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Seeing Circuits Assemble

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Abstract

Developmental neurobiology has been greatly invigorated by a recent string of breakthroughs in molecular biology and optical physics that permit direct in vivo observation of neural circuit assembly. The imaging done thus far suggests that as brains are built, a significant amount of unbuilding is also occurring. We offer the view that this tumult is the result of the intersecting behaviors of the many single-celled creatures (i.e., neurons, glia, and progenitors) that inhabit brains. New tools will certainly be needed if we wish to monitor the myriad cooperative and competitive interactions at play in the cellular society that builds brains.

Introduction

The 2008 Chemistry Nobel Prize shared by Shimomura, Chalfie, and Tsien adds an exclamation point to a revolution that biology, and most particularly neurobiology, has undergone since the dawn of molecular biology. The emergence of imaging as the tool of choice for the analysis of cellular and molecular phenomena in the nervous system has been stunningly rapid. Notably, many neuroscientists trained in electrophysiology or molecular biology have eagerly retooled to take advantage of the powerful new imaging-based approaches. Even those of us who have worked in this field for a long time are hard pressed, however, to keep up with the rapid pace of the ongoing innovations.

These new methods have been especially powerful for those researchers interested in understanding the ways in which neural circuits assemble. But new methods come with new challenges for the practicing neuroscientist. First, of course, is mastery of the diverse technologies of fluorescence-based optical imaging. Second is the challenge of learning how to turn images into data. If experiences of the two authors are any guide, neither of these challenges is trivial. Moreover, if our aim is to understand neurodevelopmental phenomena, even overcoming these challenges may be insufficient. Our aim here is to take stock of where this fast moving field is presently and where we think it might profitably head in the immediate future. We will emphasize the dominant role of imaging in modern attempts to explain the development of the nervous system. But as powerful as the new tools, which we will review briefly here, may be, we will also try to make the case that continuing efforts to develop new tools, still more powerful, will be needed to really begin to understand how the vast and intricate circuitry of the nervous system comes into being.

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1. Imaging Biology

The triumphs that led to this imaging revolution occurred largely in the 1990s and are in two general areas: molecular biology and imaging technology. By the millennium, both of these fields more or less inadvertently coalesced in the GFP revolution. Now, thanks to genetically encoded labeling strategies, scientists can label virtually any aspect of the nervous system from widespread populations of neurons (Feng et al., 2000) to selective long axon tracks (Bareyre et al., 2005) to dendrite spines (Chen et al., 2000); from neuronal mitochondria (Misgeld et al., 2007) to synaptic vesicles (Meyer and Smith, 2006); from microtubule-associated proteins (Jacobs et al., 2007) to CaM kinase II (Shen et al., 1998); etc., and can do so in the brains of living animals!

The origins of the GFP revolution stem from the powerful molecular biology toolkit developed in the 1980s. Thus, when Prasher and colleagues obtained the sequence for GFP (Prasher et al., 1992), very little time passed before Chalfie and Tsien took advantage of his clone to demonstrate the magic of genetically encoded fluorescent labels (Chalfie et al., 1994; Heim et al., 1994). Because the background fluorescence of most animal cells is so low with the visible excitation used for GFP visualization, GFP provides inherently high sensitivity. In many circumstances, even single molecules of a fluorescent probe are visible. Moreover, because genetically encoded GFP is introduced by the cell's endogenous protein synthesis machinery, many of the problems of biological perturbation and spillage background associated with earlier methods of vital staining (e.g., with absorbance dyes like methylene blue a century earlier [Lu and Lichtman, 2007]or the decades old uses of exogenous fluorescence dyes [Honig and Hume, 1989; Magrassi et al., 1987]) are automatically circumvented. Other advantages include the fact that the cell can continue synthesizing the fluorescent protein throughout its life so it is possible to monitor the same cells over arbitrarily long durations even if some of the dye degrades or is bleached by imaging. Moreover unlike small organic fluorescent molecules, GFP evolved over the eons to have relatively low phototoxicity. The fluorescent moiety is an imidazolone ring structure that is formed by the posttranslational cyclization of a tripeptide, ser65-tyr66-gly67. It is situated at the center of the cylinder created by the 238 amino acid peptide along an alpha chain that runs down the center of protein (Yang et al., 1996; Ormö et al., 1996). Because fluorescent excitation can lead to free radical formation (see Lichtman and Conchello, 2005 for discussion), this design may keep the reactive species a bit removed from nearby unrelated proteins.

Ironically the initial uses of this tool—and perhaps the majority of its current uses—relate more to histology than to molecular biology. The emergence of imaging comes as a counterpoint to the molecular triumphs in neurobiology. Synaptic function (Sudhof, 2004), synaptic plasticity (Thomas and Huganir, 2004), axon pathfinding (Charron and Tessier-Lavigne, 2007), synaptogenesis (Montgomery et al., 2004), and neuronal migration (Hatten, 2002), to name a few, have all yielded to molecular analyses giving rise to the outlines of biochemical pathways as explanations for cellular phenomena. Now these same phenomena are being revisited with tools that allow them to be directly witnessed. For the first time it is possible to see synaptic vesicle release and recycling (Schweizer and Ryan, 2006), dendritic spine plasticity (Yuste and Bonhoeffer, 2004), axon pathfinding (Hechler et al., 2006), synaptogenesis (Alsina et al., 2001; Jontes and Smith, 2000; Niell et al., 2004; Meyer and Smith, 2006), and neuronal migration (Hatta et al., 2006).

How did all these phenomena become imageable? GFP, while certainly part of the story, is not the whole story. The 1990s not only saw a maturation of molecular biological sophistication but also were marked by breakthrough after breakthrough in imaging technologies. These advances included (1) the utilization of lasers as ultra-bright light sources for laser scanning confocal microscopy (Amos and White, 2003); (2) the advancement of solidstate detectors designed for low light level fluorescence imaging (Aikens et al., 1989); (3) the realization that

nonlinear fluorescence excitation by 2- or 3-photon fluorescence excitation with a scanning pulsed laser gave optical sectioning, less photobleaching, and greater depth penetration (Denk et al., 1990); (4) the advent of a large number of small organic fluorescent probes that worked as Ca^{2+} indicator dyes (Tsien, 1989); and (5) the beginnings of the use of genetically encoded indicators such as the cameleons (Miyawaki et al., 1997; Zhang et al., 2002), the last two of these being the fundamental contributions from the lab of Roger Tsien—not to mention his central role in the development of a range of spectral variants based on GFP, for which he shared this year's chemistry Nobel.

The use of scanning microscopy techniques requires special comment. Confocal microscopy was first described by Marvin Minsky in the 1950s, but hardly anyone noticed (Minsky, 1998). In the last several years confocal imaging became commonplace when second generation spinning disc (Tanaami et al., 2002)and laser scanning approaches (Amos and White, 2003) both became robust enough to be commercially viable. This optical sectioning technique gives excellent images that are uncontaminated by out of focus light, but for imaging dynamics it has some serious drawbacks (Conchello and Lichtman, 2005). First confocal imaging has limited depth penetration in living tissue that scatters light, so it is not optimal for viewing thick volumes of in vivo. Second, confocal detection is inherently inefficient, often requiring more illumination of the live specimen than it can endure before bleaching or phototoxicity occurs. The invention of two-photon microscopy in 1990 (Denk et al., 1990) was a watershed, as this technique solved these two major problems with confocal in one stroke. Over the past 18 years many thousands of papers have used two-photon microscopy to image biological phenomena not only in neurobiology but also in immunology, developmental biology, and many other fields (Benninger et al., 2008). The rise of two-photon imaging has allowed the study of the live brain tissues in situ over periods of days to months with little or none of the phototoxicity effects that limited previous methods. Prior to two-photon microscopy, neurobiologists interested in structural dynamics of neural structures in intact organisms had to content themselves to work with accessible peripheral nervous system dendrites and synapses that could be imaged with much less sophisticated imaging tools (Purves et al., 1986; Lichtman et al., 1987).

Given the power of two-photon imaging it is remarkable that yet another revolution in imaging has also been underway to overcome what many have considered the most impenetrable barrier to understanding: the limited resolution of optical microscopy. Microscopists have traditionally accepted that imaging resolution was limited to approximately half the wavelength of light being detected due to the fundamental optical phenomenon of diffraction. This so-called hard limit in resolution hinders the ability of light microscopy to bridge the enormous chasm between the molecular interactions occurring on the scale of a few nanometers and images of neurons with resolutions that are at best several hundred nanometers. Researchers interested in molecular interactions have in some cases overcome this limitation by FRET-based imaging techniques in which fluorescence signals are modified by nanometer proximity between donor and acceptor fluorescent molecules (Roy et al., 2008). In addition, tracking single particles has long been accomplished with nanometer precision (Vale et al., 1996). But neither of these approaches produces actual images beyond the traditional diffraction limit. However, thanks to a number of new so-called “nanoscopic” fluorescence techniques (Hell, 2007), the diffraction barrier itself has been breached with what may soon provide electron microscope type resolution for standard fluorescence imaging applications. Techniques such as STED (Willig et al., 2006), PALM (Betzig et al., 2006), FPALM (Hess et al., 2006), STORM (Rust et al., 2006), and structured illumination (Gustafsson, 2005) provide the imager with a way to see fluorescently labeled structures with nanometer resolutions. Recent use of these approaches in fast modes allowed imaging dynamics in living cells at subdiffraction resolutions (Shroff et al., 2008; Hein et al., 2008).

While imaging tools have matured there has been a steady drift in the kinds of neural preparations that can be studied. Imaging neurons and glia in culture has traditionally been preferred over more intact preparations because of the greater transparency of monolayer cultures. While cellular dynamics such as growth cone behavior and dendritogenesis are much easier to image in cell cultures, the milieu is abnormally simple, motivating many developmental neuroscientists to migrate to more intact preparations such as slice cultures or acute slices. But neither acute nor cultured slices can be accepted uncritically as faithful models for an organism's development. Now, the use of two-photon imaging allows the imaging of CNS development over any time period desired.

2. Seeing Circuits Assemble: A Lot of Trial and Error

Cajal's greatest insight into the nervous system was that it behaved as a network or circuit, consisting of a vast number of interconnected neurons, where each neuron receives signals from a number of others and relays that information onward to yet other neurons. Understanding these circuits remains one of the greatest challenges in neurobiology, a subject we will come back to later. Cajal also had a keen interest in the ways these circuits were established. He was probably the first person to see axonal growth cones. Indeed at least some developmental neurobiologists see development as a strategic avenue for attaining understanding of neural circuitry—the idea being to watch circuit formation in order to ascertain what kinds of rules establish the complicated mature organization.

Unfortunately for Cajal, none of the stains or microscopes available in his time allowed neurons to be visualized in living tissue, so he could only imagine the dynamics involved in brain development. Beginning with the use of vital fluorescent dyes (Harris et al., 1987; Lichtman et al., 1987) and more recently with fluorescent proteins, researchers have overcome the limitations of static imaging in order to watch nervous system development over time. It is worth emphasizing that although development can be monitored from a time series of images from different animals there are many dynamic events (especially ones that are not monotonic) that are completely invisible with static imaging (Lichtman and Fraser, 2001).

These approaches have revealed many surprising things about the assembly of the nervous system. First is the realization that development is not a simple mechanism of accretion or building. Rather what occurs seems more akin to a series of trial and error steps or, as some have expressed this strategy, in other contexts, to blind variation and selective retention. Thus more neurons are made than ultimately survive in development, growth cones navigate to targets by exploration of potential directions rather than making a bee line, dendritic branches and spines form and are lost as a dendrite tree matures, and synapse formation and synapse elimination often occur simultaneously by the same neuron as circuitry is built (see examples of this latter idea in Figure 1). All of these building and concurrent unbuilding events argue that development is less deterministic than is, say, car manufacture. The abundance of “backwards” or unbuilding steps, as revealed only by recent live imaging experiments, may simply represent a means of developmental error correction. On the other hand, this abundant unbuilding may be at the very heart of the developmental decision making process that allows a machine as intricate as a brain to assemble itself.

These developmental decisions are presumably informed by a large number of interactions between axons and potential pathways, axons and potential targets cells, and dendrites and potential target cells. There may also be interactions between neurons and glial cells that help clear debris (Song et al., 2008) but also powerfully regulate circuit assembly processes (Stevens et al., 2007). Cell-cell interactions set off biochemical cascades that profoundly change cell behavior. In other cases the interactions may be more mechanical, corralling cells or presenting physical barriers to prevent cells from going in the wrong directions.

The remarkable thing about all these interactions is that there is no overarching topdown organizational control of these developmental decisions. Rather, the developing nervous system appears to be organizing itself based on millions of intimate cellular interactions. This raises the fundamental question of how? This is surely not a new question; a little more than a century ago Hans Dreisch gave up practicing developmental biology when he discovered the ability of a small parts of an embryo to self-organize and give rise to a whole organism presumably because cells changed their fate in these embryonic fragments. He inferred that some outside intelligence must be at work. Biologists now refute such *deus ex machina* vitalistic explanations and seek to understand how the chemical and physical interactions of cells lead to organogenesis. But in the developing nervous system we have a long way to go to get to a causal deterministic understanding of circuit formation. Indeed this is a severe challenge because there is an inherent tension between the efforts of reductionists to isolate causes and holists who seek to understand how interacting parts give rise to properties not found in the parts (e.g., the fluorescence of GFP only emerges from the assembly of the amino acids; the signaling capacity of neural circuits only emerges from the interactions of many cells).

If brain circuit development, just like the rest of embryogenesis, cannot be explained by actions of an outside agent nor by any topdown instructional master blueprint, other than the DNA common to each cell, then how do we explain it? Well we are left with the idea that the assembly of circuitry in the developing central nervous system (CNS) can only be the result of the individual and collective behaviors of the brain's individual cells—be they neuronal, glial, or precursor. That is, the physical patterns of connectivity and the molecular architectures that underlie brain function can only be the result of individual cells dividing, migrating, differentiating, growing neurites, wrestling with each other, forming and breaking adhesive and synaptic contacts, and so on. Such cellular behaviors are themselves governed by extremely complex networks of physical and chemical interactions and signaling between cells and within cells. That being said, how are we to even begin to understand such a dauntingly complex, multifaceted, and multilayered process as brain development, involving so many diverse individual cells interacting with each other?

3. The Mechanisms of Development

The evolutionary biologist Ernst Mayer has spoken eloquently about the fundamental differences between the physical and biological sciences (Mayr, 2002). This is not to say that living things are not machines or that their assembly is not based on developmental mechanisms, but rather that interactions, selection, and the dual causalities of physics and genes work in ways that are just not analogous to the mechanism at play in the inaminate world. As neurobiologists study development, they see evidence of many different, often interacting, forces at work. There is abundant evidence of orchestration of gene expression that helps to differentiate neuronal and glial cell type as well as organize groups of neurons into specific nuclei, ganglia, and lamina. There are myriads of intracellular molecular interactions that generate the structure and chemical and electrical properties of nervous system cells. There are the signals that originate in the extracellular milieu that instruct the differentiation of neurons and glia. The cell of the nervous system also have a range of intimate interactions with different categories of cells that lead to myelination, the blood-brain barrier, among other features of the nervous system. Most importantly, there are the interneuronal interactions that generate synaptic connections between appropriate pre-and postsynaptic partners and the precise alignment between presynaptic neurotransmitter release sites and postsynaptic receptor clusters that are appropriate for the particular neurotransmitter released a few nanometers away.

4. Neuron Social Psychology 101

One view about these interactions comes from the idea that cells of the developing nervous system are autonomous living creatures. They may be likened to their distant relatives the protozoa that eke out a living in the ponds of the world. They compete for limited resources, they may interact with each other, they move toward resources they need, and they are repelled by toxic agents in their milieu. Inside multicellular animals, the single-celled organisms we call neurons and glia are in a very weird pond to be sure, but still they aim to eke out a living. They surely are unaware that their calling is to help animals perceive or sense pain, or to help an animal locomote. Rather they are concerned with their own survival. They are engineered to need trophic factors that come from their targets, which are in limited supply and for which they have to compete. They are engineered to adhere to certain substrates rather than others. They fasciculate with neurons of the same type but sometimes avoid growing along with axons of different types. All of these constraints mean that these single-celled organisms will exhibit exuberant cell dynamics, exploratory behavior, trial and error based refinements, and competitive interactions leading to death of some cells and removal of some processes. Out of all this activity comes a harmonious system that reaches some equilibrium. Maybe the way humans self-organize into cities is a good metaphor. There are no top downregulators that keep the number of pianos and number of piano tuners at a constant ratio, yet one might expect that this ratio is approximately the same in city after city.

As we hinted at above, a metaphor that frames the interactions of individual cells in the developing brain in a way analogous to our life experiences with interacting individual people may be helpful. Such metaphors abound! Construction projects, ballets, dramas, politics, business, football games, and many others kinds of games. Might it be useful to liken a cell in the developing brain to a construction worker? To a football player? The construction worker metaphor is weakened by the fact that construction projects depend upon a set of external agents (construction workers) following external plans (the blueprint) to construct the product. Because brain circuits assemble themselves, the football (or some other game) player may be a more useful metaphor. Very often, games create ordered structure and function as a result of individuals following rules of behavior that do not by themselves encode any particular outcome. The orderly progress of the game does not require referees on the field or coaches on the sidelines—these are usually only present at the highest levels of play. Thus, the orderly progress of the game can reflect rules that are as internal to the individual player as DNA is to a cell. The actual outcome of the game, however, always depends critically on another set of factors including the players' diverse repertoires and their individual behavioral complexities. Thus, to really understand how the game unfolds, we need to understand the distinct characteristics and “psychologies” of the different players, as well as the rules they have in common. The goals for a developmental analysis of circuit assembly might then be conceptualized within the game metaphor as discerning both the contents of the rule book and the diverse traits and capacities of the individual cellular “players.” Humans usually learn to discern both the rules and individual player's traits by watching the game as it unfolds in real time.

How might we advance our understanding of this long and intricate game we call brain development? It seems highly likely that we'll need just about everything in the modern toolbox, ranging from genetic perturbation through computational simulation and reconstruction, and then some. It may be at this juncture that our new intravital imaging tools have their greatest impact: in vivo imaging, quantitative image analysis, and reconstructive visualization may be among the most important of tools, just as most humans learn about football by watching the game. In both cases, watching may be the most efficient route to understanding due to the predominant importance of spatiotemporal dynamics. The rationale here can be expressed (albeit speculatively) in ethological terms: our own brains, including and especially visual

systems, have evolved to discern meaning in very complex dynamic interactions between multiple individual actors and their physical and “social” surroundings. Thus, the tuning of our own brains and visual systems may stand us in good stead when it comes to exploring and interpreting the behaviors and interactions of individual neural cells during the epic turmoil that is neural circuit development.

Even with today's best imaging techniques and clever ways of visualizing and analyzing images, it will not be easy to understand how the behaviors of interacting individual cells build a circuit. Most of our descriptions of cell behavior come from circumstances where it was possible to observe the behavior of individual cells in total or relative isolation, such as in cell culture systems or in situations where only one or a few cells in a tissue express a fluorescent protein. While such observations have been extremely informative, it seems unlikely that a simple concatenation of single-cell observations will be adequate to understand the interplay of many individuals that must underlie circuit development. For instance, there have now been numerous successful analyses of the behavior of presynaptic partners during CNS synaptogenesis and also of the behavior of postsynaptic partners, but the question (undoubtedly a key one!) remains as to how presynaptic and postsynaptic cell dynamics are coordinated at the crucial periods of initial and early contact. Experimental observations of this quintessential *pas de deux* seem sure to appear soon, but then we'll have to confront the rather chilling fact that each cell in the developing brain interacts directly and powerfully with dozens of partners at any one time, not just one partner. To return to the football metaphor, the behavior of a wide receiver can only be understood with reference to the opposing defenders as well as the quarterback wishing to connect. One of the main experimental challenges to the structural neurobiologist today lies in seeing both the tree and the forest, or both the individual player and the whole game of our metaphor. This stands as one of the major challenges to today's imaging innovators.

5. Circuit Assembly: What Means to What Ends?

Neural circuits are arguably the most complex and intricate structures in the known universe. Naturally, it will be no simple task to understand how such a structure assembles itself. While developmental neurobiologists hope that the intricacies of the neural circuit emerge from the interplay of cellular players governed by rules that are simpler than the resulting circuit, there is no guarantee that any such set of rules would be simple or small in absolute terms. There are plenty of genes, cell signaling and motility systems, and cell-cell interactions to support a dauntingly large and diverse set of circuit development rules and mechanisms. We'll need much more complete descriptions of both the developmental processes and the resulting circuit before we can evaluate the adequacy of any finite set of developmental rules to predict or explain neural circuit development.

Most progress to date in understanding the dynamics of circuit development has resulted from the study of individual elements and rules of circuit development, e.g., growth cone motility and modulation, axon guidance, dendrite growth, synaptogenesis, etc., each in cleverly contrived experimental isolation. Further progress in understanding the actual development of specific neural circuits may depend, however, on better defining the end “goal” of circuit development and then working backward to understand how multiple developmental processes interact to yield that result.

One example of the success of such teleological exploration comes from the study of mammalian neuromuscular innervation development. Due to the simplicity and uncrowdedness of the neuromuscular motor arborization, the end point in maturity was well known: motor axons branch to innervate multiple muscle fibers, but each muscle fiber is contacted by *exactly* one and only one axon. This seemingly simple circuit motif actually posed a very difficult challenge to mechanistic explanation. A simple rule of random connection

where the average number of axons on one muscle fiber would be one is easy to envision but would result in a distribution of contact numbers with binomial statistics and include some zeros, twos, and threes—which are in fact never observed. Knowledge that the actual end point was always and *exactly* one led to a detailed in vivo imaging analysis that revealed the central importance of a rule of competition between axons of different motoneurons at early stages of arbor development. In the absence of detailed, quantitative knowledge of the specific circuit end point, the questions that led to the divination of this competition mechanism might never have been asked, and this key principle might never have been postulated. Will we be lucky enough to ask the right questions about the mechanisms that construct the much more complex circuitry of the CNS without some pretty clear ideas about the specifics of the patterns that emerge? The case of neuromuscular single-innervation suggests that merely qualitative information akin to “motor axon contacts muscle” would not be good enough—quantitative information (n = exactly one) was necessary to motivate the decisive investigations.

To understand the development of a circuit, it may be necessary to have detailed and quantitative information about the network architecture that is the endpoint of that circuit's development. Unfortunately, details of CNS circuit wiring diagrams remain veiled by the small size and dense packing of the axonal and dendritic “wires” and the synapses that make up these circuits. Moreover, modern molecular biology has made it extremely clear that neurons and synapses are extremely complex and diverse at the molecular level. There is no circuit in the CNS where our knowledge extends to the level of detail that motivated the exploration of neuromuscular single innervation. We know, for instance, that “hippocampal CA3 pyramidal cells” synapse onto “hippocampal CA1 pyramidal cells,” but quantitative information about such connections, and information about the diversity of interconnecting synapses, is almost completely lacking. Moreover, it is now clear that many “subtypes” of the classical hippocampal neuron “types” (such as “CA1 pyramidal”) can be readily discriminated on the basis of molecular information and information about afferent and efferent projections of specific neurons. Still less is known, for instance, about subtypes and wiring of the inhibitory interneurons that synapse upon hippocampal pyramidal cells, even though inhibition may be the real essence of the hippocampal computation. Absent better knowledge of such circuit endpoints, it is difficult to begin grappling with or even framing the challenges they pose to developmental neuroscience and to in vivo imaging analysis.

There is very little specific or quantitative information about CNS connectivity. There are only a few very isolated counterexamples. Moreover, it seems very likely today that details and diversity of component neurons' molecular identity—especially of the adhesion, guidance, and cytoskeletal signaling molecules that determine morphology and connectivity and the electropotent molecules, such as ion channels and receptors and transmitter release machinery—are extremely important to circuit functionality. Clearly, traditional (19^{th} and 20^{th} century) cell type classification falls far short of managing this molecular diversity, and it may be that new, molecular classification schemes, beyond simple notions of cell type and subtype, will be needed. Fine-grained neuronal differentiation is emerging as an extremely important element of circuit functional architecture—and almost surely not something that either functional or developmental neuroscience can safely ignore much longer! The new field of high-resolution in vivo imaging of CNS neuronal and synaptic dynamics is already marked by divergence of observation and occasional controversy, much of which may revolve around our presently poor ability to discriminate between distinct “classes” or subsets of neurons and synapses.

Fortunately, optogenetic tool constructs and tool transgenic animals are now revolutionizing opportunities both to observe developmental dynamics in vivo (as noted above) and to contact specific subsets of neurons for physiological recording and manipulation. This convergence of optogenetic tool opportunities may provide a key to managing the diversity of neurons,

synapses, and circuits and thus allow us to begin placing developmental in vivo imaging results in the context of functional circuit architectures. For one example, transgenic animals expressing fluorescent proteins or Cre recombinase in restricted subsets of neurons are now beginning to abound, and as these subsets come to be better characterized and understood in molecular, structural, and functional terms, some may be shown to offer specific and repeatable handles on neuron type subsets with well-defined functional circuit roles. As the subsets in question become better defined, subset transgenic tool lines may provide the most ready opportunity to place studies of developmental dynamics in a functional circuit context.

By completely and intensely staining sparsely distributed neurons, the Golgi stain provided 19th century anatomists with the first glimpses of complete neuronal structures and thereby gave rise to many of the key concepts of modern neuroscience. The sparseness of the Golgi stain and its modern-day analogs, including sparse fluorescent protein-expressing lines, remains a gift but is also in some ways a curse because it reveals individual neurons only by rendering neighboring neurons and thus the circuit context invisible. Newly introduced “Brainbow” and other multicolored mouse lines might provide a practical solution to this conundrum (Livet et al., 2007). By staining (eventually) every neuron, but in many different readily distinguishable colors, these color-rich transgenic lines may allow for fast and reliable imaging of most or all of a circuit's component neurons at once and thus provide for the imaging both of individual neurons and the circuit context of which they are just one small part. While present Brainbow lines do not provide cell-type-specific molecular handles directly, they provide a unique opportunity to define circuit context structurally, and this information may allow for linkage to molecular information accumulated by other methods, such as array tomography, applied to the same circuit (Micheva and Smith, 2007). The in vivo imaging of Brainbow transgenics may also be of value in allowing exploration of the kinds of cell-cell interactions that will only begin to make sense when two or more neurons are observed simultaneously.

Thus, tool transgenics such as Cre lines and Brainbows provide two different (and probably complementary) approaches to the in vivo visualization of developmental dynamics in the context of the connectivity and molecular architecture of the specific circuit under construction. This capability has no precedent (at least in the CNS) and seems likely to open the door to answering some of the questions raised above regarding the molecular mechanisms and cellular rules that govern the assembly of diverse and very numerous collections of cellular and molecular components into functional neural circuits.

Conclusions

The quest to understand how any structure as complex and intricate as a functioning neural circuit can assembles itself looms before us as one of the greatest challenges science has ever faced. At present, in fact, it is almost hard to imagine the form that any human understanding of a process involving so many interacting parts and signals could possibly take. Nonetheless, it seems inevitable that the roads toward this exalted goal will be paved with improvements in tools for visualizing the structure and dynamics of all those parts and signals. Here we celebrate two recent, revolutionary, and highly synergistic advances in our intravital imaging toolkit: one the introduction of genetically encoded fluorescent markers that began with the cloning of the jellyfish green fluorescent protein, and the other the introduction of nonlinear fluorescence imaging methods that began with the introduction of multiphoton excitation microscopy.

Looking forward, we speculate that future advances will require that we leverage these new intravital imaging tools to develop a conceptual and experimental framework for understanding the “behavioral psychology” of the neurons and glia as they cooperate to assemble themselves into functional circuits. We also propose that any satisfying conceptual synthesis of our insights

about the dynamics of individual organelles, signals, and cells will require a perspective that includes description of the structure of the developing circuit, its wiring diagram, and its molecular architecture, at levels of detail and completeness that far exceed our present knowledge. The fledgling field of connectomics (see Lichtman and Sanes, 2008 for a definition) will need to advance so that we know what the nerve cells are trying to accomplish by virtue of their developmental behaviors. The deep challenges here seem likely to require that we go beyond traditional reductionist approaches. Future advances toward understanding neural circuit self-assembly will certainly depend on progress in synthetic and computational tools, as needed both to acquire the voluminous empirical data and to reconstruct useful models of circuit structure and function.

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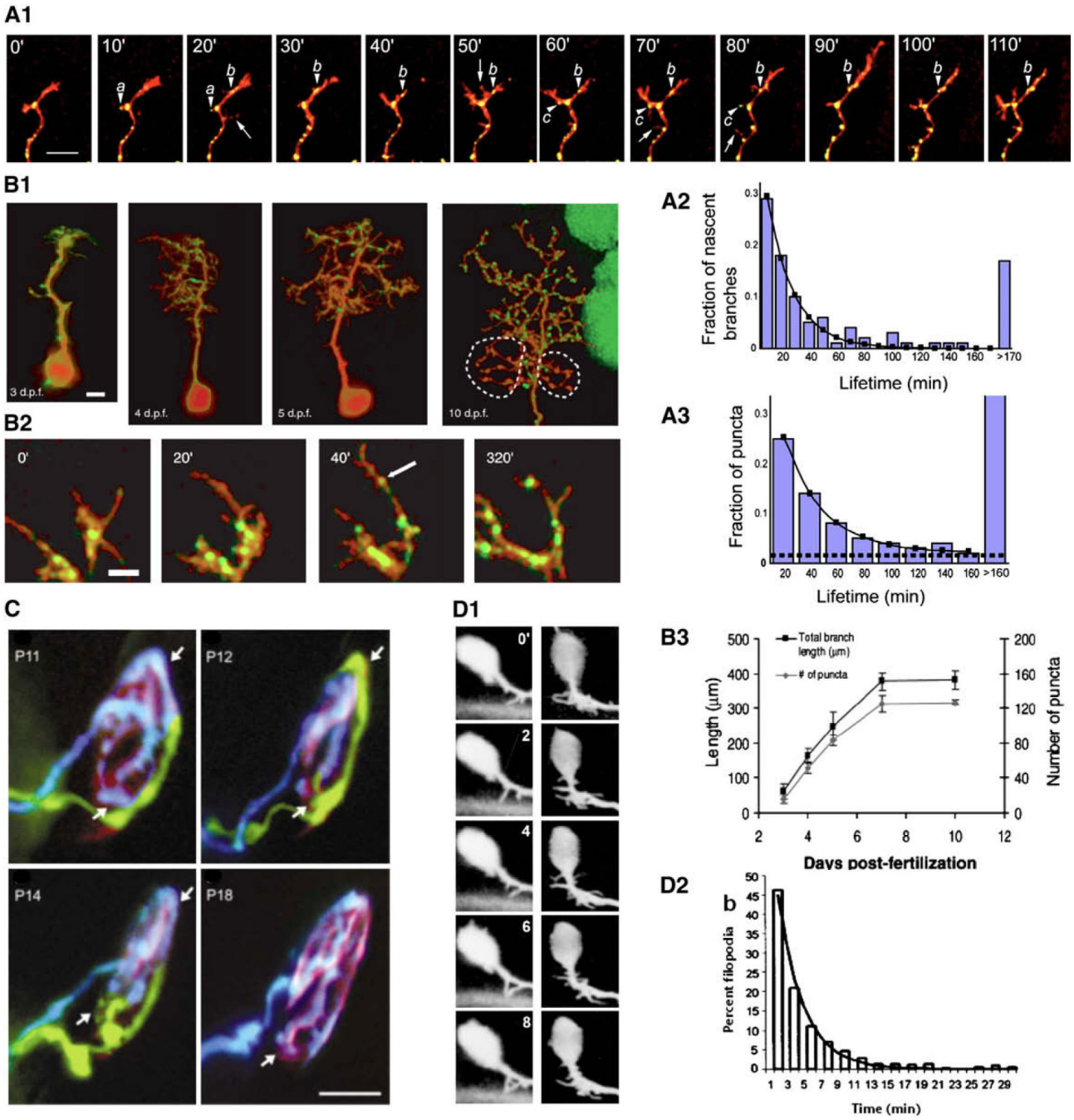

Figure 1. A Gallery Representing Discoveries of Developmental Dynamics that Were Possible only because of Intravital Timelapse Imaging Methods

(Drawn from work in the authors' laboratories.)

(A) Dynamics of growing retinal ganglion cell axon in larval zebrafish optic tectum (Meyer and Smith, 2006).

(A1) Construction of the axon arbor involves extensive and contemporaneous retraction and elimination of newly formed branches, while formation of presynaptic active zones appears to promote branch stabilization. Moreover, newly formed synaptic puncta are preferential sites of new branch formation (arrowheads). Two-photon fluorescence images were acquired at 10 min intervals. Soluble DsRed fluorescence (red) marks axonal cytoplasm; A

synaptophysin:GFP fusion protein (yellow puncta) marks cites of putative nascent presynaptic active zones. Scale bar: 10 μm.

(A2) Histogram representing observed lifetimes of newly formed branches. Most nascent axonal arbor branches have short lifetimes of less than 1 hr.

(A3) Histogram representing observed lifetimes of synaptophysin-gfp puncta. Most newly formed puncta are short lived.

(B) Dynamics of growing tectal neuron dendrite in larval zebrafish optic tectum (Niell et al., 2004).

(B1) Dendritic arbor growth occurs contemporaneously with synapse formation. Two-photon fluorescence images of the same tectal neuron were acquired at intervals as indicated in dpf (days post-fertilization). Soluble DsRed fluorescence (red) marks dendritic cytoplasm; A PSD-95:GFP fusion protein (green puncta) marks cites of putative nascent postsynaptic active zones. Scale bar: 10 μm.

(B2) Construction of a dendritic arbor involves extensive and contemporaneous retraction and elimination of newly formed branches, while formation of postsynaptic active zones appears to promote branch stabilization, often with an arrest of branch retraction at the exact site of a synaptic punctum (arrow). Time points indicated in minutes; scale bar: 3 μm.

(B3) Parallel time courses of dendritic arbor growth and synapse formation are consistent with a “synaptotrophic” model of dendrite growth, where the formation of synaptic puncta plays a causal role in the stabilization of newly formed branches. Quantitation from images similar to (B1) (six cells).

(C) Dramatic reversals in nerve terminal area during synapse elimination (Walsh and Lichtman, 2003). The four panels show four timelapse views of the same neuromuscular junction imaged between postnatal days 11 and 14. One axon expressing CFP (blue) loses and then regains postsynaptic territory. Between postnatal day (P)11 and P12 the CFP axon relinquished some of its territory to the YFP (yellow) input (compare arrows in top left and right panels). By P14, the CFP-expressing axon had reclaimed the upper right portion of the junction but continued to retreat from the lower part of the junction (compare arrows in P12 and P14 panels). At P18, the CFP input had reclaimed all of its former territory and had taken over the postsynaptic territory previously occupied by the YFP input. The thinner appearance of the junction after P11 is due to slight muscle fiber rotation. This kind of nonmonotonic behavior can only be appreciated by imaging of the same specimen over time. Scale bars equal 10 μm.

(D) Dynamics of dendritic filopodia on a DiI-labeled motorneuron in embryonic zebrafish spinal cord (Jontes et al., 2000).

(D1) Left column is time series (intervals in minutes as indicated) of images acquired using a laser-scanning confocal microscope. Right column is similar specimen (same intervals) imaged using two-photon microscope. Note the superior quality of the image acquired using two-photo excitation in comparison to the single-photon excitation confocal. In this case, image quality of the confocal was limited by the need to limit excitation energy to avoid photodamage to DiI-stained neurons. Higher excitation rates were possible without photodamage using two-photon excitation.

(D2) Rapid dynamics of dendritic filipodia are consistent with a role in exploration for suitable presynaptic partners. Histogram represents the very short lifetimes typical of motorneuron dendritic filopodia at times of developmental synapse formation. Scale: 20 μm panel width.