole mouse brains and ever-larger chunks of human brains, the approach only provides a road map of neural networks. "Of course, we need to understand the structure of the brain, but we also need to map the function. And function happens at the millisecond scale," notes Tomer. To go after neural function requires very fast, high-resolution imaging.

When it comes to resolution, speed, and depth, all microscopy techniques have trade-off rooted in optical physics. Finding the right technique for a specific research question can be fraught with difficult design decisions. Higher-resolution data can push researchers toward larger objectives. But when trying to image volumes instead of 2-D slices, larger objectives can prove limiting because they are also more difficult to move, slowing scanning speed. Another option, moving the sample instead of the objective, can jostle a living system and disrupt the processes being imaged. Light scattering within living tissues also presents fundamental physical challenges. Though increasing the light intensity can allow light to penetrate deeper into tissue, higher energy photons lead to photobleaching of fluorescent probes and damage to the tissue.

A glut of imaging data

For researchers using microscopy to study the brain, data is becoming a big problem. It's now routinely possible to acquire images of a whole mouse brain, but at medium resolution, the data acquired from a single brain can total several terabytes, says Raju Tomer. At high resolution, such imaging can produce dozens of terabytes, which makes even simple storage and file movement difficult. Beyond the problem of storage, the sheer mass of data makes it difficult to analyze or compare images from different brains. Currently, microscopy laboratories rely on expensive computers with large hard drives and parallel processors to process imaging data.

One of the major elements of the government-funded BRAIN initiative has been supporting novel solutions to these data challenges. One possible solution could be new file outputs that are easier to work with. For example, Philipp Keller has developed a new file format called KLB

that makes it easier to move and store high-resolution image files. Other tools could compress image data by up to 100-fold, Tomer says. But that means researchers have to make critical choices about what information in the image is most important. "You have to know what information you're prepared to lose," he says.

Ultimately, the answer might be found in integrative approaches where the microscope doesn't simply produce pictures. "Eventually what we want to do is to transform microscopes so that they produce knowledge rather than images as their primary output," Tomer says. Microscopes could be designed with the tools to convert image data into a readout that gives researchers important insights about their samples. And researchers are looking at visualization and other automated analysis tools that would register their data to an atlas and trace basic neural processes, says Lerner.--S.W.

Schematic of challenges associated with light-sheet microscopy imaging applications. Several recent developments in light-sheet microscopy are addressing these challenges. Credit: P. Keller.

Light-sheet microscopy excels as a tool for imaging small live model organisms, such as *Drosophila* and zebrafish, or cleared tissue samples such as whole mouse brains, Tomer notes. A pencil-thin laser light can quickly illuminate the xy-plane of a sample, and a high-end camera can take a quick snapshot of millions of elements in that plane, says Philipp Keller of Howard Hughes Medical Institute's Janelia Research Campus. As a result,

light-sheet microscopy can produce images more quickly than point-by-point methods such as confocal and multiphoton microscopy. But one drawback of traditional scanning light-sheet techniques in large specimens is that both the light-sheet and the detection focal volume can be quite thick in the z direction, leading to lower resolution in the third dimension. But two new light-sheet microscopy techniques published last year provide dif-

ferent solutions to this problem of rapid, high-resolution 3-D imaging.

Keller and his colleagues developed IsoView light-sheet microscopy, a technique that uses multiple objectives and detectors simultaneously to maximize imaging speed and resolution. The data are integrated into a full high-resolution picture of the tissue volume. Even though having two orthogonal views already leads to substantial improvements in resolution and imaging speed in small, transparent samples, the system actually uses four-view imaging, which extends these benefits to larger, partially opaque multicellular organisms, he says. In the initial paper on the IsoView method, Keller's team demonstrated whole-animal calcium imaging in *Drosophila* larvae and whole-brain functional imaging in larval zebrafish (4).

While working in the Deisseroth lab, Tomer developed another strategy for improving the volumetric imaging speed of light-sheet microscopy, an approach called spherical-aberration-assisted extended depth-of-field (SPED) light-sheet microscopy (5). Tomer's technique introduces a block of transparent material of higher refractive index between the sample and the detection objective to induce heavy, yet uniform, spherical aberrations in the detection system. This results in an extension of the detection depth-of-field while maintaining the resolution of the images. SPED acquires 3-D volumes using galvanometer scanners to quickly move the light-sheet within the sample.

Stargazing and synapses

Adaptive optics, which have been used by astronomers to improve image quality from large telescopes, are another tool that could help improve microscopy imaging depth and resolution. Just as the Earth's atmosphere can distort celestial images taken from telescopes, changes in the refractive index within tissues can disrupt microscopy images. But according to Meng Cui, a wavefront correction, which adds in additional optics, can then sharpen the image in a way similar to glasses that correct a person's blurry vision.

In one implementation of adaptive optics in two-photon microscopy, Cui and his colleagues were able to peer through an intact mouse skull (~150 μm thick) and successfully resolve the structure of microglia within the cortex at a depth of 180 μm (7).

Adaptive optics can also boost the depth and resolution of two-photon functional microscopy, where researchers remove a portion of the mouse skull and use transparent windows to view the brain. By measuring aberrations and adjusting lenses to compensate, adaptive optics can tighten the focus of excitatory wavelengths of light or reduce scattering from fluorescence emission, notes Na Ji.

Adaptive optics can also improve results for light-sheet microscopy. Recently, Philipp Keller and his team developed computer algorithms that allow their multi-view light-sheet microscopy system to detect optical aberrations in living tissues during scanning and make adjustments in real time. That information allowed them to boost fluorescence signal detection and obtain single-cell resolution in their images of whole brains from zebrafish larvae (8).--S.W.

To validate the technique, Tomer's team imaged the central nervous system of live zebrafish larvae and looked at subcellular details within cleared whole mouse brains. The speed of the technique is only limited by the camera, says Tomer, and because SPED doesn't require additional optics, it's relatively inexpensive.

Peering deep

Even as light-sheet microscopy advances continue, two-photon fluorescence microscopy remains the workhorse for studying the living brain. Though light-sheet microscopy is useful for small model organisms and cleared tissue, it's still primarily a one-photon method, according to Tomer. Two or more photons of near-infrared wavelength unleashed simultaneously produce less scattering and cause less damage to tissue than a single photon with twice the energy. As these two lower-energy photons combine, their energy is more focused, coaxing a fluorophore into an excited state within the focal plane. Those features give the technique an advantage when it comes to deep, high-resolution imaging in larger brains.

For example, the mouse neocortex typically has a thickness of approximately one millimeter, and standard two-photon approaches can provide a high-quality depth of view of ~500 μm , says Meng Cui of Purdue University. Researchers continue to work on approaches to improve the depth of imaging, including an approach known as adaptive optics (see "Stargazing and synapses" sidebar). Cui says that these efforts are pushing the limits of high-resolution optical imaging throughout the cortex and toward deeper brain structures, such as the hippocampus, and offer the opportunity to study information storage processes. And Yuste and others are developing methods that allow researchers to perform high-resolution volumetric imaging using two-photon microscopy.

One of the most important developments for neuroscientists within the last 30 years is in vivo calcium imaging, Cui says. While working on his PhD, Yuste discovered that he could use calcium indicators to monitor changes in intracellular free calcium as a way to follow neuronal spiking. Now widely used,

this approach allows researchers to follow the activity of every neuron within an optical field of view, Yuste says.

Snapshots of the future

Calcium imaging is powerful, but it is also limiting. "As much as I love the method, I would be the first one to drop it," when a better alternative becomes available, says Yuste. Although calcium imaging measures action potentials, the technique doesn't allow researchers to follow synaptic potentials or signaling inhibition. "The activity of the brain is written using voltage," he adds. Yuste's group and others are now developing genetic, chemical, and nanoparticle approaches that can follow voltage in much the same way calcium can be followed. However, the signal-to-noise ratio of these techniques would need to increase by up to two orders of magnitude for the spatial and temporal resolution to be competitive with calcium imaging.

The limited brightness of fluorescent probes places an upper boundary on imaging speed, says Na Ji of HHMI's Janelia Research Campus. Currently available fluorescent probes are relatively faint and inefficient, requiring a longer exposure to laser light to get a measurable signal. Chemists and protein engineers are working on this problem, so she expects improvements that will boost temporal resolution. For example, Cui says, GCaMP6, the most sensitive genetic calcium indicator, has a 20% signal variation with a single action potential; therefore 25 photons of light are needed to resolve images that follow this probe. A GCaMP indicator with a 50% efficiency would take the number of necessary photons down to 4, he says. With the many improvements in imaging speed and resolution across all microscopy methods, storage, management, and analysis of data are also looming problems

for the brain imaging community (See "A glut of imaging data" sidebar).

Though there are many pathways for improving the temporal and spatial resolution of functional imaging (6), Ji cautions that methods developers have to keep their work focused on the eventual users, the neuroscientists. To encourage adoption of new techniques, it's important to design them in a way that allows them to integrate easily within an existing microscope system. "If you want to convince people to throw out their existing microscopes and use your new machine, you'd better make sure that the final results are really, really revolutionary," says Ji.

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BioTechniques 61:285-291 December 2016
doi: 10.2144/000114480

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