genesis 49:534–545 (2011)

REVIEW

Time-Lapse Imaging of Neural Development: Zebrafish Lead the Way Into the Fourth Dimension

Sandra Rieger, Fang Wang, and Alvaro Sagasti*

Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, California

Received 19 November 2010; Revised 24 January 2011; Accepted 25 January 2011

Summary: Time-lapse imaging is often the only way to appreciate fully the many dynamic cell movements critical to neural development. Zebrafish possess many advantages that make them the best vertebrate model organism for live imaging of dynamic development events. This review will discuss technical considerations of time-lapse imaging experiments in zebrafish, describe selected examples of imaging studies in zebrafish that revealed new features or principles of neural development, and consider the promise and challenges of future time-lapse studies of neural development in zebrafish embryos and adults. genesis 49:534–545, 2011. © 2011 Wiley-Liss, Inc.

Key words: zebrafish; imaging; time-lapse; neural development; neurons

INTRODUCTION

During development cells move and change their shape as they integrate into functional tissues and organs; they divide in precise orientations, grow in a polarized fashion, gain or lose adhesive contacts, migrate, project appendages, form specialized connections with other cells, trim off pieces of themselves, die in an orderly manner, or alter their structure in response to physiological activity. Nowhere are these dynamic processes more complicated, nor is the need for their precision more crucial, than in the developing nervous system, where cells must connect with one another to form neural circuits. It is difficult to describe these behaviors or understand how they contribute to neural development solely by examining static developmental snapshots. Trying to reconstruct complicated cellular behaviors from still images of different animals would be like trying to decipher the rules of baseball from still photographs of several different games, or attempting to appreciate the grace of a dance from images of several different performances. Repeatedly imaging the same cells over time to create a time-lapse movie is a powerful approach for ordering events into a comprehensive, coherent sequence.

Zebrafish have emerged as the premier model for directly imaging dynamic cellular processes. Time-lapse imaging of cellular development during embryonic stages is difficult in mammals. To study embryonic development in mice, a pregnant mother must be sacrificed and each embryo removed from the uterus and dissected, providing a single snapshot of a particular developmental stage. As a proxy for investigating dynamic developmental behaviors in mammals, cultured cells, tissue slices, and organ explants have been used for time-lapse imaging. However, since cellular behaviors are often influenced by the embryonic environment, these preparations do not always recapitulate *in vivo* developmental processes with complete accuracy. The zebrafish model provides an opportunity for imaging development in live, intact vertebrate animals. Zebrafish embryos are optically clear and fertilized externally. Because they develop in water, the entire process of development can be observed over many hours without molesting the embryo. These advantages, combined with the availability of many mutants and an always-improving molecular toolkit, make zebrafish embryos a

Sandra Rieger and Fang Wang contributed equally to this work.

*Correspondence to: Alvaro Sagasti, Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA.
E-mail: sagasti@mcdb.ucla.edu

Contract grant sponsors: National Science Foundation; National Institute of Dental and Craniofacial Research
Published online 8 February 2011 in
Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/dvg.20729

powerful setting in which to study dynamic developmental events by time-lapse. Time-lapse imaging is a complement, rather than a substitute, for traditional analyses in fixed animals, which make it possible to collect larger sample sizes and to stain for endogenous messenger RNAs and proteins. Together time-lapse and fixed tissue experiments make possible a more complete view of neural development. This review will discuss technical considerations of time-lapse imaging experiments in zebrafish, describe selected examples of imaging studies in zebrafish that revealed new features or principles of neural development, and consider the promise and challenges of future time-lapse studies of neural development in zebrafish embryos and adults.

Making Movies: Tools and Methods for Time-Lapse Imaging in Zebrafish

Making time-lapse movies of zebrafish development is conceptually simple: a labeled embryo is placed under a microscope and images are captured repeatedly at varying intervals. To position a cell for imaging, embryos are anaesthetized or paralyzed, and typically embedded in agarose. Zebrafish embryos are surprisingly resilient and can develop normally in agarose for hours. Laser scanning confocal microscopy is the most common method for imaging. However, mulitiphoton microscopy is better for imaging deep tissues, and if scanning speed is the major concern, spinning disk confocal microscopy, which can capture millisecond dynamic events, may be the best choice. Several technical parameters must be considered when designing each experiment, including how long the imaging session should be, what magnification will be used, what mounting method will be used (which depends on the developmental stage and whether an upright or inverted microscope is used), how to balance imaging speed (which affects image quality) with the number of time-lapse intervals, and how to quantify time-lapse data. Although this review does not focus on these technical aspects of microscopy, optimizing imaging parameters for each particular neuronal population and developmental stage is a major challenge that must often be accomplished through trial-and-error.

Labeling-Specific Cells

The first step in designing zebrafish imaging experiments is choosing a method for labeling specific cells and, if desired, manipulating gene expression within those cells. The optimization of molecular tools, many of them borrowed from the *Drosophila* system, has made these tasks easier and provided a wealth of options for creating sophisticated transgenes tailored for specific experimental questions.

There are two general methods for visualizing cells in zebrafish with transgenic reporters: expressing transgenes transiently (sometimes called somatic transgenesis) or making stable transgenic lines (germline transgenics). Because extrachromosomal DNA is unstably inherited during cell division, simply injecting a DNA transgene into fertilized eggs results in its mosaic inheritance (Fig. 1a). This makes it possible to perform analyses at a single-cell level, even when a transgene drives reporter expression broadly (Downes *et al.*, 2002). If, on the other hand, labeling an entire population of cells is the goal, stable transgenic lines can be generated (Fig. 1b). Making stable transgenic fish used to be a somewhat burdensome undertaking, but the development of transposon-based methods, which increase the frequency at which exogenous DNA integrates into chromosomes, has greatly facilitated the process (Kikuta and Kawakami, 2009). As an alternative to genetic labeling, cells can be filled with fluorescent dye by injection or electroporation (Fig. 1c), (Cox and Fetcho, 1996;

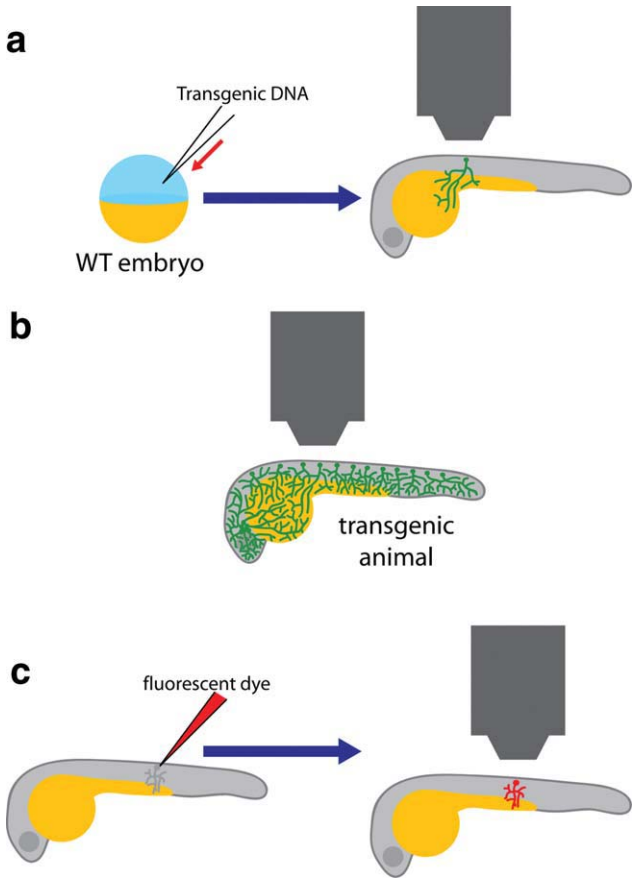

FIG. 1. Three approaches to cell labeling. (**a**) DNA transgenes injected into early stage embryos are mosaically inherited, resulting in stochastic expression of the transgene in a subset of cells. Embryos expressing the reporter in a single cell can thus be identified for imaging. (**b**) An entire population of cells can be imaged in embryos harboring a stable, integrated transgene. (**c**) Electroporation or pressure injection of fluorescent dyes makes it possible to image a selected cell.

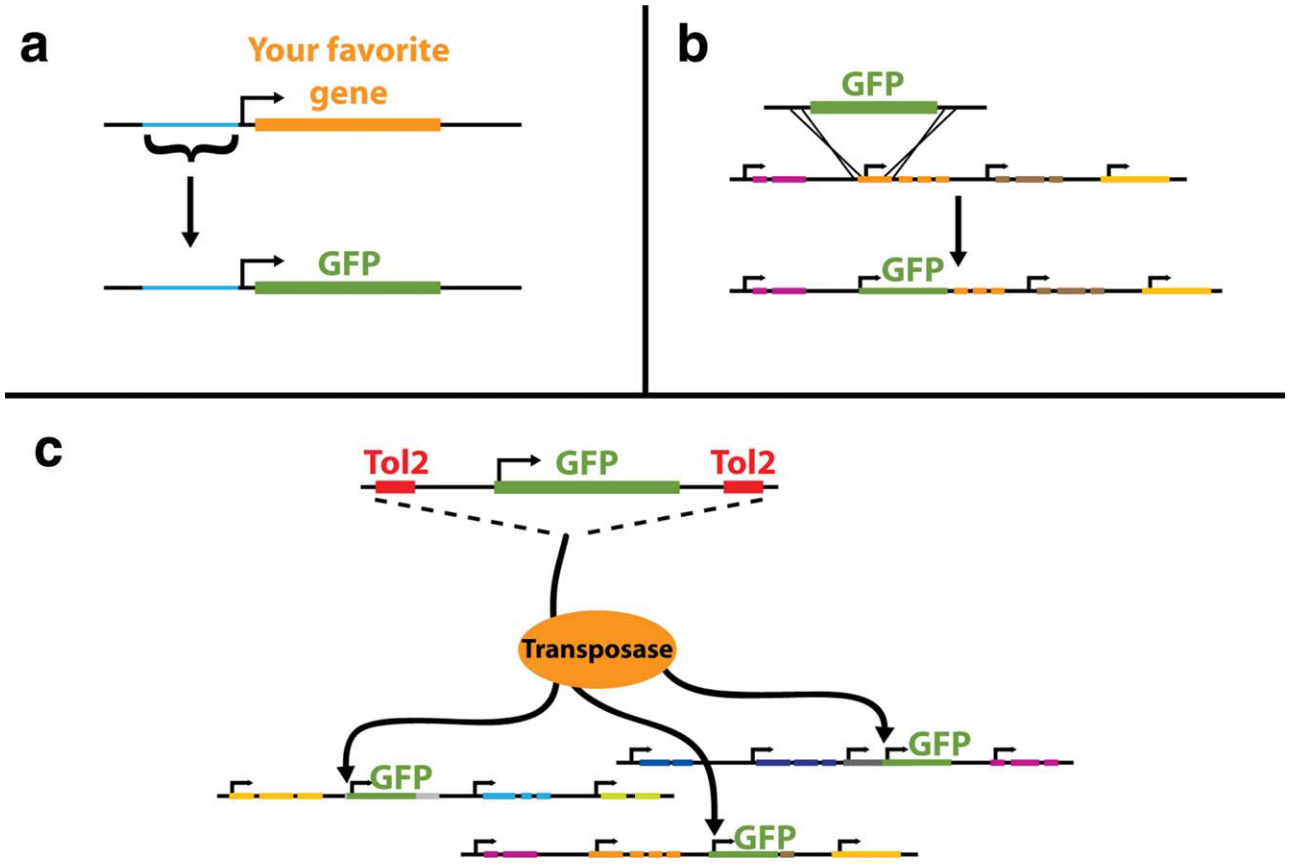

FIG. 2. Three methods for obtaining cell type-specific reporters. (**a**) A region upstream of a selected gene can be subcloned upstream of a fluorescent reporter, and often drives expression in a pattern approximating the endogenous gene. (**b**) To improve the chances of faithfully recapitulating an endogenous expression pattern, a fluorescent reporter gene can be recombined into a targeted location in a BAC. (**c**) Tol2 transposition can be used to randomly integrate a reporter throughout the genome. The reporter will occasionally land near regulatory elements that drive expression in a useful pattern.

Hutson and Chien, 2002; Tawk *et al.*, 2009). Although this is an older method, it is still in frequent use, since it eliminates the necessity for creating a transgene and makes it possible to target specific cells.

There are several approaches to finding enhancers/promoters for creating a cell type-specific reporter transgene. If a gene expressed in the cells of interest is known, it can be relatively easy to clone regulatory elements from the region surrounding that gene (Fig. 2a). Most commonly regions upstream of the gene are chosen, but cross-species comparative analysis can aid in the identification of conserved regulatory regions anywhere near the gene (e.g., Allende *et al.*, 2006). This approach, however, does not guarantee a fully accurate reporter, since regulatory elements can be located quite far from the start of a gene. A more reliable method for obtaining faithful reporter expression is to recombine a fluorescent reporter gene into a bacterial artificial chromosome (BAC) containing the target gene (Fig. 2b) (Yang *et al.*, 2009). The larger sequence context of a BAC reporter improves the fidelity of reporter gene expression. Recently, transposon (Tol2)-mediated BAC transgenesis has been developed in zebrafish and mice to precisely deliver single-copy BAC transgenes (Suster *et al.*, 2009). As an alternative to creating reporters based on specifically selected genes, many labs have recently performed enhancer- and gene-trap screens (Asakawa *et al.*, 2008; Balciunas *et al.*, 2004; Davison *et al.*, 2007; Kawakami *et al.*, 2004; Kikuta *et al.*, 2007; Laplante *et al.*, 2006; Lobo *et al.*, 2006; Parinov *et al.*, 2004; Scott *et al.*, 2007). In these screens, a reporter gene is randomly inserted into the genome, where it may land near specific regulatory elements (Fig. 2c). These screens have together created a large collection of reporter transgenes with novel expression patterns.

Reporters and Effectors

Many fluorescent proteins can be used as reporters. In addition to the common ones, such as GFP and RFP, a few photoconvertible fluorescent reporters have proven valuable for tracking cell fate and analyzing the

morphology of single cells (Aramaki and Hatta, 2006; Arrenberg *et al*., 2009; Caron *et al*., 2008; Davison *et al*., 2007; Hatta *et al*., 2006; Marriott *et al*., 2008; McLean and Fetcho, 2009; Sato *et al*., 2006; Scott *et al*., 2007). Kaede (Ando *et al*., 2002), KikGR (Tsutsui *et al*., 2005), and Dendra (Gurskaya *et al*., 2006) are all photoconvertible fluorescent proteins with emission spectra that can be shifted from green to red upon exposure to UV or blue light. Dronpa is also a GFP-like protein, but 405- and 488-nm light can reversibly switch the fluorescent "on"-state and nonfluorescent "off"-state of Dronpa (Ando *et al*., 2004). Such reversible photoswitching could be repeated more than 100 times with a millisecond response time, making it possible to monitor dynamic subcellular events (Habuchi *et al*., 2005).

The goal of many experiments is not only to image a cell, but also to simultaneously express an effector gene that alters its function. Several strategies have been developed for tandem co-expression of two genes. For examples, a fluorescent reporter and effector gene can both be placed under control of the Gal4 upstream activation sequence (UAS) and activated with the same Gal4 driver (Koster and Fraser, 2001). Alternatively, two separate proteins can be translated from one messenger RNA with an internal ribosome entry site (IRES) (Fahrenkrug *et al*., 1999), or, to ensure equimolar co-expression, a single fusion protein can be split into two autonomous proteins with viral 2A peptides, which, though often called "self-cleaving," create two peptides by a "ribosome-skipping" mechanism (Provost *et al*., 2007). These techniques can be used to overexpress wildtype, dominant negative, or constitutively active proteins, along with a fluorescent reporter, making it possible to compare the behavior of molecularly manipulated cells to wild-type cells.

Gal4/UAS Transgenes

The yeast Gal4/UAS transcriptional activation system has been an extraordinarily successful tool for manipulating gene expression in *Drosophila*. With modifications, this system has also been widely incorporated into zebrafish transgenes (reviewed in Halpern *et al*., 2008). The beauty of this binary approach is that Gal4 drivers and UAS reporters can be produced separately and put together in different combinations, providing powerful versatility. Fusion of Gal4 to the VP16 transcriptional activation domain provides substantial amplification, allowing abundant expression even from weak enhancers/promoters (Koster and Fraser, 2001). A fusion of Gal4 to the ligand-binding domain of the ecdysone receptor allows hormone-mediated temporal control of gene expression (Esengil *et al*., 2007). Silencing and variegation has been a significant problem with the Gal4/UAS system in zebrafish, but the creation of non-repetitive UAS elements alleviates this problem (Akitake *et al*., 2011). In addition to using known tissue-specific regulatory elements to direct Gal4 expression, several labs have also used Gal4 for enhancer trap screens, creating an enormously versatile set of tools for imaging cells and misexpressing genes in tissues throughout the animal (Asakawa and Kawakami, 2009; Asakawa *et al*., 2008; Davison *et al*., 2007; Scott *et al*., 2007).

What Have We Learned From Watching Movies?

Time-lapse imaging provides at least three kinds of insights into mechanisms of neural development. First, it can reveal a link between a cell's behavior at one moment and a previous experience, suggesting a mechanistic connection between two discrete events. For example, the ultimate fate of newborn cells can sometimes be predicted by the orientation of a cell division, a relationship that can only be definitively deduced by watching division occur and tracking the daughter cells over time. Second, time-lapse imaging is the most direct way to identify a migratory pathway, and may be the only way of deducing these routes if migratory cells are intermingled with a heterogeneous group of cells exhibiting different behaviors. Third, time-lapse imaging can reveal transient cellular behaviors that are characteristic features of developmental processes. For example, developing axons often project branches that are later pruned away; if degeneration is rapid or stochastic it may be impossible to know that pruning occurred without time-lapse imaging. Identifying linked sequences of events, migratory pathways and transient phenomena is fundamental to understanding how the nervous system is constructed.

Watching Cells Divide and Differentiate

During central nervous system development, highly organized multi-layered structures, such as the cortex and retina, emerge from single-layered neuroepithelia. Recent time-lapse imaging studies of the developing zebrafish retina and hindbrain have challenged several aspects of widely accepted models describing how these laminar brain regions develop (see Fig. 3). These studies exemplify the powerful ability of live imaging to order discrete events into a progressive sequence.

Before the onset of neurogenesis in the central nervous system, elongated progenitor cells span the width of the neuroepithelium. Their nuclei are positioned all along the apical-basal axis, giving the neuroepithelium a pseudostratified appearance. According to a prevalent model, the nuclei of progenitor cells were thought to migrate from the apical to the basal surface during G1 and S-phase, return toward the apical pole during G2, and divide at the apical surface, a sequence termed interkinetic nuclear migration (IKNM) (Miyata, 2008). Though the model relating nuclear position to cell cycle stage was widely accepted, IKNM was never visualized

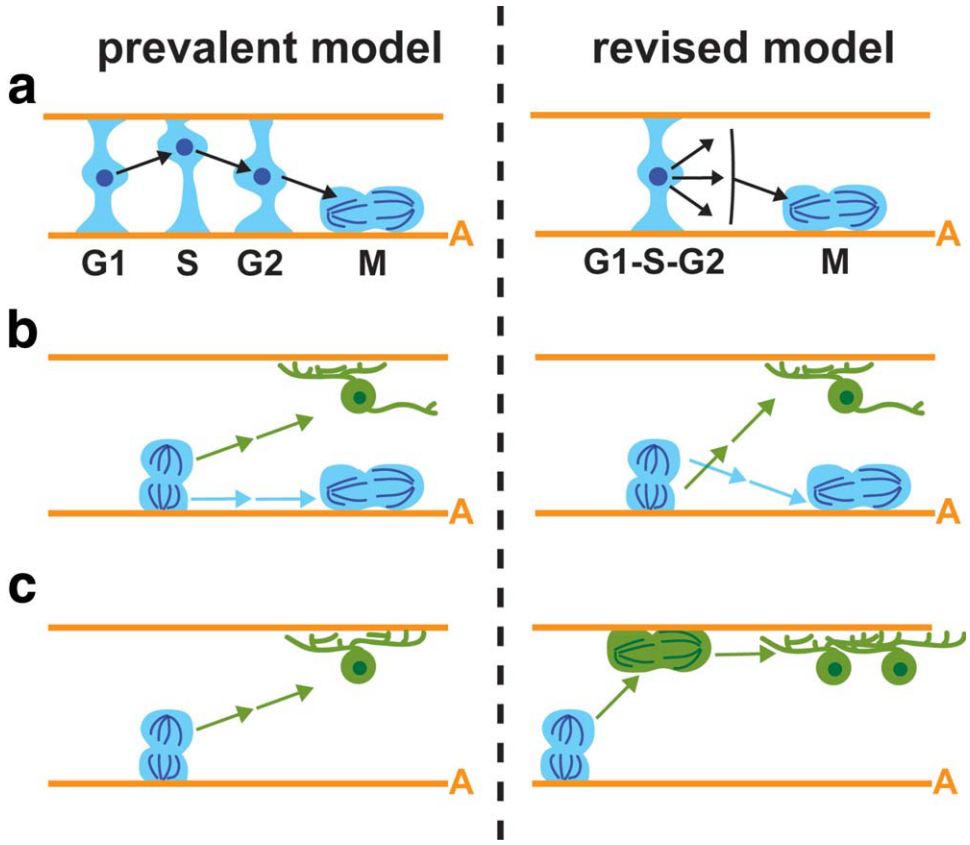

FIG. 3. Studies in the retina and hindbrain have revised models for the formation of laminar structures from simple neuroepithelia. In all images, "A" indicates the apical surface; for simplicity, basal processes are not shown in dividing cells. (**a**) Interkinetic nuclear migration was thought to be a smooth movement of nuclei in concert with the cell cycle, but a recent study in the zebrafish retina showed that nuclear movements are stochastic (Norden *et al*., 2009). (**b**) It was believed that during asymmetric cell division in simple neuroepithelia, apical daughters become progenitors that continue to divide and basal daughters differentiate into neurons, but a recent study found the opposite to be true in the developing zebrafish hindbrain (Alexandre *et al*., 2010). (**c**) A recent study in the zebrafish retina found that daughters of cells that divided at the apical surface often migrated to the inner nuclear layer to divide again and generate horizontal cells (Godinho *et al*., 2007).

directly in living animals. To image IKNM, Norden *et al*. tracked progenitor nuclei in the developing zebrafish retina labeled with the nuclear marker H2B:RFP using confocal microscopy (Norden *et al*., 2009). Rather than observing smooth, directed nuclear movements in concert with the cell cycle, the authors found that nuclear movements were mostly stochastic, only becoming rapidly directed towards the apical surface just before mitosis (Fig. 3a). Thus, IKNM movements, at least in the zebrafish retina, are not as predictable or organized as was once thought.

Once at the apical surface, cells can divide parallel or obliquely to the apical surface. Symmetric divisions generating two proliferative progenitor cells occur when the cleavage plane is parallel to the apical-basal axis (in other words, the long axis of dividing cells is parallel to the apical surface), whereas asymmetric divisions generating one progenitor and one neuronal daughter cell occur when the cleavage plane is perpendicular to the apical-basal axis (Chenn and McConnell, 1995; Miyata, 2008). The prevalent model relating cell division with asymmetric cell fates proposed that inheritance by the apical daughter cell of an apical protein complex caused it to retain the progenitor fate, whereas the basal cell that did not inherit the complex adopted a neural fate (Huttner and Brand, 1997). A recent study tested this model directly by imaging asymmetrically dividing cells and their progeny in the developing posterior hindbrain (Alexandre *et al*., 2010). Expressing an apical complex marker (Par3-GFP), a marker for the basolateral compartment (Numb-GFP), and a reporter for neuronal cell fate (HuC-GFP), in neuroepethelial cells, allowed the authors to monitor cell division, inheritance of the apical complex, and the fate of daughter cells. Consistent with previous models, progenitors that cleaved parallel to the apical-basal axis mostly generated two proliferative progenitor daughter cells, whereas cleavages more than 15° off this axis generated a progenitor and a neuron. As expected, the "apical footprint" (Par3-GFP) was inherited by both proliferative

daughters during symmetric divisions, but was asymmetrically inherited by one daughter during asymmetric division. The surprising finding was that during asymmetric division the cell fated to become a neuron, as judged by the fact that it turned on the HuC:GFP reporter, was apically positioned and inherited the apical Par3 complex, while the basal daughter cell became the progenitor and re-established Par3-GFP expression after several minutes (Fig. 3b). These compelling results challenge the prevalent model, which posits that the apical cell becomes the progenitor, and provide a striking example of time-lapse analysis clarifying an issue that was difficult to resolve in analyses of static images.

Following cell division, developing neurons migrate from the apical surface to the appropriate laminar position. In the retina, the frequent appearance of mitotic cells in the inner nuclear layer raised the interesting possibility that neurons could also be born away from the apical surface, either close to or within their respective cell layers (Smirnov and Puchkov, 2004). Godinho *et al*. addressed this idea with time-lapse multiphoton imaging of fluorescently-labeled horizontal cells from early stages, when only a few horizontal cell precursors were present, to stages when the horizontal cell layer was completely formed (Godinho *et al*., 2007). Strikingly, rather than directly generating horizontal cells, many asymmetric cell divisions at the apical surface yielded unipotent proliferative cells that migrated to the inner nuclear layer and divided again to generate horizontal cells (Fig. 3c). In fact, the majority of horizontal cells (~85%) were formed by symmetric division of progenitor cells in the layer where mature horizontal cells reside, rather than by migration of terminally fated daughter cells from an apical division. Together, these studies of IKNM, cell division, and differentiation in the retina and hindbrain, using cell type-specific transgenic reporters, revised long-standing models of laminar development (see Fig. 3).

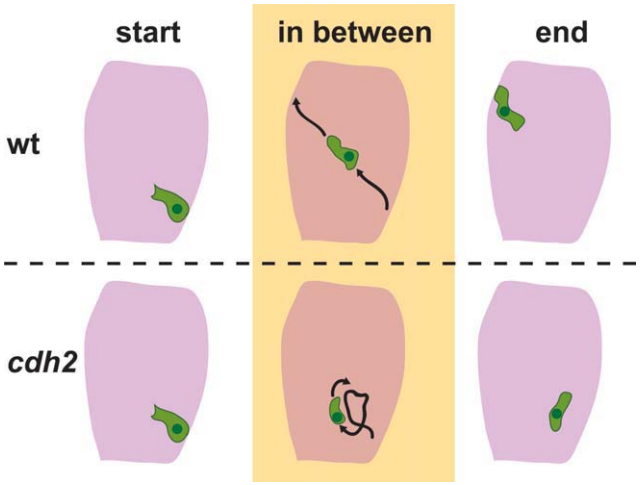

FIG. 4. Granule cell precursors in wild type and cadherin 2 (*cdh2*) mutants start out in the same area of the upper rhombic lip (start), but although wild-type cells migrate a substantial distance, mutant cells stay close to their starting point (end). Time-lapse imaging revealed that *cdh2* mutant cells are defective in the directionality of migration, rather than the speed of movement (in between) (Rieger *et al*., 2009).

Watching Neurons Migrate

Many neurons migrate long distances from their birth site to their final residence. Live imaging reveals migratory pathways, since a neuron's trajectory from its birthplace to its destination can be traced directly. A study of cell migration in the developing zebrafish cerebellum illustrated how the migratory paths of individual cells can be distinguished, even within a heterogeneous population of cells, by time-lapse imaging (Volkmann *et al*., 2008). In the cerebellum, granule cells migrate from the upper rhombic lip, a proliferative neuroepithelium, deep into the cerebellar cortex and ventral hindbrain, where they form neuronal clusters that connect with various parts of the brain to mediate motor control and other functions (Wingate, 2001). Although diverse cells emerge from the rhombic lip, the neuroepithelium displays no anatomically obvious regional divisions. Imaging granule cell precursor migration and tracking cell trajectories with software-assisted tracing revealed that cells emerge from distinct spatial subdomains along the mediolateral axis of the upper rhombic lip (Volkmann *et al*., 2008); cells born in a lateral subdomain of the rhombic lip migrated along a different route than cells born in a dorsomedial subdomain and formed distinct anatomical structures. Thus, time-lapse imaging made it possible to distinguish the origin and migratory paths of different subpopulations of cells without requiring distinct molecular markers for each cell type.

Comparing migratory pathways in mutant and wild-type cells can illuminate the molecular mechanisms controlling cell motility. For example, another study of cerebellar cell migration revealed an unexpected function for Cadherin 2 (Cdh2), a major constituent of adherens junctions in neuronal migration (Rieger *et al*., 2009). Previous work in chick and zebrafish had shown that loss of Cdh2 function resulted in mispositioned neurons and neural crest cells close to their place of origin in the brain. This observation suggested that the motility of migratory cells was compromised in the absence of Cdh2 (Kasemeier-Kulesa *et al*., 2006; Lele *et al*., 2002). To re-examine this idea, Rieger *et al*. traced individual granule cells over time in Cdh2 mutants. Unexpectedly, cell velocities were similar in mutant and wild-type animals, but in Cdh2 mutants the direction of migration was randomized, resulting in circling behaviors of granule cells (see Fig. 4). The lack of directionality of migrating mutant granule cells was confirmed with the expression of a red-fluorescent centrosome

marker. In migrating wild-type cells, the centrosome was located predominantly anteriorly, in the direction of migration, but its location within the cell was random in Cdh2-deficient granule cells. Moreover, the position of an adherens junction marker (a fusion of the Cdh2 protein to mCherry) within migrating neurons correlated with the distinct migratory steps of granule cells and was predominantly located at the anterior cell cortex during forward movement, close to the centrosome. Thus, rather than controlling the rate of migration, adherens junctions control the direction of cell migration, an insight that would have been difficult to glean from still images of migrating cells.

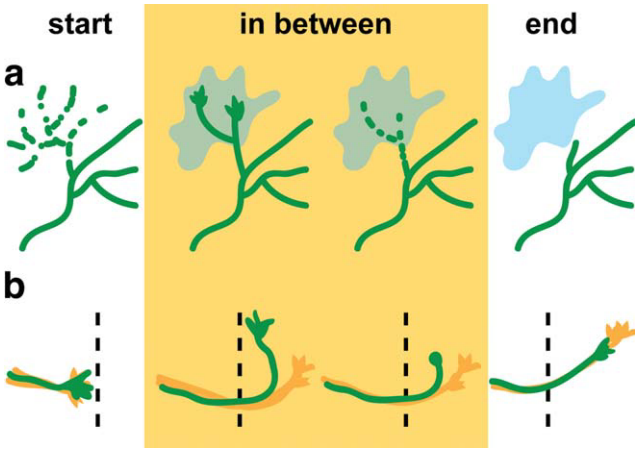

FIG. 5. Time-lapse imaging can reveal transient events in growth cone development and regeneration. (**a**) Following axotomy of the peripheral axon arbor of a trigeminal neuron (start), regenerating axons never reinnervate former territory (blue patch), despite the fact that severed portions of the axons are quickly cleared (end). Examining regeneration by time-lapse imaging revealed that sometimes axons do grow into former territory, but always degenerate or retract out of it (in between) (O'Brien *et al*., 2009a). (**b**) Wild-type retinal ganglion cell axons projecting into the brain (dotted line represents the midline) almost never misroute (end). However, examining intermediate stages of migration by time-lapse revealed that some axons (green axon) leave the common path (orange axons), but these errors are almost always corrected (in between) (Hutson and Chien, 2002).

Watching Growth Cones Navigate

Once a neuron has chosen its fate and its cell body has reached the appropriate location, it projects neurites to establish synaptic connections. Watching dendrites and axons grow in real time can reveal aspects of their navigational strategies that are not possible to deduce simply by determining whether or not they reached their destination. For example, transient growth cone behaviors, including changes in their speed, shape or size are most clearly detected by time-lapse imaging and often indicate axon guidance choice points. Comparing these behaviors in wild type and mutant growth cones can reveal how guidance molecules regulate these choices (e.g., Hutson and Chien, 2002; Jing *et al*., 2009; Sato-Maeda *et al*., 2006). Time-lapse imaging can also reveal whether guidance decisions result from attraction to a particular path or the avoidance of alternative paths. For example, the peripheral axons of somatosensory neurons are complex branched structures that are segregated from those of their neighbors in a "tiled" arrangement. In principle, this segregated arbor arrangement could result from competition between adjacent axons for a limiting positive factor or from the active avoidance of neighboring cells. Time-lapse imaging of the developing peripheral arbors of trigeminal neurons, which innervate the head, and Rohon-Beard neurons, which innervate the trunk and tail, clearly demonstrated that when growth cones contacted a neighboring axon they collapsed and turned away (Liu and Halloran, 2005; Sagasti *et al*., 2005), providing a mechanistic explanation for the formation of a tiled pattern.

Perhaps the most dramatic transient events are the degeneration or rerouting of selective neurite branches. These events leave no trace, making them impossible to detect by examining mature arbor structures. For example, time-lapse imaging of trigeminal sensory neuron arborization in zebrafish revealed that sporadic pruning of axonal arbors and spontaneous neuronal death are common during the course of peripheral sensory axon development (Sagasti *et al*., 2005). These sporadic pruning events often occurred when axon branches crossed over one another, suggesting that pruning may be a backup mechanism for ensuring a tiled arrangement when repulsion between neighboring arbors fails. Quantitative analyses of these local degeneration events demonstrated that axon branch detachment, fragmentation, and clearance were all complete in less than three hours, which makes this process difficult to detect in still images (Martin *et al*., 2010). Imaging the regeneration of trigeminal peripheral axons that were precisely severed with intense laser power on a multiphoton microscope revealed that related behaviors regulate the ability of sensory axons to successfully reinnervate their former territory (O'Brien *et al*., 2009a). Following axotomy, injured axons could reinitiate growth but were often repelled by their former territories, in much the same manner that neighboring axon branches repel one another during tiling. Strikingly, time-lapse movies also showed that when regenerating axons did reenter their former territories they invariably retracted back out of the region, degenerated locally, or the parent neuron died (Fig. 5a). Together these observations suggested that local inhibitory factors persist in denervated skin after a severed axon branch has degenerated.

As with studies of migration, comparing transient events in axon development between mutant and wild-type neurons can uncover unexpected molecular

mechanisms regulating axon guidance. A study of a mutant in a receptor of the Robo family illustrates this point. Robo proteins are receptors for secreted guidance signals of the Slit family and their activation is thought primarily to induce repulsive growth cone behaviors. Hutson and Chien investigated the phenotype of the *astray* mutant, which has a mutation in the robo2 gene, using time-lapse imaging of dye-filled retinal ganglion cells to study its role in the establishment of retinotectal axon pathways (Hutson and Chien, 2002). These time-lapse analyses revealed that in wild-type zebrafish, retinal ganglion cell (RGC) axons growing across the optic chiasm occasionally made pathfinding errors, which were always eventually corrected (Fig. 5b). In the *astray*/robo2 mutant, RGC axons made more errors, and those errors were rarely corrected. Thus, in addition to its guidance role, robo2 is unexpectedly also required for correcting erroneous projections—both functions that may depend on sensing repulsive gradients. These misprojections can be detected in fixed specimens, but their correction in wild-type animals would have been difficult to detect without time-lapse imaging, and defects in the *astray* mutant may have been interpreted solely as guidance defects.

Into the Future: More to Watch

The use of zebrafish time-lapse imaging studies for identifying links between developmental events, tracing migratory routes, and revealing transient cellular behaviors, perhaps constitutes the model's most valuable contribution to the field of neural development. These studies are continually becoming more sophisticated as microscopy techniques and molecular tools improve. Although the studies we have described here demonstrate that zebrafish imaging experiments are already making significant contributions, several aspects of neural development have only just begun to be explored with this powerful approach. These new frontiers include studies of subcellular events, the relationship between physiological states and development, and the regulation of plasticity in the mature nervous system.

Watching Events Within Cells

Some of the studies mentioned in this review employed genetically-encoded fluorescent reporter fusions to image subcellular structures (Alexandre *et al*., 2010; Norden *et al*., 2009; Rieger *et al*., 2009). As more of these fusion proteins are created, it becomes possible to track an increasing variety of organelles, subcellular compartments, and protein complexes. Imaging techniques that minimize exposure of the sample, such as multiphoton or spinning disk microscopy can help reduce bleaching of these sometimes-vulnerable fusion proteins. One caution with these studies is that overexpression of fusion genes could drive localization to ectopic locations within a cell or interfere with normal development, but appropriate controls such as co-staining with an antibody for an endogenous protein, or using the fusion protein to rescue a mutant phenotype, can alleviate these concerns. Live imaging studies of the development of synapses, perhaps the most interesting subcellular specializations in neurons, using both pre- and post-synaptic reporter fusions, have already yielded insights into the assembly of synapses and the establishment of connections (Jontes *et al*., 2004; Meyer and Smith, 2006; Niell *et al*., 2004). Synapse formation is likely to become a growing focus of zebrafish research, and these live imaging studies are poised to make substantial contributions in new areas, such as how organelles and vesicles are trafficked in axons and dendrites, or how cytoskeletal dynamics are regulated during cell and growth cone migration.

Linking Physiology and Development

Many developmental events are influenced by a neuron's physiological state. For example, neuronal activity can influence processes as diverse as neurite arborization and the initiation of cell death (Ben Fredj *et al*., 2010; Hua *et al*., 2005; Paulus *et al*., 2009; Svoboda *et al*., 2001). Calcium sensors are faithful indicators of calcium transients and, indirectly, of action potentials. For several years now, imaging calcium in live zebrafish larvae with calcium-sensitive dyes or genetic indicators, such as GCaMP and cameleon, has helped decipher neural circuit organization (reviewed in McLean and Fetcho, 2008). Combining these indicators with long-term imaging of developmental processes could help detect correlations between activity and specific cellular behaviors. The causality of these relationships could then be tested with drugs that alter cellular activity, or more precisely with genetically encoded proteins that either stimulate or block cellular activity, such as the light-activated channels channelrhodopsin, halorhodopsin, and LiGluR (reviewed in Baier and Scott, 2009). The development of indicators for other physiological phenomena, such as synaptic transmission (Dreosti *et al*., 2009), the presence of reactive oxygen species (Niethammer *et al*., 2009), pH (Miesenbock *et al*., 1998), voltage (Baker *et al*., 2008), or the activity of specific channels (Richler *et al*., 2008), will make it possible for zebrafish researchers to ask a broader array of questions about the influence of physiological states on development.

Imaging Cellular Plasticity in Mature Animals

Dynamic cellular behaviors do not cease when neurons attain their mature structures. Cells grow and change as animals age, they undergo regulated responses to cellular damage, and they establish new neural connections as animals learn. A dramatic example of extreme cellular plasticity was provided by a

recent zebrafish time-lapse study that documented the transdifferentiation of cells from dorsal root ganglia neurons into sympathetic neurons (Wright *et al*., 2010). It is likely that many other surprising transformations occur in the nervous system as animals grow and transition from embryonic to larval, and larval to adult forms.

At any age, structural plasticity is particularly dramatic after cellular damage. Molecules that regulate the ability of axons to regenerate are conserved between fish and mammals (Bhatt *et al*., 2004; O'Brien *et al*., 2009a), giving these studies particular medical relevance. The ability to create precise damage to cells, dendrites or axons with lasers makes it possible to image regeneration in live animals, but this method is only just beginning to be exploited for zebrafish studies (Martin *et al*., 2010; O'Brien *et al*., 2009b).

Characterization of the adult zebrafish brain is also just beginning, with studies of adult neurogenesis leading the way (e.g., Adolf *et al*., 2006; Chapouton *et al*., 2006; Grandel *et al*., 2006). One of the next challenges is to create fluorescent reporters expressed in adult neurons, of which there are currently few. This could be accomplished by screening enhancer trap lines for adult expression or creating BAC transgenic reporters of genes expressed in the adult. Even enhancers that drive early transient expression can be used to permanently mark neurons, either in combination with the Cre-lox recombination system or with a self-maintaining Gal4-based positive feedback system (dubbed "Kaloop") (Collins *et al*., 2010; Distel *et al*., 2009; Thummel *et al*., 2005). The development of the tol2 transposon system in fish, which greatly increases the integration of transgenes into the genome of somatic cells, has even made it possible to perform imaging experiments in transient transgenic adults (e.g., Tu and Johnson, 2010). It should be possible to do studies in zebrafish analogous to recent tour-de-force multiphoton imaging studies of the adult mouse cortex (Pan and Gan, 2008). Combining adult imaging studies with new reporters for subcellular structures and physiological indicators would provide a powerful opportunity to gain deeper insights into the structural plasticity of adult brains.

ACKNOWLEDGMENTS

The authors thank members of the Sagasti lab and reviewers for their comments on the manuscript.

LITERATURE CITED

Adolf B, Chapouton P, Lam CS, Topp S, Tannhauser B, Strahle U, Gotz M, Bally-Cuif L. 2006. Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. Dev Biol 295:278–293.

Akitake CM, Macurak M, Halpern ME, Goll MG. 2011. Transgenerational analysis of transcriptional silencing in zebrafish. Dev Biol. [Epub ahead of print].

Alexandre P, Reugels AM, Barker D, Blanc E, Clarke JD. 2010. Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. Nat Neurosci 13:673–679.

Allende ML, Manzanares M, Tena JJ, Feijoo CG, Gomez-Skarmeta JL. 2006. Cracking the genome's second code: Enhancer detection by combined phylogenetic footprinting and transgenic fish and frog embryos. Methods 39:212–219.

Ando R, Hama H, Yamamoto-Hino M, Mizuno H, Miyawaki A. 2002. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc Natl Acad Sci USA 99:12651–12656.

Ando R, Mizuno H, Miyawaki A. 2004. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. Science 306:1370–1373.

Aramaki S, Hatta K. 2006. Visualizing neurons one-by-one in vivo: Optical dissection and reconstruction of neural networks with reversible fluorescent proteins. Dev Dyn 235:2192–2199.

Arrenberg AB, Del Bene F, Baier H. 2009. Optical control of zebrafish behavior with halorhodopsin. Proc Natl Acad Sci USA 106:17968–17973.

Asakawa K, Kawakami K. 2009. The Tol2-mediated Gal4-UAS method for gene and enhancer trapping in zebrafish. Methods 49:275–281.

Asakawa K, Suster ML, Mizusawa K, Nagayoshi S, Kotani T, Urasaki A, Kishimoto Y, Hibi M, Kawakami K. 2008. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proc Natl Acad Sci USA 105:1255–1260.

Baier H, Scott EK. 2009. Genetic and optical targeting of neural circuits and behavior—Zebrafish in the spotlight. Curr Opin Neurobiol 19:553–560.

Baker BJ, Mutoh H, Dimitrov D, Akemann W, Perron A, Iwamoto Y, Jin L, Cohen LB, Isacoff EY, Pieribone VA, Hughes T, Knopfel T. 2008. Genetically encoded fluorescent sensors of membrane potential. Brain Cell Biol 36:53–67.

Balciunas D, Davidson AE, Sivasubbu S, Hermanson SB, Welle Z, Ekker SC. 2004. Enhancer trapping in zebrafish using the Sleeping Beauty transposon. BMC Genomics 5:62.

Ben Fredj N, Hammond S, Otsuna H, Chien CB, Burrone J, Meyer MP. 2010. Synaptic activity and activity-dependent competition regulates axon arbor maturation, growth arrest, and territory in the retinotectal projection. J Neurosci 30:10939–10951.

Bhatt DH, Otto SJ, Depoister B, Fetcho JR. 2004. Cyclic AMP-induced repair of zebrafish spinal circuits. Science 305:254–258.

Caron SJ, Prober D, Choy M, Schier AF. 2008. In vivo birthdating by BAPTISM reveals that trigeminal

sensory neuron diversity depends on early neurogenesis. Development 135:3259–3269.

Chapouton P, Adolf B, Leucht C, Tannhauser B, Ryu S, Driever W, Bally-Cuif L. 2006. her5 expression reveals a pool of neural stem cells in the adult zebrafish midbrain. Development 133:4293–4303.

Chenn A, McConnell SK. 1995. Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82:631–641.

Collins RT, Linker C, Lewis J. 2010. MAZe: A tool for mosaic analysis of gene function in zebrafish. Nat Methods 7:219–223.

Cox KJ, Fetcho JR. 1996. Labeling blastomeres with a calcium indicator: A non-invasive method of visualizing neuronal activity in zebrafish. J Neurosci Methods 68:185–191.

Davison JM, Akitake CM, Goll MG, Rhee JM, Gosse N, Baier H, Halpern ME, Leach SD, Parsons MJ. 2007. Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. Dev Biol 304:811–824.

Distel M, Wullimann MF, Koster RW. 2009. Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. Proc Natl Acad Sci USA 106:13365–13370.

Downes GB, Waterbury JA, Granato M. 2002. Rapid in vivo labeling of identified zebrafish neurons. Genesis 34:196–202.

Dreosti E, Odermatt B, Dorostkar MM, Lagnado L. 2009. A genetically encoded reporter of synaptic activity in vivo. Nat Methods 6:883–889.

Esengil H, Chang V, Mich JK, Chen JK. 2007. Small-molecule regulation of zebrafish gene expression. Nat Chem Biol 3:154–155.

Fahrenkrug SC, Clark KJ, Dahlquist MO, Hackett PB Jr. 1999. Dicistronic gene expression in developing zebrafish. Mar Biotechnol (NY) 1:552–561.

Godinho L, Williams PR, Claassen Y, Provost E, Leach SD, Kamermans M, Wong RO. 2007. Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. Neuron 56:597–603.

Grandel H, Kaslin J, Ganz J, Wenzel I, Brand M. 2006. Neural stem cells and neurogenesis in the adult zebrafish brain: Origin, proliferation dynamics, migration and cell fate. Dev Biol 295:263–277.

Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, Lukyanov S, Lukyanov KA. 2006. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol 24:461–465.

Habuchi S, Ando R, Dedecker P, Verheijen W, Mizuno H, Miyawaki A, Hofkens J. 2005. Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. Proc Natl Acad Sci USA 102:9511–9516.

Halpern ME, Rhee J, Goll MG, Akitake CM, Parsons M, Leach SD. 2008. Gal4/UAS transgenic tools and their application to zebrafish. Zebrafish 5:97–110.

Hatta K, Tsujii H, Omura T. 2006. Cell tracking using a photoconvertible fluorescent protein. Nat Protoc 1:960–967.

Hua JY, Smear MC, Baier H, Smith SJ. 2005. Regulation of axon growth in vivo by activity-based competition. Nature 434:1022–1026.

Hutson LD, Chien CB. 2002. Pathfinding and error correction by retinal axons: The role of astray/robo2. Neuron 33:205–217.

Huttner WB, Brand M. 1997. Asymmetric division and polarity of neuroepithelial cells. Curr Opin Neurobiol 7:29–39.

Jing L, Lefebvre JL, Gordon LR, Granato M. 2009. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. Neuron 61:721–733.

Jontes JD, Emond MR, Smith SJ. 2004. In vivo trafficking and targeting of N-cadherin to nascent presynaptic terminals. J Neurosci 24:9027–9034.

Kasemeier-Kulesa JC, Bradley R, Pasquale EB, Lefcort F, Kulesa PM. 2006. Eph/ephrins and N-cadherin coordinate to control the pattern of sympathetic ganglia. Development 133:4839–4847.

Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M. 2004. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Dev Cell 7:133–144.

Kikuta H, Fredman D, Rinkwitz S, Lenhard B, Becker TS. 2007. Retroviral enhancer detection insertions in zebrafish combined with comparative genomics reveal genomic regulatory blocks—A fundamental feature of vertebrate genomes. Genome Biol 8 (Suppl 1):S4.

Kikuta H, Kawakami K. 2009. Transient and stable transgenesis using tol2 transposon vectors. Methods Mol Biol 546:69–84.

Koster RW, Fraser SE. 2001. Tracing transgene expression in living zebrafish embryos. Dev Biol 233:329–346.

Laplante M, Kikuta H, Konig M, Becker TS. 2006. Enhancer detection in the zebrafish using pseudotyped murine retroviruses. Methods 39:189–198.

Lele Z, Folchert A, Concha M, Rauch GJ, Geisler R, Rosa F, Wilson SW, Hammerschmidt M, Bally-Cuif L. 2002. parachute/n-cadherin is required for morphogenesis and maintained integrity of the zebrafish neural tube. Development 129:3281–3294.

Liu Y, Halloran MC. 2005. Central and peripheral axon branches from one neuron are guided differentially by Semaphorin3D and transient axonal glycoprotein-1. J Neurosci 25:10556–10563.

Lobo NF, Fraser TS, Adams JA, Fraser MJ Jr. 2006. Interplasmid transposition demonstrates piggyBac

mobility in vertebrate species. Genetica 128:347–357.

Marriott G, Mao S, Sakata T, Ran J, Jackson DK, Petchprayoon C, Gomez TJ, Warp E, Tulyathan O, Aaron HL, Isacoff EY, Yan Y. 2008. Optical lock-in detection imaging microscopy for contrast-enhanced imaging in living cells. Proc Natl Acad Sci USA 105:17789–17794.

Martin SM, O'Brien GS, Portera-Cailliau C, Sagasti A. 2010. Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. Development 137:3985–3994.

McLean DL, Fetcho JR. 2008. Using imaging and genetics in zebrafish to study developing spinal circuits in vivo. Dev Neurobiol 68:817–834.

McLean DL, Fetcho JR. 2009. Spinal interneurons differentiate sequentially from those driving the fastest swimming movements in larval zebrafish to those driving the slowest ones. J Neurosci 29:13566–13577.

Meyer MP, Smith SJ. 2006. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. J Neurosci 26:3604–3614.

Miesenbock G, De Angelis DA, Rothman JE. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394:192–195.

Miyata T. 2008. Development of three-dimensional architecture of the neuroepithelium: Role of pseudostratification and cellular "community." Dev Growth Differ 50 (Suppl 1):S105–S112.

Niell CM, Meyer MP, Smith SJ. 2004. In vivo imaging of synapse formation on a growing dendritic arbor. Nat Neurosci 7:254–260.

Niethammer P, Grabher C, Look AT, Mitchison TJ. 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature 459:996–999.

Norden C, Young S, Link BA, Harris WA. 2009. Actomyosin is the main driver of interkinetic nuclear migration in the retina. Cell 138:1195–1208.

O'Brien GS, Martin SM, Sollner C, Wright GJ, Becker CG, Portera-Cailliau C, Sagasti A. 2009a. Developmentally regulated impediments to skin reinnervation by injured peripheral sensory axon terminals. Curr Biol 19:2086–2090.

O'Brien GS, Rieger S, Martin SM, Cavanaugh AM, Portera-Cailliau C, Sagasti A. 2009b. Two-photon axotomy and time-lapse confocal imaging in live zebrafish embryos. J Vis Exp 2:pe30.

Pan F, Gan WB. 2008. Two-photon imaging of dendritic spine development in the mouse cortex. Dev Neurobiol 68:771–778.

Parinov S, Kondrichin I, Korzh V, Emelyanov A. 2004. Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. Dev Dyn 231:449–459.

Paulus JD, Willer GB, Willer JR, Gregg RG, Halloran MC. 2009. Muscle contractions guide rohon-beard peripheral sensory axons. J Neurosci 29:13190–13201.

Provost E, Rhee J, Leach SD. 2007. Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. Genesis 45:625–629.

Richler E, Chaumont S, Shigetomi E, Sagasti A, Khakh BS. 2008. Tracking transmitter-gated P2X cation channel activation in vitro and in vivo. Nat Methods 5:87–93.

Rieger S, Senghaas N, Walch A, Koster RW. 2009. Cadherin-2 controls directional chain migration of cerebellar granule neurons. PLoS Biol 7:e1000240.

Sagasti A, Guido MR, Raible DW, Schier AF. 2005. Repulsive interactions shape the morphologies and functional arrangement of zebrafish peripheral sensory arbors. Curr Biol 15:804–814.

Sato T, Takahoko M, Okamoto H. 2006. HuC:Kaede, a useful tool to label neural morphologies in networks in vivo. Genesis 44:136–142.

Sato-Maeda M, Tawarayama H, Obinata M, Kuwada JY, Shoji W. 2006. Sema3a1 guides spinal motor axons in a cell- and stage-specific manner in zebrafish. Development 133:937–947.

Scott EK, Mason L, Arrenberg AB, Ziv L, Gosse NJ, Xiao T, Chi NC, Asakawa K, Kawakami K, Baier H. 2007. Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. Nat Methods 4:323–326.

Smirnov EB, Puchkov VF. 2004. Characteristics of cellular proliferation in the developing human retina. Neurosci Behav Physiol 34:643–648.

Suster ML, Sumiyama K, Kawakami K. 2009. Transposon-mediated BAC transgenesis in zebrafish and mice. BMC Genomics 10:477.

Svoboda KR, Linares AE, Ribera AB. 2001. Activity regulates programmed cell death of zebrafish Rohon-Beard neurons. Development 128:3511–3520.

Tawk M, Bianco IH, Clarke JD. 2009. Focal electroporation in zebrafish embryos and larvae. Methods Mol Biol 546:145–151.

Thummel R, Burket CT, Brewer JL, Sarras MP Jr, Li L, Perry M, McDermott JP, Sauer B, Hyde DR, Godwin AR. 2005. Cre-mediated site-specific recombination in zebrafish embryos. Dev Dyn 233:1366–1377.

Tsutsui H, Karasawa S, Shimizu H, Nukina N, Miyawaki A. 2005. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. EMBO Rep 6:233–238.

Tu S, Johnson SL. 2010. Clonal analyses reveal roles of organ founding stem cells, melanocyte stem cells

and melanoblasts in establishment, growth and regeneration of the adult zebrafish fin. Development 137:3931–3939.

Volkmann K, Rieger S, Babaryka A, Koster RW. 2008. The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments. Dev Biol 313:167–180.

Wingate RJ. 2001. The rhombic lip and early cerebellar development. Curr Opin Neurobiol 11:82–88.

Wright MA, Mo W, Nicolson T, Ribera AB. 2010. In vivo evidence for transdifferentiation of peripheral neurons. Development 137:3047–3056.

Yang Z, Jiang H, Lin S. 2009. Bacterial artificial chromosome transgenesis for zebrafish. Methods Mol Biol 546:103–116.