Title
Tools for Analyzing, Repairing, and Simulating the Brain

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Abstract
Understanding how the brain generates complex functions like decisions and emotions, and how it can be repaired from a disease state, would benefit from tools for characterizing the fundamental components of the brain, and how they interact with one another. Towards the goal of mapping the structure and composition of the brain, expansion microscopy (ExM), a technology that physically swells preserved biological specimens through a chemical process, is opening up the possibility of scalably mapping the wiring and molecules of the brain. Towards the goal of controlling and observing the dynamical signals of the brain, genetically encoded tools such as optogenetic tools, fluorescent voltage indicators, and multiplexable signal reporters, are enabling brain circuit signaling dynamics to be perturbed and imaged with single-cell and millisecond timescale precision. Applying these toolsets in an integrated way may enable biologically accurate computer simulations of the brain to be constructed, starting with small organisms such as the worm C. elegans and the larval zebrafish, and potentially scaling to the human brain. Such simulations may be useful in exploring how specific brain components contribute to complex emergent functions, in discovering better therapeutic targets and strategies, and in generating new hypotheses regarding mysteries such as the nature of subjective experience.

Main text

1. Introduction: towards ground truth
Scientific fields, historically, have often undergone revolutions when they achieved a "ground truth" level of understanding. I here use this term to mean a sufficiently detailed description of the fundamental building blocks or primitives at hand, and the principles governing how they interact with each other, in the context of generating emergent systems of interest. Perhaps the concept of "abstraction layer" is synergistic. When "ground truth" is reached, seeking further detail (e.g., breaking down the fundamental building blocks into yet-smaller entities) is not very helpful, but coarse-graining (e.g., lumping together building blocks into larger entities) can be very detrimental. Of course, context is key – vastly different kinds of system might require different kinds of ground truth. Think of the field of chemistry. As the periodic table (the list of building blocks, namely the elements) and the description of chemical bonds and reactions (the list of interactions), emerged, it became much easier to design and analyze, chemicals - even very complex ones. The context is that of chemical reactions on earth (if you go into the middle of a star, of course, one might need to think instead about subatomic particles and their very different way of reacting with one another). Or think of physics. After the discovery of particles such as electrons and protons (the building blocks), and the understanding of quantum mechanics (the interactions), it became possible to devise powerful inventions such as computers, LEDs, and mobile communication devices. The context is that of a certain range of temperatures and size scales (for a much larger system, one might need to consider gravity as well). In both cases, an era of "ground truth" scientific understanding was followed by an era of

great capability to analyze and create, even yielding many practical and useful outcomes for everyday life. The risk associated with scientific unknowns was greatly reduced, since a fundamental description of the components and how they work together, at least in the context of interest, had become available.

Contrast this state of affairs to that of brain science. Analyzing how the brain generates complex functions like decisions and emotions, and creating therapeutic interventions to help people with brain disorders (which affect perhaps a billion people around the world), are hit-or-miss enterprises – the immense complexity of the brain, and the large number of unknowns associated with it, make such work very challenging. An idea for a project may sound good, but then it could fail, sometimes for an entirely unexpected reason. We do not have a full explanation or understanding for any brain function, such as a sensation, movement, decision, or emotion. In the clinical realm, it was reported in 2010 that brain therapies were taking ~10 years for clinical development and approval, the approval rate was meeting with >90% failure, and the cost was almost $1 billion each (Miller 2010) – and even if a therapy passed all those hurdles, often it did not work very well, and/or presented serious side effects. (These numbers, by the way, have gotten even worse in the time since, by some reports.) Why is it so hard to understand a brain function? And why is it so hard to develop a therapy for a brain disease?

The fundamental complexity of the brain is daunting – a cubic millimeter of the human brain might contain 100,000 brain cells (such as neurons; other cell types like glia also exist), with complexly shaped arborizations, connected by a billion connections called synapses. The diameter of the branches of these arborizations (axons and dendrites), and the precision of these connections, exhibit nanoscale feature sizes, making them hard to observe, especially in the context of the densely packed, spatially extensive, 3-d volume of the brain. Furthermore, different cells exhibit different molecular compositions – of the 30,000 or so genes in the human genome, different subsets of these genes are expressed, in the form of gene products (e.g., proteins, and other biomolecules), in different cells. Indeed, even different locations within a cell may use a protein in different ways, connecting it via molecular contacts to different scaffolding proteins or downstream signaling proteins, so that a given protein may serve different (and sometimes even opposite) functions in different cells, depending on the identity of its neighbors. We do not have good maps of the molecules and wiring of any brain, to date. On top of that immense molecular and structural complexity, the time dynamics of the brain is also daunting. A brain process like learning a language or generating a creative insight, or a brain disorder like Alzheimer's disease or Parkinson's disease, might develop over days, months, or even years. In contrast, the dynamic signals of the brain – for example, electrical pulses (known as spikes or action potentials) generated within brain cells during computation, and chemical exchanges between brain cells at synapses, are fast – lasting on the order of a millisecond. Furthermore, there are many other kinds of dynamic signal, mediated by the aforementioned very long list of gene products – biomolecules can change conformation, bind to one another, and undergo other chemical interactions, to support neural computation. Observing and controlling these fast signals, across extended 3-d brain circuits, remains a challenge.

Given this complexity, and its inaccessibility to scientific investigation, perhaps it should be no surprise that understanding and repairing the brain is currently difficult. Understanding a decision or emotion at the level of fundamental mechanism requires the analysis of how many different cells, of different shapes and molecular organizations, and organized in a complex network, use molecular signals within cells and between cells to support computational operations. In the medical arena, even if a drug is devised to bind a specific target protein, or a neural electrical stimulator is positioned within a specific site in the brain, applying such an intervention to the brain will kick off a complex downstream cascade of events, as molecules

and cells, respectively, are engaged downstream of the intervention point. These downstream events could in turn result in unpredictable emergent effects. The net outcome of such emergent effects is that a therapy may not work as well as hoped for, or a therapy could cause an undesired side effect. Furthermore, such lack of efficacy or presence of side effects could be hard to anticipate, and compensate for, because they are the result of emergent effects that result from the operation of many interacting parts, which we do not understand.

Thus, ideally we would have a list of the fundamental building blocks of the brain, and how they work together. This could be considered a potential first pass at aiming for "ground truth" for the brain. Because there are so many kinds of molecule and cell, and their spatial organization is so complex - and their high-speed signals are so diverse and interconnected in complex networks - the best way to see if we indeed have attained "ground truth" would probably be to make a computer simulation of a brain, based upon the molecular, structural, and signaling data hypothesized to be "ground truth". Such a simulation could be run under many conditions, including conditions with external constraints imposed (e.g., with simulated stimuli or environments provided, or with simulated pathological insults or candidate therapies added), which thus could be used to make predictions of how given inputs or states contribute to emergent functions or dysfunctions. Such predictions could then be tested, perhaps through classical causal perturbation and observation experiments. Over time, if the models consistently make predictions that are borne out by experimentation, the models, and the datasets supplied to generate them, could come to be considered a "ground truth" description of how the brain works.

Practically speaking, such models could be very useful for generating interesting hypotheses to be tested through causal intervention: if you wanted to stimulate every possible sequence of 5 neurons in a network with 100,000 neurons, that means on the order of $100{,}000^5$ experiments (even ignoring the different stimulus timings possible) – an intractable number. But simulating a network in order to predict which sets of neurons would be interesting to perturb, in service of exploring the mechanism underlying some function or dysfunction, and then generating a small number of hypothesized sets of neurons to perturb in a real-life experimental context, would be quite actionable. In contrast to causal perturbation, imaging and observation technologies can be applied quite scalably – think about how DNA sequencing (which in the end, often reduces to a kind of imaging) is getting cheaper faster than Moore's law. Once one has enough imaging data, computational simulation also, in principle, could scale quite well.

It is important to emphasize that we do not have a complete list of all the components of the brain, even at the gene product level – and thus, the sheer number of possible molecular interactions within the brain, is enormous. However, by designing technologies so that they can be extended to image and control new molecular building blocks as they are discovered – or to analyze them even when they have not been previously described (the same way that genome sequencing, because it sequences entire genomes, can be used to analyze sequences of genes that have not been previously characterized) – one might make it possible to attempt a "ground truth" understanding of the brain relatively soon (Marblestone and Boyden 2014). In small neural systems like the crab stomatogastric ganglion, which contains just a few dozen neurons, knowing the wiring, molecular composition, and high-speed electrical and synaptic signals, has been sufficient to generate biologically meaningful computational models, which can make powerful predictions to be tested by experiment, and which also have yielded fundamental insights into the mechanisms of neural computation, such as revealing the principle of homeostatic neural plasticity (Marder and Bucher 2007). Analyzing entire brains in this way has been limited by a lack of technology capable of obtaining such datasets for large scale neural circuits. But with such technology, it would in principle be possible to acquire such

datasets for entire brains, and then to attempt to simulate them. At the very least, such simulations would allow us to test whether the kinds of data our technologies yield, are sufficient to make a model. If the modeling effort fails, one could still consider that outcome to be a constructive failure if it tells us what kind of data is missing, or what kind of technology we need to build next. For example, if the model works for some stimuli but not others, or accounts for the activity of certain cells but not others – these deficiencies could point to a class of molecular or cellular mechanism that we are missing, and thus we could hypothesize what that mechanism was, or generate a tool to probe for it. It is reassuring to think that even a constructive failure could still be very useful, by helping direct our experimental and innovative attention in specific directions in the future.

In the following sections, I outline three families of neurotechnologies that we, and our colleagues and collaborators, have been working on, to try to address the above challenge. Throughout, I discuss thoughts about neural simulations, and how they might be empowered by the tools here described. Hopefully this essay, while probably opening up more questions than it answers, will at least paint a picture of what might lie ahead, in terms of our fundamental understanding of the brain.

2. Mapping: expansion microscopy

The brain is an extended 3-d object – brain cells are by far the largest cells in the body, with their arborizations often extending many centimeters in the human brain, and with some axons projecting perhaps a meter in distance, down the spinal cord. In contrast, the axons and dendrites of neurons, and the synapses between neurons, and the molecules along these wires and at these synapses, are nanoscale. Imaging with nanoscale precision is hard – for example, due to the wavelength of visible light, conventional light microscopes have a best resolution of ~300 nm, much larger than a single biomolecule (which might be ~5 nm in size, and often much smaller). Advanced technologies like electron microscopy and super-resolution microscopy exhibit high spatial resolution, but are difficult to scalably apply to extended 3-d objects like brains, and, in the case of electron microscopy, struggle to reveal the identity of molecules. How could one image a large structure like a brain, with the spatial precision and molecular contrast needed to map its fundamental molecular building blocks?

For over 300 years, the way biologists imaged cells was to magnify images of the cells, obtained by transforming signals broadcast, or modulated, by the biological specimen. We wondered – what if we instead physically magnified the biological specimen itself?

In 2015, we announced the discovery that physically magnifying biological specimens was possible – and presented a technique that we call expansion microscopy (ExM; Chen, Tillberg, Boyden, 2015). ExM proceeds in four steps. By (1) chemically synthesizing a dense (~1-2 nm spacing), 3-d spider-web-like mesh of what is essentially the active material in baby diapers – the swellable polymer sodium polyacrylate – throughout a preserved biological specimen (this strategy will not work on living specimens), (2) anchoring biomolecules to the polymer mesh through covalent molecular linkers, (3) softening the specimen by chemical treatment (e.g., with enzymes, heat, and/or detergent), and (4) adding water to swell the polymer, we can pull biomolecules apart from each other, in an even way. Thus, two biomolecules that were touching will now be some minimum distance apart, and two biomolecules that were some distance apart will have their distance scaled up by a linear factor. The biomolecules can then be labeled (e.g., proteins can be stained with fluorescent antibodies, nucleic acids can be labeled with fluorescent hybridization probes, and so forth), and imaged on a conventional microscope. The net result is that the conventional microscope used becomes a nano-imaging device: expanding a specimen 20x, for example, in linear dimension, would result in a ~300 nm resolution

microscope now having an effective resolution of ~300 nm / 20 or ~15 nm. Expansions of 50x have been reported (Chang et al 2017), and the fundamental resolution of ExM has been estimated to potentially be better than one nanometer (Shaib et al. 2022). Custom microscopes can also be developed to take advantage of the unique properties of ExM specimens – they are not only transparent (because they are mostly water, after having been swollen 100x or more in volume, in water), but they have very homogeneous optical properties (i.e., their refractive index becomes basically the same as water, since all the biomolecules have been diluted in water by 100x or more relative to their original concentration), making them amenable to high-speed yet high-resolution methods of imaging such as lattice lightsheet imaging (Gao et al. 2019). Different forms of ExM have been developed, with different expansion factors (and thus resolutions), and optimized for different species and contexts. ExM is very easy to use, and has rapidly spread throughout biology, having been used in over 600 experimental papers (peer-reviewed or in preprint form) to date.

By labeling the boundaries of neurons (i.e., the lipid membrane; Shin et al. 2024) or the overall distribution of proteins in neurons (i.e., through nonspecific protein staining; Tavakoli et al. 2024), or by expressing fluorescent proteins, or proteins that can later be fluorescently stained, in combinatorial fashion (i.e., so that every neuron gets its own “barcode”, defined as a different combination of exogenous proteins expressed in a given cell; Shen et al. 2020), in the context of expansion microscopy, it becomes possible to trace the shapes of neurons, and how they connect. Importantly, staining synaptic proteins with fluorescent antibodies enables the pinpointing of the synaptic connections and their type and state. In addition, staining with fluorescent antibodies against specific ion channels, receptors, neurotransmitters, and neuromodulators, amongst other key biomolecules, helps identify and locate the molecules that determine how the cells operate in a biophysical sense. A potentially unlimited number of antibodies could be applied to image a large number of different targets within the same expanded sample, either by repeatedly staining a specimen with different sets of antibodies against different sets of targets, imaging the specimen, and then washing away those antibodies (Ku et al 2016; Valdes et al. 2024), or by using DNA-barcoded antibodies that can then be read out through multiplexed DNA-based readout (Saka et al. 2019). One tantalizing possibility is that some biomolecules, such as those made of DNA or RNA, could be sequenced in situ (i.e., while still inside the expanded brain; Alon et al. 2021; Payne et al. 2021), whereas others, such as proteins, might be recognizable from their shape (Shaib et al. 2022), facilitating the identification of many kinds of biomolecule at once, within the same neural circuit, or even entire brain.

The net result would be a map of the molecules throughout cells, and the cells throughout a brain. Could such a map be used as the basis of a biologically accurate computer simulation of the brain? In principle, using cable equations for the branches of neurons, with elements of these differential equations representing specific ion channels, receptors, and so forth, should recapitulate the essential processes needed to emulate brain function, at least over short timescales (Koch and Segev 1998). But one must know, of course, how to represent each biomolecule in the equations – meaning that one must have data indicating the physiological contributions of each molecule. Such data could be acquired by reading the literature, by high-throughput assessment of the molecules in heterologous expression systems (e.g., cultured human cell lines, expressing key receptors and ion channels and then assessed by patch clamp or voltage imaging, e.g. Huang et al. 2006), and/or by machine learning association of molecular recipes (e.g., attained by the aforementioned multiplexed antibody staining or in situ RNA sequencing methods) with physiological observations from the living state, performed through the dynamical imaging and control of neural activity throughout entire small brains (see below).

3. Control: optogenetics

ExM, while precise, multiplexable, fast, scalable, and inexpensive, has one major limitation that might never be overcome – it will not work on a living system, since it works by separating biomolecules from each other, which prevents them from achieving their function. Thus we must invent other toolsets to image and control the signaling dynamics of brain cells while the brain is alive. Then, one might imagine an experimental workflow where one would first image and control signals in the brain while it is alive, perhaps while an animal is performing a behavior of interest, or while it is in a brain disorder state that one seeks to overcome. After such an experiment is completed, one would then preserve the brain, and map its structure and molecular composition by ExM, as above. Such datasets, combined, could be used to support the computational modeling of the brain.

In order to control the high speed electrical activity of neurons, one of the brain's most important and ubiquitous signals, especially when it comes to the high-speed propagation and transformation of information in the brain, we have developed a toolset known as optogenetics (opto meaning light, and genetics because the tools are genetically encoded; reviewed in Piatkevich and Boyden 2024; the content in this paragraph and the following few paragraphs is summarized from this review). In optogenetics, we borrow molecules from nature – using light-activated ion channels and pumps from organisms such as bacteria, fungi, algae, and archaea, to mediate the optical control of neurons. Microbial rhodopsins are naturally occurring seven-transmembrane proteins that bind, at a specific site, a vitamin A variant called all-trans-retinal. They reside in the membranes of many kinds of microbe, where they either pump ions to store solar energy in the form of ionic gradients, or open up a channel pore to let ions pass through in order to drive downstream signals to guide the behavior of the organism. Biophysically, when hit by light of the appropriate color, the all-trans-retinal isomerizes to the 13-cis form, and then the protein undergoes a rapid series of conformational changes, which either transport a specific ion (such as a proton, or a chloride ion) from one side of the membrane to the other, if the rhodopsin is a light-driven ion pump, or that open up a pore that selectively conducts one or more kinds of ion (such as protons and sodium, or chloride, or potassium) from one side of the membrane to the other, if the rhodopsin is a light-driven ion channel.

We and others have found that certain rhodopsins are fast enough, safe enough, and powerful enough, to function in neurons of the mammalian brain – a truly serendipitous finding. Such molecules could have been toxic in mammalian neurons, or might not have functioned properly – but, they worked. Most serendipitously, perhaps, all-trans-retinal appears in the mammalian brain at concentrations sufficient to enable rhodopsins to function, without requiring any exogenous chemical supplementation, greatly simplifying ease of use. After taking the gene that encodes for a brain-appropriate microbial rhodopsin, and delivering it genetically (e.g., with a virus or other gene therapy vector) to specific neurons in the brain of an animal, those neurons will express the gene, manufacturing the protein that is encoded by the gene, and installing it on the membrane of the neuron. Various tricks from the field of gene therapy (e.g., the design or choice of regulatory promoter) can be used to insure that a specific, well-defined subset of the cells transduced, will express the gene. The all-trans-retinal naturally found in the mammalian brain is incorporated into the rhodopsin. Then, light of the appropriate color to engage the rhodopsin, delivered from a laser or LED, potentially implanted in the brain or connected to an optical fiber implanted in the brain, will cause the rhodopsin to be activated.

For example, if a light-driven proton pump such as archaerhodopsin-3 from *H. sodomense*, or a light-driven chloride pump such as the halorhodopsin from *N. pharaonis*, is expressed in a set of neurons, and then green or yellow light of appropriate power (e.g., in the tens of milliwatts per square millimeter) is delivered to the brain, then protons will be pumped out of the neuron or

chloride will be pumped into the neuron, respectively, causing the membrane voltage of the neuron to undergo hyperpolarization, blocking it from spiking. By silencing neurons thus, it is possible to find out what their electrical activity is necessary for: one can silence a set of neurons and see if that prevents a behavior from occurring, or cancels out a pathological pattern of activity of the brain. If a light-driven cation channel that passes sodium and protons, such as channelrhodopsin-2 from *C. reinhardtii,* is expressed in a set of neurons, and then blue light is delivered to the brain, then sodium and protons will flow into the neuron, causing it to spike. By activating neurons thus, it is possible to find out what their electrical activity is sufficient for: one can activate a set of neurons and see if that causes the initiation of a behavior, or causes a pattern of activity that can help overcome a brain disorder state.

Optogenetics, like ExM, is easy to use, and rapidly has found widespread use in neuroscience, with perhaps thousands of scientists using it to study how the electrical activity of specific neurons contributes to specific behaviors, or how it can cause therapeutic effects for specific brain disease states. These experiments have almost entirely been done on animals such as mice, including mouse models of human brain diseases. But they are yielding many surprising discoveries, some of which have led to therapeutic ideas in human trials for brain diseases. As one example, my collaborator Li-Huei Tsai led a team that used optogenetics to discover that driving neurons at a specific frequency, 40 Hz (also known as gamma frequency), in the brains of Alzheimer's model mice, causes a reversal of some of the molecular pathologies associated with Alzheimer's – reducing microglial inflammation, and cleaning up amyloid plaques, for example (Iaccarino et al., 2016). The team went on, led by Li-Huei, to find that such 40 Hz oscillations could be recruited by having mice watch flickering lights and hear clicking sounds – basically, a movie; no need for any gene therapy or implant (Martorell et al., 2019). Alzheimer's model mice, exposed to such stimuli for an hour a day or so, had improved cognition, and reduction in the molecular hallmarks of Alzheimer's disease. Now, multiple clinical trials (including one run by a company cofounded by Li-Huei and myself), with actual Alzheimer's patients, are showing a slowing of Alzheimer's progression in patients treated daily with such gamma stimulation. While it is early days, and the therapy is not approved by any regulatory body yet, as trials are still ongoing, it is exciting to think that one of the most intractable diseases of our time might be treatable by a sensory stimulus.

Optogenetics, most recently, has begun to find potential uses in the human nervous system. Millions of people are blind, because they have lost the photoreceptors in the retina that capture light and translate the signals into forms that can be relayed to the brain, for example due to the genetic conditions known as retinitis pigmentosa. In summer 2021, it was announced that a blind man, who received an adeno-associated virus (AAV) gene therapy vector encoding for the light-activated cation channel ChrimsonR (discovered by us in 2014; Klapoetke et al. 2014), and expressing it in the retinal ganglion cells of the eye, had a partial restoration of functional vision – he could make out household objects, see doors on a hallway, and make out the lines of a crosswalk (Sahel et al. 2021). It was not perfect vision – he could not recognize faces or read text – but it was a striking improvement, compared to any earlier technology. The expression of the gene persisted, and there were no serious adverse events. Thus, although the impact of optogenetics will likely to continue to be felt primarily in the space of scientific discovery, in the time to come, a second, exciting arena is opening up – the potential of using light to enter information into the nervous system.

4. Dynamical imaging: voltage and other signals

Many groups, including ours, have been designing, synthesizing, and evolving genes that encode for proteins that can perform the inverse function of optogenetics: when such genes are expressed in neurons, they generate membrane proteins whose fluorescence is proportional to

the voltage of the neuron in which they are expressed. Rhodopsins, for example, can be found that weakly fluoresce in proportion to membrane voltage (Kralj et al. 2012); mutated, they can become high signal-to-noise fluorescent reporters of membrane potential (e.g., the molecule Archon, when illuminated with red light, will glow in the infrared, in a fashion that is proportional to the membrane potential; Piatkevich et al., 2018). They can also be fused to other fluorescent proteins (Gong et al. 2015), or coupled to small molecule dyes (Abdelfattah et al. 2019), to enable brighter fluorescence than enabled by opsins alone. Such molecules, used in conjunction with high-speed microscopes optimized to image 3-d volumes at high speeds (e.g., >200 Hz), while maintaining high resolution (e.g., enough to resolve adjacent single cells from one another) and good sensitivity (e.g., enough to see individual action potentials), are starting to enable the imaging of the voltage of neurons throughout entire small brains (Wang et al. 2023).

Of course, brain cells take in many dynamical signals – neurotransmitters, neuromodulators, hormones, nutrients – and generate many kinds of outputs - releasing new transmitters and modulators in turn, changing gene expression, and altering shape and connectivity. In between these inputs and outputs is a sea of molecules that interact in complex networks, such as kinases, transcription factors, and other proteins that move, bind one another, alter each other, and change shape. How can we image such molecular signaling networks, ideally all the way from cellular input to cellular output, and capturing the essential computation in between? The traditional answer was to make fluorescent reporters of different signals, based upon fluorescent proteins of different colors – then, one could express the genes encoding for all these proteins in the same cell, and image all the signals at once while the cell was alive, using a multicolor microscope. But what if you want to image more than 2 or 3 such signals at once? Recently we put forth two new ideas to this end – one which uses molecules embodying the concept of spatial multiplexing (Linghu et al. 2020), and one which uses molecules embodying the concept of temporal multiplexing (Qian et al. 2023), to measure many signals at the same time, in the same living cell.

In spatially multiplexed imaging (SMI), fluorescent reporters of different cellular signals (e.g., proteins that are fusions between a fluorescent protein and a protein that binds a specific messenger like Ca+2 or cAMP, and that change brightness in proportion to that messenger), are fused to different self-assembling peptides. Such proteins, expressed in cells, will self-assemble safely at random, but stable, points in space throughout the cell – close enough to sample the relevant biology, but far enough apart to be resolved under a microscope. Indicators of different signals will, because of their distinctive self-assembling peptides, assemble at different points in space. Each protein is also equipped with an epitope which, when stained with a fluorescent antibody, will identify which protein is found at that point. The net result is that when many different thus-equipped fluorescent reporters are expressed in the same cell, they will cluster at random points in space, from which they can broadcast information while the cell is alive. When the cell is fixed, it can then be stained against the epitopes and imaged, potentially over many cycles, so that the identity of the reporter at each site can be identified. Using this strategy, we imaged four different signals at once in the same cultured neuron (Ca+2, cAMP, PKA activity, and PKC activity), using just one color – green – and was able to discover relationships between cAMP, PKA, and Ca+2 in cultured neurons challenged with forskolin.

In temporally multiplexed imaging (TMI), different proteins are fused to different reversibly switchable fluorescent proteins (rsFPs) – fluorescent proteins that, when illuminated with one color of light, go dim, and when illuminated by a second color of light, go bright again. Please note – this is not photobleaching, or fluorescence lifetime – this is a reversible process that occurs over timescales of milliseconds to seconds. Many such fusion proteins can be

expressed in the same cell, with each protein of interest fused to a rsFP with distinct kinetics. The amount of each protein, at a given place in a cell, can be identified, even if all the rsFPs are the same color: simply take a brief movie of a cell expressing many such proteins. Each pixel of the movie will be a linear combination of the exponential decay traces characteristic of each rsFP. Take the movie, and simply perform a standard linear unmixing: then, the coefficients of the exponentiatl decay traces will represent the amount of each fluorophore present at that pixel. Using TMI, we imaged six different cytoskeletal and organelle markers, in the same cell, using just one color – green – and we discovered relationships between different cell cycle markers, different kinases, and other members of complex signaling networks.

Dynamical voltage imaging tools, along with older technologies such as fluorescent calcium indicators (Chen et al. 2013), can be used to image neural activity across entire nervous systems such as those of worms, while specific neurons are optically perturbed using optogenetic tools (Randi et al. 2023), enabling neuronal input-output functions to be derived. One possibility is that such neuronal input-output functions could be a key ingredient in constructing simulations of entire small brains, in the near future (Haspel et al. 2023). Of course, deep learning methods can also be used to try to predict future neural activity across entire brains, based upon past brainwide neural activity histories alone (Simeon et al. 2024). Combined with anatomical and molecular maps, as described above, dynamical imaging and control technologies may enable very detailed biophysical models of the brain to be made, taking a cue from successful efforts to combine molecular, connectomic, and dynamical data from entire small neural circuits such as those of the crab stomatogastric ganglion (Marder and Bucher 2007). It is interesting to speculate that in such simulations, interesting patterns of neural activity may emerge, that do not correlate with an obvious sensory input or behavioral output, and may provoke novel hypotheses about philosophically challenging topics like subjective experience.

Acknowledgments
I have been supported by Lisa Yang, John Doerr, and the Howard Hughes Medical Institute. I would like to thank all the members of our group at MIT, over the years, who have generated valuable insights, discoveries, and technologies that have informed this essay.

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