The Big and the Small: Challenges of Imaging the Brain’s Circuits

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The relation between the structure of the nervous system and its function is more poorly understood than the relation between structure and function in any other organ system. We explore why bridging the structure-function divide is uniquely difficult in the brain. These difficulties also explain the thrust behind the enormous amount of innovation centered on microscopy in neuroscience. We highlight some recent progress and the challenges that remain.

A central theme of biology is the relation between the structure and function of things. By structure, we mean the physical form of something, a property that humans can apprehend by touch (if the object is big enough) or by sight. Right now, the leading edge of this effort is the field known by the general name “structural biology” but is focused narrowly on the shapes of molecules in order to provide insights into how proteins such as channels, enzymes, and transcription factors do their jobs. The x-ray crystallography approach commonly used in structural biology does not generate images per se (producing instead diffraction patterns), but with the help of computers humans can change the data into a form that is interpretable intuitively by the visual system. Indeed, all of “imaging” is a means of generating data about an object that depend on location and that are presented so they can be seen and hence interpreted by vision, our most powerful sense.

Going back in history, of course, there was a time when structural biology dealt with larger things: Discovering the functions of whole organs was center stage (1). Imaging was a critical part of the endeavor too. With the exception of the heart, which in many animals can be functionally understood without the need for magnification, most organs require microscopical analysis of structure and functional dynamics for the cellular organization (measured in micrometers) to be visible to the eye, whose limiting resolution several orders of magnitude coarser. Spanning 350 years, the microscope-enabled exploration of the cellular structure of organs has been extremely fertile.

Indeed, for all organ systems, save one notable exception, it is now pretty much settled how the structure of an organ is related to its function—the exception being the nervous system, where much progress has been made at the molecular and functional level. But notwithstanding the extraordinary insights of neurobiology’s foremost structural biologist, Cajal, our understanding of the relation between the structure and function of the brain remains primitive, especially when compared to other organ systems. There is no other organ system where so many common diseases and disorders have no known histological trace. There is no other organ system for which we still debate how many cell types there are. There is no other organ system for which one would seriously propose to image the entire organ’s volume with an electron microscope. And there is also no other organ system for which the complexity of the structure is so great that earnest arguments can be made against delving into structural analysis because such an effort might well provide an unprecedentedly gigantic, yet totally incomprehensible, mass of data.

Here, we will explore why bridging the structure-function divide is uniquely difficult in the brain. These difficulties also explain the thrust behind the enormous amount of innovation centered on microscopy in neuroscience, innovation that has been motivated by the special challenges of understanding how the brain’s functions are related to its especially complicated structure.

Problem Number 1: Immense Diversity of Cell Types

One unique feature of the brain that frustrates easy understanding of the relation between its structure and function is the immense structural and functional diversity of its cells. The nervous system of Caenorhabditis elegans is miniscule, only ~300 neurons (2), and yet nearly every single cell is unique in shape and function. In the far larger vertebrate brains, there are certainly structural classes of neurons that, although not having identical morphologies, have enough similarities within a class to be easily identifiable. The retina is a good example. With its iterated tiled structure, its cell classes are easy to recognize because they are repeated at regular intervals and have particular morphologies and molecular properties. Despite this, it is an area of active research to determine the full extent of cell-type diversity in this small part of the nervous system, because the range of cell types continues to grow as the analysis becomes more refined. Moreover, neuronal cellular architecture is so variable from one region to the next that no single area of the brain serves as a guide for anything other than itself. Few believe that learning the full extent of retinal cellular diversity will be of much use in trying to understand the diversity in the cerebral cortex.

Researchers have found ways to explore this diversity by developing imaging-based modalities to categorize cells and synapses. On the basis of the molecular peculiarities of cell types, molecular biologists have engineered means of generating tissue-specific expression of fluorescent markers in a wide and ever-growing range of neuronal cell types. In some cases the rationale is clear: The enzymes responsible for the synthesis of inhibitory transmitters are expressed only in inhibitory cells (3). In other cases, the molecular markers are useful but not well understood (4). The perhaps most powerful approach to cell classification uses antibodies. The well-known field of immunohistochemistry has had an extraordinary impact on cellular and subcellular analysis. However, there are special challenges in using antibodies to identify neuronal processes. First, antibodies do not penetrate well in thick tissues. Second, even if they did, the sheer density of epitopes makes it nearly impossible to unambiguously associate the epitopes with a particular process of a particular cell. Third, the range of markers necessary to delineate the many different cell and synapse types is extraordinarily large. These problems all have the same solution. By labeling arrays of serial sections with different antibodies (Fig. 1A), the three-dimensional (3D) distribution of an array of epitopes can be mapped in a volume and potentially assigned to different cells (5, 6). The z resolution is given by the section thickness of 50 to 100 nm, with the exciting possibility of a similar lateral resolution achievable by using one of the new super-resolution techniques (7) (Fig. 1, B and C).

Problem No. 2: Imaging Electrical and Chemical Activity

A second unique feature of the nervous system is that the structural connectivity may ultimately determine function but does not constitute a map of function. The nervous system depends on rapid reversals of membrane potential, known as action potentials, to transmit signals between one part of a neuron and another distant part and depends on smaller, slower changes in member potential at sites of synaptic contact (i.e., synaptic potentials) to mediate the exchange of information between one cell and the next. Action potentials are typically only a few milliseconds in duration, which means direct optical readout of action-potential voltage is a challenge, because, for whatever optical signal one might devise, only...
relatively few photons would likely be associated with each impulse. Detecting action potentials from many cells in a volume simultaneously would be even more difficult. Synaptic potentials can be orders of magnitude longer in duration than action potentials but are smaller in size, which poses other technical challenges. The development of small-molecule fluorescent calcium indicators, begun in the 1980s (8), showed that the rise in intracellular calcium, a consequence of neuronal activity, could in many cases serve as a substitute for the direct optical readout of action potential activity. Although in many cases it is only a rough approximation of the number of action potentials, “functional imaging,” as it is called, is a mainstay of linking neuronal function to individual neurons. Used often in conjunction with two-photon (2P) microscopy (9) and with its unique ability to image with high resolution and high sensitivity inside scattering tissue (10), calcium imaging is now performed routinely in vivo (11, 12), in awake animals that are head-fixed and virtually (13) or truly (14) freely moving. Functional imaging has received an additional boost from the development of genetically encoded calcium indicators (15), which are finally becoming competitive (16, 17) with chemically synthesized indicators in sensitivity and speed. The genetically encoded indicators have the added advantage of targetability to specific cell classes. Researchers still await equivalently powerful genetically encoded voltage sensors, because these might provide the high temporal resolution required to understand certain details of circuit function. Tantalizing new proteorhodopsin-based probes are being developed that may be useful in this regard (18). The fact that most brain structures extend over considerable volumes requires imaging into a tissue volume rather than a thin sheet, another impediment to functional imaging.

Scattering of light blurs wide-field images and strongly attenuates signals based on confocal detection. The attenuation is much less for signals based on 2P excitation. Nonetheless, imaging deep into volumes remains an area requiring new innovations. Penetration toward the deeper layers of cerebral cortex has been shown to be aided by combining 2P microscopy with adaptive optics (19, 20), amplified pulses (21), and longer excitation wavelengths (22). But at best, these approaches can extend the depth to about 1 mm. The imaging of subcortical structures requires the removal of overlying brain tissue (23) or the insertion of penetrating optical elements such as gradient-index lenses (24).

One recently rediscovered approach, which has some of the advantages of 2P imaging in that it excites fluorescent molecules in a restricted optical section without excitation of fluorescence above or below that region, is the so-called “light-sheet” microscopy, in which a plane of a volume is illuminated by a narrow sheet of light and detected via a wide-field camera (25, 26). One advantage of this approach is that it can acquire images of many cells simultaneously, giving higher throughput for functional imaging than otherwise possible (27) but with less depth penetration than 2P imaging.

Activity or its suppression can be optically triggered with high time resolution in cells expressing light-activated channels or transporters with different ion selectivities (28, 29). In addition to controlling the illumination pattern, targeting to certain cell classes is possible by using the same molecular tricks used for fluorescent proteins. This allows, for example, the testing of hypotheses such as whether particular cells whose activity is correlated to a particular behavior are central or peripheral to its generation (30).

Problem No. 3: Neurons Extend Over Vast Volumes

Since Cajal’s insight that nerve cells are functionally interconnected in a directional signaling cascade, with axons playing the role of transmitter and dendrites and somata that of receivers,
it has been appreciated that neural connectivity holds the key to function. Although neuronal somata are not unusually large, a neuron’s synaptic connections are distributed all through their dendritic and axonal branches. These branches often extend through tissue volumes that are enormous when compared to the actual volume of the cell itself. A pyramidal neuron in the cerebral cortex can have axonal branches that cross to the other hemisphere or go down to the brain stem or even the spinal cord. The dendrites of one pyramidal cell may be circumscribed by a volume of nearly a cubic millimeter. All told, the length of all the branches of one such cell may exceed a centimeter in a mouse and more than a meter in human brain. Thus, fully describing the shape of a single cortical neuron could require sampling a substantial percentage of an entire brain’s volume. But if one wanted to document the sites of all its synaptic connections, the sampling would need to be done at very high resolution to identify all the fine branches containing pre- and postsynaptic sites. Indeed, if one wished to “just” image the complete cell geometry of one neuron along with the complete geometry of the set of all the neurons that are directly pre- and directly postsynaptic to it, that volume would probably require imaging neuronal processes that span nearly the entire brain volume. As already mentioned, seeing into a volume of brain, even in fixed material, is a long-standing challenge. Indeed, the confocal microscope was invented because of attempts to peer into a tissue block of Golgi-stained cerebral cortex, where out-of-focus and scattered light undermined a clear view (31). Recent progress with clearing agents may be quite useful as we try to understand ever larger volumes of brain (32, 33).

But is it possible to image such a large volume at very high resolution? In general in imaging, there is a tradeoff between volume and resolution: Large-volume imaging is done with low spatial resolution techniques, and high spatial resolution is accomplished over small volumes. Magnetic resonance imaging (MRI) can provide an image of a whole brain with ~1-mm³ voxels, whereas an electron microscope can render the structure of a synapse impinging on a dendritic spine by using

Fig. 2. The brain is organized over sizes that span 6 orders of magnitude. (A) The macroscopic brain, at the cm scale, is organized into regions such as the lobes of the cortex, the cerebellum, the brainstem, and the spinal cord. (B) At the millimeter scale, it is apparent that each brain region has neurons arranged in columns, layers, or other kinds of clusters. Often the axons communicating between brain regions (e.g., in the white matter beneath the cortex, which is tinted pink in this drawing) are separated from the sites where neuronal somata and their synaptic interconnections reside (e.g., in the gray matter of the cerebral cortex that lies above the white matter). (C) At the 100-μm scale, it is apparent that within each region neurons extend dendrites locally and send axons into the same or more distant sites. The most common excitatory neuron in the cerebral cortex is the pyramidal cell (shown here), whose dendrites can span a cubic millimeter. (D) At the 10-μm scale, the structure of the individual branches of a neuron become apparent. Many dendrites are studded with small processes (spines) where the axons of other neurons establish excitatory synaptic connections. (E to G) The detailed structure of the brain visualized by using EM. Successively higher resolution images of the same region of cerebrum acquired from a 29-nm section imaged on tape with a SEM detecting secondary electrons. In (E), which spans 100 μm, many neuron cell bodies (N) and their exiting processes can be seen along with occasional blood vessels, such as the capillary in the bottom right (BV). In (F), more cellular detail is apparent. The dark rings are cross sections of myelinated axons, such as the one labeled MY. The small dark objects are mitochondria (Mi), and the large polygonal objects are dendrites in cross section (D). Occasional somata of neurons and glia are visible (S). Most of the remaining tissue falls into three categories: glial processes (one highlighted in blue), presynaptic terminals (one tinted red), and dendrite spines (green). (G) The synapse is shown in red at 10 times higher resolution. At 3 nm per pixel, the synaptic vesicles (SV) are visible, along with the synaptic cleft (SC) and a membranous spine apparatus (SA) in the postsynaptic dendritic spine. [Artwork by Julia Kuhl, and SEM images are from Richard Schalek, Ken Hayworth, and Bobby Kasthuri, Harvard University.]
voxels of less than 100 nm$^3$, a trillion-fold smaller volume. Thus, it is an extraordinary challenge to image large brain volumes at a resolution sufficient to find all the synapses (Fig. 2).

The solution requires the automation, even industrialization, of imaging. Once the imaging is accomplished by machines, without the need for direct human oversight of each step, it becomes possible to increase the throughput by running an instrument around the clock and potentially many imaging machines working in parallel. Automation also increases reliability, which is essential because loss of even a small amount of data can invalidate the entire data set. Over the past several years there have been a number of techniques developed to optimize the automation of imaging: optical ablation (34), optical (35) and electron microscopic (36, 37) block face imaging (Fig. 3), and increased automation of stage motion and imaging steps in both standard scanning light and electron microscopes.

**Problem No. 4: The Detailed Structure Cannot Be Resolved by Traditional Light Microscopy**

The critical details of neuronal connectivity occur at the level of the synapse. Synapses are densely packed, and in cerebral cortex synapses often connect axons thinner than 100 nm to dendritic spines whose necks are thinner still. The resolution of diffraction-limited light microscopy is insufficient even when used in conjunction with confocal detection, 2P excitation, or—for fast imaging in clear specimens—selective-plane illumination (see above), all so-called optical sectioning techniques that eliminate signals from out-of-focus structures.

Optical super-resolution techniques that truly break the diffraction barrier and rely on the strong nonlinearities of stimulated emission–based deterministic (38) or light activation–based stochastic (39, 40) molecular switching have matured in recent years (41, 42) and are beginning to engage questions about synaptic function (43) and other neurobiological problems (7, 44). Particularly important problems that are not yet fully overcome include the ability to image multiple colors and to observe dynamic processes at sub-wavelength resolution, even when they are located hundreds of micrometers below the brain’s surface. There are also other serious limitations related to the facts that the overall signal from small structures is proportional to their size and that often labeling densities, especially in vivo, are not high enough to give a sufficiently low noise image of the smallest structures. Moreover, often it is not the physics of the imaging process per se or the label density but rather intrinsic properties of the tissue, especially refractive index inhomogeneities, that distort the wave front and degrade resolution. Adaptive optics, a trick borrowed from astronomy, in which it is used to counteract the effects of Earth’s atmosphere, can improve resolution, signal, and depth penetration, in particular for in vivo imaging (19, 20). Lastly all these techniques are fluorescence-based,
which allows very selective labeling but can make us forget the structural context.

Problem No. 5: Need for Dense or Saturated Reconstruction

Our present notions of neural circuits are still largely informed by Cajal’s ideas that showed with small arrows the directional flow of information through circuits and pathways. For his insights, the intrinsic inefficiency of the Golgi stain, which labels less than 5% of the neurons, was crucial. This sparse labeling allowed him to propose correctly the law of dynamic polarization, in which axons send signals to dendrites and somata and that information is then passed along to the next neuron by a cell’s axon. But the sparse labeling and his inability to see the signs (inhibitory or excitatory) of connections undermined an understanding of how information was actually processed by the circuits. Such fundamental questions as the number of different inputs a cell receives and the number of target cells a neuron innervates remain unknown even a century later. A small muscle that wiggles the mouse ear is the only place in the mammalian nervous system for which, in some sense, the entire connectivity network is revealed to the next neuron by a cell’s axon. But the sparse labeling and his inability to see the signs may help decipher the code by which neuronal processes determine the connections. The links may be on the horizon with better detectors and new scanning strategies.

Advances in instrumentation and labeling are giving us an unprecedented flood of data, which, partly because they are not perfect, need sophisticated computer science for analysis. Computers can learn how to best analyze the data by giving them a sufficient number of examples even if we do not understand the computational function that determines whether a voxel is inside or outside a cell. In the end, artificial neural networks may help us to analyze the data with which we are then able to understand the brain.

As circuit analysis finally moves forward, serious questions concerning its utility will be raised. One obvious question concerns the variability in the structure of the brain at the synaptic level. During the study of the mouse ear muscle described above, it became clear that every instantiation of the wiring diagram was different from every other one (45). Some will take such variability to mean that nothing can be learned from doing this kind of tedious, data-intensive, and highly expensive work. Alternatively, it is likely that one could learn a great deal about the game of chess by watching one game, despite the fact that it is highly unlikely that any two games are identical. It is certainly possible that certain cellular motifs will be recognizable and will be interpretable in a number of contexts. The key may be to make successful use of orthogonal data, such as from functional imaging, to link structure to a cellular or organismal behavior. The links may help decipher the code by which neuronal connections underlie all that we do.

References and Notes

1. P. M. Motta, M. Malpighi, in VIIth International Symposium on Morphological Sciences (Liss, Rome, Italy, 10 to 15 July 1988), vol. 295.


33. H. Hama et al., Nat. Neurosci., published online 30 August 2011 (10.1038/nn.2928).


The Cell Biology of Synaptic Plasticity

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Synaptic plasticity is the experience-dependent change in connectivity between neurons that is believed to underlie learning and memory. Here, we discuss the cellular and molecular processes that are altered when a neuron responds to external stimuli, and how these alterations lead to an increase or decrease in synaptic connectivity. Modification of synaptic components and changes in gene expression are necessary for many forms of plasticity. We focus on excitatory neurons in the mammalian hippocampus, one of the best-studied model systems of learning-related plasticity.

The circuitry of the human brain is composed of a trillion (10^{12}) neurons and a quadrillion (10^{15}) synapses, whose connectivity underlies all human perception, emotion, thought, and behavior. Studies in a range of species have revealed that the overall structure of the nervous system is genetically hard-wired but that neural circuits undergo extensive sculpting and rewiring in response to a variety of stimuli. This process of experience-dependent changes in synaptic connectivity is called synaptic plasticity.

Studies of synaptic plasticity have begun to detail the molecular mechanisms that underlie these synaptic changes. This research has examined a variety of cellular biological processes, including synaptic vesicle release and recycling, neurotransmitter receptor trafficking, cell adhesion, and stimulus-induced changes in gene expression within neurons. Taken together, these studies have provided an initial molecular biological understanding of how nature and nurture combine to determine our identities. As a result, research on synaptic plasticity promises to provide insight into the biological basis of many neuropsychiatric disorders in which experience-dependent brain rewiring goes awry.

Here we focus on long-lasting forms of plasticity that underlie learning and memory. We consider, in turn, each component of the synapse: the presynaptic compartment, the postsynaptic compartment, and the synaptic cleft, and discuss processes that undergo activity-dependent modifications to alter synaptic efficacy. Long-lasting changes in synaptic connectivity require new RNA and/or protein synthesis, and we discuss how gene expression is regulated within neurons. We concentrate on studies of learning-related plasticity at excitatory chemical synapses in the rodent hippocampus because these provide extensive evidence for the cell biological mechanisms of plasticity in the vertebrate brain. Space constraints prevent us from addressing any single mechanism in depth; instead, our aim is to provide a framework for understanding the cell biology of synaptic plasticity.

Hippocampal Synaptic Plasticity

The successful study of the cell biology of synaptic plasticity requires a tractable experimental model system. Ideally, such a model should consist of a defined population of identifiable neurons and be amenable to electrophysiological, genetic, and molecular cell biological manipulations. A well-studied model system for studying plasticity in the adult vertebrate nervous system is the rodent hippocampus (Fig. 1). Critical for memory formation, the anatomy of the hippocampus renders it particularly suitable for electrophysiological investigation. It consists of three sequential synaptic pathways (perforant, mossy fiber, and Schaffer collateral pathways), each with discrete cell body layers and axonal and dendritic projections (Fig. 1). Synaptic plasticity has been studied in all three hippocampal pathways. Distinct stimuli elicit changes in synaptic plasticity that underlie learning and memory.

**Fig. 1.** Hippocampal synaptic plasticity. The rodent hippocampus can be dissected and cut into transverse slices that preserve all three synaptic pathways. In the perforant pathway (purple), axons from the entorhinal cortex project to form synapses (yellow circles) on dendrites of dentate granule cells; in the mossy fiber pathway (green), dentate granule axons synapse on CA3 pyramidal neuron dendrites; and in the Schaffer collateral pathway (brown), CA3 axons synapse on CA1 dendrites. The dentate, CA3, and CA1 cell bodies form discrete somatic layers (dark blue lines), projecting axons and dendrites into defined regions. Electrodes can be used to stimulate axonal afferents and record from postsynaptic follower cells, as illustrated for the Schaffer collateral (CA3-CA1) pathway.