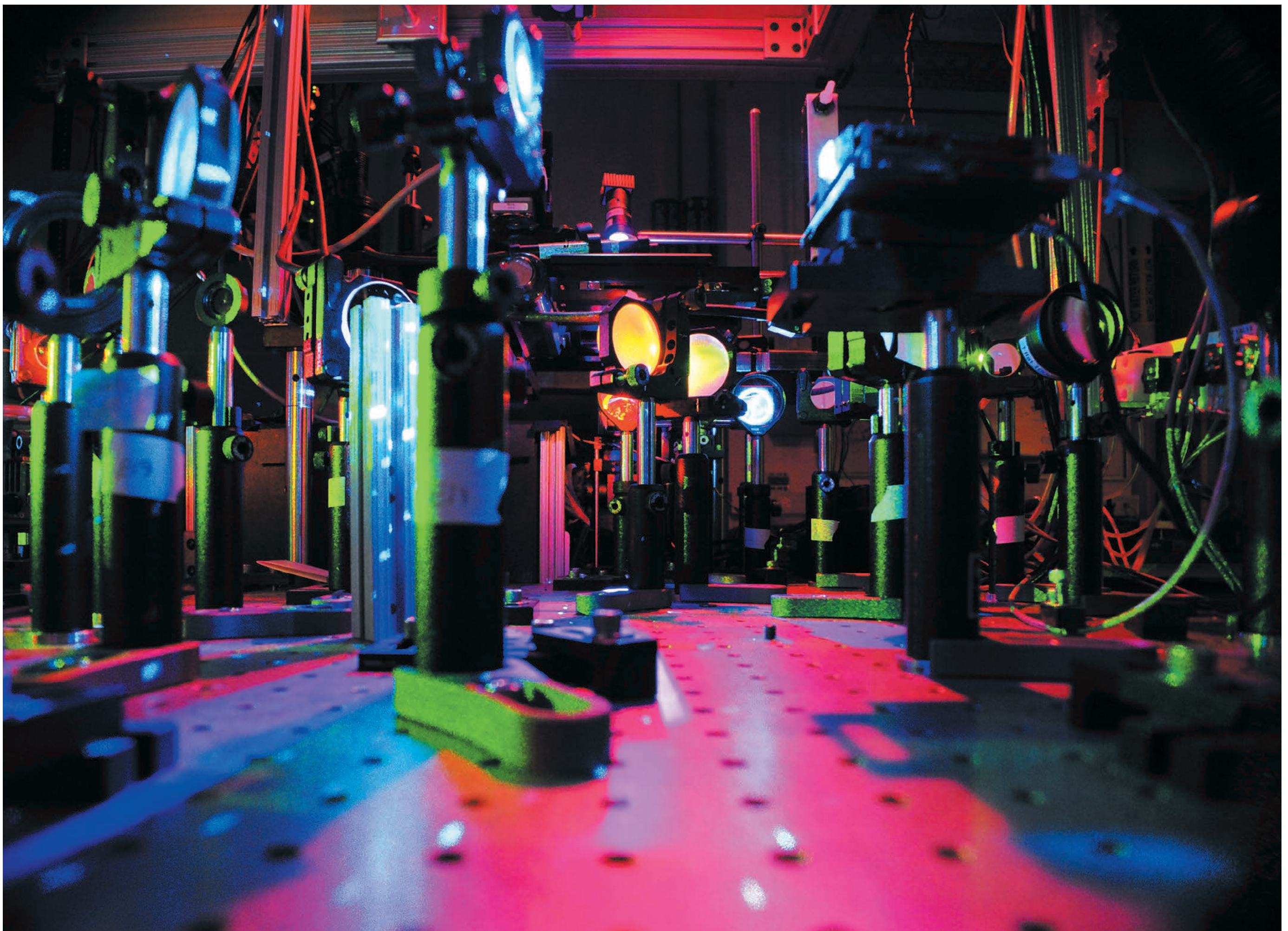
THE REAL-TIME TECHNICOLOUR LIVING BRAIN

Neurobiologists are coming up with innovative ways to get high-resolution pictures of the whole brain at work.

HE TIAN



Elaborate arrangements of lasers, mirrors and optics are allowing researchers to probe the brain in ever more creative ways.

BY AMBER DANCE

Rosa Cossart thinks she knows what a memory looks like.

In a study published in *Science* in September, Cossart, a neurobiologist at the Institute of Neurobiology of the Mediterranean in Marseilles, France, opened up mouse brains to visualize their neural activity as the animals raced on treadmills and rested. As the mice ran, some 50 neurons in their hippocampi fired in sequence, possibly to help the animals measure the distance travelled. Later, when the mice were resting, certain subsets of those neurons turned on again[1]. This reactivation, Cossart suspects, has to do with encoding and retrieving memory — as if the mouse is recalling its earlier exercise.

"The power of imaging is really to be able to see the cells, to see not only the active ones but also the silent ones and to map them on the anatomical structure of the brain," she says.

It has not yet provided proof for Cossart's hypothesis, but the microscope and neural-activity markers behind the techniques represent the very latest in methods to study brain connectivity. In the past, researchers studied just a few neurons at a time using electrodes implanted into the brain. But that gives a fairly crude picture of what is going on, like ▸

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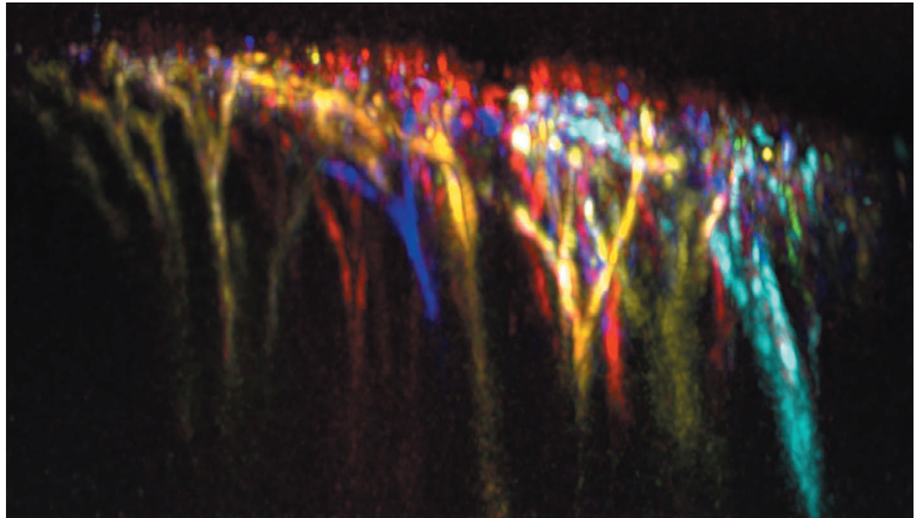
looking at a monitor with just a couple of functioning pixels, says Rafael Yuste, director of the NeuroTechnology Center at Columbia University in New York City.

But new techniques are fleshing out the picture. Scientists can now watch neurons live and in colour, helping them to work out which cells work together. Methods such as Cossart's zoom in at the microscopic scale to catch individual neurons in the act; others provide a whole-brain, or mesoscopic, view. And although it is possible to perform these experiments with an off-the-shelf microscope, scientists have been customizing them to suit their specific purposes; these devices are in various stages of commercialization.

The field of live-brain imaging is flourishing thanks to innovations such as two-photon microscopy, which allows scientists to image deeper into brain tissue, and indicators that flash as neurons fire; Cossart combined the two in her study.

Major funding initiatives are also pushing the field forward, particularly the US Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative, which aims to improve researchers' ability to map the brain. The US National Institutes of Health has partnered with groups in Canada, Australia and Denmark to co-fund investigators from other countries involved in the BRAIN Initiative. In Japan, the Brain Mapping by Innovative Neurotechnologies for Disease Studies (Brain/MINDS) programme includes funding for projects such as functional magnetic resonance imaging (fMRI) analysis of the marmoset brain.

Nevertheless, the scientists involved in these projects face major challenges. The biggest is the brain matter itself. "Brain tissue has the optical properties of milk," Yuste says. The light waves that microscopists use to visualize neurons tend to bounce off surrounding tissue and scatter in multiple directions. That means that most studies cannot penetrate much more than a millimetre below the brain's surface. But researchers can now use both crude surgical techniques (removing part of the brain to discern what happens underneath, or poking in fibre optics, for example) and tricks of light to sneak their laser beams deeper into the tissue.


ELIZABETH HILLMAN AND RANDY BRUNO, COLUMBIA UNIV.

New methods such as SCAPE are making it possible to visualize 3D neural activity in animals as they move, as in these apical dendrites in the brain of a living mouse.

Other challenges include the incredible speed at which mammalian neurons communicate as well as how to integrate data all the way from the meso- to the microscale. "The dream is obviously every neuron — every axon, dendrite, synapse — in the whole brain flashing away," says Columbia biomedical engineer Elizabeth Hillman. "We can do it in the fruit-fly brain, and in the zebrafish, just not yet in the mouse."

But despite its limitations, live-mouse-brain imaging is already starting to reveal how neural connections can be silenced, or regrow, in studies of brain disease and ageing.

OF CALCIUM AND CIRCULATION

Take the work that has been done on stroke at the mesoscale, for instance. Blood clots in the brain damage neurons and thus the routes of neural communication. The damage can easily be seen in people: fMRI has shown that stroke affects the flow of blood between mirror-image parts of the two hemispheres, a cross-talk that is crucial for activities such as coordinated movement. But probing the details of stroke is difficult to do in people, so researchers including neurologist Jin-Moo Lee at Washington University School of Medicine in St. Louis, Missouri, are keen to use mice as a model to study the disease and possible treatments.

However, mouse brains are so small that fMRI signals get lost in the noise, so Lee had to turn to a different technique to track blood flow. His colleague Joe Culver, a biomedical engineer, introduced him to a technique called optical intrinsic signal imaging (OIS), which picks up colour alterations that are linked to changes in blood oxygen levels. Oxygenated blood is reddish and deoxygenated blood bluish, and the different colours can be detected through the thin skull of a mouse using fairly basic scientific equipment, or even a wearable consumer camera known as a GoPro. Well-oxygenated areas are likely to be more active than others. To study neural connectivity, Culver and his colleagues zoomed out to look at the entire cortex, and presumed that highly oxygenated spots that are flashing in sync are likely to be connected. He calls the new method "functional connectivity optical intrinsic signal imaging", or fcOIS[2].

In 2014, Culver and Lee used this technique to show that strokes in mice affect connections between mirror-image parts of the two hemispheres, just as they do in people[3]. Culver has also applied fcOIS to a mouse model of Alzheimer's disease and found that cross-hemisphere communication not only drops, but is also correlated with plaque deposition and with ageing. The loss of connectivity seems to happen first, foreshadowing which areas might be vulnerable to plaque accumulation[4].

The technique provides a good first-pass screen for changes in connectivity, Culver says, because it works on any mouse; some markers, including those used by Cossart, need to be genetically engineered into mouse neurons. Yet it's still only a surrogate marker of brain activity. A step closer is calcium indicators. When neurons receive a signal, an electrical current passes through them. That depolarizes

AN IMAGING PALETTE

A selection of the genetically encoded neural-activity indicators used in live-brain imaging.

Indicator	Mode	Excitation (nm)	Emission (nm)	Addgene ID
Calcium				
GCaMP5G	2-photon	900–1000 (near infrared)	509 (green)	31788
GCaMP5G	1 -photon	488 (blue)	509 (green)	31788
GCaMP6f	2 -photon	900–1000 (near infrared)	509 (green)	40755
GCaMP6f	1 -photon	488 (blue)	509 (green)	40755
Voltage				
QuasAr2-mRuby2	1 -photon	561 (yellow)	600 (red)	59174
QuasAr2-Citrine	1 -photon	488 (blue)	529 (yellow)	59172
QuasAr2-mOrange2	1 -photon	532 (green)	565 (orange)	59173

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the plasma membrane and opens ion channels, allowing calcium to flood into the cell. Indicators in the cytoplasm change shape and fluoresce when calcium flows in, providing more-immediate visual feedback. Among the most popular calcium indicators are the GCaMP proteins (see 'An imaging palette') developed at the Janelia Research Campus in Ashburn, Virginia, which are now in their sixth generation. "We're switching everything we can over to calcium," says Culver.

FLASHING QUASARS

Calcium indicators have become the workhorses of live-brain microscopy. Scientists can see every neuron — at least in the plane imaged by their microscope — and follow their activity over time. For this kind of focus, scientists often use two-photon microscopy. In standard microscopy, a fluorophore is excited by just one packet of light, so any fluorophore that receives a packet will light up, even if it is outside the focal plane. In two-photon microscopy, scientists use a longer-wavelength laser, so the fluorophore must absorb two photons simultaneously to fluoresce. Because the chance of two photons hitting the same spot is high only at the laser's focal point, the signal is effectively limited to the focal plane. As an added bonus, the longer-wavelength, lower-energy light can penetrate deeper into the tissue. By scanning the laser across the brain, microscopists can build up a high-resolution picture of the brain at a depth of up to one millimetre, Yuste says.

Yet calcium indicators are still only a proxy for the electrical spikes that mediate neural signalling. And they're relatively slow to reflect neural communication — "the smeared-out remnant of a spike", says David Kleinfeld, a neurophysicist at the University of California, San Diego. It takes about 100 milliseconds after the membrane depolarizes for the calcium to bind to the indicator and cause it to change shape and fluoresce, estimates Karel Svoboda, a neurobiologist and biophysicist at Janelia. It also takes half a second or so for the fluorescence signal to decay back to the unlit state, so two or three electrical impulses, or 'action potentials', could pass in the time that the calcium system can indicate only one. "You probably miss stuff," Svoboda says.

The seventh generation of GCaMP indicators, anticipated within the year, should improve the response speed by an order of magnitude, as well as boost its sensitivity, Svoboda says. But no calcium indicator will ever measure action potentials with the same speed and range as electrodes, he says.

Adam E. Cohen, a biophysicist at Harvard University in Cambridge, Massachusetts, is pursuing a faster type of visual indicator — one that fluoresces as the membrane depolarizes.

The sensors — called genetically encoded voltage indicators (GEVIs) — are based on a protein that allows a Dead Sea microbe to harvest solar energy. Luckily for Cohen and his collaborators, that protein — called Archaerhodopsin — also fluoresced in response to changes in membrane voltage. And Cohen's team was able to enhance the mechanism to create a pair of GEVIs called QuasArs. But the QuasArs were fairly dim, so the group fused them to brighter fluorophores to create pairs that perform fluorescence resonance energy transfer (FRET), with one fluorophore influencing the emission of the other[5].

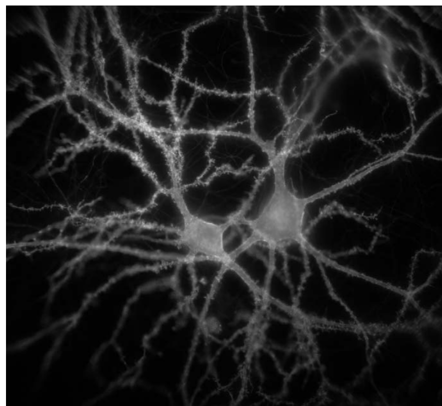
"The dream is obviously every neuron — every axon, dendrite, synapse — in the whole brain flashing away."

Cohen's team used these indicators, which are available in a handful of colours (see 'An imaging palette'), to monitor spontaneous and induced voltages in cultured rat neurons. Yet, like calcium indicators, GEVIs are neither fast enough nor bright enough to faithfully report neural firing, Cohen acknowledges. And the available colours are restrictive: ideally, he says, GEVIs should fluoresce in the far-red part of the light spectrum, leaving the rest of the rainbow open for the use of other proteins. In particular, neuroscientists often use light-activated proteins to control neuronal activity, using a technique called optogenetics.

ADVANCING MICROSCOPY

Other researchers are focusing on the microscopy itself, and specifically on imaging in 3D. Because the neurons that work together are not conveniently organized in a single plane, the scanning process must be able to keep pace with signalling across the volume of the brain. Ten frames or 'volumes' per second is a good benchmark, says Fritjof Helmchen, who co-directs the Brain Research Institute at the University of Zurich in Switzerland. "This is one of the clocks the brain is working on" — millisecond resolution would be even better, he adds.

That means that microscope designers must minimize the moving parts that slow things down, says Diego Restrepo, co-director of the Center for NeuroScience at the University



SAMOUIL FARHI

Microscale images can reveal neural activity in a rat hippocampus.

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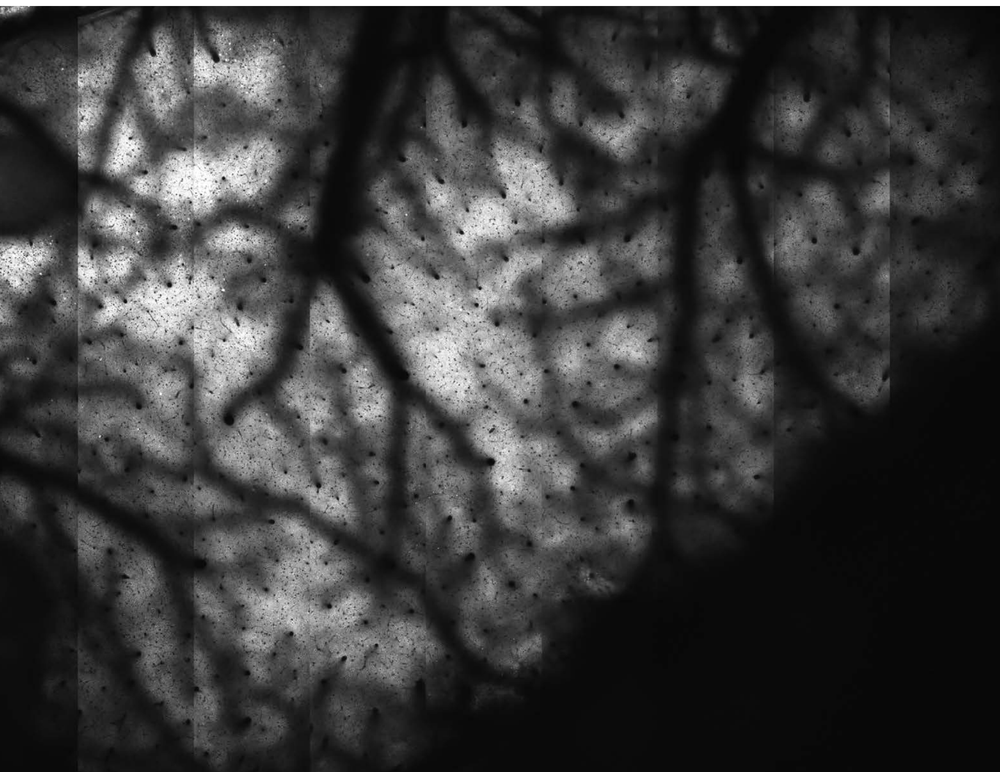

NICHOLAS SOFRONIEW, DOI.ORG/10.7554/ELIFE.14472.015/CC BY 4.0

Two-photon microscopy can image cells as deep as 1 mm. These cortical neurons were imaged at 200 μm.

of Colorado Anschutz Medical Campus in Aurora. He and his collaborators have eliminated the up-and-down motion required for focusing by using a liquid objective lens that is controlled by electric field. "When you throw oil on water, you form a lens," explains Restrepo. By making the lens very small, he and his colleagues have managed to make it very stable, so that it doesn't bobble about as an animal moves. And they can change the lens' shape and focal plane by altering the electrical field. Restrepo's team has used this lens in combination with a confocal microscope and a fibre-optic system to image brain slices[6], and now plan to attach the device to a mouse's head.

"We're literally still scratching the surface."

At University College London, neuroscientist Angus Silver found a way to accelerate the focus changes while imaging across multiple focal planes. He uses an acousto-optic lens that transmits megahertz sound waves through tellurium dioxide crystals to focus the laser beam. "The limitation to speed is the speed of sound across crystal, basically," Silver says. The technique still isn't ideal for quickly imaging every neuron in a volume, he says, but it can move from one region to the next in about 25 microseconds[7]. That makes it useful for viewing all of a sparse population, such as inhibitory interneurons in a volume of brain, he suggests.

Another solution to quickly sampling different depths is a modification of light-sheet microscopy, which typically involves moving multiple lenses to continually refocus a sheet of light. The technique can image one or two volumes per second, Hillman estimates. But by turning the sheet on an angle and using a single mirror to sweep it across the volume of interest, Hillman's group achieved a rate of 20 times per second. Hillman calls the technique swept confocally aligned planar excitation, or SCAPE, and her team has used it to visualize dozens of distinct firing patterns in the brains of awake mice[8]. The technology has been licensed to Leica Microsystems in Wetzlar, Germany.

Yuste's group offers yet another option. It uses a spatial light modulator, which splits the laser beam into many beamlets, each of which is aimed at a different part of the tissue. "Imagine a comb of light that's hitting the sample," Yuste explains. The microscope picks up any light that comes back, so it can capture multiple planes at once[9]. It can collect about ten sets of images per second, and the researchers are already speeding that up, Yuste says. Yuste has licensed the technology to Bruker in Billerica, Massachusetts, and Olympus in Tokyo, and is contemplating starting his own company.

ZOOMING IN, ZOOMING OUT

Most 2D and 3D techniques remain hampered by how the brain scatters light, but scientists have ways of circumventing that limitation, too. At Cornell University in Ithaca, New York, applied physicist Chris Xu and his colleagues reasoned that if two photons could push the imaging depth to a millimetre or so, then three should go even deeper. Indeed, Xu's three-photon imaging can reach two or three times further down than two-photon imaging can, he says, although the limits depend on the properties of the tissue being imaged. His group managed to use the technique to image the mouse hippocampus, without removing any of the cortex above[10].

Xu's team still can't penetrate all the way through the brain — "We're literally still scratching the surface," he acknowledges — but there's plenty of room for improvement, he says.

There's also room to develop live-brain imaging in other ways. A number of researchers, including Kleinfeld and Svoboda, have devised systems that combine the wide mesoscopic field of view with the single-cell resolution achieved by two-photon imaging, allowing them to zoom out on much of the brain or zoom in, Google Earth-style, on individual neurons[11,12]. Kleinfeld's field-of-view covers an 8×10 millimetre section of cortex; Svoboda's group can manage a cylinder of brain about 5 millimetres in diameter and 1 millimetre deep, and that's about 25 times the typical field-of-view in two-photon microscopy, he says. Svoboda has now trained several labs to build their own versions of his microscope, and licensed the technology to Thorlabs in Newton, New Jersey.

Ultimately, these diverse technologies could realize Yuste's dream for neuroscience: to "crack the code" that links neural firing patterns with behaviour and sensation. The technology can't yet be used to look at and interpret the activity in a mouse's visual cortex, for instance, but it has certainly added plenty of pixels to the screen. ■

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CORRECTION

The Technology Feature 'The dark side of the human genome' (*Nature* **538,** 275–277; 2016) implied that Ran Elkon was solely responsible for performing the first screen using the advanced editing system. In fact, he was part of the team headed by Reuven Agami.

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