Imaging the Neural Symphony

By Karel Svoboda, Ph.D.

Editor’s Note: Since the start of the new millennium, a method called two-photon microscopy has allowed scientists to peer farther into the brain than ever before. Our author, one of the pioneers in the development of this new technology, writes that “directly observing the dynamics of neural networks in an intact brain has become one of the holy grails of brain research.” His article describes the advances that led to this remarkable breakthrough—one that is helping neuroscientists better understand neural networks.
South African biologist and Nobel Prize winner Sydney Brenner once said “progress in science depends on new techniques, new discoveries, and new ideas—probably in that order.” The mammalian brain is a case in point. It is an incredibly complex organ, and major new insights into its function have typically followed on the heels of novel scientific instruments and new methods. To illustrate the brain’s complexity, scientists often use numbers: One cubic millimeter of gray matter, about the size of a grain of rice, contains 100,000 of neurons (the overall number of neurons in the brain is similar to the number of stars in the Milky Way.) Each of these neurons connects to approximately 1,000 other neurons at synapses, tiny communication channels between brain cells.

But numbers alone do not do justice to the brain. Neurons fall into multiple classes, a dozen or more clustered in each of the approximately 1,000 regions of the brain, and they take elaborate shapes. They sprout large tree-like appendages at both ends—dendrites, which receive inputs, and axons, which send outputs. These diverse neuron types hook up in extensive and intricate neural networks that link our senses to our musculature and produce intelligent behavior.

Similar to digital computers, these neural networks use electrical signals to process information. Neural computations can happen faster than the blink of an eye (after all, the blink reflex is caused by electrical signals zipping through a three-neuron chain), so that we can act quickly in a rapidly changing world. However, the brain also accumulates information gradually as we learn. Our memories allow our brains to construct a model of the world, which influences quick, millisecond processing. Memories can last a long time; for example, most of us remember our first day of school or our first kiss. This is because neurons process information over times of milliseconds to years.

Meanwhile, our abilities to navigate dynamic environments and store information are based on a sort of symphony of electrical signals that neurons produce, each with its own melody, timbre, and rhythm. Each neuronal “tune” organizes into orchestras, which ultimately conduct our perception of the world and our actions within it. Until the turn of the century, scientists had only one option to interrogate neurons in the intact brain—insert a wire into the brain, close to a single neuron. But the isolated electrical readout from a single neuron is a pale reflection of the grander symphony.
Even using a far more elaborate setup, such as magnetic resonance imaging (MRI), one of only a few techniques routinely used in humans, only shows average activity in large regions of the brain, not nearly detailed enough to reveal neuronal signals. Directly observing the dynamics of neural networks in an intact brain has become one of the holy grails of brain research.

Now, through a confluence of optical physics, protein engineering, and molecular biology, it is possible to observe directly the neural symphony in the intact brain. A method called two-photon microscopy allows researchers an unprecedented view of the living brain in action, from individual synapses to entire neurons and neural circuits.

**Imaging Neurons: Why Two Photons Are Better than One**

Neurons and their synapses are microscopic. To study them, scientists often use biological microscopes that utilize a molecular process called fluorescence to generate detailed images. In fluorescence microscopy, scientists add special molecules to the tissue, either as chemicals or by reprogramming the genomes of cells to coax them into producing fluorescent proteins. Samples are then illuminated with colored light that is absorbed by the fluorescent molecules, which, in turn, emit fluorescence. The molecules absorb the high-energy illumination photons, or particles of light, and then in response emit lower-energy fluorescence photons (Figure 1). The fluorescent substances can then be detected with exquisitely sensitive electronic sensors. Fluorescence microscopy produces clear images of thin samples, such as brain sections or neurons growing in a dish.

![Figure 1](image-url). Diagrams illustrating different modes of fluorescence. Left, standard fluorescence. A blue illumination photon excites a green fluorescent molecule (green circle). The molecule subsequently emits a green photon. Right, two-photon excitation of fluorescence. Two infrared illumination photons combine together to excite the green fluorescent molecule. The infrared photons have to arrive nearly simultaneously for two-photon excitation to occur. The molecule subsequently emits a green photon, identical to the standard case.
The situation is a lot more challenging for imaging neurons in the intact, living brain. Because the brain’s gray matter is so densely packed with cell bodies, neurites, supporting cells, and blood vessels, traditional microscopy falls short of capturing clear, useful images. The brain is as impenetrable to light as a glass of milk, and mostly for the same reasons: When visible light enters the brain, myriad refractile structures randomly deflect it, causing the images to lose contrast and appear hazy or distorted.

Fortunately, a different method, called two-photon microscopy, has provided a solution to the opacity problem. Using the weird physics of quantum mechanics and extremely intense illumination, two-photon microscopy produces crisp images, even in living tissue. The key difference with respect to standard microscopy is the type of illumination light. In two-photon microscopy, invisible infrared light is used to excite fluorescent molecules in the sample, whereas in standard microscopy visible light excites the same types of molecules. In both cases, the resulting excited molecules emit light that we can see. Because infrared photons have lower energy than visible photons, two infrared photons have to effectively combine into one to be energetic enough to excite the fluorescent molecule, resulting in “two-photon” excitation of fluorescence (Figure 1). This strange phenomenon was predicted 85 years ago by the German physicist Maria Goeppert in her doctoral thesis.¹

Because the process of two-photon excitation of fluorescence is extremely inefficient, producing images for microscopy in reasonable time requires illumination light more intense than that on the surface of the sun. A special property of lasers is that they can be focused to a uniquely tiny spot, boosting the concentration of photons, or light intensity. For this reason, two-photon excitation was observed experimentally only after the laser was invented in 1961.

Despite the groundbreaking invention of the laser, standard laser light still was not sufficiently bright for two-photon microscopy. It took about another 20 years to develop specialized lasers that produce trains of extremely short pulses of light (one tenth of a trillionth of a second). These potent laser beams effectively concentrate light in time and, at the point of focus of the two-photon microscope, produce a brightness that exceeds all other light intensities in the known universe.
Aside from the cutting-edge lasers, the remaining mechanics of two-photon microscopy are fairly simple. The laser beam is shaped to a tiny point and scanned in three dimensions over the specimen of interest. Then the fluorescence emitted by the sample is collected as a signal. This fluorescent signal, which represents a single point of the sample illuminated by the laser, is converted into a pixel, showing the corresponding location in the image. Thus the final, complete image is built one laser focal point and corresponding pixel at a time. Though it sounds painstaking, the lasers can move over the brain at high speeds, like a laser light show, producing images at rapid rates, comparable to a motion picture.

Where It Began
Two-photon microscopy was born more than two decades ago at Cornell University, where experts in microscopy and laser physics worked under one roof. But before it took off, many scientists were skeptical about its use in biology, surmising that the kind of extremely intense light was unsuitable to image living biological specimen. On top of that, the high cost and complexity of the early lasers presented a formidable obstacle to biologists. Most importantly, biologists had yet to find a standout application for two-photon microscopy.

The skepticism evaporated with developments in the mid-1990s at Bell Laboratories. Winfried Denk, one of the inventors of two-photon microscopy, and his collaborators noticed they could actually use the technique to image thick tissues while preserving the wonderful properties of fluorescence microscopy: high spatial resolution and high contrast. Two-photon microscopy is advantageous for imaging thick tissue for multiple reasons. First, the infrared excitation light travels much farther in tissue without having its path perturbed, so the tiny laser focus can be maintained much deeper for imaging. Second, if tissue deflects a photon, it simply bounces out of the brain without producing fluorescent background noise. Third, fluorescence photons can be gathered efficiently to produce a bright signal. And, as it turns out—rather remarkably—the intense infrared excitation light is harmless to biological tissue. As a result, two-photon microscopy produces spectacularly detailed images of living neurons in intact nervous systems (Figure 2).
Lighting Up the Brain

By itself the brain is nonfluorescent and appears black when viewed under a fluorescence microscope. Visualizing neurons with a two-photon microscope requires that fluorescent substances be added to the brain. Biologists had figured out how to make many distinct parts of neurons fluorescent, for example using antibodies with attached fluorescent molecules, but these methods were not usable in the intact brain. At Bell Laboratories in 1997, Denk, David Tank, David Kleinfeld and I produced the first clear images of neurons in the intact brain using two-photon microscopy. We threaded a tiny glass tube into the brain and injected fluorescent dye into the cytoplasm of a single neuron (similar to Figure 2). Trapped inside the neuron, the dye spread throughout the cell’s axons and dendrites, making it light up like a Christmas tree. Using two-photon microscopy, we saw the neuron in its native habitat in exquisite detail.

But injecting one neuron at a time in an intact brain is a difficult and tedious procedure, and it often disrupts the targeted neuron. Introducing fluorescent substances to living neurons in the intact brain in an efficient and nondestructive manner created a new technical challenge. An unexpected solution emerged from the sea when scientists discovered a naturally occurring fluorescent protein in crystal jellies, corals, and anemones. Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien received a Nobel Prize in Chemistry in 2008 for their “discovery and development of the green fluorescent protein (GFP).” These proteins are fully encoded by DNA sequences, meaning that they
can be manipulated using the tools of molecular biology. Scientists now modify the genomes of neurons, or infect neurons with engineered viruses, to coax them into producing fluorescent proteins. Though GFP is the most famous of the fluorescent protein family, others can glow blue, orange, or red. The combination of two-photon microscopy and fluorescent proteins has made imaging in the intact brain routine.

**Imaging Neuronal Structure**

Since the early days of brain research, scientists thought that neurons changed structurally during long-term memory formation. While experiments in invertebrate nervous systems suggested that simple memories are encoded in the growth of new synaptic connections between neurons, no evidence suggested the same for complex mammals, such as humans. Imaging tools weren’t sophisticated enough to test the hypothesized changes. In 2002, Brian Chen, Josh Trachtenberg, and I, then working at Cold Spring Harbor Laboratory, decided to image the same neurons in the brains of mice for a period of months. We used mice with modified genomes in which one hundredth of one percent of neurons expressed GFP. The labeled neurons clearly stand out in two-photon microscopy, with virtually no background from the unlabeled neurons. The images were sufficiently crisp that we were able to track changes in even the smallest neuronal structures (Figure 3).

![Figure 3](image)

*Figure 3.* Long-term imaging of neuronal structure in the intact brain. Left, view of the dendritic arbor of a single neuron expressing green fluorescent protein. The fluorescent striations on the right are the surface of the brain. Yellow boxes correspond to the zoom-in on the right. Right, two high-magnification time-lapse sequences showing structural changes at the level of dendritic spines. Yellow arrows indicate persistent spines, orange indicate new spines, and green indicate lost spines. Data from Holtmaat et al., *Nature*, 2006.
The dendrites of most neurons, for example, are studded with tiny protrusions called spines, about as long as one hundredth of the width of a human hair. Spines harbor the synaptic inputs to neurons and bridge the gap across neurons, from dendrites to axons. A series of time-lapse images captured over days and weeks revealed that a subset of the spines appear and disappear, connecting and disconnecting neurons. During the experiment, we would change the experience of the animals through behavioral training; for example, mice had to use their whiskers to distinguish, progressively, differences in locations of an object. We saw the rate of new connections increase as the mice learned new tasks, but only in the parts of the brain relevant to learning and with a time course similar to learning. What’s more, mutant mice with learning deficits showed reduced ability to make new connections. These experiments, and many others like them, have provided support for the hypothesis that structural changes in neural networks support information storage in the brain.6

These long-term imaging experiments revealed structural changes at the level of synapses, but from a broader perspective, they also indicated remarkable structural stability. For example, the dendrites on which the spines sit persist over the entire life of the animal; this is also largely true for the axons. It’s likely that, in the dense mesh of intermingled axons and dendrites, those nearby one another are neighbors for life. Between neighbors though, these synapses fluctuate between formation and elimination to support the making of new memories.

**Imaging Neural Activity**

In the neuronal symphony, each neuron’s tune is made up of sequences of electrical signals called spikes, which represent information. Through synaptic communication, these spikes influence the pattern of spikes in other neurons. Each neuron produces a characteristic pattern of spikes, like each instrument in a gigantic symphony orchestra. The flow of spikes through large collections of connected neurons underlies the processing of information in the brain. Imaging the structure of neurons alone is a bit like analyzing the shapes of the instruments in a symphony orchestra: suggestive and informative, but not sufficient to perceive the music. Recently it has become possible to use two-photon microscopy, together with fluorescent probes of calcium, to measure spikes in large numbers of neurons simultaneously and literally produce movies of the neural symphony in action.
Neurons have specialized mechanisms to keep their calcium levels very low, and, for reasons we do not yet understand, neuronal spikes cause rapid and large calcium changes in neurons. Scientists find calcium, a ubiquitous intracellular signal in most cells, an alluring target to monitor. Shortly after accomplishing fluorescent protein cloning, protein engineers started tinkering with DNA sequences to make fluorescent proteins that change in response to calcium binding. In the past decade, a variety of calcium-reporting proteins have been iteratively fine-tuned in efforts to image the spikes found in neural populations. The GENIE project at Howard Hughes Medical Institute’s Janelia Research Campus, a highly collaborative project involving multiple investigators, solely strives to engineer the next generation of genetically encoded indicators for neuronal function. The GENIE project’s “GCaMP6” proteins, which consist of GFP fused with the calcium-binding protein calmodulin, have proved particularly useful. Upon binding to calcium, GCaMP6 becomes fifty-fold brighter. Quiescent neurons expressing GCaMP6 are essentially black but produce bright flashes of green fluorescence immediately after a spike. Using two-photon microscopy, researchers can readily image these temporal changes in fluorescence and interpret them in terms of spike trains (Figure 4). GCaMP6 is so sensitive that it can track activity in thousands of neurons when imaged using two-photon microscopy.

![Figure 4. Left, image of the neurons with schematic of a pipette (red) that is used to directly record spikes. Right, comparison of fluorescence changes (top) and spike trains (bottom) for the neuron. Numbers below the spike train indicate the number of spikes in a burst; asterisks indicate single spikes. Data from Chen, Wardill et al., Nature, 2013.](image)

In the past decade, imaging neural activity has progressed into a critical method in brain research. GCaMP6 is used by more than one thousand laboratories worldwide to study fundamental questions in neuroscience, but also to understand mechanisms underlying neurodevelopmental and
neurodegenerative disorders. Now, imaging large populations of neurons while animals perform behavioral tasks is routine. Researchers are even combining two-photon imaging simultaneously in multiple brain regions with virtual reality, in which mice might navigate a visual or tactile environment in search of a reward (View video). Through statistical analysis of activity patterns in neurons, movies of GCaMP6-expressing nerve cells are beginning to reveal the relationships between behavior and activity of specific neurons. In this way, scientists are learning how spike trains represent information in the brain and how these representations change with learning.

What’s Ahead

Brain research, which relies on progress in computation, electron, and light microscopy, as well as DNA and RNA sequencing, molecular biology, genetics, chemistry, and applied physics, is entering a rapid phase of discovery. Research methods are evolving and combining in interesting ways to boost brain research. Rapid sequencing methods developed for cancer biology now measure gene expression in single neurons and are beginning to provide a catalog of the brain’s neuron types. New anatomical methods derived from virology enable a mapping of the connections between those neuron types. Optogenetic reagents allow scientists to manipulate the electrical signals in neurons with light and evaluate how these neurons contribute to animal behavior.

The story of two-photon microscopy illuminates several points with respect to technology development and scientific discovery. New technologies ring in rapid phases of discovery. Often these technologies bring together multiple disciplines in unexpected ways. Two-photon microscopy would have struggled to take off without the boost of laser technology. And without the cloning and engineering of GFP, imaging the intact brain would have remained an artisanal pursuit of interest to relatively few biologists. Finally, so-called “incremental” improvements to technology can open up new vistas and shape science just as much as completely new advances. Multiple rounds of quantitative improvements to GFP-based sensors ultimately made the once unattainable holy grail of brain research—images of neural activity in the living brain—possible in many laboratories throughout the world.
Bio

Karel Svoboda, Ph.D., who was born in the Czech Republic, received a doctoral degree in biophysics from Harvard University and performed his postdoctoral work at Bell Laboratories. From 1997 to 2006, he was a professor at Cold Spring Harbor Laboratory, and he has been a Howard Hughes Medical Institute (HHMI) investigator since 2000. At Cold Spring Harbor Laboratory, the Svoboda lab developed and exploited methods to track synaptic transmission and plasticity at the level of individual connections, even in the intact brain. In 2006, the Svoboda lab moved to the newly established HHMI’s Janelia Farm Research Campus, where Svoboda is a group leader. His current work focuses on how neocortical circuits produce our perception of the world and our actions within it. He also has a long-standing interest in new biophysical and molecular methods for brain research. In 2015, he was awarded the Brain Prize from the Grete Lundbeck European Brain Research Foundation. Svoboda is a member of the National Academy of Sciences (USA).

References

1. Masters, B. R. and P. T. So (2004). "Antecedents of two-photon excitation laser scanning microscopy." Microsc Res Tech 63(1): 3-11.
2. Denk, W., J. H. Strickler and W. W. Webb (1990). "Two-photon laser scanning microscopy." Science 248: 73-76.
3. Denk, W. and K. Svoboda (1997). "Photon upmanship: why multiphoton imaging is more than a gimmick." Neuron 18: 351-357.
4. Tsien, R. Y. (1998). "The green fluorescent protein." Annu Rev Biochem 67: 509-544.
5. Trachtenberg, J. T., B. E. Chen, G. W. Knott, G. Feng, J. R. Sanes, E. Welker and K. Svoboda (2002). "Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex." Nature 420(6917): 788-794.
6. Holtmaat, A. and K. Svoboda (2009). "Experience-dependent structural synaptic plasticity in the mammalian brain." Nat Rev Neurosci 10(9): 647-658.
7. Chen, T. W., T. J. Wardill, Y. Sun, S. R. Pulver, S. L. Renninger, A. Baohan, E. R. Schreiter, R. A. Kerr, M. B. Orger, V. Jayaraman, L. L. Looger, K. Svoboda and D. S. Kim (2013). "Ultrasensitive fluorescent proteins for imaging neuronal activity." Nature 499(7458): 295-300.
8. Peron, S. P., J. Freeman, V. Iyer, C. Guo and K. Svoboda (2015). "A Cellular Resolution Map of Barrel Cortex Activity during Tactile Behavior." Neuron 86(3): 783-799.
9. Zeisel, A., A. B. Munoz-Manchado, S. Codeluppi, P. Lonnerberg, G. La Manno, A. Jureus, S. Marques, H. Munguba, L. He, C. Betsholtz, C. Rolny, G. Castelo-Branco, J. Hjerling-Leffler and S. Linnarsson (2015). "Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq." *Science* **347**(6226): 1138-1142.

10. Luo, L., E. M. Callaway and K. Svoboda (2008). "Genetic dissection of neural circuits." *Neuron* **57**(5): 634-660.

11. Deisseroth, K. (2011). "Optogenetics." *Nature methods* **8**(1): 26-29.