Chapter 5
Live Imaging of Connectivity in Developing Neural Circuits in *Drosophila*

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Abstract How neural circuits assemble during development influences functional adult circuit architecture, specificity, and variability. Live observation of brain development reveals stochastic and dynamic processes that help to understand functional constraints in the adult circuitry. In the first part of this chapter, we will explore what live imaging tells us about how dynamic processes create and constrain circuit specificity. In the second part of this chapter, we provide a current view of how live observation can be achieved in intact *Drosophila* brains in comparison to developmental imaging in other species. The goal of this chapter is to provide both the context and tools to understand neural circuits as a function of their developmental context.

5.1 The Developmental Context: From Dynamics to Synaptic Specificity

Key questions in modern neuroscience include "*how do neural circuits work?*" and "*how do neural circuits form?*". Approaches to answer these questions require overlapping information concerning neuronal morphology and connectivity. When Roger Sperry referred to an "unadaptable rigidity" of mechanisms that drive the development of visuomotor connectivity (Sperry 1943), he provided a theory that was to define developmental neuroscience for decades to come. The chemoaffinity theory has evolved to include the idea of wiring codes that neurons use to make connections with their targets based on specific molecular markers. In this section, we will discuss a conceptual framework that builds on and expands the chemoaffinity theory to include dynamic and stochastic processes, which are best studied through developmental live imaging.

The search for guidance molecules and the elucidation of their molecular mechanisms has been hugely successful, especially during the last 20 years

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(for review see Kolodkin and Tessier-Lavigne 2011; Raper and Mason 2010; Yogev and Shen 2014). As more molecules and mechanisms were discovered, the ideas of how guidance cues and codes function became more nuanced. Numerous molecules have been shown to function repeatedly at different places and times, in combinations and in gradients (Sanes and Zipursky 2010; Yogev and Shen 2014). Importantly, not all molecules that exhibit attractive or repulsive binding function as guidance cues; for example, thousands of isoforms of the repulsive, homophilic cell adhesion molecule Dscam ensure self-avoidance of dendritic branches from the same neuron, but provide no specific directional cue for any neuronal extension where to grow or make synapses. Instead of functioning as cues, Dscam isoforms (similar to vertebrate Protocadherins) execute a simple pattern formation process based on self-avoidance (Kise and Schmucker 2013). In another example, the widely expressed cell adhesion molecule N-Cadherin is not required as a targeting cue in *Drosophila* photoreceptors, but can function in the stabilization of growth cones (Ozel et al. 2015). Hence, attractive and repulsive molecules can play important roles in neural circuit assembly without specifying target areas or cells.

The two examples above highlight another important extension of the original chemoaffinity theory: both Dscam-mediated self-avoidance and N-Cadherin-mediated stabilization contribute to wiring specificity using dynamic and stochastic processes. Apart from the random Dscam isoform choice of individual neurons, self-avoidance leads to spreading of dendritic branches only if individual branches non-deterministically grow such that self-avoidance can act on them. As a consequence, every neuron, like every snowflake and every apple tree, has a uniquely different branching pattern. Similarly, every growth cone has a unique branching pattern of dynamically extending and retracting filopodia. In the case of *Drosophila* R7 photoreceptor axons, this random pattern of extensions and retractions seems to be required for N-Cadherin-mediated growth cone stabilization (Ozel et al. 2015). These cases exemplify how dynamic and stochastic growth is in fact necessary for the molecules to execute their function. This observation significantly extends on the early versions of the chemoaffinity theory based on molecular matchmaking cues. The idea of matchmaking is more deterministic: from the perspective of a specific cue a stochastic process is more likely to represent noise that the system would try to minimize, rather than a necessary part of the molecular mechanism. Neural circuit assembly is likely to employ both mechanisms: pattern formation based on stochastic growth as well as molecular specification through matchmaking; examples for both have been firmly established (Hassan and Hiesinger 2015; Kolodkin and Tessier-Lavigne 2011; Yogev and Shen 2014).

To understand the role of dynamic and stochastic processes as part of neural circuit assembly, we have recently proposed a rules-based framework to help incorporate the mechanisms and roles of molecules like Dscam and N-Cadherin as part of developmental algorithms underlying brain wiring (Hassan and Hiesinger 2015). In the example of Dscam, in this framework, the focus is on the level of the rule ‘self-avoidance’ as part of a larger developmental program, rather than the molecular mechanism of homophilic, repulsive binding that executes that rule (or the molecule itself for that matter: the same rule can be carried out by different

molecules, as exemplified by Protocadherins in vertebrates). In this case, the rule can be formulated as: (1) grow stochastic filopodial extensions and branches, (2) stop individual filopodia from growing further when contacting other filopodia from the same neuron. This rule is not sufficient without additional constraints, e.g., probability of branching, inter-branch spacing, etc., but it captures the essence of the developmental process executed by the homophilic repulsion function of Dscam.

To understand the interplay of stochastic growth and molecular mechanisms in executing a rule like self-avoidance, it is beneficial to think of neurons and neuronal processes as individual entities that explore the environment, advance to targets and compete with each other. Lichtman and Fraser (2001) proposed the analogy between such a setting and a football (or similar sports) game. Each player has its abilities, restrictions as well as his or her own agenda. If the players each follow the rules, these games will create an ordered structure without any external supervision; however, the exact outcome is not scripted. Referees and game plans can help structure the game, but are not strictly required: even a backyard football game with variable numbers of players and imprecise playing field can work out wonderfully.

How do we figure out the rules of the game called 'brain wiring', which is arguably much more complicated than any sports we have ever invented? An established approach is to disrupt specific genes, in particular those encoding presumptive guidance cues, and study the end results of the effects on the circuit. *Drosophila* has been a particularly useful system using this approach, in part due to the development of a technique to render individual neurons mutant in an otherwise wild-type animal (Lee and Luo 1999). If the disrupted gene is indeed a molecular matchmaking cue, then loss or gain in individual neurons predictively rewire connectivity, as has been shown in several examples, e.g., the teneurins in Drosophila (Hong et al. 2012) or type II Cadherins in the vertebrate retina (Duan et al. 2014). In many cases, however, loss and gain of function studies for single genes led to surprising, less instructive outcomes. Following up with the soccer analogy, a perturbation may, for example, restrict the usage of hands for each player one by one, revealing that only in the case of the goalie this causes the eventual score to change. Here, the analogy highlights what gene perturbations may indirectly provide insight into the rules of the game. Many rules have been discovered through carefully conducted gene perturbation experiments, including the discovery of self-avoidance through mutant analyses of Dscams. If the goal is the characterization of the rule, however, it is not a priori clear that a gene perturbation experiment is the shortest path to uncover the rule. For example, the rule on usage of hands in a soccer game may also be deduced from observation of the unperturbed game. Comparing static pictures from different points of different games would not easily reveal this rule. Static pictures can provide important information on the game but will fail to capture stochastic and dynamic actions that do not stereotypically happen at an exact time point for every single member of a 'player' type. Since stochastic and dynamic actions are key to pattern formation rules like self-avoidance, live observation is of particular importance to the discovery of rules

underlying brain wiring. Ultimately, a combination of gene and cell perturbation experiments with live observation yields the highest likelihood of uncovering principles underlying the development of connectivity.

As we will discuss in the second section of this chapter, during the past 15 years our ability to image live neurons forming circuits in their natural environments has significantly improved, especially in the model organisms worm, fly, zebrafish, and mouse. Yet, we have only begun to characterize the dynamic properties of developing neural circuits. In the early parts of brain development, live imaging has already been very useful for the study of neural stem cell migration (Lerit et al. 2014; Ortega and Costa 2016). When it comes to studying the development of circuit connectivity, we need to look at neurons that develop two types of dynamic structures: axonal growth cones and dendritic extensions. In both cases stochastic extension and retraction dynamics of filopodia underlie what appear to be robust choices in the adult connectivity. The next subsections will address our current state of knowledge about the role these subcellular filopodial structures have in vivo and how they might relate to the establishment of synaptic specificity.

5.1.1 Growth Cone Guidance and Early Filopodia

When Cajal studied chick embryos to show that axons grow out of neurons, he discovered what he called a "cone-like lump with a peripheral base" with thorny processes at the tips of commissural axons (Ramón y Cajal 1890). This description came to define the features of the 'textbook growth cone': a widened terminal with filopodia and lamellipodia at its tip. The growth cone has received plenty of attention over the decades as the presumptive structure that detects guidance cues and actively advances to the target (for review see Raper and Mason 2010; Vitriol and Zheng 2012). Filopodia have been suggested as agents for the detection of guidance cues (Rajnicek et al. 2006; Zheng et al. 1996) and in vitro evidence was provided in favor of filopodia forming a clutch mechanism for growth cone movement by acting as 'sticky fingers' (Chan and Odde 2008; Heidemann et al. 1990). It has largely remained unclear, why and how stochastic extension and retraction dynamics execute these processes.

Most of our understanding of the dynamics of growth cones is based on in vitro systems. However, some in vivo live imaging data has already provided glimpses into the functions of filopodia that do not easily fall into the categories 'searching agents' or 'sticky fingers'. Some of the first live imaging studies in intact tissue revealed that growth cones tend to adapt simple, streamlined forms while extending and more complex forms (like the classical growth cone) when they pause or reach their targets, e.g., in mammalian retina preparations (Godement et al. 1994) and intact zebrafish embryos (Jontes et al. 2000; Kaethner and Stuermer 1994). These observations have been interpreted as growth cones adapting to complex, high filopodial activity forms at 'decision regions', where there are multiple cues and the axon needs to make a decision on which direction to go; then it quickly advances

towards that direction without many filopodia (Mason and Erskine 2000). On the other hand, it has been shown that filopodia are at least partially dispensable for axon navigation but essential for terminal arborization of retinal axons (Dwivedy et al. 2007). Recent findings on developing R7 photoreceptor growth cones in intact *Drosophila* eye–brain complexes revealed a similar pattern (Ozel et al. 2015). These axons exhibit streamlined structures while extending, but expand into more complicated filopodial structures once they stabilize at the target layer. Interestingly, when their attachment to that layer was genetically impaired, these growth cones went, at unpredictable time points, through a gradual filopodial collapse followed by regaining of motility by the axon tip. The close temporal link between filopodia formation and axon stabilization suggests that in this case filopodia might function as an adhesion surface for stabilization, rather than being important for guidance or extension. However, a direct causal link between the two processes is yet to be established.

Finally, rather than directing the growth cone to the target, filopodia can also function in guidance by extending to a target, expanding and becoming the new axon terminal/growth cone. This was observed in vitro (O'Connor et al. 1990) and in intact *Drosophila* embryos (Murray et al. 1998). A similar behavior is observed for developing R8 photoreceptor growth cones in *Drosophila* as they relocate from their temporary position to the medulla layer M3 at mid-pupal development (Ozel et al. 2015). These growth cones extend a single filopodium to deeper layers, which is initially very dynamic with almost complete retractions and re-extensions but eventually stabilizes its tip in the correct target layer, expands, and ultimately forms the adult R8 axonal terminal prior to synaptogenesis.

The few selected examples discussed here highlight the origin of an important aspect of adult circuit connectivity: The precise axon positions, dendritic branch points, and their contacts in the adult may not only be slightly imprecise due to biological noise, but be the result of necessary stochastic processes based on filopodial dynamics during growth cone guidance, stabilization, and synaptic partner identification. Especially on this latter aspect, important insight comes from filopodia on axons and dendrites at later developmental stages, as discussed in the following section.

5.1.2 Synapse Specification and Late Axonal and Dendritic Filopodia

After a growth cone reaches its target area, the main body of the axon no longer advances; therefore, it may be more accurate to classify the filopodia at this stage as 'axonal filopodia'. These are not limited to the tip of the axon and have been linked to axonal branching (reviewed in Gallo 2011). Dendrites also form filopodia, and these perhaps constitute the class of filopodia that has so far been most closely linked to the establishment of synaptic connectivity.

Work by Stephen Smith and colleagues have provided pioneering insights into the roles of axonal and dendritic filopodia during brain wiring. Live imaging of tectal neuron dendritic arbors in intact developing zebrafish revealed that young arbors create many transient filopodia, some of which become the sites of de novo synapse formation. In turn, formation of these synapses directly stabilizes the respective filopodia, turning it into a stable branch (Niell et al. 2004). Live observation helped to establish a link between the processes of synapse formation and filopodial stabilization without perturbation experiments. Filopodia without a synapse never persisted longer than an hour and the stabilized filopodia only retracted if its synapses were eliminated. Similarly, at the presynaptic partners of these cells (retinal axons), new branches extend preferentially from newly formed synaptic sites and no branch is stabilized over an hour without a synaptic site present (Meyer and Smith 2006). Together, these observations support the synaptotropic model (Vaughn 1989), whereby stabilization through synapse formation guides axonal and dendritic extension. Importantly, this process can only work if axonal and dendritic arbors provide initially stochastically extended filopodial processes to select from. A process based on the rule of selection and stabilization precludes precise positioning of pre- and postsynaptic partners, but not synaptic specificity, in adult circuit connectivity.

These data showcase links between synapse formation and filopodial dynamics and how the former can direct the latter to bias axonal and dendritic arbors towards stabilized connection sites. However, they are only our first glimpses into the roles of filopodial dynamics in brain wiring in intact, developing brains—and they have largely been limited to wild type. Important questions remain, for example: How are some synapses selected to stabilize while others are lost? What are the rules and mechanisms underlying synapse-mediated branch stabilization? Watching the dynamics of axonal and dendritic dynamics in wild-type and mutant neurons are necessary approaches to answer these questions and understand the rules that establish adult connectivity.

5.2 The Imaging Approach: Watching Circuit Assembly Live

Live imaging protocols for *Drosophila* are available for embryos (Evans et al. 2010; Reed et al. 2009), larval neuromuscular junctions (Schmid and Sigrist 2008) and brains (Cabernard and Doe 2013). Investigating the dynamics of how adult neural circuits form requires long-term imaging of developing pupal brains at high spatiotemporal resolution. Until recently, fast and high-resolution imaging in developing *Drosophila* brains was largely restricted to short (about 1 h) imaging periods (Williamson and Hiesinger 2010). In this section, we will describe two techniques that we recently described for long-term, high-resolution imaging of intact, developing pupal brains: unobtrusive intravital imaging in intact pupae and

ex vivo imaging in developing eye–brain complexes. We will also discuss available microscopy options along with technical concerns related to the imaging. Finally, we will provide a brief overview of techniques in other model systems that allow long-term imaging of neural circuit formation.

5.2.1 *Intravital Imaging of the Developing Drosophila Brain*

We refer to intravital imaging as a completely non-invasive technique that does not interfere measurably with development or function. We have recently performed intravital imaging of developing brains through the eyes of pupae (Langen et al. 2015). This method is, to our knowledge, the only known example of completely non-invasive imaging of pupal to adult brain development in flies, and may allow imaging of deeper brain structures as well.

During the first half of pupal development the *Drosophila* eye is mostly transparent and the center of the eye is largely not obstructed by lipid droplets or light-scattering tissues. In addition, during the pupal stages there are no muscle contractions, eliminating the need for anesthesia or physical immobilization. This allows monitoring of the developing optic lobe in a completely non-invasive manner for a limited time window of brain development (Fig. 5.1). We exploited these facts to build an imaging chamber (Fig. 5.2), which allowed us to perform long-term, high-resolution imaging of photoreceptor terminals in the lamina neuropil (Langen et al. 2015). The method described here allowed imaging of these growth cones at the resolution of filopodial dynamics for up to 24 h, and the pupae continued normal development thereafter to adulthood.

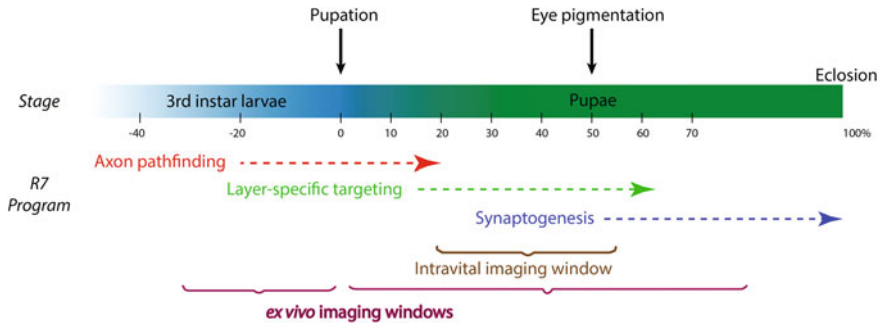

Fig. 5.1 Timeline of *Drosophila* development from late larval stage to adulthood. As a reference, different developmental steps of R7 photoreceptor axons are overlaid. Intravital imaging can be performed between $P + 15\%$ and 55% of the pupal development, while *ex vivo* imaging can be performed throughout the pupal development as well as during 3rd instar larval stage

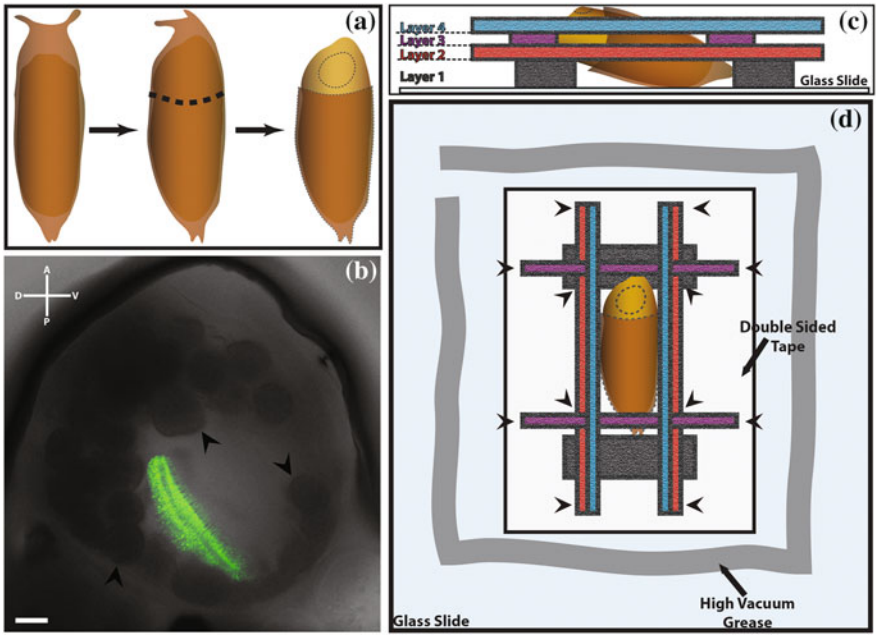

Fig. 5.2 **a** Pupa is aligned such that the right eye is imaged after the cuticle around the head is dissected. **b** Transmitted light image of the right eye of a pupa. Lamina neurons are genetically labeled with GFP, revealing the position of the lamina layer of the optic lobe. *Arrowheads* mark the lipid bodies. **c** *Side* view of the imaging chamber. Four layers of filter paper are shown in different *colors* for clarity. Pupa is kept in position as the whole body axis makes an angle with the plane of the glass slide. **d** *Top* view of the imaging chamber. Layers are shown in the same *colors* as in **c**. *Arrowheads* show the positions where 4% agarose is applied. Images courtesy of E. Agi

A brief protocol summary for intravital imaging through the pupal eye is provided here: First, a part of the pupal case is removed from the head section (Fig. 5.2a); the pupa is mounted on its side at an angle that exposes the eye as the highest point (Fig. 5.2c). Around the pupa an elevated barrier is constructed with filter paper to keep the chamber moist; agarose is used as cement at junctions of the layers (Fig. 5.2d). Vacuum grease holds the cover slip in place. A drop of HL3 solution is put on a cover slip before it is placed on the eye. It is important to make sure that the eye makes firm contact with the cover slip by applying gentle pressure on the cover slip with the forceps while avoiding bursting the eye. Additional technical details have been published (Langen et al. 2015, Supplementary Experimental Procedures).

The chief advantage of this method is the ability to remove the pupae from the chamber after imaging and rear them to be healthy adults, which can then be analyzed to test whether normal development was affected by the imaging conditions. This level of non-invasiveness, however, comes with certain limitations.

First, after $P + 50\%$ of pupal development the fly eye starts to accumulate pigment, which effectively prevents brain imaging. Using white eyed flies (which can be generated even in the presence of *white+* transgenes through a combination of *brown* with either *cinnabar*, *scarlet,* or *vermillion*) (Kim et al. 2013), can extend the imaging window up to $P + 60\%$. However, the thickening of the eyes increases light scattering, complicating deep brain imaging after this point even in white-eyed flies. Early in development, the intravital imaging time window through the pupal eye is limited to around $P + 15\%$. Until this time point, the pupal case is attached to the cuticle; as a result, removal of the pupal case to expose the eyes before that stage is very difficult.

Second, having to image through a defined window, i.e., the eye, spatially limits the accessible brain areas. Imaging deep in the optic lobe and beyond significantly reduces the signal-to-noise ratio. Finally, both sample movement as well as the intrinsic morphological rearrangements inside the developing pupae often results in significant drift, complicating the experiment and requiring time consuming post-processing.

In summary, the currently available method for intravital imaging of the developing *Drosophila* brain is perfectly suited to study developmental processes of the eye, lamina or distal medulla between $P + 15\%$ and $P + 55\%$.

5.2.2 *Ex Vivo Imaging of Developing Eye–Brain Complexes*

It has long been known that pupal eye–brain complexes of *Drosophila* can recapitulate certain events of metamorphosis when removed from the body and placed under well-controlled culture conditions (Gibbs and Truman 1998). This has been exploited to maintain brains in culture over long periods (Ayaz et al. 2008). An adaptation of this method to perform high-resolution time-lapse imaging required particular consideration of sample drift and phototoxicity as chief problems over long periods at subcellular resolution. To tackle these issues, we recently developed an imaging chamber that can be built with minimal effort (Ozel et al. 2015).

A **brief protocol summary for ex vivo brain imaging is provided here**: We use a combination of embedding in low-melting agarose and a closed system that virtually eliminates sample drift. In addition, placement of the brain right under a coverslip allows access to high-powered objectives with minimal working distance to maximize signal and therefore reduce the required laser power, which is crucial for keeping the tissue healthy during extended periods of imaging (Fig. 5.3b). A detailed step-by-step protocol for building the culture imaging chamber has been published (Ozel et al. 2015). Briefly, pupal eye–brain complexes are dissected in chilled Schneider's *Drosophila* Medium and placed within a drop of 0.4% (in culture medium) low-melting agarose on a Sylgard layer in a petri dish lid. Next, a circular coverslip (4 cm diameter) is placed on the drop; 200–250 µm-thick (depending on the brain's age) spacers are used to prevent the coverslip from crushing the brain. After the polymerization of agarose, the remaining space under the

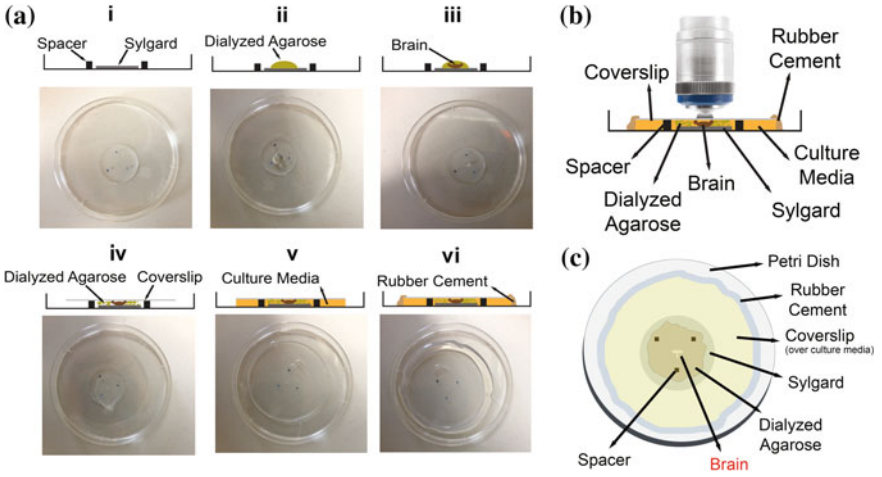

Fig. 5.3 **a** Step-by-step construction of the imaging chamber. (*i*) Spacers are placed on the Sylgard layer in a *triangle* formation. (*ii*) A drop of diluted dialyzed agarose is pipetted onto the Sylgard. (*iii*) Dissected eye-brain complex is placed into the agarose drop. (*iv*) The mix is covered with a coverslip. (*v*) After the agarose polymerization, remaining space under the coverslip is filled with the culture media; (*vi*) and sealed completely with rubber cement. Schematic of the final chamber **b** from the *side* and **c** the *top*. Adapted from Ozel et al. (2015). DOI:10.7554/eLife.10721.004

coverslip is filled with oxygenized culture medium before the chamber is sealed using rubber cement (Fig. 5.3a). We use a culture medium that is based on Schneider's medium and includes 10% fetal bovine serum (FBS), 10 μg/ml insulin, 1% penicillin/streptomycin mix and 1 μg/ml 20-Hydroxyecdysone (20-HE). Usage of a closed system limits the amount of available oxygen and nutrients to the amount of culture media that fits under the coverslip (approximately 500 μl). We have found this is sufficient to support normal development for 24 h. A similar approach has recently been published using an open chamber and culture medium that included ascorbic acid and higher concentrations of ecdysone (Rabinovich et al. 2015). If longer imaging times are necessary, both systems can be adapted to a perfusion chamber (Williamson and Hiesinger 2010).

The ex vivo system addresses two main limitations inherent to intravital imaging. First, every part of the brain is accessible to high-resolution imaging. Second, brains at all stages of pupal development as well as larval brains can be imaged using this technique. However, we found that cultures that start after $P + 60\%$ are often less healthy, possibly because it becomes increasingly difficult to dissect intact eye–brain complexes as the eyes start to fuse with the head cuticle. Furthermore, it does not seem possible to recapitulate the events at the onset of metamorphosis in culture and cultures that start earlier than $P + 10\%$ require a higher concentration of 20-HE (Rabinovich et al. 2015). Finally, previous measurements showed that ecdysteroid levels during metamorphosis peak around $P + 40\%$ and then start to drop (Paul Bainbridge and Bownes 1988). Consistent

with this data, we have found that it is necessary to exclude 20-HE from cultures that start after $P + 50\%$ (Ozel et al. 2015). Failure to do so appears to slow down eye pigmentation and induces aberrant filopodial formations on R7 photoreceptor axonal terminals.

Despite its advantages, ex vivo imaging inherently retains one main drawback: not being in vivo. Certain developmental events depend on surrounding tissues, e.g., eye disc expansion or lamina rotation. In addition, some developmental processes occur slower or faster compared to in vivo controls, requiring careful calibration and controls (Ozel et al. 2015).

5.2.3 *Microscopy Systems for Developmental Live Imaging*

Despite the recent advances in newer technologies like super-resolution or light-sheet microscopy, imaging of small regions deep in living tissue currently still remains the domain of multiphoton microscopy. However, our ex vivo imaging chamber is also compatible with other light microscopy approaches, including standard confocal systems (Zschatzsch et al. 2014), particularly for short-term experiments and superficial brain areas (close to the coverslip). Resonant scanning systems, which provide 10–20 times higher scan speeds compared to conventional systems (Art and Goodman 1993), reduce phototoxicity and are preferable for live imaging. Increased noise that emanates from high laser scan speeds typically need to be reduced by averaging; importantly, even if the total scan time may not be improved significantly when using high averaging, photobleaching is significantly lower at the same time-lapse speed. It is better to scan the same point 20 times for x amount of time rather than scanning it once for $20x$ amount of time because the former allows fluorescent molecules to relax back to the ground state, preventing them from potentially being hit by another photon in their excited, unstable state and rendering them dysfunctional (Borlinghaus 2006).

Confocal microscopes use a pinhole to exclude out-of-focus light. As a result, it excites fluorophores all over the sample, but detects only a fraction of them (Webb 1996). Multiphoton microscopes, on the other hand, utilize simultaneous absorption of two lower energy (higher wavelength) photons. Because excitation rate in this case depends on the second power of light intensity, excitation outside of the focal plane becomes extremely unlikely (Denk et al. 1990), i.e., all of the excited fluorophores can now be detected. The elimination of the need to spatially restrict detected light is the key advantage of two-photon microscopy. First, sending the fluorescence light back through the scanning pathway (descanning) is no longer necessary. By using non-descanned detectors (NDD) close to the objective, valuable photons that would otherwise be lost at every mirror, lens, and filter can now be detected. Second, scattered light, which is very common in thick, living samples and would be excluded by a confocal pinhole, can now be detected. Detection efficiency of a two-photon microscope does not decrease with depth as dramatically as with confocal microscopy, because scattered photons eventually leave the sample

and can be counted. Finally, infrared light used for two-photon excitation scatters less than visible light, further increasing depth penetration.

Together, these advantages currently allow higher spatiotemporal resolution for small regions of interest in deep tissue than light-sheet microscopy. In contrast, light-sheet microscopy offers much faster image acquisition of large fields of view at high and even super-resolution (Heddleston and Chew 2016; Hu et al. 2014; Lemon and Keller 2015). However, multi-channel imaging with a two-photon microscope can be complicated. Traditionally, most commercial NDD units have a maximum of two channels, but quad-channel NDD units have recently become commercially available. The maxima for most red fluorophores fall outside the range where standard Ti::Sapphire lasers provide sufficient power (700–1000 nm). Far-red excitation can be achieved with a dedicated second laser or solutions such as OPO (optical parametric oscillator), which extend the excitation range to 1300 nm and beyond.

5.2.4 Fluorescent Markers for Developmental Live Imaging

Along with the advancements in microscopy, the so-called 'GFP revolution' (Chalfie et al. 1994; Heim et al. 1994; Prasher et al. 1992) is responsible for the fact that live imaging as presented in this chapter is possible. Here, we will briefly discuss genetically encoded fluorescent proteins best suited to image neural circuit development using multiphoton microscopy in *Drosophila* brains.

5.2.4.1 Blue/Green Fluorescent Proteins

Despite the development of a plethora of new variants of GFP, the early original EGFP (Heim et al. 1995) remains one of the best fluorophores for multiphoton microscopy. It is a monomer, has low phototoxicity and high two-photon brightness. In vitro measurements place its two-photon excitation maxima at 927 nm (Drobizhev et al. 2011), but in practice we observed only little variation between 900–940 nm in *Drosophila* tissue. A more recent CFP variant, mTFP1 (Ai et al. 2006), has twice the measured two-photon brightness of GFP at the excitation maxima of 875 nm (Drobizhev et al. 2011), but we currently have no experimental data on its use as a protein tag. For imaging of thin membrane processes, we also require a good membrane tag along with a bright fluorophore. Popular tags include myristoylation (myr), CD4 and CD8. In our experience, CD4 performs best in labeling thin structures among the three (Han et al. 2011).

5.2.4.2 Red/Far-Red Fluorescent Proteins

Most available red fluorescent proteins are variants of DsRed, which unfortunately is a multimer and therefore not suitable as a protein tag. However, several newer variants (e.g., mCherry) behave like (or close to) monomers. tdTomato has the highest two-photon brightness of available red fluorophores. Its measured two-photon excitation maxima is 1050 nm (Drobizhev et al. 2011), but we have observed a stronger signal around 1100 nm in fly tissue. This could be related to lower tissue attenuation at these wavelengths. Fortunately, tdTomato can also be excited at 950 nm at decent (but not optimal) levels. This is close to the optimum excitation range for GFP and permits two-color imaging even with a single laser. However, tdTomato is a dimer-like DsRed and is therefore not recommended as a protein tag. In contrast, mKate2 is a monomeric far-red fluorescent molecule that is suitable for protein labeling. It is an improved, threefold brighter version of TagFP635 (mKate) (Shcherbo et al. 2009). It has a measured two-photon excitation maxima at 1140 nm and higher two-photon brightness than any other monomeric red protein (Drobizhev et al. 2011). It also has very high photo- and pH stability. On the other hand, highly red-shifted excitation properties make its usage impractical without a high wavelength laser.

5.3 Developmental Brain Imaging in Other Model Systems

In the final section of this chapter, we will provide a brief, non-comprehensive comparison to similar imaging approaches in other model systems where long-term live imaging of neural circuit development is feasible.

5.3.1 Caenorhabditis Elegans *Embryo Mount*

The nematode *C. elegans* is an excellent genetic model system and the entire embryonic development takes only 14 h. The simplicity of the system (even the adult animals have an invariant set of 302 neurons), small size, fast life cycle, and powerful genetic tools make *C. elegans* an excellent model to study molecular mechanisms of fundamental events (e.g., synaptogenesis). Due to their transparency, live imaging of these embryos is very feasible and the methods have been available for almost 20 years (Mohler and White 1998). As opposed to the *Drosophila* techniques presented in this chapter, live imaging in worms is scalable to very high sample sizes to perform screens. However, to our knowledge, the available methods are limited to the embryonic stage. The particularly small size of *C. elegans* is an advantage when imaging the entire animal, well suited for

light-sheet microscopy, and does not require multiphoton microscopy. On the other hand, *C. elegans* provides only a limited model for synaptic specification processes in dense brain regions.

5.3.2 Zebrafish Embryos and Larvae

Zebrafish (*D. rerio*) is an excellent model system for developmental live imaging and comes with the chief advantage (over *Drosophila*) of being a vertebrate. Both its embryos (Dynes and Ngai 1998) and larvae (Niell et al. 2004) are transparent and amenable to long-term live imaging. As discussed in the first part of this chapter, the key studies that provided insight into the relationship between filopodial dynamics and synaptogenesis in vivo came from this system (Meyer and Smith 2006; Niell et al. 2004). Zebrafish embryos develop rapidly; axon growth and synapse formation can already be observed by 24 hpf. Larvae are free swimming and need to be immobilized by embedding in 1% agarose. It may be necessary to include PTU (phenylthiocarbamide) to inhibit pigmentation but live imaging can be performed at least up to 10 dpf for 24 h sessions after which the larvae remain healthy and continue to develop normally (Niell et al. 2004). The challenges of the zebrafish system include fewer genetic tools compared to *C. elegans* or *Drosophila*. For example, sparse labeling of individual, genetically manipulated neurons is not easy. Despite an adaptation of the Gal4/UAS system over 15 years ago (Koster and Fraser 2001), most imaging protocols require injection of plasmid DNA encoding the fluorescent markers to embryos and then screening potentially large numbers of them to identify those suitable.

5.3.3 Frog Tadpoles

Xenopus tadpoles, like zebrafish larvae, are mostly transparent (though their embryos are not), making it possible to perform imaging on the intact animal (Keller 1978). This model has been extensively used to study axonal, dendritic, and synaptic dynamics, particularly during retinotectal circuit formation (Alsina et al. 2001; Elul et al. 2003; Li et al. 2011; Manitt et al. 2009); but also in other circuits, for review see (Erdogan et al. 2016). Traditionally, it has been limited to the early stages of development where tadpoles do not have significant locomotion because the requirement for anesthetics at the later stages is detrimental to long-term imaging. However, a technique that involves constant flow of the anesthetic MS-222 and thereby allowing imaging up to 48 h has recently been described (Hamilton and Henry 2014). Historically, one of the main reasons for imaging *Xenopus* development has been the significantly larger sizes of their cells (as well as their growth cones) than virtually any other model system; making it very suitable for studying subcellular events and protein localization. Its drawbacks are similar to the zebrafish system, i.e., limited selection of genetic tools.

5.3.4 *In Ovo Imaging of Chick Embryogenesis*

It is possible to image the development of chick embryos by inserting an imaging window into the egg, whose details are discussed elsewhere (Kulesa and Fraser 2000). Chick embryos have a great tradition for developmental biology, thanks to their evolutionary vicinity to mammals and easy accessibility of the embryos. Nevertheless, aside from not having a particularly robust genetic toolbox, in ovo imaging is limited to a time window from stage-8 embryos to 5-day old embryos. This window allows the imaging of early nervous system development such as neural crest patterning and somite formation, but not the formation of neural circuits. Extending the imaging window beyond 5 days requires tackling the challenges associated with natural movements of the embryo due to development, causing drastic drifts, as well as the movements due to the heartbeat (Kulesa et al. 2010).

5.3.5 *Imaging in Mammalian Systems*

Mammalian systems such as mice or rats have the highest biological relevance to human physiology and development. Live imaging protocols for nervous system development are available in particular for mice. In utero development may obstruct high-resolution imaging of intact developing mammalian brains; however mouse embryos can be cultured ex vivo until the E10 stage, for up to 24 h periods (Jones et al. 2002). With this technique, processes like cell migration and differentiation can be observed and it has been used to study neurulation (Pyrgaki et al. 2010); but more advanced stages of brain development, i.e., the time when neurons start to establish connections, are not easily accessible.

An important exception is the retina, which is particularly accessible in retinal whole-mount cultures of the mouse eye (Williams et al. 2013). The mammalian retina has an extensive neural circuitry comparable in complexity to the *Drosophila* optic lobe lamina and medulla combined (Sanes and Zipursky 2010). Live imaging can be performed while these circuits develop. Retinal whole-mounts preserve the intrinsic circuitry and can be maintained for several days, but it is recommended to stay within the first 36 h of initial dissection (Williams et al. 2013). Several dynamic processes have been studied with great success in this system, including (1) cell migration and the establishment of horizontal cell territories during early neonatal development (Huckfeldt et al. 2009), (2) dynamics of bipolar cell layer-specific axonal targeting and stabilization at P5 stage (Morgan et al. 2006), and (3) activity-dependent synapse formation between bipolar and retinal ganglion cells at the P9 stage, in combination with electrophysiology (Kerschensteiner et al. 2009).

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