

Optogenetics: Tools for Controlling Brain Cells with Light

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The brain is made out of an incredible diversity of cells called neurons, which have different shapes, are made of different molecules, and that change in different ways in diseases. In optogenetics, microbial opsins, natural proteins that convert light into electrical signals, are genetically expressed in neurons. Then, light pulses can be used to turn neurons on, revealing how they trigger behaviors, disease states, or therapeutic effects, or to turn neurons off, revealing what functions or dysfunctions they are necessary for.

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Our brains generate our thoughts and feelings, and make us who we are. Brains are incredibly complex networks of cells called neurons. A cubic millimeter of the brain contains perhaps 100,000 neurons, connected by a billion connections called synapses. These neurons work together as a network to process information and generate our behaviors. Within neurons, electrical pulses called action potentials propagate throughout complex treelike branches (Fig. 1), eventually reaching synapses, where they trigger chemicals called neurotransmitters to be released onto proteins called receptors on downstream neurons, mediating neural communication (Fig. 2).

Some neurons receive sensory information from the outside world, from organs like our eyes and ears, and in turn project to downstream neurons that help make decisions, bring in information from memory, and take account of emotional states. Those neurons then project to neurons that generate movements. Thus, the neural networks of the brain exhibit incredible spatial complexity, which determines how information is conveyed and transformed, so that an organism can respond to changes in the world by generating appropriate behaviors.

In addition, amongst biological systems, the brain exhibits a very high speed of operation. The action potentials generated by neurons last about a millisecond in duration, which in turn cause the release of neurotransmitters at synapses, with millisecond precision. When the neurotransmitters land on the receptors of downstream neurons, they cause new electrical signals to be generated, which in turn can cause new high-speed action potentials to be generated, and the cycle continues.

Thus, understanding how the neural networks of the brain compute our thoughts and feelings, and make us who we are, requires us to be able to map the neural networks of the brain, and to control their electrical activity with great spatial and temporal precision.

Although understanding how the brain generates the mind is of immense human interest, this quest is not one of pure curiosity. Brain disorders affect over a billion people around the world. These conditions, including Alzheimer's, schizophrenia, Parkinson's disease, chronic pain, and depression, cannot currently be fully cured. The available treatments, in addition, are partial in efficacy, and often cause side effects.

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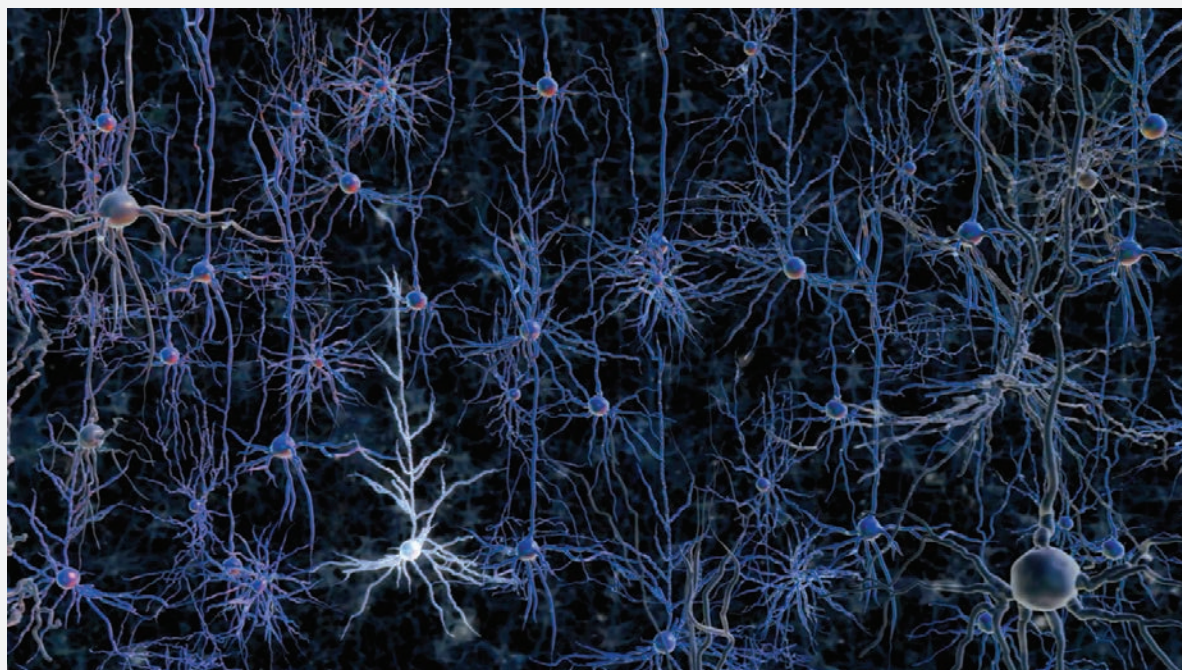


Figure 1. **Cartoon of brain cells (neurons), with one neuron “lighting up,” representing its electrical activity.** In the real brain, there is no space between the neurons, as in this illustration; they are densely packed into intertwined neural networks.



Figure 2. **Cartoon of a neural connection (synapse) between two neurons.** Chemicals called neurotransmitters are released by one neuron in response to an action potential, and are sensed by receptors on a downstream neuron. These receptors, in turn, drive electrical signals within the downstream neuron, which can generate a new action potential, and the cycle continues.

Why? The spatial and temporal complexity of the brain means that we don't fully understand any brain disorder. In contrast to other diseases like infections, where the disease-causing agent is clearly distinct from the body and thus can be destroyed by targeting its specific properties (for example, we take antibiotics to treat a bacterial infection; the antibiotics, in contrast, are almost entirely harmless to human cells), brain disorders are due to changes in the body's own cells, and thus are harder to understand, and harder to selectively treat. Furthermore, many brain disorders are extremely subtle, being due to minuscule changes — such as alterations in the electrical activity patterns, and the patterns of connectivity, of brain cells. Perhaps not surprisingly, making new treatments for the brain is difficult. New brain drugs take a decade to develop, the cost is about a billion US dollars each, and the failure rate to get regulatory approval for clinical use is over 90%¹. And as noted, the drugs often don't work very well.

Neurons come in many kinds: some are large and others are small, some excite their downstream neuronal targets and some inhibit them, and some generate action potentials quickly and others slowly. Different kinds of neurons contain different sets of biomolecules, due to their expressing different subsets of the ~30,000 genes of the genome. And, different kinds of neuron are altered, in different disease states. For example, a specific kind of neuron dies off in Parkinson's disease, other kinds of neurons atrophy in schizophrenia, and still other kinds of neurons are affected in narcolepsy.

In 1999, Francis Crick, who helped decipher the structure of DNA, commented that a great need in neuroscience "...is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. The ideal signal would be light... This seems rather far-fetched but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way"². By turning on the electrical activity of one kind of neuron, one could figure out how it triggers, or sustains, a given behavior, or a given disease state, or a potential therapeutic effect. And by turning off the electrical activity of a specific kind of neuron, one could figure out what behaviors, disease states, or therapeutic effects it was necessary for. If you could discover a set of cells that, when activated or silenced, improves the health of a mouse engineered to simulate a human brain disease, then maybe that set of cells could be targeted by new drugs, or by noninvasive brain stimulation devices, to remedy the disease in humans.

Around the same time as Crick's essay³, I began doing research in the neurosciences PhD program at Stanford University, having previously trained as a chemist, physicist, and engineer. There I met a fellow student, Karl Deisseroth, who was also interested in the problem of how to control neurons. We began brainstorming about every possible way you might

turn specific kinds of neurons on or off. It was clear that to be temporally precise, one would need to deliver high-speed pulses of energy to the brain. It turns out there is a pretty short list of ways to deliver energy to the brain: mechanical force, ultrasound, magnetic fields, electric fields, light, and a few other modalities — you can write the entire list down on a sheet of paper.

We began doing calculations in order to determine what would be the most powerful, precise way to control neurons with pulses of energy. I became very interested in using light, especially when I read a paper that described a very curious class of molecule — the microbial opsin. Microbial opsins are proteins that sit in the membranes of single-celled organisms, and convert light into electrical signals. The first of these to be found, in 1971⁴, was a light-driven proton pump, found in a species of microbe (in the kingdom archaea) that lives in very salty water (such microbes are known as halophiles). The discoverers named it bacteriorhodopsin. Bacteriorhodopsin pumps protons out of the cell, in response to green light. The resultant proton gradient powers a protein known as ATP synthase, resulting in chemical energy stored inside the microbe in the form of ATP. (In our own cells, something analogous happens. Mitochondria produce ATP through the creation of a proton gradient, which powers ATP generation by ATP synthase. In contrast, however, our proton gradient is produced by chemical metabolism, rather than light.)

Around a decade later, multiple groups found light-driven chloride pumps in such microbes, which respond to yellow light by pumping chloride ions into the cell, and which they named halorhodopsins⁵⁻⁹. A paper from 1999 caught my attention, because it showed that although these molecules come from organisms that live in very salty water, one of these molecules, the halorhodopsin from the species *N. pharaonis*, for whatever evolutionary reason, functions best at low salt concentrations¹⁰. I noted that this molecule could, in principle, work in the (relatively) low salt environment of the brain, if it could be expressed safely, and if it mediated large enough light-induced currents. Thus, Karl and I started collecting such molecules from colleagues.

The first one we collaboratively tried out in neurons was a light-driven cation channel from the green alga *C. reinhardtii* (Fig. 3), discovered by Peter Hegemann and colleagues, which responds to blue light by transporting positively charged ions through the membrane of a small organelle within the alga, called the eyespot^{11,12}. This molecule, channelrhodopsin-2 (Fig. 4), helps the algae navigate around in water to optimize their photosynthesis, by sensing the amplitude of sunlight, and controlling the flagellae (tails) of the algae. Karl delivered the gene into cultured mammalian neurons, and I shined blue light on the neurons while recording the electrical activity with an electrode. Amazingly, the neuron fired action potentials on the first try (Fig. 5). Serendipity had struck.



Figure 3. **Cartoon of the green alga *C. reinhardtii*, with the eyespot (brown) highlighted.**

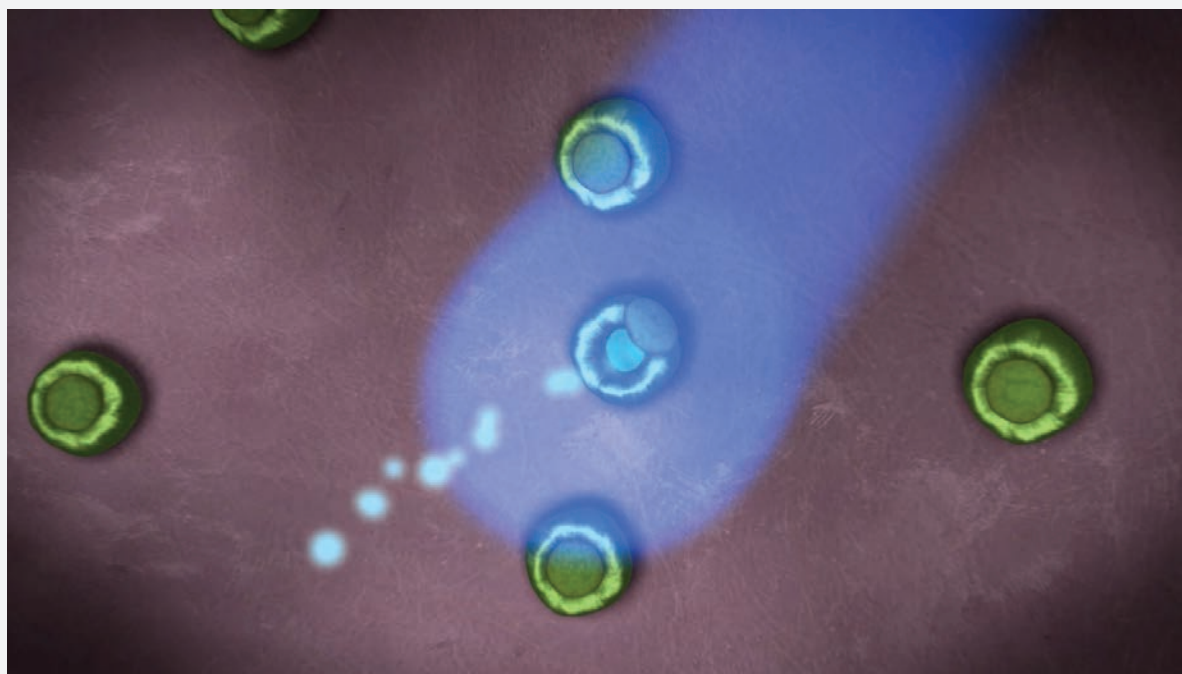


Figure 4. **Cartoon of the membrane of the eyespot.** When illuminated, the light-gated cation channel channelrhodopsin-2 (green cylinders) lets positively charged ions through the membrane of the eyespot.

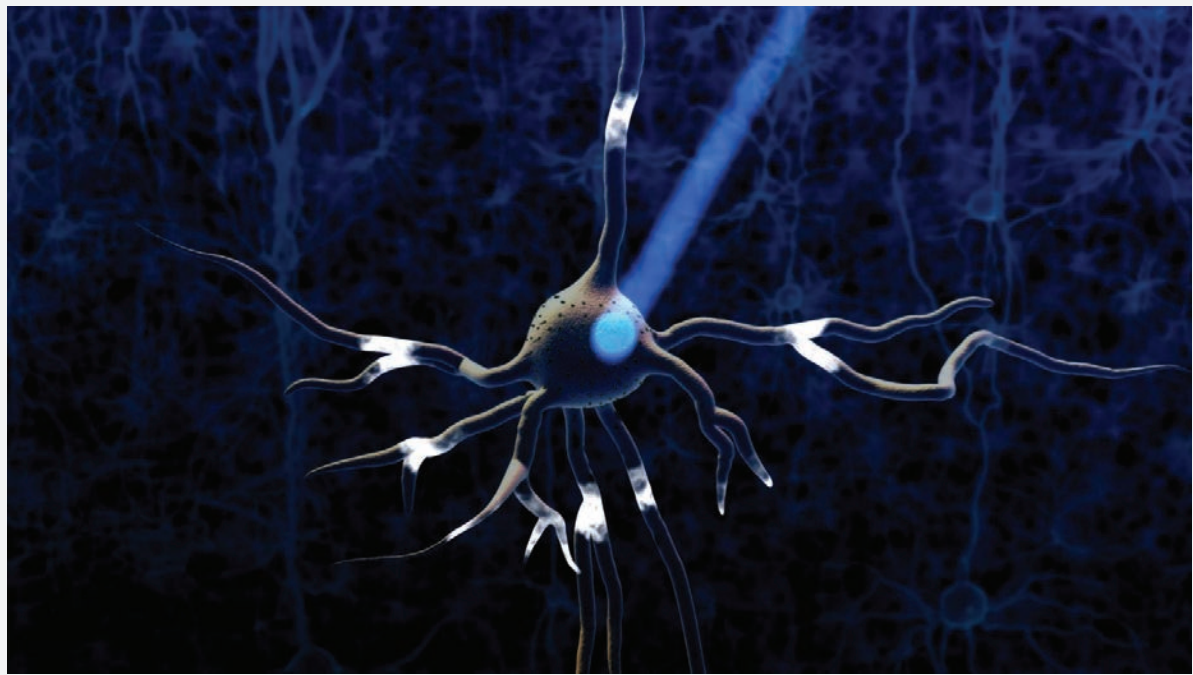


Figure 5. **Cartoon of a neuron expressing channelrhodopsin-2 (small green dots) and illuminated by blue light.** The light-gated ion channels open, letting positive charge into the neuron.

This protein, taken from algae, somehow possessed all the right properties to safely and effectively express in delicate mammalian neurons. It even went to the right part of the cell, the outer membrane (not a sure-fire thing, since in the algae it goes to an intracellular organelle, the eyespot). And the speed of the protein, and the magnitude of the currents generated, were sufficient to enable action potentials to be generated.

Furthermore, the protein generated by the gene was able to work without adding any external chemicals. This was not guaranteed, since the protein requires, for its light-sensing capability, a vitamin A-related small molecule called all-trans-retinal. Serendipitously, mammalian neurons seem to have enough all-trans-retinal lying around that no supplementation with external all-trans-retinal is needed. We had a way to activate a set of cells with light, simply by expressing a gene in them.

My group at MIT went on to show that light-driven chloride pumps (such as the *N. pharaonis* halorhodopsin mentioned before) and proton pumps (such as a bacteriorhodopsin-related protein called archaerhodopsin-3) could be used to turn neurons off with green or yellow light^{13–15}. These tools are now in widespread use in neuroscience, where they help reveal how activating or silencing neurons can trigger behaviors, pathological states, and therapeutic states in model organisms such as the worm *C. elegans*, the fly *D. melanogaster*, the fish *D. rerio*, and the mouse.

As an example, one study placed channelrhodopsin-2 in neurons in a deep region of the mouse brain called the hypothalamus¹⁶. This can be done by using a virus, as is done in gene therapy. By inserting the gene for channelrhodopsin-2 into the virus, and injecting the virus into the brain, neurons will take up the virus, and thus the gene for channelrhodopsin-2. Neurons will then manufacture the protein, as in Fig. 5, making them light-activatable. By implanting an optical fiber in the brain, with the free end connected to a blue laser, one can deliver pulses of light into the brain, activating the opsin-expressing cells below the end of the optical fiber. In this study, the scientists found a set of cells in the hypothalamus that, when activated, caused mice to become aggressive. They would attack other mice, or even a rubber glove. Thus, activating a set of cells lets you see if they can trigger a behavior — in this case, even aggressive behavior.

As a second example, one study used channelrhodopsin-2, targeted using a virus to a specific subset of cells called parvalbumin-positive interneurons (Fig. 6), and drove these interneurons at a high speed — 40 times per second (40 Hz)¹⁷. In mice genetically engineered to get Alzheimer's-like symptoms, activating neurons at this speed caused the mice to get better. For example, beta-amyloid, and phosphorylated tau, molecular changes associated with Alzheimer's disease, both improved. In other words, a specific pattern of brain



Figure 6. **Cartoon of a neural network with the parvalbumin-positive interneurons expressing channelrhodopsin-2.** Exposing the brain circuit to blue light causes the parvalbumin-positive interneurons to fire.

activity might be able to treat one of the most devastating brain diseases. The study went on to show that this pattern of brain activity could be induced noninvasively, by showing the mice visual stimuli or playing auditory stimuli, timed to cause this rhythm. Now, human trials are under way of noninvasive ways (e.g., watching flickering lights and hearing clicking sounds) to induce this high-speed pattern of brain activity, with actual Alzheimer's patients.

As a final example¹⁸, a study asked whether a memory is stored in different parts of the brain, at different times after the memory was initially formed. After delivering a light-driven proton pump to neurons of the prefrontal cortex in such a way that the gene would be expressed only in cells that were active during learning, the scientists trained mice to fear a specific place. They then silenced the neurons that were active during learning. They found that silencing these neurons impaired the fear memory 12 days after learning, but not 2 days after learning. Thus, the neurons involved with learning in the prefrontal cortex are necessary for long-term memories, but not for recently learned memories.

Perhaps thousands of scientists are now using this toolset (thanks to free dissemination of the tools through non-profit repositories; see <http://synthneuro.org/protocols>), now known as optogenetics (opto- meaning light, and -genetics because the tools are genetically encoded) to study the brains

of mice and other model organisms used in neuroscience, figuring out how specific kinds of neuron contribute to sensations, actions, decisions, and emotions, and figuring out new patterns of neural activity, new cell types, and new therapeutic targets, for treating brain disorders. Optogenetics is a testament to the richness of the natural world in yielding new tools for understanding and repairing the brain: these molecules, after all, are from single celled microbes. Ecological diversity is an endless source of new tools for biology — think of the green fluorescent protein (GFP) and the genome editing tool CRISPR, as two other examples. These stories highlight the importance of conserving the natural world in order to preserve potential new biotechnology innovations for future generations.

In our group, we have continued to develop this toolset, aiming to make optogenetic tools that reach their maximum physical performance. Much of this effort involves identifying new molecules from the natural world, from new organisms, sometimes in new ecological niches, that have unique new properties. For example, one goal is to make red light-sensitive molecules, because red light can go deeper in the brain than other colors of visible light. That is why blood looks red, because it doesn't absorb red light. By using red light-sensitive molecules, and delivering red light to the brain, we can control a larger volume of the brain than possible with

other colors of visible light, and even achieve noninvasive optogenetics in awake behaving mice. We discovered a halorhodopsin that is sensitive to red light, which we named Jaws¹⁹, as well as a channelrhodopsin that is sensitive to red light, which we named Chrimson²⁰. Using Jaws or Chrimson, one can silence or activate, respectively, a larger volume of brain tissue than possible with earlier tools.

Another physical limit that we are approaching is speed. We found a channelrhodopsin which we named Chronos, which has extremely fast kinetics, causing electrical currents that turn on very quickly in response to light, and that turn off very quickly when light ceases²⁰. This is important because neural action potentials are brief and very precisely timed, and can occur at fast rates, especially in systems such as the auditory system.

We also worked to develop optogenetic tools that can enable individual cells to be precisely targeted, or for sets of cells to be controlled with single-cell resolution. The studies discussed above would deliver light to many cells at once, and thus activate or shut down the whole set of neurons at the same time. In contrast, in the awake behaving brain, even nearby neurons of the same type are often doing very different things. Ideally, one would be able to activate or silence each cell individually, specifying its own pattern of activity, even quite different from that of its neighbor. Groups have invented microscopes that can project holograms — essentially, 3-D sculptures made of light — into the brain. We developed a powerful optogenetic tool, soCoChR, which enables holographically aimed laser light to activate individual cells with millisecond precision, without the light activating nearby cells²¹. Thus, the optogenetic toolset has begun to reach its physical limits, not just in terms of color of light and speed, but also spatial and temporal precision.

Optogenetics is great for perturbing specific cell types within specific neural circuits. But how do you know which ones to perturb? There are so many kinds of cells, and how they are connected into networks is very difficult to characterize. Ideally you would have a map of the brain, so that you would know which cells are changed in a given disease, or how cells are connected in a network that generates a behavior. Because neural wires — axons, the branches of neurons that convey outgoing electrical pulses towards synapses, and dendrites, which bear receptors that receive neurotransmitters from synaptic inputs — are nanoscale in dimension, mapping the brain has commonly been done with a technology called electron microscopy, which has very high spatial resolution. But it is very difficult to use electron microscopy to map the identity and location of biomolecules, such as the neurotransmitters, ion channels, and receptors, which make neurons do what they do. These biomolecules are also nanoscale in dimension, and often organized with nanoscale precision with respect to one another, raising the question of

how best to map their 3-D organization across the neurons of a brain circuit.

Light microscopes can see in multiple colors, and thus can image dyes that are targeted to specific biomolecules by specific labels (for example, one can buy or make antibodies that will selectively bind to specific proteins; if those antibodies are equipped with a fluorescent dye, and delivered to a preserved specimen of the brain, you can then see the location of a given kind of protein within a brain), but due to a phenomenon called diffraction, light microscopy cannot resolve objects much smaller than the wavelength of light, resulting in resolution limits of around 300 nanometers or so. In recent years, super-resolution microscopes, which use clever physical tricks to overcome this diffraction limit, have been able to achieve much finer resolutions, down to around 20 nm or so, but such microscopes are slow, expensive, and/or difficult to apply to extended 3-D objects like brain circuits.

In our group, we began to think about taking an approach that is quite different from the last 300 years of microscopy, where one uses a lens to magnify an image of an object: what if we physically magnified the object itself? Then you could use fast, conventional, ubiquitous microscopes to map the brain. We discovered that one could take a preserved specimen of brain tissue, chemically synthesize a dense and evenly created spiderweb-like mesh of swellable polymer throughout it (binding to biomolecules within the specimen through anchors), chemically soften the specimen, and add water. The water would then be absorbed by the swellable polymer, and through the anchors, move biomolecules apart from each other, thus causing the brain specimen to expand.

Because the polymer is so dense — the spacing between polymer threads is just a few nanometers in size — and because we synthesize it so evenly throughout the cells of the brain, and soften the specimen, the expansion process is very even (Fig. 7), and preserves information down to the nanoscale. We call the process expansion microscopy (ExM), and have trained hundreds of groups on how to expand biological specimens so they can map their nanostructure, on microscopes they already have²² (protocols are downloadable at <http://expansionmicroscopy.org/>).

As a result of expansion microscopy, the fine wiring of neurons can be mapped using ubiquitous, conventional light microscopes. For example, one can label neurons with genetically encoded fluorophores from organisms like jellyfish and coral, by delivering the genes that encode for these fluorophores in a random fashion to different neurons, using viruses. The resulting physical color coding of neurons was named “Brainbow” by its inventors²⁴. Antibodies bearing small molecule fluorophores can be delivered to bind to these genetically encoded fluorophores, boosting their fluorescence further (Fig. 8). Upon expansion, the net result is that axons and dendrites that are too small to be resolved (Fig. 8d) can be

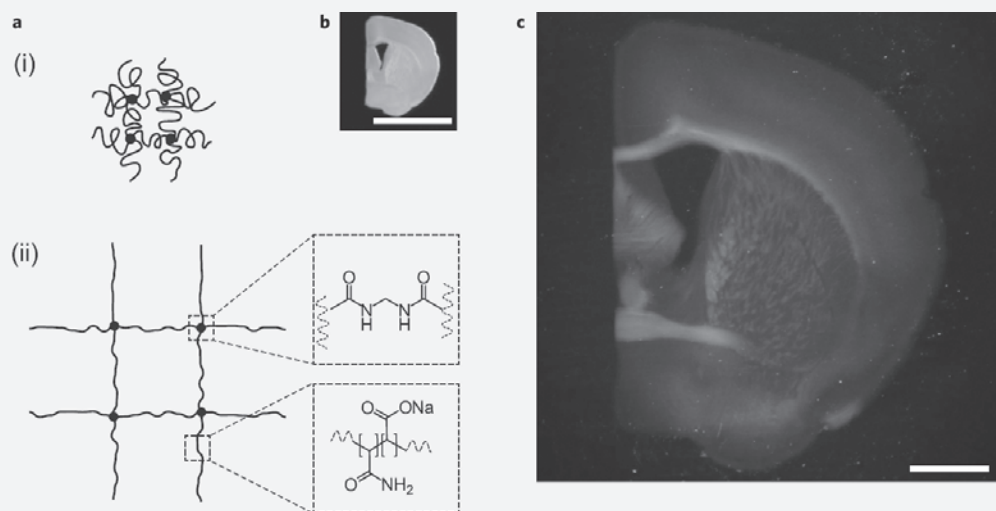


Figure 7. **Concept and example of expansion microscopy.** (a) Schematic of (i), collapsed polyelectrolyte network, showing crosslinker (dot) and polymer chain (line), and (ii) expanded network after H₂O dialysis. (b) Photograph of fixed mouse brain slice. (c) Photograph, post-ExM, of the sample (b) under side illumination. Adapted from Ref. 23.

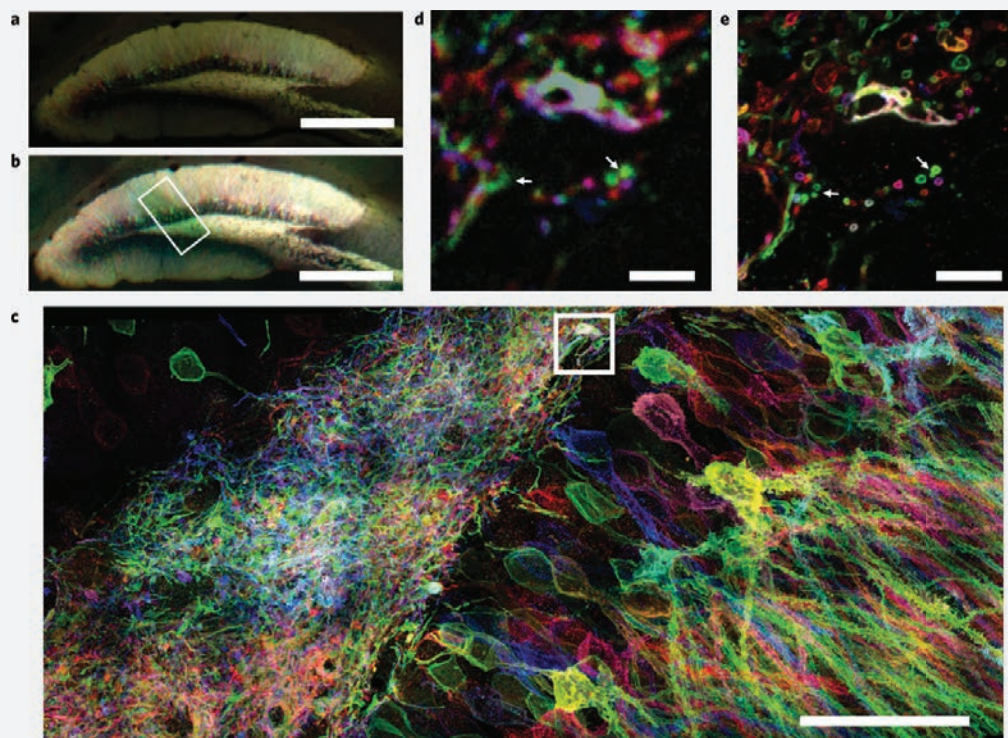


Figure 8. **Expansion microscopy of Rainbow-labeled brain.** (a) Low-magnification widefield fluorescence imaging showing immunostained mouse hippocampus expressing virally delivered Brainbow3.0. (b) Post-expansion widefield image of the sample in a. (c) Maximum intensity projection of high-resolution confocal microscopy stack following expansion of membrane labeled Brainbow3.0 neurons from the boxed region in b. (d) Pre-expansion confocal image showing one optical section of the boxed region in c. (e) Post-expansion image of d. Scale bars: (a) 500 μm , (b) 500 μm (in biological units), (c) 50 μm (in biological units), (d) 5 μm , (e) 5 μm (in biological units). Adapted from Ref. 25.

easily distinguished, and traced (Fig. 8e). The color codes help by disambiguating nearby neurons from each other: if two neural wires are nearby, and get confused with one another as they pass by one another, one can use the color codes to help tell them apart.

In summary, we are excited that in the years to come, we will be able to make maps of brain circuits in healthy and diseased states, and then use these maps to derive strategies for optogenetically probing how specific neurons contribute to behaviors, pathological states, and therapeutic states. By generating new hypotheses about how the brain works through observation, and then testing these hypotheses through causal perturbation, we hopefully can systematically probe neural circuits across scales, ultimately aiming to make computational models of how neural systems work.

CONFLICT OF INTEREST

ESB is an inventor on multiple patents on the technologies described, as well as a co-founder of companies seeking to pursue medical applications of 40 Hz oscillations to Alzheimer's disease, and of expansion microscopy to medical diagnosis.

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