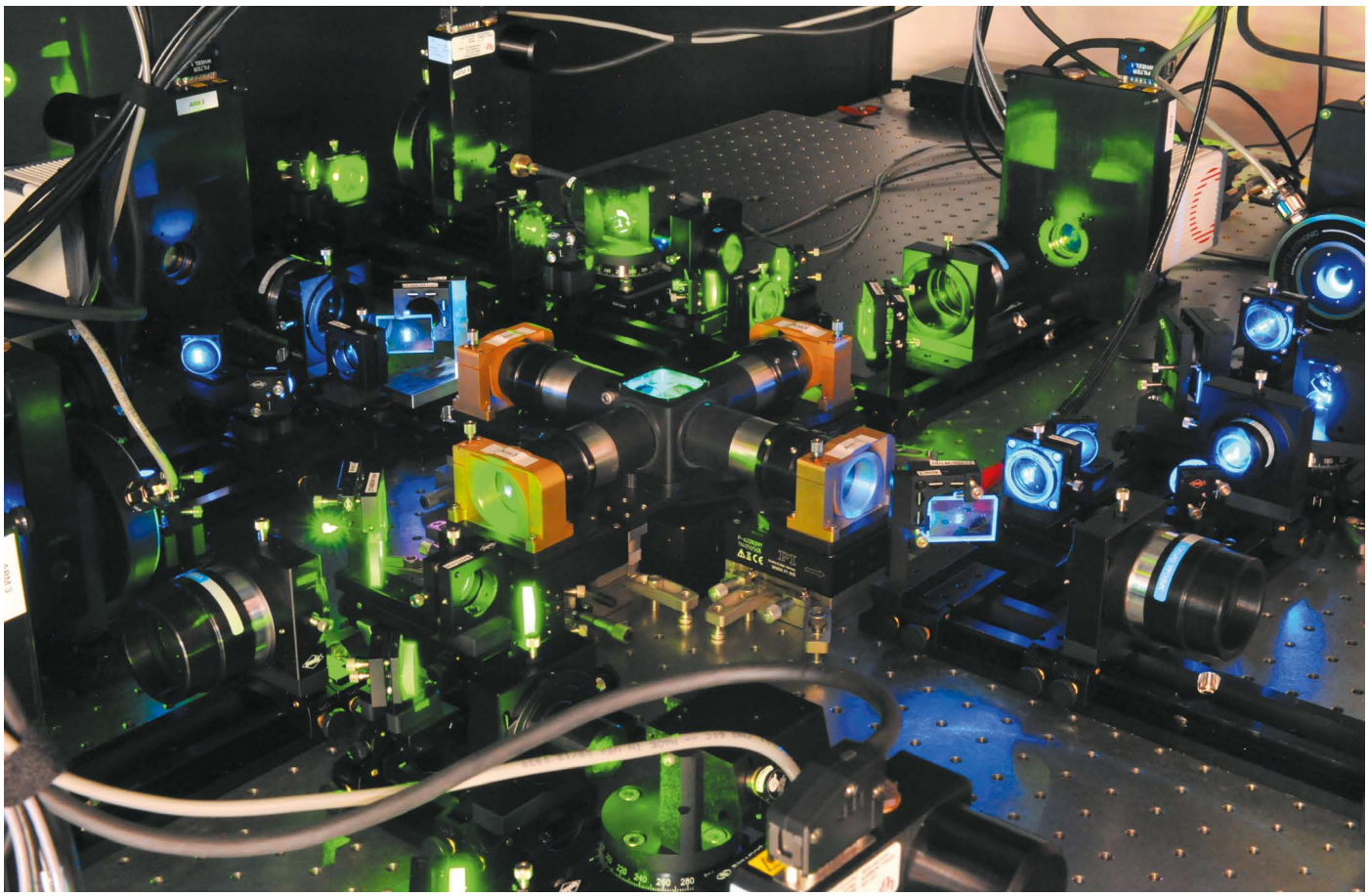


RAGHAV CHHETRI, MATT STALEY AND PHILIPP KELLER/HHMI, JANELIA RESEARCH CAMPUS

Light-sheet microscopes use low-intensity lasers to study live tissue for extended periods.

GO BIG OR GO HOME

Microscopy is turning into 'mesoscopy' as researchers set their sights on ever-larger biological samples. **By Jeffrey M. Perkel**

In a sunny third-floor office at the Howard Hughes Medical Institute's Janelia Research Campus in Ashburn, Virginia, Philipp Keller is showing off the optics for his latest microscope. They're not much to look at – yet.

"What we have right now in the building is this," Keller says, pointing to his computer screen. "Basically, these are slabs of glass as they would be drawn out of a continuous-flow furnace."

The slabs resemble book-sized blocks of ice, standing as if on a library shelf. Keller, a physicist, hopes to use them to build a new type of microscope, one that can achieve high resolution and that can handle specimens of a size that has long been on biologists' wish list.

In biological microscopy, he says, researchers can either look at big samples at low resolution, or small samples at high resolution. It hasn't really been possible to look at big samples – larger than about one cubic millimetre – and pick out cellular or even finer details.

"It's usually a trade-off," he says. "You can either get a macroscopic view, or you can get a high-resolution, zoomed-in view. And then the only way to combine them both is that you do some kind of massive tiling strategy where you take a tiny imaging volume and you just raster it through the entire sample in 3D" – a time-consuming and computationally demanding process.

Increasingly, researchers are developing 'mesoscopes' – mesoscale microscopes – to circumvent that challenge. These instruments can capture cellular and even subcellular processes across samples that can exceed one centimetre in size. The resulting data sets provide an unprecedented perspective. As Gail McConnell, an optical physicist at the University of Strathclyde in Glasgow, UK, who developed one such mesoscope, puts it: "It's almost macro photography, but with higher resolution."

Biological microscopy is all about compromise. To follow a fast-moving process, for instance, researchers typically capture many images in quick succession, with short exposure times. The sample must therefore be very bright to provide as many photons as possible in the time available. That requires more input light energy, which can kill (or at least bleach) the sample. As a result, such imaging usually cannot be done for long.

Big objectives

Similarly, systems that can capture fine cellular detail tend to have a narrow field of view. Point-scanning confocal microscopes produce sharp images of subcellular structures by scanning a tightly focused laser beam across a sample, exciting fluorescence in the sample pixel by pixel.

Such imaging is "fabulous for very tiny tissue volumes", McConnell says, but it cannot be applied to large specimens – such as

late-stage mouse embryos — "because of the low numerical aperture of a low-magnification lens" that would be needed for such large samples. Numerical aperture (NA) refers to the ability of a lens to capture light; a higher NA usually corresponds to higher magnification and a shorter working distance between the objective lens and the sample.

To overcome that problem, McConnell teamed up with confocal-microscopy developer Brad Amos, also at Strathclyde, to build a macro-scale objective lens with the unusual combination of a high NA and low magnification, capable of providing both a wide field of view and high spatial detail. The result is the 'Mesolens', a custom optic that can image over a 6-millimetre-wide field of view with 0.7-micrometre lateral and 5-micrometre axial resolution, and a 3-mm working distance — sufficient to distinguish objects that are about one-tenth the diameter of a typical mammalian cell[1].

The Mesolens looks like an objective lens on steroids: "I would liken it to roughly the same length and width as an adult human arm," McConnell says. It took a decade to build, and its sheer size — its glass elements are nearly three times the diameter of the typical microscope objective lens — makes it incompatible with many off-the-shelf components, McConnell notes. "The tolerance with which they have to be ground and polished" — not to mention aligned relative to one another — "becomes much more stringent" than with conventional lenses, she says.

Suitable detectors were a problem, too. One reason it took McConnell and Amos so long to build the Mesolens was that they had to wait for wide-field sensors that could capture the resulting photons, she says. Amos co-founded a spin-off company to commercialize the Mesolens, but "we're not really in a commercial space at the moment", says McConnell (who holds no stake in the company). "We are working on a lens prescription that will hopefully be easier to make."

Still, McConnell's team has used its design to begin to address biological questions, including the architecture of bacterial biofilms, which are collections of microbes that grow on a surface inside a film that they secrete, and which are often associated with disease. "We're seeing new and emergent properties of these biofilms that could potentially inform our knowledge about tackling antimicrobial resistance," she says.

Qionghai Dai, an information scientist at Tsinghua University in Beijing, has also been tackling the problem of imaging centimetre-scale samples. Recognizing a huge gap between high-resolution microscopy and macro-scale techniques such as functional magnetic resonance imaging and computed tomography, Dai's team set about developing a gigapixel microscope with high spatio-temporal resolution, says Lingjie Kong, a physicist in the lab.

The result of that work is RUSH, an instrument that features a custom objective lens, a curved array of 35 cameras and a computational system that can capture and analyse data in real time. Whereas the Mesolens can produce some 4 million pixels' worth of data per second, Kong says, RUSH produces 5.1 billion, imaging a 10×12 mm^2 field of view at 30 frames per second. That's enough to image the entire surface of the mouse brain in a single shot, and to resolve individual subcellular organelles called mitochondria[2].

Dai's team used its system to track fluorescently labelled blood cells travelling across the surface of a mouse brain, and to monitor neural activity in freshly prepared human-brain slices. Studies in non-human primates are being planned. A second-generation RUSH, featuring higher resolution and data throughput, is in development, Kong says, as is a commercial version of the instrument. "It is expected to be available by next year."

Mirror mirror

To tackle its big-sample challenges, Keller's team took a cue from astronomy. Large telescopes use mirrors because they are lighter and easier to fabricate than lenses. Mirrors also lack many of the aberrations that can degrade performance as lenses get larger. So why has nobody used them to build a microscope? "To be honest, I don't know exactly why they haven't done it," Keller concedes.

> **"It's almost macro photography, but with higher resolution."**

His team is no stranger to custom microscopes. Over the past decade, it has developed a succession of increasingly complex 'light-sheet' systems, in which a plane of low-intensity laser light is projected through the sample. Images are captured from the side (that is, at 90 degrees to the plane of light) to maximize imaging time and minimize photodamage. These microscopes, assembled on optical workbenches that dominate the small rooms in Keller's lab, can record the cellular dynamics and neural activity of developing fruit flies and zebrafish for hours at a time.

In October 2018, Kate McDole, a research scientist in Keller's group, described a combination microscope and environmental-chamber design that extended that period to two days. She has used it on a much more challenging subject: in tandem with a powerful suite of custom analysis software, she was able to image and track every cell in a mouse embryo over 48 hours. During that time, the embryo grows 250-fold in volume[3].

Custom microscopes provide extraordinary flexibility, but aren't necessarily easy to use. McDole estimates that only seven of ten experiments run to completion. Sometimes the microscope misbehaves, and sometimes the sample does. Sometimes the carbon dioxide tank, required for maintaining the animal's growth, gets shut off, and sometimes the microscope's computer system decides to update during an experiment — a not-infrequent occurrence, she says. "Murphy" — as in Murphy's Law — "follows me around like a puppy," McDole quips.

The key element to the lab's in-development 'mirror microscope' project, led by optical engineer Dan Flickinger and postdoc Benquan Wang, is a concave mirror 168 mm in length, which Keller says cost about US$6,000 to make. With a 14×14 mm^2 field of view, a high NA of 1.0 and a detection array comprising a dozen cameras, the system should capture some 200 times more of the sample than most commercial microscopes, yet still resolve subcellular detail, Keller says.

Working with another team at Janelia, he hopes to use the mirror-based system to capture the neural connectivity, or 'connectome', of an entire mouse brain. And he hopes to record the brain activity of several zebrafish simultaneously as they interact and swim in a small dish. Data sets could run to petabytes, Keller says — equivalent to what his team currently generates in a year. McDole's first mouse data set was 20 terabytes, she says, "and it broke every piece of imaging software that we had".

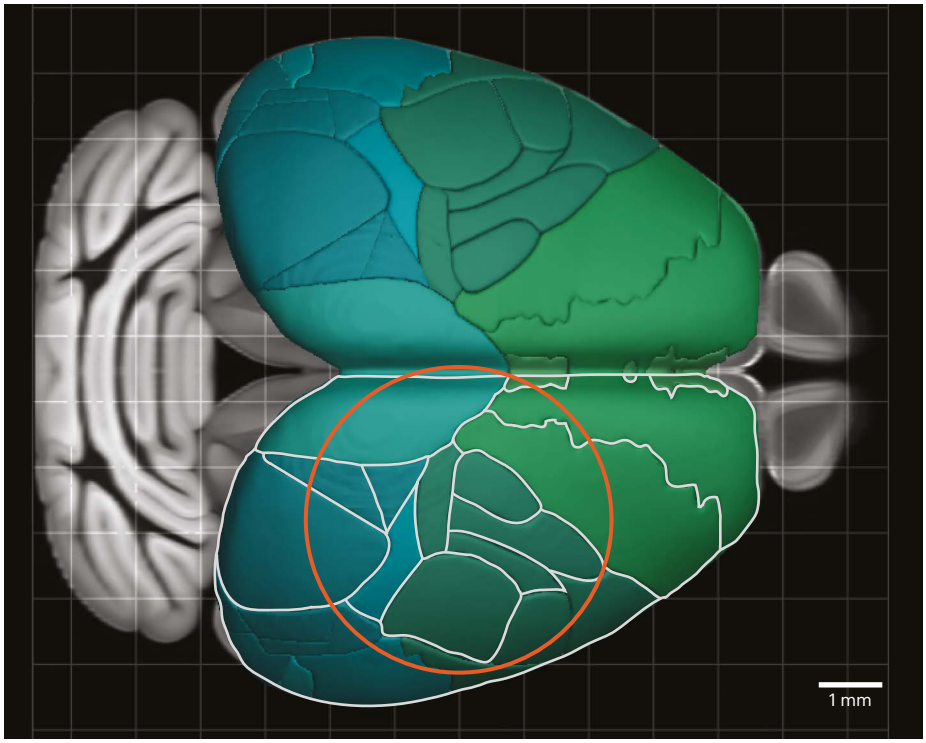
First, however, the team will have to show that the design works. Preliminary testing is set to kick off this month. "I am confident that by the end of the year we will have the first image that has been formed with an entirely mirror-based detection system," Keller says.

Two photons are better than one

Light-sheet microscopy works best for relatively transparent samples. More opaque specimens call for other techniques, and researchers are making progress in applying them to large fields of view, too.

In 2016, teams led by microscopy developers Fritjof Helmchen at the University of Zurich in Switzerland, Spencer Smith, then at the University of North Carolina, Chapel Hill, and Karel Svoboda at Janelia independently described innovative 'two-photon' microscopes that can image macro-scale samples[4–6]. These devices use ultrafast, long-wavelength laser pulses that excite fluorescence in the sample, but only in a sharply defined plane. The resulting images are high-contrast up to 1 mm deep, but cover only about 1 mm^2. Svoboda's system, called the 2p-RAM mesoscope, provides a 5×5 mm^2 field of view — enough to take in the entire surface of the mouse brain. Helmchen's and Smith's systems image 1.8×1.8 mm^2 and more than 9.5 mm^2, respectively.

When going big, Svoboda says, it's not



NICK SOFRONIEW AND KAREL SVOBODA

The 2p-RAM instrument's field of view (orange) can access much of the mouse brain at once.

enough to super-size the objective; multiple parts must be reengineered to accommodate how light propagates through such optics. In fact, the reason his team settled on a 5×5 mm^2 field of view, he says, is that larger detectors weren't available: "5 mm is right now the limit, unless you want to throw away a lot of signal. And in this business, we hate to do that," he says.

Such systems allow neuroscientists to see the forest for the trees. Trying to decode neural communication by studying one brain region at a time, Svoboda says, is like concentrating on only one section of an orchestra. But with larger fields of view, "a good chunk of the cortical surface of a mouse" becomes open to scrutiny. Neurons flicker like grainy greyscale fireflies, and by tracing those flashes over time, researchers can identify correlations between cells, circuits and larger brain areas. "We can now interrogate what we refer to as multi-regional circuits or multi-regional interactions in real time."

The 2p-RAM system has been licensed to Thorlabs, a microscopy vendor in Sterling, Virginia. One of the first commercially available instruments was bought by neuroscientist Mackenzie Mathis, at Harvard University in Cambridge, Massachusetts. At the Society for Neuroscience annual conference last month in Chicago, Illinois, Mathis presented data to a Thorlabs user-group meeting showing that she could use that system, plus some home-built deep-learning software, to study mice interacting with a video game. "Joystick pulls can be accurately decoded from neural activity," she told the audience.

The RAM in 2p-RAM stands for 'random-access mesoscopy': the system can rapidly move the laser around in the overall field of view. "What that means in practice is that I can, say, go to the motor cortex and record layer-5 output neurons, and then go to another area of cortex and simultaneously image layer 2/3," Mathis says. Such data can reveal how brain regions interact and communicate as the mice play the game.

But the 2p-RAM cannot scan those regions literally simultaneously; there is a delay of several milliseconds as the laser hops from place to place. Helmchen's and Smith's designs use beam-splitters to provide effectively simultaneous imaging of multiple points — a process called temporal multiplexing. Smith and neuroscientist Jerry Chen, who was the lead author of Helmchen's paper and is now at Boston University in Massachusetts, are collaborating on a second-generation system that they say will be able to access four regions within a 2p-RAM-sized field of view simultaneously. Thorlabs, which has already installed more than 25 mesoscopes in various labs, is developing an updated system capable of simultaneous imaging at multiple depths, says Sam Rubin, the company's general manager.

The oblique approach

At Columbia University in New York City, biomedical engineer Elizabeth Hillman has worked out a way to apply light-sheet microscopy to opaque samples. In conventional light-sheet microscopes, the laser beam is focused by its own objective lens, which is perpendicular to the detection objective. This arrangement limits the size of the sample that the system can accommodate, and precludes its application to live mice.

Hillman's design, called SCAPE 2.0, eliminates one of the objectives, projecting a plane of light obliquely into the sample and capturing the resulting fluorescence with the same lens. The only moving element is a steering mirror, and the system can record volumes at blazing speeds when used in tandem with a fast camera. "We can do three dimensions faster than [point-scanning microscopes] can do two," Hillman says[7].

But light sheets still cannot penetrate opaque samples well. So Hillman is now developing a two-photon variant that will be able to probe hundreds of micrometres into opaque samples, as well as a mesoscale version for larger samples.

The system can also be applied to another rapidly growing area of microscopy: the imaging of large, 'cleared' tissues, such as mouse brains, that have been made transparent through chemical treatment. Hillman's team was able to image a piece of mouse brain measuring $8.4 \times 9.1 \times 0.4$ mm^3 in just 4 minutes[7]. Other light-sheet designs can also tackle such samples. One, called the mesoSPIM, can image a 21-mm field of view[8]; another, developed by physicist Reto Fiolka at the University of Texas Southwestern Medical Center in Dallas, and his colleagues, tiles millimetre-sized fields to capture centimetre-sized samples with subcellular resolution[9]. Neuroscientist Raju Tomer, a Keller lab alumnus and colleague of Hillman's at Columbia, has developed yet another geometry, called 'light-sheet theta microscopy', in which the excitation objective is positioned at an oblique angle to remove light-sheet microscopy's lateral constraint[10]. This design provides a 1-mm field of view, but can accommodate samples of theoretically any width.

As these and other designs percolate through the microscopy community, new research avenues will open. But, Keller warns, to have a truly broad impact, such developments will need to be paired with better sample preparation and handling, ease of use and affordability. "If the goal is to image a large sample at high resolution, the microscope can only do so much," he says.

Jeffrey M. Perkel is technology editor at *Nature*.

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