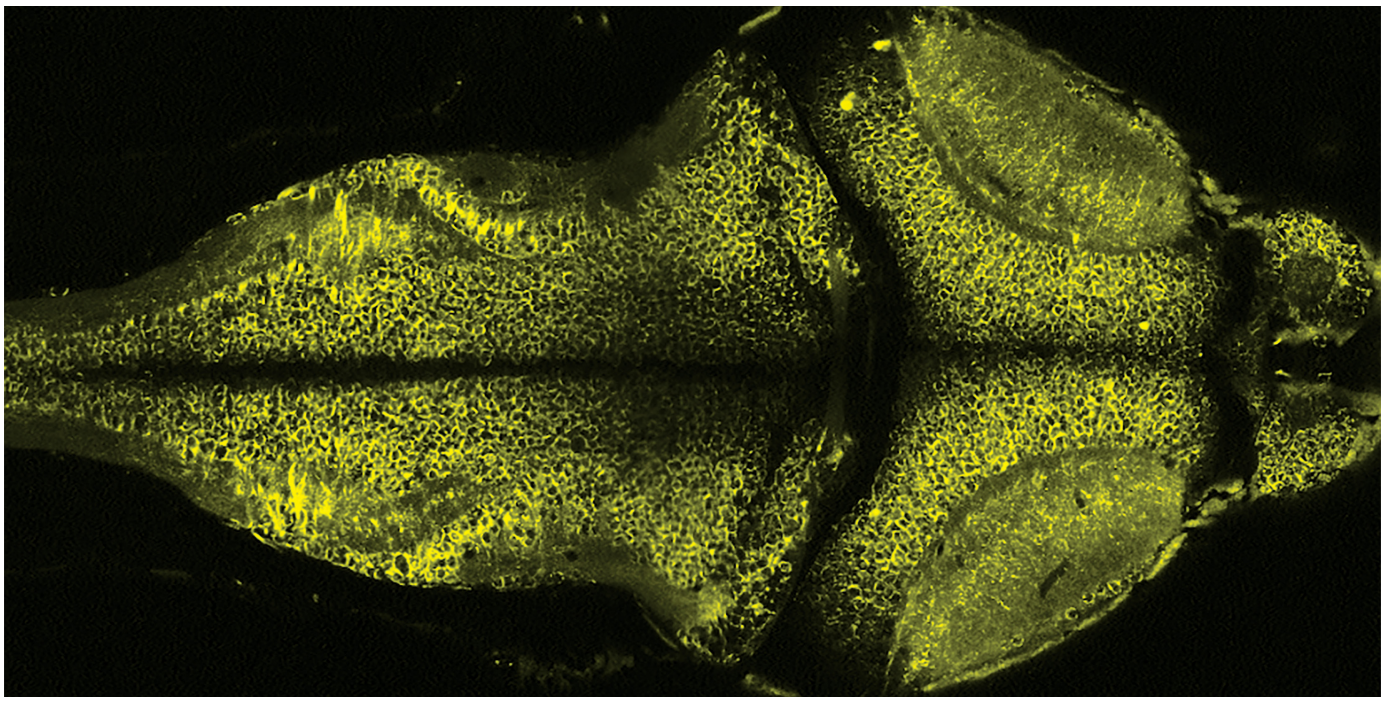
TECHNOLOGY FEATURE

GENETIC LIGHT BULBS ILLUMINATE THE BRAIN

Genetically encoded voltage indicators change colour in real time when neurons transmit electrical information, offering unprecedented insight into neural activity.

A. S. ABDELFATTAH *ET AL./SCIENCE*



Fluorescent proteins that react to voltage changes show signalling between cells in the brain of a zebrafish (*Danio rerio*).

BY JYOTI MADHUSOODANAN

Douglas Storace still has the dollar bill that he triumphantly taped above his laboratory bench seven years ago, a trophy from a successful wager. His postdoctoral mentor, Larry Cohen at Yale University in New Haven, Connecticut, bet that Storace couldn't express a protein sensor of voltage changes in mice back in September 2012. Storace won.

The bill is a handy reminder that the experiments he aims to try in his new lab can work. And it's a testament to just how tricky it is to correctly express these sensors and track their signals. Storace, now an assistant professor at Florida State University in Tallahassee, plans to use these sensors, known as genetically encoded voltage indicators (GEVIs), to study how neurons in the olfactory bulb sense and react to smells.

GEVIs are voltage-sensitive, fluorescent proteins that change colour when a neuron fires or receives a signal. Because GEVIs can be targeted to individual cells and directly indicate a cell's electrical signals, researchers consider them to be the ideal probes for studying neurons. But they have proved frustratingly difficult to use. "Being able to visualize voltage changes in a cell has always been the dream," says neuroscientist Bradley Baker at the Korea Institute of Science and Technology in Seoul. "But probes that looked great often didn't behave in ways that were useful."

Early GEVIs disappointed on several levels. They were bright when a cell was resting and dimmed when the cell fired an action potential, producing signals that were tough to distinguish from the background. And they failed to concentrate in the nerve-cell membranes, where they function. But researchers are beginning to solve these issues. Some are turning to advanced fluorescent proteins or chemical dyes for better signals; others are using directed evolution and high-throughput screens to make GEVIs more sensitive to voltage changes. Meanwhile, biologists are putting these molecules through their paces. GEVIs, says neuroscientist Katalin Toth at Laval University in Quebec City, Canada, are not yet widely used, but they're getting there. "They are becoming brighter and faster — and growing in popularity," she says. "I think this is the dawn of GEVIs."

GLIMMER OF PROMISE

When a mouse smells a banana and races towards the treat, it is the inevitable result of a well-organized orchestra of neural circuits. Researchers can tap into these pathways using patch-clamping (in which electrodes and pipettes are placed on cells to track electrical activity in a given brain region) and voltage-sensitive dyes (which can reveal overarching electrical changes).

Genetic probes are another option. In a similar way to dyes, these molecules fluoresce in response to electrical signals. But researchers ▶

can use genetic tricks to limit the probes' expression to specific cells. Genetically encoded calcium indicators (GECIs), such as GCaMP proteins, are made by fusing a fluorescent protein to one that can bind to calcium. Calcium floods a nerve cell after it has fired an electrical signal, causing a change in the binding protein's shape that triggers a change in fluorescence.

But GECIs are only proxies for neural electrical activity. Although they are sensitive to action potentials, which are the basic units of neural communication, they cannot capture the smaller, sub-excitatory cues that help nerve cells to compute and integrate different kinds of information.

In 1997, Ehud Isacoff at the University of California, Berkeley, developed the first GEVI, named FlaSh, by fusing green fluorescent protein with a voltage-sensitive potassium channel[1]. Imperial College London neuroscientist Thomas Knöpfel, then at RIKEN in Wako, Japan, followed suit in 2010 by fusing a voltage-sensing phosphatase enzyme derived from *Ciona intestinalis*, a marine invertebrate, to a fluorescent protein[2]. Other designs followed, including the 2012 discovery that a random mutation in one protein made it 14-fold more sensitive to voltage changes, leading to one of the biggest early GEVI successes[3], ArcLight.

Today, there are three major classes of GEVI (see 'Flavours of fluorescence'). Probes such as ArcLight fuse voltage-sensitive protein domains (VSDs) to fluorescent proteins, whereas others such as Archer and QuasAr2 rely on fluorescent membrane-spanning bacterial proteins known as rhodopsins. Ace–mNeon represents a third group known as opsin–FRET (fluorescence resonance energy transfer) probes. These molecules combine light-sensitive opsins that are similar to rhodopsin with a second fluorescent protein to create an energy transfer — detectable as a change in fluorescent colour — when the proteins are excited. "Unlike GCaMP, where everyone was focused on one scaffold, each GEVI has its own developmental path," says neuroscientist Michael Lin at Stanford University in California.

Ideally, a GEVI will yield a bright, stable signal that consistently follows a change in voltage or action potential, and produce minimal background fluorescence. But this doesn't always happen. Unlike GCaMPs, which can fill a cell's volume, GEVIs must be localized to the cell membrane to be effective. They cannot be tested in bacteria, because it is difficult to maintain a membrane potential in these cells. And the change in fluorescence when a GEVI fires is much smaller than that seen with GECIs.

The millisecond pace of neural electrical activity is also a problem, both for GEVIs and the cameras that image them. And generating a sufficiently bright signal requires intense excitation light, which can overheat cells and cause the GEVI to bleach within minutes.

As a result, most biologists still look to GCaMPs to study fine-scale neuronal activities. "What the field would love to have is a solution like GCaMP. Calcium imaging works consistently in anyone's hands," says neuroscientist Eric Schreiter at the Howard Hughes Medical Institute's Janelia Research Campus in Ashburn, Virginia. "There are very few reports of existing GEVIs being used *in vivo*, and they're quite limited in their scope."

BRIGHTER, FASTER … BETTER?

But that is beginning to change, thanks to directed-evolution approaches, high-throughput screening strategies and more-stable fluorescent molecules.

Schreiter and his team, for instance, removed the fluorescent portions of rhodopsin-based sensors and replaced them with a protein that binds to a synthetic dye molecule in response to voltage changes. Synthetic dyes are significantly brighter and more photo-stable than fluorescent proteins. One such probe, dubbed Voltron, produced a signal that was several-fold brighter than its parent GEVIs and lasted upwards of 15 minutes without bleaching[4].

Voltron's signal is a flare of bright light against a background of unbound dye that is also fluorescing. This 'negative' signal is much harder to spot under a microscope than a 'positive' one, where the background remains dark. In subsequent experiments, Schreiter's team discovered that three specific mutations in the rhodopsin proton-transport domain reduce the protein's fluorescence when a cell is resting and thus result in a 'reverse Voltron' that produces this kind of positive signal[5]. "It's one of the rare instances in my career where trying something rational actually worked on a protein," says neuroscientist Ahmed Abdelfattah, a postdoctoral researcher in Schreiter's lab.

These mutations could also help to tweak the bright-on-bright signals from other rhodopsin-based GEVIs, says neuroscientist Yuki Bando at Hamamatsu University in Japan.

Other researchers have made GEVIs that use red fluorescent proteins instead of green ones, because red light can penetrate deeper into tissues and causes less cellular damage. Recent examples include VARNAM (voltage-activated red neuronal-activity monitor), which blends a red fluorescent protein named mRuby with an opsin-based probe; nirButterfly, a variant of the FRET-based GEVI called Butterfly that swaps the paired fluorescent proteins with bacterial near-infrared proteins; and Ilmol, which uses one of the brightest red fluorescent proteins available to produce a signal three times stronger than that of FlicR1, the VSD probe on which it is based[6–8].

Expanding the GEVI spectrum helps researchers to combine probes or techniques in the same study. For example, chemical biologist Adam Cohen at Harvard University in Cambridge, Massachusetts, and his colleagues developed QuasAr3, a near-infrared probe that surpasses its predecessors in terms of signal-to-noise ratio, membrane-specific expression and other properties. The team combined QuasAr3 with optogenetics — the use of different wavelengths of light to control neuronal activity — to study voltage changes correlated with behaviour and movement in mice[9].

"Some of the largest improvements in opsin-based probes have been in membrane localization," Lin says. "That alone has been very useful. Others such as nirButterfly and VARNAM also show improvements in brightness and responsiveness."

Lin's own work has focused on a series of GEVIs dubbed ASAPs for their fast responses. The latest iteration, ASAP3, has a signal that is significantly stronger than its predecessor, ASAP2 (ref. 10).

SPEEDIER SCREENS

Many of these protein improvements stem from directed-evolution techniques, in which proteins are randomly mutated and improved versions selected over multiple cycles. But it is one thing to create a GEVI, and another thing to test it. When it comes to GEVIs, says protein engineer Robert Campbell at the University of Alberta in Edmonton, Canada, screening remains a "bottleneck".

Yale University neuroscientist Vincent Pieribone's team, which created VARNAM, uses a 96-well-plate set-up in which a field electrode moves from well to well to excite cells that carry GEVIs harbouring different mutations, linkers or fluorescent proteins. The system lets researchers quickly study each GEVI's response to voltage changes. In another approach, Edward Boyden at the Massachusetts Institute of Technology in Cambridge enriched for brighter rhodopsin-based probes using a microscopy-guided, robotic process[11].

Lin's group turned to a classic genetic technique — electroporation — to quickly screen its probes. In electroporation, a quick pulse of electricity reduces the resting membrane potential of cells to zero, creating temporary holes in the cell membrane so that DNA can enter. But because electroporation creates a defined voltage change, it can also be correlated to a probe's fluorescence signal. "It's a very simple idea," Lin says, "But it hadn't been used before to screen fluorescent probes."

These high-throughput methods help researchers to screen thousands of GEVI variants in a matter of hours. "Perhaps a major push to apply such screening systems to the right templates" is all that's needed to propel GEVI technology forward — and into more widespread use, Campbell suggests.

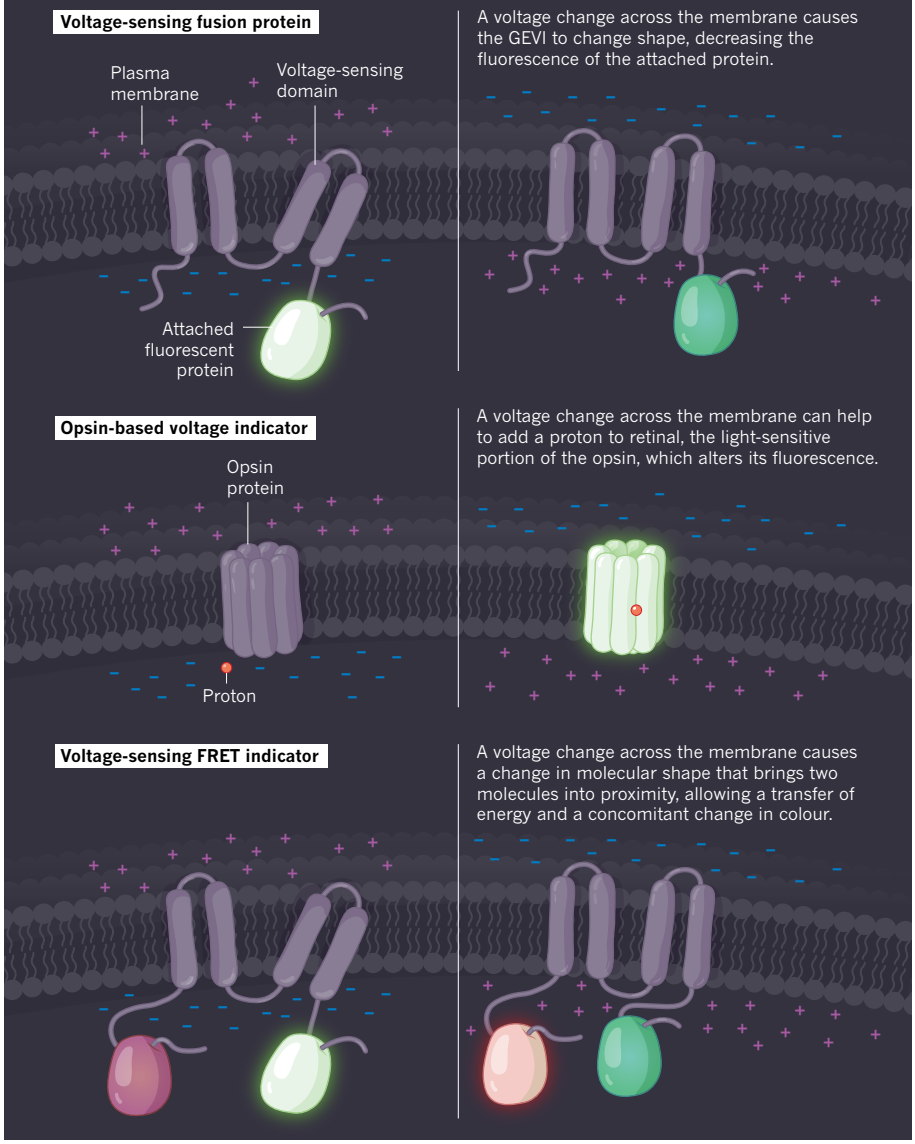
BRIGHT APPLICATIONS

GEVI developers usually work with collaborators to test their probes in flies, mice or cultured human cells. Although independent researchers can order and use any GEVI from the non-profit repository Addgene, based in Watertown, Massachusetts, there have been few published reports from external users who have applied GEVIs to their experiments.

In part that could be due to instrumentation, Toth says. Her lab works with the ASAP

FLAVOURS OF FLUORESCENCE

Scientists have built different types of genetically encoded voltage indicator (GEVI). One major category (top) uses a membrane-bound portion of a voltage-sensing protein, such as a sodium channel, fused to one or more fluorescent proteins. Another category (middle) uses an opsin protein, such as a microbial rhodopsin, a membrane channel that directly changes its fluorescent properties in response to an electric field. Other categories rely on fluorescence resonance energy transfer (FRET) to produce signals (bottom).



probes, and uses random-access two-photon microscopy to capture signals. But these systems can prove expensive and thus difficult to access for many researchers, she says.

Another hurdle has been the difficulty researchers have in directly comparing GEVI performance. Most labs report GEVI performance using a few standard metrics, which can help users to decide how different probes stack up against each other, Lin says. But until this year, only one study had compared a range of GEVIs in parallel using the same experimental conditions[12]. "Each GEVI has very specific characteristic properties," says Bando, who led that research. "But nobody had compared the indicators with the same experimental conditions."

Bando and his colleagues compared eight GEVIs in cultured neurons and mice, using both widefield and two-photon imaging. The team tested the proteins for their ability to track action potentials, synaptic input, photobleaching and other properties. Some probes, they found, emitted a dim baseline fluorescence and thus needed a very-high-powered excitation laser, which could overheat and damage cells. Others produced fast, reliable signals under conventional imaging, but failed with two-photon microscopy, which can visualize deeper brain regions *in vivo*. Still others produced strong, but short-lived signals. Overall, they found that "no indicators could detect both action potential and synaptic inputs *in vivo*", Bando says. And, "only ArcLight worked with two-photon imaging *in vivo*".

But ArcLight's fluorescent responses are slow — too slow to track a neuron's action potential, which lasts only about a millisecond. Nonetheless, this GEVI's consistency and clear signal led Bando to focus on optimizing ArcLight for his own studies. When selecting a voltage indicator, consider the purpose of the experiment, he suggests. Archer, QuasAr and Ace2N–mNeon are ideal for one-photon (that is, conventional) imaging of cultured cells or brain slices. To study deeper brain regions in live animals using multiphoton approaches, ArcLight might be a researcher's best choice for now.

Baker concurs that for GEVI novices, ArcLight is the easiest option. Probes can fail for several reasons, he explains, including incompatible cameras, photodamage, and poor or aberrant protein expression. "So many things can go wrong, and you need a probe that gives you the confidence that the imaging works," he says. "It might not be the right probe for you, but if you don't see a signal with ArcLight, you will not see a signal with whatever other GEVI you try."

Indeed, ArcLight's sluggishness might be the reason it works so well, Storace says, because it ends up integrating input from various neurons into a single signal that is easy to distinguish from background noise. "I strongly feel the reason it's better is because it's slower," he says.

The brain region being studied is also a factor. Storace focuses on the olfactory system, which is physically organized into bulbs, each of which responds to a single odorant. Applying GEVIs to this region reveals useful data, he says, because even if the signals from individual cells are indistinguishable, the population-level data can be revealing. "It's a useful strategy in the olfactory system, but I'm not sure it's easily translated to other brain areas such as the cortex," he says.

Indeed, as researchers continue refining and exploring these probes, fresh strategies are likely to emerge, as will insights into their strengths and weaknesses. Different probes might well be ideal for different questions, Storace says. "In about four or five years, we'll have a better idea of how easy it is to use GEVIs." ■

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