Going for GECIs	878
Extending electrophysiology	881
All together now	882
Box 1: More signals than calcium	878

From promising to practical: tools to study networks of neurons

Monya Baker

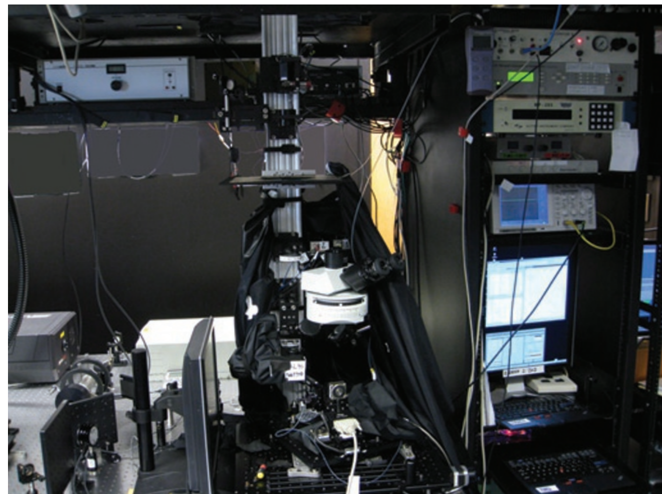
Combinations of electrophysiology, two-photon microscopy and new tools for detecting neural activity show how neurons function in circuits.

© 2010 Nature America, Inc. All rights reserved.

Researchers who want to understand how neurons work together in networks can now conduct experiments that are at just the right scale. Until recently, the tools researchers had for studying neural activity were either too big or too small to let them track the simultaneous activity of many individual cells. Techniques such as patch clamping can precisely monitor small changes in electrical activity but follow only a few neurons at a time. Techniques such as functional magnetic resonance imaging can be used to detect activity in large regions of the brain but cannot pinpoint the activity of an individual neuron. Advances in imaging and electrophysiology, however, are now allowing researchers to examine how neural circuits function at the cellular level.

Clay Reid of Harvard Medical School, who studies how neurons convey information within the visual cortex, estimates he can now routinely monitor the activity of as many as 1,000 individual neurons simultaneously by using fluorescent indicators and laser microscopy. "It's a dream come true," he says. "We can see the whole ensemble and begin to hunt for ways to see connections between neurons."

Many tools for monitoring neural activity have moved from promising to practical in just the last few years. Perhaps a dozen companies supply two-photon microscopes that let researchers peer into intact ensembles of neurons, and researchers can tell which cells are doing what using an expanding repertoire of sensors that fluoresce in response to neural activity. Advances are also happening outside imaging in the form of dense arrays of extracellular electrodes that make sensitive recordings of neurons' electrical activity.



Members of Clay Reid lab, Harvard Medical School

A two-photon microscope that can track dozens of neurons while taking 30 images a second.

But although the advances are clear, so are the hurdles. Imaging techniques can be used to identify particular cell types and their anatomical locations, yet capturing events other than action potentials is still difficult (**Box 1**). Multielectrode arrays excel in time resolution and sensitivity but not in pinpointing activity to individual neurons. Researchers are overcoming these drawbacks not only by improving the techniques themselves but also by using different methods to observe the same sample and so glean advantages of separate techniques.

Two photons forward

The rise in neural imaging owes much to the maturation of two-photon microscopy. Invented in the 1990s by Winfried Denk, James Strickler and Watt Webb, two-photon microscopy can peer relatively deep into tissues and use less-damaging light to generate fluorescent signals. Soon after its invention, Denk and his postdocs Karel Svoboda and Rafael Yuste demonstrated that two-photon microscopy could be used to watch activity in neural networks. Shortly after, Michael Szulczewski, founder of Prairie Technologies, began getting requests to build two-photon microscopes specialized for this purpose. At the time, he says, the larger microscope companies were concentrating on building two-photon instruments to study individual cells, and Szulczewski could collaborate with Svoboda and other leading scientists to build the first commercial two-photon microscopes for *in vivo* use.

Initially, Szulczewski thought demand would be limited. "Eight years ago, I would have thought the market size would be

CORRECTED AFTER PRINT 10 NOVEMBER 2010.

© 2010 Nature America, Inc. All rights reserved.

BOX 1 MORE SIGNALS THAN CALCIUM

Much more happens in neurons than electrical action potentials racing along axons. Action potentials are usually triggered by signals neurons receive across a synapse. However, these 'subthreshold' voltage events can be orders of magnitude smaller than action potentials and so are much harder to detect.

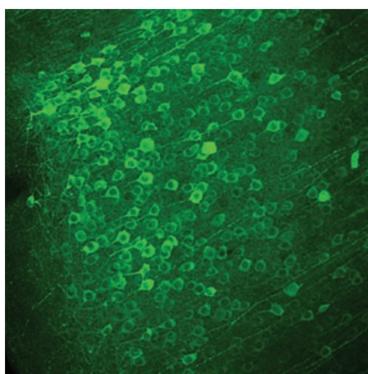
The commonly used calcium sensors provide only a slow, indirect measure of electrical signaling in a neuron. While a change in calcium level may last hundreds of milliseconds, a depolarization event at the synapse lasts only a few milliseconds. To provide a more direct readout of electrical activity, researchers are working hard to develop new voltage sensors that could not only track such minute depolarization events but could provide faster and more direct reporting of action potentials. A genetically encoded voltage sensor developed by Thomas Knöpfel of the RIKEN Brain Science Institute was recently shown to work in anesthetized mice[11], and researchers are making progress on small organic dyes as well, says Michael Häusser, of University College London, who lists "a sensitive, fast genetically encoded voltage sensor" as one of his top three most-wanted tools.

The field could also benefit from developing entirely new kinds of sensors, both recombinant and synthetic. For example, Häusser says we could learn a great deal by using well-behaved molecules that respond to sodium or chloride. It is unfortunate, he says, that nearly all of the probe development work is happening in academia. "The science would move forward much more quickly if there were more companies making new indicators."

18 instruments a year," he says. Now he estimates that perhaps 200 laboratories a year buy or build a two-photon microscope to study neural circuits. Prairie Technologies offers some half-dozen varieties of two-photon instruments, specialized to provide, for example, lenses and lasers that point up through tissue or down onto mouse brains. Much of the growth, he says, comes from laboratories acquiring second or third instruments. At $150,000 and up, lasers are by far the most expensive element of a two-photon microscopy station, so the cost of adding additional setups using the same laser drops quickly.

When choosing a microscope, the most difficult choices are between how many neurons to watch and how often, because there is a tradeoff between the speed of imaging and the number of neurons that can be imaged. The amount of signal collected at video rates, about one-thirtieth of a second per image, cannot cover a wide field of view, so researchers are limited to watching only a few dozen neurons if they hope to catch every neuron as it fires.

Reid and others have developed approaches to widen the field of view and study more neurons. Still other researchers, including Fritjof Helmchen, Peter Saggau and Angus Silver, are working to speed up the rate of scanning by improving detectors, for example, using acousto-optic deflectors that focus on different depths within a sample without requiring mechanical parts or by scanning neurons when they are most likely to be active. These approaches, though very promising, are not yet widely adopted. Meanwhile, improved versions of the charge-coupled device cameras used to collect fluorescence signals are steadily improving the images captured, and scientists who are experts in the technique can improve the performance of their systems with better optics, phase-compensating lasers or other tricks.



Lin Tian, Janelia Farm Research Campus

The calcium sensor GCaMP3 fluoresces brightly in mouse brain cortex.

Going for GECIs

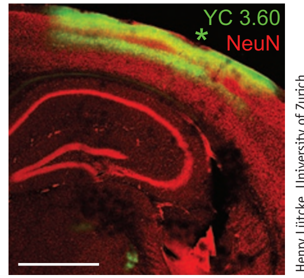
All forms of two-photon microscopy depend on the detection of optical signals. Neurons' communication is invisible, so researchers must introduce moieties that convert chemical and electrical signals into light. The most popular is an organic dye known as Oregon

Green that fluoresces in response to calcium fluctuations and thus indirectly signals action potentials. Newer options include several genetically encoded protein calcium indicators (GECIs, pronounced 'geckies'), which are generally made by fusing calcium-binding proteins with fluorescent proteins such that a change in conformation of the former changes fluorescence in the latter.

GECI-expressing neurons can be generated in a wide variety of animals using engineered viruses, and although transgenic mouse lines expressing GECIs have been produced, researchers are still waiting for mouse lines expressing a GECI with the right characteristics. Compared to synthetic dyes, GECIs have long promised greater control and ease of use—their first demonstration *in vivo* was reported in worms in 2000 (ref. 1)—but researchers generally stick to synthetic dyes because they yield clearer signals. "The whole field is waiting for the GECIs to provide similar performance to the classical indicators, and as soon as that happens, everyone will switch over," says Michael Häusser, who studies the link between neural networks and behavior at University College London.

That switch has started, says Svoboda, now at the Howard Hughes Medical Institute's Janelia Farm Research Campus. "It's been on the horizon for more than a decade," he says. "Until the advent of the latest-generation sensors, they were not very usable." Within the next few months, he predicts, there will be a surge of papers describing not just the advancement of GECIs as tools but the use of GECIs to understand neural biology.

A spate of recent papers report improved versions of GECIs and demonstrations of their reliability. Indeed, researchers including Häusser and Svoboda are now using them routinely in their experiments. Late last year, a team of Janelia Farm researchers described a third generation GECI dubbed GCaMP3 and showed it could be used to image neuronal activity in living mouse brains over the course of months[2]. The protein also had stronger calcium-dependent fluorescence signals and a wider dynamic range compared to its predecessor. In April of this year, researchers reported that a previously reported sensor, yellow cameleon 3.60 (YC3.60), could be reliably expressed in a subset of neurons in the mouse cortex and detect single action potentials in anesthetized mice as well as bulk calcium signals in freely moving mice[3].



Henry Lütcke, University of Zurich

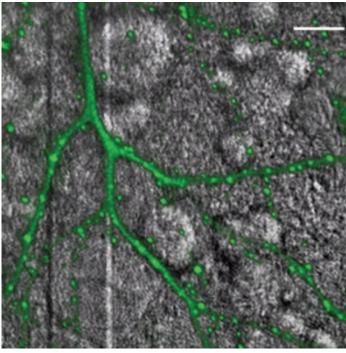
The calcium sensor YC3.60 glows brightly in mouse neocortex. Scale bar, 1 mm.

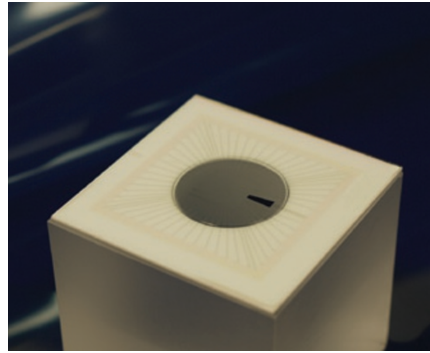
As GECIs move past feasibility studies, researchers will need to be cautious that engineered proteins do not affect cell behavior, says Loren Looger, a protein engineer at Janelia Farm who led the work on GCaMP3 and is now working on assays to predict undesired effects. GCaMP3, like most GECIs, consists of a fluorescent protein fused with calmodulin, a protein involved in multiple signaling cascades. Adding GCaMP3 to the cellular milieu could change the effective concentration of calmodulin and present important signaling proteins with an aberrant binding partner.

Although neurons expressing GCaMP3 seem to tolerate it quite well, at least one GECI has been designed to avoid calmodulin. TN-XXL, designed by Oliver Griesbeck at the Max Planck Institute of Neurobiology, Martinsried, uses the muscle-specific protein troponin as a calcium sensor[4]. One problem GECIs cannot avoid, says Looger, is that they bind calcium. Too little calcium binding and researchers have nothing to see, but if too many calcium sensors bind too much calcium, the cell cannot transmit its own signals, which could cause the cell to commit apoptosis or disrupt communications with other neurons in the network under study.

Although researchers have not yet reported major new biological discoveries using GECIs alone, Harvard University's Reid recently used both YC3.60 and Oregon Green to demonstrate an imaging technique for tracking neural activity over three dimensions while mice responded to visual stimuli[5]. Reid, who is now using GCaMP3 in his lab, predicts newcomers to imaging will find GECIs easier to use than synthetic dyes, which can be difficult to load without harming cells. Getting synthetic dyes to work reliably, he says, "takes luck and a group of people who help each other, and you have to have good hands, too."

© 2010 Nature America, Inc. All rights reserved. npg





Multi Channel Systems

Multi Channel Systems makes ThinMEAs, which can be used with imaging experiments that visualize neurons with green fluorescent proteins or other techniques. Scale bar, 10 μm.

© 2010 Nature America, Inc. All rights reserved. npg

Other advantages of GECIs include the ability to target expression of the sensor to specific cell types or even particular parts of a neuron, like dendrites and synapses. In addition, imaging can be conducted over an animal's lifetime and not just in a single experiment. Of course, synthetic dyes also have advantages. Their signals are typically brighter. Because there is no need to wait for transduction and protein expression, researchers can start imaging experiments shortly after loading dyes into their subjects' brains.

For most researchers, however, the biggest advantage of dyes is the speed of their signals. Although not as sensitive as electrical recordings, synthetic dyes detect individual action potentials more consistently than protein sensors. GECIs are often too slow to discriminate between several action potentials in quick succession. "Under battlefield conditions, they don't have the sensitivity to detect one action potential or to distinguish three from four," says Svoboda. "The issue for future generations is to inch closer to the gold standard of electrophysiology."

Extending electrophysiology

Compared to imaging techniques, electrodes are much better at tracking neural activity in real time. And although calcium sensors track only a proxy of action potentials, electrodes sense the actual underlying activity. In fact, electrophysiological recordings are essential to calibrate optical signals from calcium sensors. But as intracellular electrodes used for calibration can monitor only a few neurons at once, researchers have turned to collections of extracellular electrodes that can profile a few hundred neurons simultaneously.

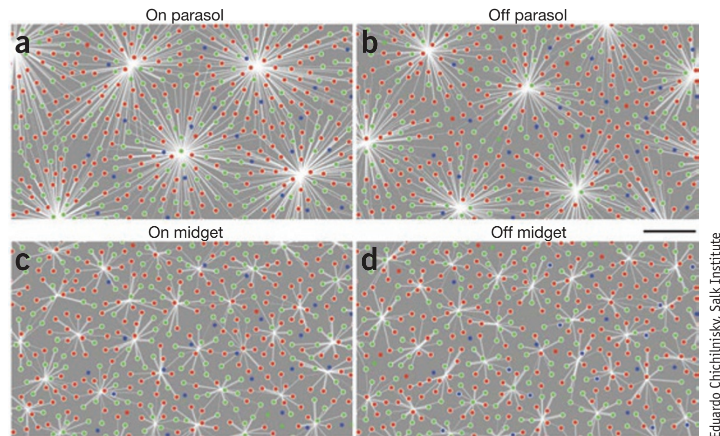
Rather than tracking the activity of an individual neuron, each extracellullar electrode collects signals from whichever neurons are nearby. This makes it hard to know precisely which cells are being monitored, particularly when many types of neurons are intermingled. Nonetheless, many researchers are working on tiny devices for gleaning information about circuits in living brains. Researchers in Germany and the Netherlands placed 1-mm electrodes within 10-μm polymer foil, which could be cut into 'fingers' and laid over monkeys' brains to record from 252 sites in many cortical areas at once[6]. Researchers at Niigata University School of Medicine recently designed what they called a 'multichannel electrode mesh' from the organic polymer used to coat printed circuit boards. They laid the mesh onto the visual cortex in rats to collect electrocorticograms for as long as two weeks[7]. Researchers at the Universiteit van Amsterdam recently showed that a very lightweight device containing 24 electrodes could allow recording for up to 12 weeks when implanted into the orbitofrontal cortex and hippocampus of mice[8]. Companies such as Intan Technologies specialize in producing amplifier arrays and circuits tiny enough for such experiments.

Standard wire electrodes record signal at their tips, and although their wires can be twisted into groups of four 'tetrodes', these often require painstaking individual adjustment to monitor multiple sites, and their blunt ends can cause damage when inserted into animals' brains. In contrast, silicon probes, which have been developed by Ken Wise and his group at the University of Michigan and commercialized by companies such as NeuroNexus, can be manufactured as fork-like devices with tapered tines, called shanks, each containing multiple recording sites covering a small area. A typical unit recording array that is only 12.5 μm thick has four to eight shanks spaced about 200 μm apart. Shanks are 5 μm at the tip and widen up to 40 μm at the top. Each contains eight recording sites (an 'octrode') spaced 20 μm from top to bottom, yielding 32 or 64 channels per probe. These probes are based on work by György Buzsáki at Rutgers University, Newark, who is currently working on ways to allow recording from more sites within even smaller volumes in the brain. One probe in development features eight shanks, each with 32 recording sites, for a total of 256 channels.

Whereas silicon probes are designed to be inserted into animals' brains, denser arrays of metal electrodes are designed for *in vitro* studies. These are used to study neural networks that originate spontaneously in cultured neurons, networks in slices of tissue from brain and spinal cord and networks from the retina, which are particularly amenable to study because they can be maintained relatively intact when the tissue is laid flat over an array. The standard arrays sold by Multi Channel Systems have diameters of 30 μm and are spaced from 100 μm to 500 μm apart, but Karl-Heinz Boven, chief executive officer of Multi Channel Systems, says demand for very-high-density electrodes is growing, and the company now also sells high-density multielectrode arrays (MEAs) with electrodes that are 10 μm in diameter and 30 μm apart. The first high-density array that the company sold had 60 electrodes; the standard array now has 256 electrodes, and the company currently has a prototype containing as many as 1,024 electrodes.

Eduardo Chichilnisky at the Salk Institute is already working with dense arrays of tiny metal electrodes to study information processing in the retina. These arrays, developed by researchers at the University of California, Santa Cruz, and the University of Glasgow, have 519 electrodes of about 5 μm in diameter spaced about 30 μm apart and can monitor several hundred cells. The advantage of having many smaller electrodes is that the signal from each neuron can be seen clearly on several electrodes. By having many tightly packed electrodes, researchers can literally follow the flow of electrical signal over the array and triangulate the location of individual cells.

Members of Chichilnisky's lab recently produced the first high-resolution maps showing the initial stages of information processing that occur when primates see color: interleaved lattices of color-sensing cone cells feed information to ganglion cells,



Cone cells that feed signals into receptive field centers of four types of retinal ganglion cells were identified in a single recording. Scale bar, 50 mm, Reprinted from reference 9.

allowing signals to be compared before further information processing[9]. Using these arrays, Chichilnisky tracked the functional input of each cone cell into each of a subset of ganglion cells over a 0.25 mm^2 area in the retina. This captured how signals from the collection of individual cone cells were transformed into signals in individual ganglion cells. "What we've been able to do with this approach is to study the complete transformation of input signals to output signals over the observed region of retina," he says.

All together now

With more tools available, researchers can combine sensors with other techniques to get even more detail on which neurons are interacting in an ensemble. Reid, for example, is combining different types of microscopy. He used mice engineered so that interneurons expressed a fluorescent protein, then used organic dyes to follow calcium flux in the visual cortex. Neurons in fixed tissue from the same specimen were then stained to distinguish three subtypes of interneurons[10]. In other experiments, he is correlating electron microscopy of postmortem tissue with images from live-cell studies to understand exactly how inhibitory interneurons and other cells are connected.

Salk's Chichilnisky is combining fluorescent proteins, calcium-sensing dyes and very dense electrode arrays. He hopes the dyes will allow him to know what types of cells he is looking at, and the electrodes will record the neural activity with precise time resolution. Multi Channel Systems has begun producing very thin, transparent arrays (called ThinMEAs) that can be used with optical imaging. Arrays and amplifiers built so that they fit into microscopes will, Boven expects, be a big growth area over the next few years.

Researchers are also combining two-photon calcium imaging with electrophysiology in other ways. "Where imaging has a huge advantage over electrophysiology is because it provides access to structure and to genetic labels, and where it still lags is in time resolution and for recording from large numbers of cells," remarks Häusser. Moreover, says Chichilnisky, the puzzles even small networks present are boggling. "When information is transmitted, it's not one cell or a dozen cells, but probably hundreds or thousands of cells, and the cells are of different types," he says. It is only logical that a variety of methods will be necessary to understand the process.

1. Kerr, R. *et al. Neuron* **26**, 583–594 (2000).
2. Tian, L. *et al. Nat. Methods* **6**, 875–881 (2009).
3. Lütcke, H. *et al. Front. Neural Circuits* **4**, 9 (2010).
4. Mank, M. *et al. Nat. Methods* **5**, 805–811 (2008).
5. Andermann, M.L., Kerlin, A.M. & Reid, R.C. *Front. Cell Neurosci.* **4**, 3 (2010).
6. Rubehn, B., Bosman, C., Oostenveld, R., Fries, P. & Stieglitz, T. *J. Neural Eng.* **6**, 036003 (2009).
7. Toda, H. *et al. Neuroimage* advance online publication, doi:10.1016/j.neuroimage.2010.08.003 (7 August 2010).
8. Battaglia, F.P. *et al. J. Neurosci. Methods* **178**, 291–300 (2010).
9. Field, G. *et al. Nature* **467**, 675–677 (2010).
10. Kerlin, A.M., Andermann, M.L., Berezovskii, V.K. & Reid, R.C. *Neuron* **67**, 858–871 (2010).
11. Akemann, W., Mutoh, H., Perron, A., Rossier, J. & Knöpfel, T. *Nat. Methods* **7**, 643–649 (2010).

Monya Baker is technology editor for *Nature* and *Nature Methods* (techfeatures@nature.com).

© 2010 Nature America, Inc. All rights reserved.
npg

SUPPLIERS GUIDE: COMPANIES OFFERING PRODUCTS AND SERVICES TO STUDY NEURONS IN NETWORKS

Company	Web address
ALA Scientific	http://www.alascience.com/
Alpha Med Scientific	http://www.med64.com/
AnaSpec	http://www.anaspec.com/
Andor Technology	http://www.andor.com/
AutoMate Scientific Inc	http://www.autom8.com/
Ayanda Biosystems	http://www.ayanda-biosys.com/physiology.html
Bio-Logic Science Instruments	http://www.bio-logic.info/electrophysiology/biomea.html
BioRad Laboratories	http://www.bio-rad.com/
Carl Zeiss	http://www.zeiss.de/micro
Chroma Technology	http://www.chroma.com/
Coherent	http://www.coherent.com/
Conoptics	http://www.conoptics.com/
Evrogen	http://www.evrogen.com/
Graftek Imaging	http://www.graftek.com/
GRINTECH GmbH	http://www.grintech.de/
Hamamatsu	http://www.hamamatsu.com/
HOLOEYE Photonics	http://www.holoeye.com/
Intan Technologies	http://www.intantech.com/
Intelligent Imaging Innovations	http://www.intelligent-imaging.com/
LaVision BioTec GmbH	http://www.lavisionbiotec.de/
Leica Microsystems	http://www.leica-microsystems.com/
Mauna Kea Technologies	http://www.maunakeatech.com/
Molecular Devices	http://www.moleculardevices.com/
Molecular Probes (part of Invitrogen)	http://www.invitrogen.com/
Multi Channel Systems	http://www.multichannelsystems.com/
Neuralynx	http://www.neuralynx.com/
NeuroNexus	http://www.neuronexustech.com/
Newport	http://www.newport.com/
Nikon Instruments Inc.	http://www.nikoninstruments.com/
Olympus	http://www.olympusamerica.com/
Omega Optical	http://www.omegafilters.com/
Optical Imaging	http://www.opt-imaging.com/
Optronics	http://www.optronics.com/
PerkinElmer	http://www.perkinelmer.com/
Photometrics	http://www.photometrics.com/
Physik Instrumente	http://www.physikinstrumente.com/
Prairie Technologies	http://www.prairie-technologies.com/
QImaging	http://www.qimaging.com/
Semrock	http://www.semrock.com/
Sigma-Aldrich	http://www.sigmaaldrich.com/
Sutter Instrument	http://www.sutter.com/
TEF-Labs	http://www.teflabs.com/
TILL Photonics	http://www.till-photonics.com/
Vincent Associates	http://www.uniblitz.com/
World Precision Instruments	http://www.wpiinc.com/

© 2010 Nature America, Inc. All rights reserved.

Erratum: From promising to practical: tools to study networks of neurons

Monya Baker

***Nat. Methods* 7, 877–883 (2010); published online 28 October 2010; corrected after print 10 November 2010.**

In the version of this article initially published, a scale bar showing 1 mm was mislabeled on page 879 as showing 10 mm. The error has been corrected in the HTML and PDF versions of the article.

© 2010 Nature America, Inc. All rights reserved.

npg