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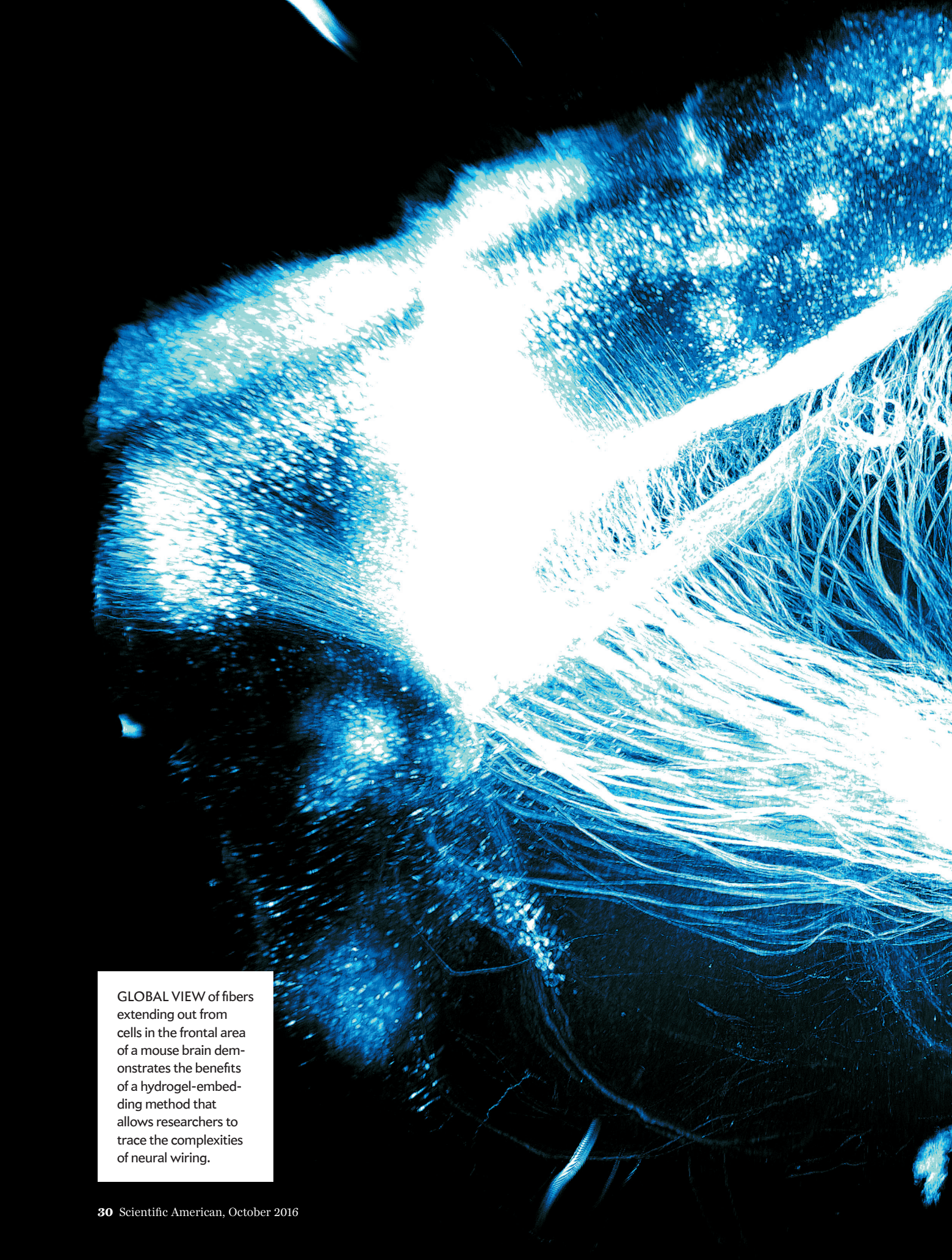
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SEE- THROUGH BRAIN

A new look at how
we're wired to think

SPECIAL REPORT
STATE OF THE
WORLD'S
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— 2016 —



GLOBAL VIEW of fibers extending out from cells in the frontal area of a mouse brain demonstrates the benefits of a hydrogel-embedding method that allows researchers to trace the complexities of neural wiring.



NEUROSCIENCE

A LOOK INSIDE THE BRAIN

A NEW EXPERIMENTAL APPROACH AT
THE INTERFACE OF CHEMISTRY AND
BIOLOGY LETS SCIENTISTS PEER
INTO THE DEEPEST REACHES OF THE
BODY'S MASTER CONTROLLER

By Karl Deisseroth

Karl Deisseroth is a professor of bioengineering and psychiatry at Stanford University. He was the recipient of the 2015 Lurie Prize in Biomedical Sciences for the development of CLARITY and optogenetics.



OUR NERVOUS SYSTEM is like a tapestry of sorts, woven with interconnecting threads. These threads, the thin fibers known as axons that extend out from neurons, carry electrical information from individual nerve cells to other neurons that receive the signals. Long-range projecting axons, like the structural “warp” threads in a textile, interweave with

the brain’s own version of crossing, or “weft” fibers: axons that wind back and forth over short distances, transmitting signals to perform computations.

To understand the inner workings of the brain, scientists need to decipher how this neural tapestry is organized at the level of individual elements, such as an individual axon. But to understand the role of an axon, we would also like a global perspective spanning the entire brain that somehow does not lose sight of the single, threadlike axon and its context. To gain such a view, one needs a special kind of tool because the brain is not flat like woven cloth, nor is it transparent. Fat molecules (lipids) throughout the brain, particularly in cell membranes, cause light from imaging devices to scatter and thus greatly hinder our view beyond the most superficial layer of cells into the profound depths of the brain.

Now a new technology has opened exciting vistas for neuroscientists, creating a way to see into the intact brain—and to both determine the trajectories and define the molecular properties of individual connecting fibers that weave through the brain’s intricate inner workings. This method is built on the chemistry of hydrogels, polymers that form a three-dimensional network of connected compartments able to retain water

without dissolving. It is used to create 3-D polymer endoskeletons within biological tissue. In this three-step process, a transparent gel is first formed within the laboratory animal or post-mortem human brain itself, linked to and thus protecting the brain’s key information-rich molecular parts, including proteins and nucleic acids (DNA and RNA). This step is followed by the removal of the tissue components that are not of interest or that scatter light, such as lipids. Finally, by introducing a multitude of fluorescent labels and other markers throughout this structure—in addition to being transparent, the gel is designed to allow fast infusion of these probes—scientists can light up and directly visualize diverse fibers and molecules of interest at very high resolution throughout the intact brain.

This newfound ability to see into the depths of the body’s master controller is leading to numerous insights. Scientists are using this approach to link physical form with behavioral function of neural pathways involved in action and cognition, ranging from movement to memory. This method has also helped elucidate processes that contribute to parkinsonism,

IN BRIEF

The brain’s inner workings will only yield themselves to neuroscientists through close inspection of individual cells combined with large-scale surveys of the entire organ.

Optical imaging methods in routine use cannot penetrate the opaqueness of brain tissue because of light scattering at the interfaces of water and the fat molecules in cell membranes.

New techniques that remove lipids and replace them with a substance that holds brain parts in place furnish a window to gaze past the typical barriers that block an internal view.

Hydrogel-embedding methods, as these techniques are called, allow researchers to examine the wiring of specific neural circuits that control various behaviors.

Alzheimer's disease, multiple sclerosis, autism, drug abuse, and fear and anxiety disorders. We even helped start a company to explore tissue-hydrogel applications for cancer diagnosis. This method is now being applied beyond the brain to diverse organs and tissues across the entire body.

GOING CLEAR

MAKING A SEE-THROUGH BRAIN is so difficult that even evolution, over hundreds of millions of years, has not achieved that feat in the lineage of large animals. Invisibility, of course, could provide major advantages, and some species have been evolutionarily selected for a certain amount of transparency to adapt to their environment (for example, to avoid predators). Certain fish even lack the reddish hemoglobin protein, essentially doing without blood as most vertebrates know it and thus achieving a modicum of invisibility. Yet even these animals cannot seem to make their central nervous systems transparent, despite intense evolutionary pressure. In partially transparent fish or shrimp, nervous systems remain at least partially opaque; evolution can go even as far as giving up on red blood cells, but nothing, it seems, lets light move unimpeded through a large living brain.

This opaque quality results from light being scattered in neural tissue. Photons bounce off interfaces of fat and water (because of differences in the rate at which light travels in the two substances) and in seemingly random directions (because of the structural complexity of neural wiring). This effect cannot be easily engineered or evolved away. The lipid barriers that constitute cell membranes and internal structures within a brain cell also play key roles as insulating material for the ions that mediate the flow of electrical impulses along intricately intertwined axons. Ironically, the organ that biologists most need to keep intact to understand is also the one that we have been least able to render transparent.

In 2009 I turned to the unresolved challenge of making the intact, mature, mammalian brain transparent—while still allowing detailed labeling of diverse molecules within. By then, hundreds of labs around the world had begun using a technology my colleagues and I had developed between 2004 and 2009 for turning specific brain circuit components off and on with light. The technique, called optogenetics, combines lasers, fiber optics and genes for light-sensitive proteins called microbial opsins from algae and bacteria to control neural activity precisely in specific cells within whole living brains as animals run, jump, swim, socialize and carry out complex behaviors. By the summer of 2009, five years after the initial July 2004 experimental demonstration with microbial opsins in neurons, key challenges in optogenetics were largely resolved, and the technique could be easily and generally applied. Although thousands of new insights on the causal neural mechanisms of behavior have since been discovered with this method, optoge-

netics alone cannot provide another key type of information: a high-resolution picture that furnishes insight into the brain-wide wiring of the individual cells being controlled by light.

Linking the big picture of a system to its individual basic components is an aspiration common to many fields of science, although this goal often (and appropriately) gets sacrificed. Separating out the individual parts of a complex system for isolated analysis has always been essential to science because removing a component from its context allows one to determine which properties are intrinsic and do not depend on other elements. But for a richly interconnected structure such as the brain, taking the system apart, like separating all the threads of a tapestry, is not always the best strategy for understanding and appreciating the big picture.

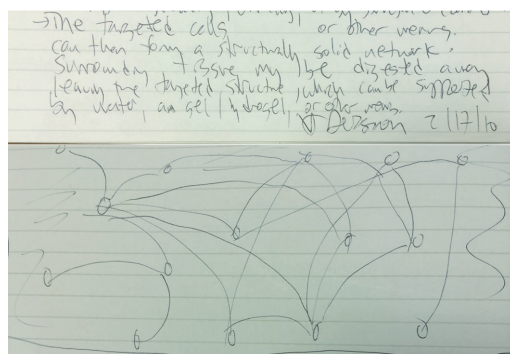
For visualization and labeling, the opaque nature of adult mammalian brains had long dictated the necessity for disassembly, typically via slicing the brain, thus turning the three-dimensional volume of tissue into hundreds or thousands of virtually two-dimensional slices. This process consumes prohibitive amounts of time and expense, especially when many brains are required to produce meaningful statistical results (as is common in the study of mammalian behavior). Moreover, key information is irreversibly lost. Because, with optogenetics, we were already building new functionality within the intact brain, in 2009 I began to consider what else we could build within a brain to help us with this problem.

The seed of the idea had been planted 15 years earlier. In the mid-1990s I had become intrigued with the idea of trying to build brainlike circuits in the lab, start-

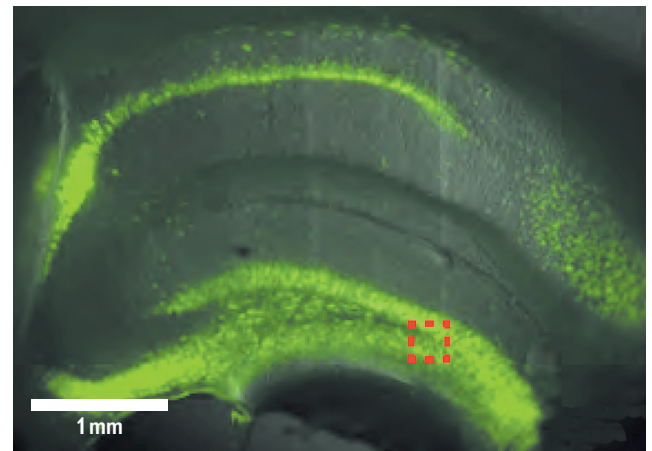
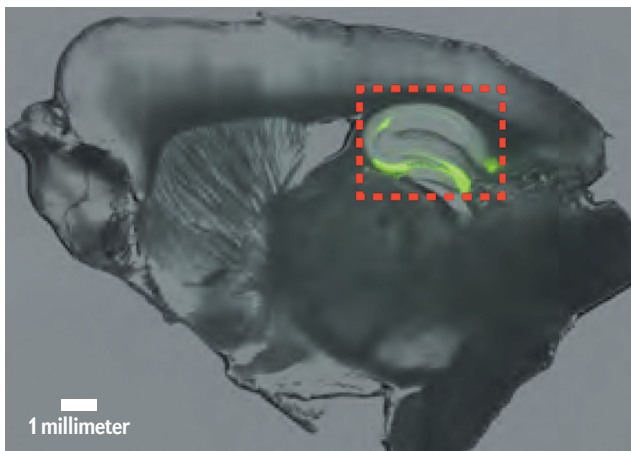
ing from individual cells. One way to do this might be by seeding neural stem cells onto polymer scaffolds, where they could be biochemically coaxed to turn into neurons. In pursuing this effort, I had delved into the science and engineering literature of hydrogels that appeared to be particularly appealing as scaffolds by virtue of their biocompatibility and transparency.

In later years I would eventually carry out only simple pilot experiments, seeding stem cells onto polymeric scaffolds and turning them into neurons, but I never got to the point of making an intact brainlike structure from single cells—a devilishly challenging undertaking. Still, I dutifully lugged my increasingly dusty folder of carefully stapled papers labeled “hydrogels” as I moved from lab to lab during the next 15 years and from step to step in my career (receiving my Ph.D. in neuroscience in 1998, completing my psychiatry residency and postdoctoral fellowship, and launching my engineering lab at Stanford University in 2004). But the mental scaffolding was in place, and the idea took root and eventually evolved, with the critical involvement of some amazingly talented people in the lab, into a workable strategy for building a transparent and accessible brain.

A sketch I made in February 2010, while sitting at my desk



INITIAL SKETCH in the author's lab notebook in early 2010 traces the idea for building a hydrogel in tissue and removing other components.



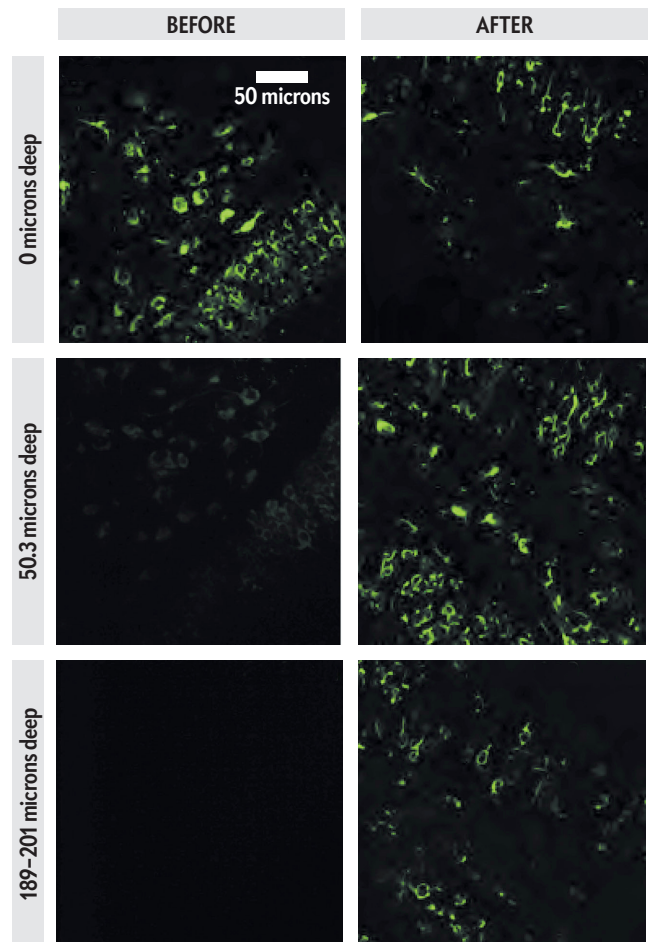
after a long period of considering the problem of brain-wide visualization, depicted the basic idea [see illustration on preceding page]. It was the initial concept turned on its head—instead of starting from a hydrogel and building a brain within, we would start from a brain and build a hydrogel within. The hydrogel would serve as a support structure and preserve spatial placement of brain components we cared about, such as proteins and nucleic acids, but allow removal of everything else that kept us from seeing deep within. It would, meanwhile, prevent the brain from collapsing into a shapeless soup as structural but less interesting components were dissolved or digested away.

The very first experiments, which bridged separate fields and brought initial tentative shape to what had been mere possibility, can be best appreciated years later with the broad perspective that passage of time brings. Two creative and courageous researchers then at the lab—Viviana Gradinaru and lab manager Charu Ramakrishnan—were the first ones willing to take on this daunting project. The risk of failure was so high that I decided not to involve the whole group; I thought that these two experienced researchers (who had been very successful already with other projects) could handle the risk and disappointment if the project ultimately did not work out.

Beginning in early 2010, Gradinaru and Ramakrishnan sought to make neurons invulnerable to damage from the agents that would disrupt fine tissue structure and cell membranes. In theory, filling brain cells with a durable polymer of some kind might do the trick, and the neurons would then remain intact if supported by the hydrogel. The two tried a number of strategies, including the introduction of genes encoding certain enzymes to allow neurons to manufacture durable polymers such as chitin and cellulose. The best approach, a creative idea from Gradinaru, turned out to be a process to make another biopolymer, keratin, inside cells. She had shown that keratin in cultured neurons could protect cell structure from disruption and reasoned that for intact brain tissue (with the neurons stabilized with keratin and hydrogel added for external support) the lipids might be washed out with detergent to reveal the targeted brain structures of interest, suspended in the transparent hydrogel.

At that point, building the hydrogel in the intact brain existed as a pure idea. I decided to make the project move more quickly by seeking deeper experience from a chemical engineer. Although no

MOUSE BRAIN embedded with a transparent hydrogel—after removal of light-scattering tissue—glows green when a fluorescent protein linked to keratin illuminates marked cells. Zooming in from a view spanning the brain (upper left), the curves of the hippocampus substructure (upper right) appear, followed by close-ups of individual cells (lower panels). Prior to implementing the CLARITY process, cells at a depth of more than 50 microns from the surface were invisible because of light scattering (left panels below). Once the process is complete, as shown in this 2010 experiment from Viviana Gradinaru, Kwanghun Chung and Charu Ram.



COURTESY OF DEISSEROTH LAB STANFORD UNIVERSITY AND VIVIANA GRADINARU, KWANGHUN CHUNG AND CHARU RAMAKRISHNAN

one outside the lab knew of the project, I searched my in-box for e-mails from prospective postdoctoral fellows who might have the right background in hydrogels. The name of Kwanghun Chung, a remarkably talented chemical engineer, then at the Georgia Institute of Technology, came up. Chung had heard of our optogenetics and stem cell work and was interested in joining the lab.

In early March 2010, only a few weeks after making my original sketch shown in the illustration on page 33, I set up our first brief conversation over the phone while I was at a meeting in Utah. Then I did something that I had never done before (or since) because I was so sure about this new direction. I invited Chung to join our team without even a lab visit or face-to-face interview. Strange times for a neuroscience lab—a chemical engineer appearing out of nowhere.

On his arrival, Chung launched immediately into the under-the-radar project. By the end of 2010 the three-member team in my lab had created transparent blocks of a mouse brain in which the preserved keratin-containing and hydrogel-embedded cells could be seen clearly, even hundreds of microns deep within tissue, a far greater depth than would have been possible using existing methods [*see illustration on opposite page*]. The first fully functional hydrogel that Chung produced was based on acrylamide, commonly used in the lab to separate nucleic acids or proteins. The gel-tissue hybrids produced from this creative work were designed so that we could introduce fluorescent markers and other labels directly to visualize preserved proteins and structures, such as axons, over many rounds of labeling, and we found that we no longer needed a keratin component to keep cellular structures in place—the hydrogel alone was enough. Despite pioneering work with other approaches from Hans-Ulrich Dodt and Atsushi Miyawaki (the 3DISCO and Scale methods, respectively), such transparency and accessibility in the adult mammalian brain had not been previously achieved.

This particular acrylamide-based variant of the hydrogel-built-in-tissue idea (there are now many other published variants) was named CLARITY (for clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel). Since our 2013 publication of the technique, even this single version of the tissue-hydrogel technique has been adopted for diverse basic science applications and also applied clinically (for example, to postmortem brains of individuals with autism or Alzheimer's), as well as to spinal cords and brains of mice (for example, in discovery of previously unknown pathways for control of fear and anxiety behavior). Many papers from labs around the world have now been published using this general approach to understand the basic structure of the nervous system, often in combination with optogenetics, and to provide fresh ideas for understanding adaptive and maladaptive brain circuitry.

Just as the first five years of optogenetics with microbial opsins brought forth numerous innovations enabling broad applicability of that method, the technique for building tissue-hydrogels inside

brains has likewise advanced dramatically over the first few years of that method's existence. For example, the earliest version of the hydrogel technique described a step with an imposed electric field to accelerate rapid clearance of electrically charged detergent particles bound to lipids. This step took some practice to master, and tissue could be damaged if the voltage had been turned up too high. To tackle this issue, beginning in early 2014 Raju Tomer, Brian Hsueh and Li Ye, all then lab members, published two papers (one co-authored with our colleagues in Sweden) defining a simplified version of this step. It became known as passive CLARITY because it does not use electric fields. Tomer and the team also described specialized brain-hydrogel imaging using a high-resolution fast form of light sheet microscopy, adapted to the unique challenges of rapidly imaging large hydrogel volumes by scanning planes—light sheets—instead of points of light.

Gradinaru and Chung were both running their own thriving labs at this point (at the California Institute of Technology and the Massachusetts Institute of Technology, respectively), each gener-

Tissue-hydrogel techniques enable access to the brain's deepest reaches, giving insight into the biology of the brain and its disorders.

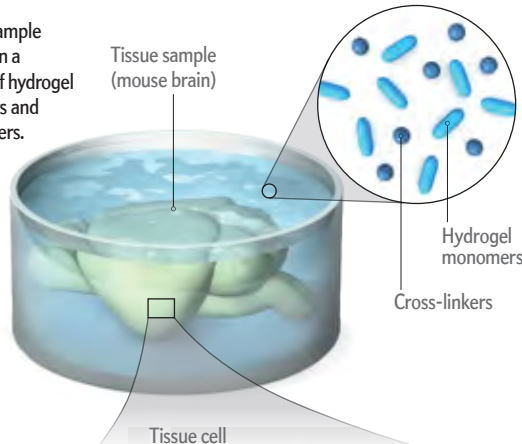
ating major new innovations. Indeed, subsequent developments have come quickly not only from these but also from many other investigators. Gradinaru independently developed and published a CLARITY strategy suited for whole organisms called PARS. Both Gradinaru and Chung published new hydrogel formulations called PACT and SWITCH, respectively, and now a large variety of tissue-hydrogel composites have been described from labs around the world. Yet when it comes to exploring possible hydrogels experimentally, we have only scratched the surface. In 2013 Chung and I disclosed a very long list of possible hydrogel variant compositions, from acrylates to alginates and beyond, and my lab and our collaborators are now exploring ways in which the polymers can even become active—modified, for example, with elements that could create tunable electrical conductivity or chemical reactivity, opening up new possibilities.

Another challenge related to a property of tissue-hydrogel composites, which, as we described in our 2013 and 2014 papers, causes the hydrogel-embedded tissues to physically expand substantially. This property of the composite is not always a problem and can be compatible with imaging at high resolution, either in the original CLARITY or in later, similar hydrogel-in-brain formulations (each with its own identifying acronym: PACT/ePACT

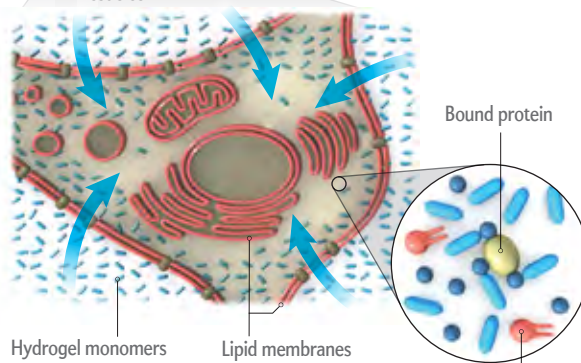
Making a Tissue-Hydrogel

Cursory sketches of a technique for making a brain transparent gradually evolved into a new chemistry-based method for creating a novel kind of material, a tissue-hydrogel hybrid that stabilizes neurons and molecules within the intact brain before removing lipids in cell membranes that prevent researchers from getting an unimpeded view. Many such hydrogel-embedding methods are now being adopted in neuroscience laboratories globally to study intact tissue in ways that were until now impossible.

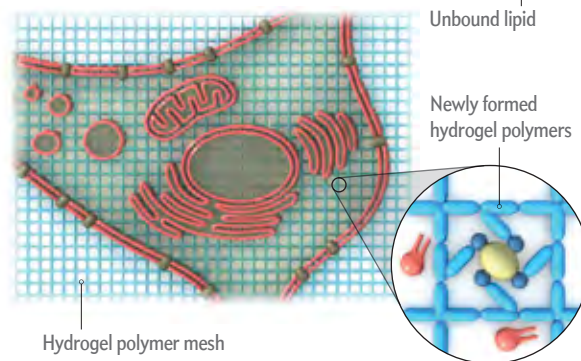
- 1** A tissue sample is placed in a solution of hydrogel monomers and cross-linkers.



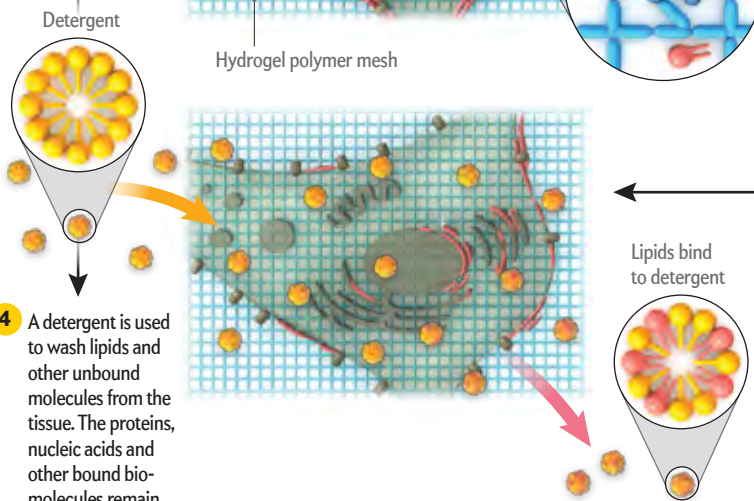
- 2** The monomers and cross-linkers diffuse into the tissue's cells and bind to biomolecules such as proteins and nucleic acids but not to the light-scattering lipids.



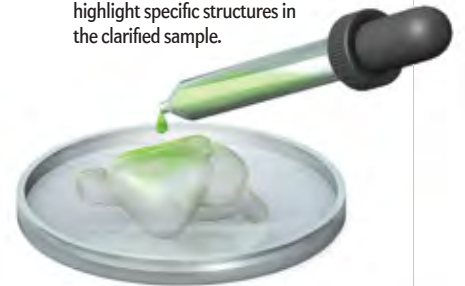
- 3** After diffusion, the temperature is raised to 37 °C, causing the hydrogel monomers to polymerize into a cross-linked mesh.



- 4** A detergent is used to wash lipids and other unbound molecules from the tissue. The proteins, nucleic acids and other bound biomolecules remain embedded within the hydrogel mesh.



- 5** If desired, antibody-based immunostaining or labeling for many nucleic acids (RNA/DNA) at once can be used to highlight specific structures in the clarified sample.



- 6** The tissue is placed in a mounting solution for imaging with a confocal or light sheet microscope or another 3-D technique.



- 7** The same detergent-mediated clarifying process can be used to wash out staining, allowing for multiple rounds of molecular labeling and imaging.

beginning in 2014, followed in 2015 and 2016 by ExM/proExM and MAP) developed by other groups that promote the basic swelling effect. But to be able to compare our transparent brains with those in academic brain atlases, which requires a precise, undisturbed rendition of the original tissue, we developed a final, optional step for shrinking enlarged tissue back to original size.

With Ye and another team member, Will Allen, my lab also developed and published high-speed and automated imaging and analysis software that can be downloaded and used by anyone. The group of our colleague Marc Tessier-Lavigne, then at the Rockefeller University and now president of Stanford, did so as well for its new iDISCO method. These two complementary papers were published in the same issue of *Cell* just this year. My group, including Emily Sylwestrak, Priya Rajasethupathy and Matthew Wright, has also been able to make a crucially important type of fluorescent labeling of many RNAs at once work reliably within intact brains using yet another tissue-hydrogel formulation, as we earlier reported in a *Cell* paper in March.

The ability to label multiple types of molecules, including nucleic acids such as RNA, turns out to be a special advantage of the hydrogel approach and opens up vast realms of gene-expression analyses. With all these challenges resolved—many of them only this year—the technique has now matured to where it is used by labs across the world.

BRINGING THE THREADS TOGETHER

IT IS REMARKABLE to look back and compare the initial humble sketch in 2010 with its fully functional implementation and integration just six years later [see illustration on pages 30 and 31]. A key goal driving this progression of the tissue-hydrogel vision has been to complement intact-brain optogenetics with intact-brain structural information—a goal already realized and reported on in several papers, including one in the June 16 issue of *Cell*. The work described in that paper focused on the brain's prefrontal cortex, a region responsible for regulating high-level cognitive processes and emotions. Scientists hope that understanding how this structure controls such diverse behaviors may provide insight into psychiatric disorders such as autism and schizophrenia.

With Ye, Allen and Kim Thompson, all then in my group, along with colleagues in other labs, including those of Liqun Luo and Jennifer McNab, both at Stanford, my team first used optogenetics to define a cell population in the prefrontal cortex that is active during (and also controls appropriate behavioral responses to) rewarding experiences such as highly palatable food or even cocaine. We next found a complementary population of prefrontal cells for negative (aversive) experiences. And finally, using our latest tissue-hydrogel methods, we were able to show that these two different populations of cells each wire up differently across the brain—the positive ones preferentially

send connections to a deep-brain structure called the nucleus accumbens [see illustration on pages 30 and 31], whereas the negative ones are more connected to a deep structure called the lateral habenula. In this way, the tissue-hydrogel and optogenetic approaches are allowing scientists to study intact biologi-

After creating a transparent brain, our group could look at an area called the prefrontal cortex and see how cell populations for positive and aversive experiences were wired differently.

cal tissues in consequential ways never before possible and to make headway in understanding the basic biology of health and disease.

The fullest appreciation of complex systems emerges with the ability to exchange information at both local and global scales, whether the system in question is a whole brain or an intricate tapestry. In neuroscience, enormous amounts of data can now be collected with rich and diverse detail illuminating intact-organ structure, molecular components and cellular activity. As a result, a broad yet nuanced perspective on brain function is starting to take shape.

Achieving such global perspective with local resolution is difficult—and uncommon—but it is important to meet this challenge. Emergent properties of complex systems often arise from local interactions, like the weave of a tapestry and like the process of science itself. Only with a sweeping perspective does the role of each kind of thread become clear. ■

MORE TO EXPLORE

Methods and Compositions for Preparing Biological Specimens for Microscopic Analysis. Filing date: March 13, 2013. www.google.com/patents/US20150144490
Structural and Molecular Interrogation of Intact Biological Systems. *Nature*, Vol. 497, pages 332–337; May 16, 2013.
Optogenetics: 10 years of Microbial Opsins in Neuroscience. Karl Deisseroth in *Nature Neuroscience*, Vol. 18, No. 9, pages 1213–1225; September 2015. www.ncbi.nlm.nih.gov/pmc/articles/PMC4790845
CLARITY Resources Web site: clarityresourcecenter.org

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Controlling the Brain with Light. Karl Deisseroth; November 2010.

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