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Live Imaging of Nervous System Development and Function Using Light-Sheet Microscopy

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SUMMARY

In vivo imaging applications typically require carefully balancing conflicting parameters. Often it is necessary to achieve high imaging speed, low photo-bleaching, and photo-toxicity, good three-dimensional resolution, high signal-to-noise ratio, and excellent physical coverage at the same time. Light-sheet microscopy provides good performance in all of these categories, and is thus emerging as a particularly powerful live imaging method for the life sciences. We see an outstanding potential for applying light-sheet microscopy to the study of development and function of the early nervous system in vertebrates and higher invertebrates. Here, we review state-of-the-art approaches to live imaging of early development, and show how the unique capabilities of light-sheet microscopy can further advance our understanding of the development and function of the nervous system. We discuss key considerations in the design of light-sheet microscopy experiments, including sample preparation and fluorescent marker strategies, and provide an outlook for future directions in the field.

"Using light-sheet microscopy, dynamic events can be investigated in a completely non-invasive manner, even for relatively large, living biological systems."

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INTRODUCTION

Developmental neurobiologists are generally interested in seeing and understanding the dynamic processes that lead to the formation of the nervous system. The most commonly used approach to visualize these processes is to acquire a series of static images from a number of fixed specimens at various time points throughout development and connect the dots to infer a stereotyped developmental trajectory (Yang et al., 2013; Yu et al., 2013). The less commonly used alternative approach is to directly observe the development processes as they occur by means of live imaging a developing animal (Keller, 2013a). Until recently, however, the technology has not been available to image the entire nervous system of an early embryo, such as those of the fruit fly *Drosophila melanogaster* or the mouse *Mus musculus*, over a developmentally meaningful duration with the spatial and temporal resolution required to accurately track the underlying cellular dynamics. Recent advances in light microscopy, in particular the emergence of light-sheet fluorescence microscopy (Huisken and Stainier, 2009; Khairy and Keller, 2011; Mertz, 2011; Tomer et al., 2011; Hockendorf et al., 2012), have resulted in a technology that can now be used to image the entire embryonic nervous system with resolution that allows the fate of individual cells to be unambiguously determined.

This review will discuss the capabilities of state-of-the-art light microscopy techniques and provide a few representative examples of their application to the study of the development and function of the nervous systems in several model systems, such as Drosophila, mice, and the zebrafish *Danio rerio*. In addition, we will outline new ways that emerging light-sheet microscopy techniques can be used for advancing our understanding of neural

development and for functional imaging of nervous system activity in the developing embryo.

LIVE IMAGING APPROACHES TO THE STUDY OF DEVELOPMENT AND FUNCTION OF NEURAL TISSUES

The most common type of imaging studies in developmental neurobiology investigates the formation of neuronal morphology and identity. These studies have typically used fixed tissues and have not relied on in vivo imaging. One productive approach is to label a small number of neurons early in development, and then examine their fate in later developmental stages. The labeling is performed with a variety of techniques, from genetically encoded fluorescent proteins to injection of dyes. These techniques have produced a remarkable wealth of information on neuroblast lineages and the developmental fate of neural progenitors.

Reporters in Living Animals

A recent example of the dye-injection technique demonstrated how injection of lipophilic dyes and subsequent confocal imaging of fixed specimens were used to identify the neuroectodermal cells that give rise to the mushroom body neuroblasts in Drosophila (Kunz et al., 2012). Individual mushroom-body neuroblasts in the early embryo were injected with the lipophilic dye DiI, and then imaged at later stages of development to see which neurons were derived from each neuroblast (Fig. 1). The authors used this technique to describe, for the first time, the complete cell lineages for a class of stems cells in the Drosophila central nervous system.

Another example from Drosophila shows the utility of genetically encoded labels in determining the developmental origins and fates of individual neurons (Yu et al., 2010). In this study, all of the daughter cells from a neuroblast were individually labeled with green fluorescent protein, and the final morphologies of the adult neurons were observed with a confocal microscope (Fig. 2). While this technique is very useful, it only shows the endpoint of the developmental process rather than the dynamic processes that produced the result. Following individual cells from birth to adulthood could be much more instructive. We propose that in vivo imaging of the embryonic nervous system using light-sheet microscopy now has the potential to be a very fruitful approach to the study of neurodevelopment, owing to the outstanding capabilities of this technique for in vivo imaging of complex dynamic processes at unprecedented spatiotemporal resolution and over long periods of time.

Recently, in vivo imaging has been artfully used to examine the function of the nervous system using optical indicators of neural activity. For example, Seelig et al. (2010) used two-photon microscopy to observe the activity-triggered changes in calcium concentration in visual interneurons of an intact fruit fly brain (Fig. 3). Calcium transients in motion detecting neurons, reported by the genetically encoded calcium sensor GCaMP, were strongly correlated with visual motion stimuli and with the optomotor response of the walking fly. This study demonstrated that it was possible to visualize neural activity from a defined population of neurons that were responding to natural stimuli in a behaving animal. In a similar study, Ahrens et al. (2012) performed brain-wide functional imaging during behavior in the zebrafish (Fig. 4). In these experiments, immobilized fish were induced to produce fictive motor patterns in response to visual stimuli. Both of these studies show the remarkable potential for using new techniques while live imaging to study the function of the intact nervous system.

To date, there has been relatively little effort made to combine studies of nervous system development and function using live imaging, despite the wealth of hypotheses that neural function and development are intimately connected. To resolve a complete picture of nervous system development, it is important to understand the relative roles of activity-dependent and -independent processes in specifying final neural architecture and connectivity. These types of studies would require live imaging of a developing nervous system with simultaneous monitoring of neural activity using an optical indicator of calcium concentration or voltage.

Live Imaging Methodology

Conventional imaging techniques, such as wide-field microscopy or point-scanning confocal microscopy (Pawley, 2006), have limitations that fundamentally constrain their potential for live imaging of neural development. Chief among those limitations are photo-bleaching and photo-damage (Tsien et al., 2006).

Conventional wide-field microscopy, which takes advantage of state-of-the-art camera technology for fluorescence detection of millions of pixels simultaneously, is much faster than confocal microscopy, but suffers from limitations caused by non-specific illumination of the entire specimen and an inherent lack of axial resolution. In addition, the out-of-focus light contribution from thick biological specimens has the potential to completely obscure the signal of interest.

Confocal microscopes illuminate the entire thickness of a specimen and achieve optical sectioning by blocking light emitted by fluorescent molecules outside the focal volume. Since the laser light for fluorescence excitation effectively passes through the entire specimen, photo-bleaching of fluorescent markers and photo-toxic changes inside cells occur in a large fraction of the specimen, even when only a single plane is being imaged. When that procedure is repeated on many planes to image the entire volume of the nervous system, the light-induced changes in the specimen can be substantial. In addition, the sequential point-scanning approach of most confocal microscopes limits the imaging speed and requires the use of high laser power to achieve a sufficiently high signal-to-noise ratio.

These problems are partially addressed in confocal systems that use lower laser power and increase the speed of image acquisition while sacrificing some spatial resolution. Multi-aperture confocals, such as spinning disk confocal microscopes (Graf et al., 2005), use many pinholes simultaneously to collect images extremely rapidly. A

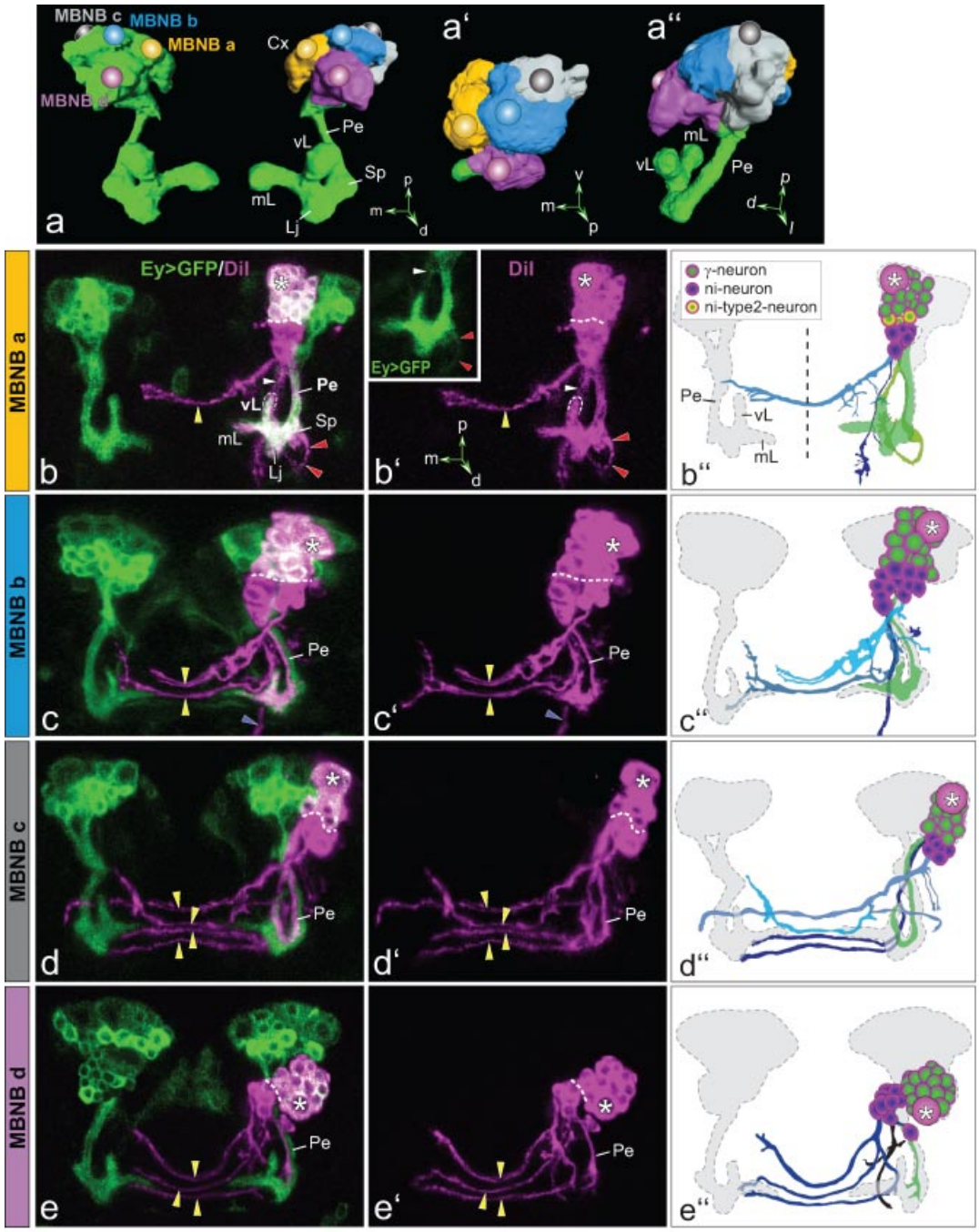


Figure 1. Confocal projections and reconstructions of mushroom-body neuroblast cell lineages in late Drosophila embryos (b–e) and early larvae (**a**). In (a), (a′), and (a″) the different colors represent the mushroom body areas occupied by neurons derived from different neuroblasts, based on dye injections. In (b) through (e), individual mushroom-body neuroblasts were injected with the lipophilic dye DiI (magenta) in early embryos expressing GFP (green) throughout the mushroom bodies, then imaged at a later embryonic stage. **b**–**e**: shows the mushroom-body label in green and the progeny of the injected mushroom-body neuroblast in magenta. (b′), (c′), (d′), and (e′) show only the dye-injected mushroom-body neuroblast lineages. (b″), (c″), (d″), and (e″) are illustrations of the mushroom-body neuroblasts (asterisks) and their progeny. Reprinted from Kunz et al. (2012), with permission from The Company of Biologists Ltd.

standard confocal microscope must scan a single point across the specimen, whereas one rotation of the multi-pinhole disk creates a complete optical section. Light from many rotations can be summed to create an image over a longer period of time using lower intensities of excitation light. These systems are thus capable of generating brighter images with lower rates of photo-damage. Image quality in spinning-disk confocal microscopes is reduced

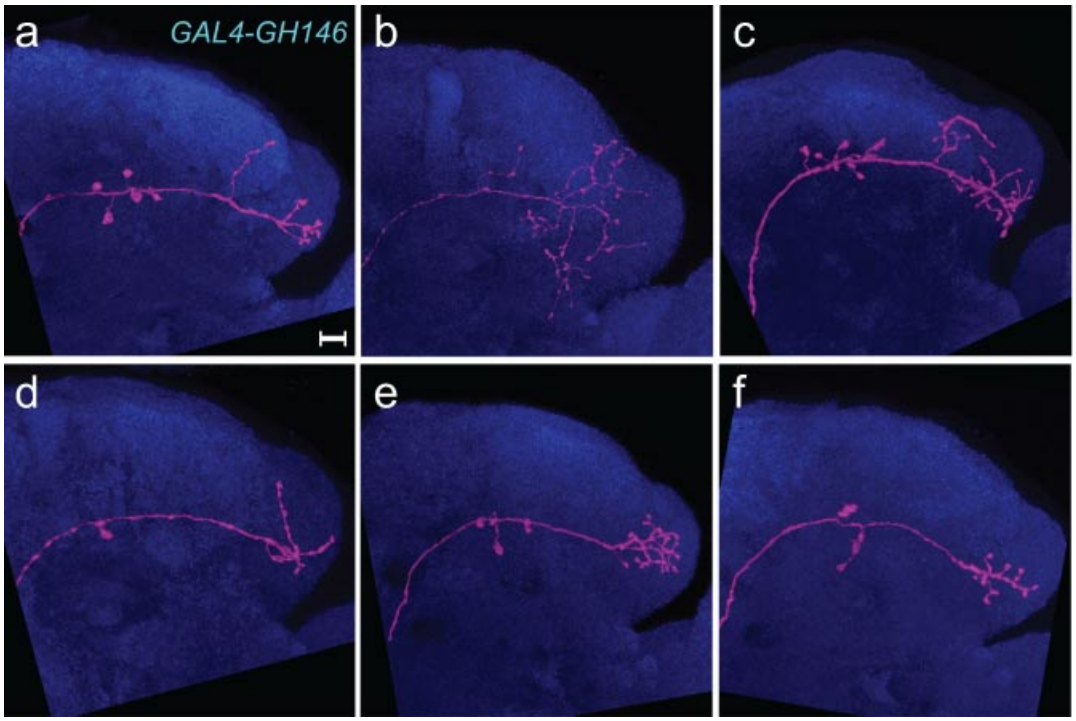
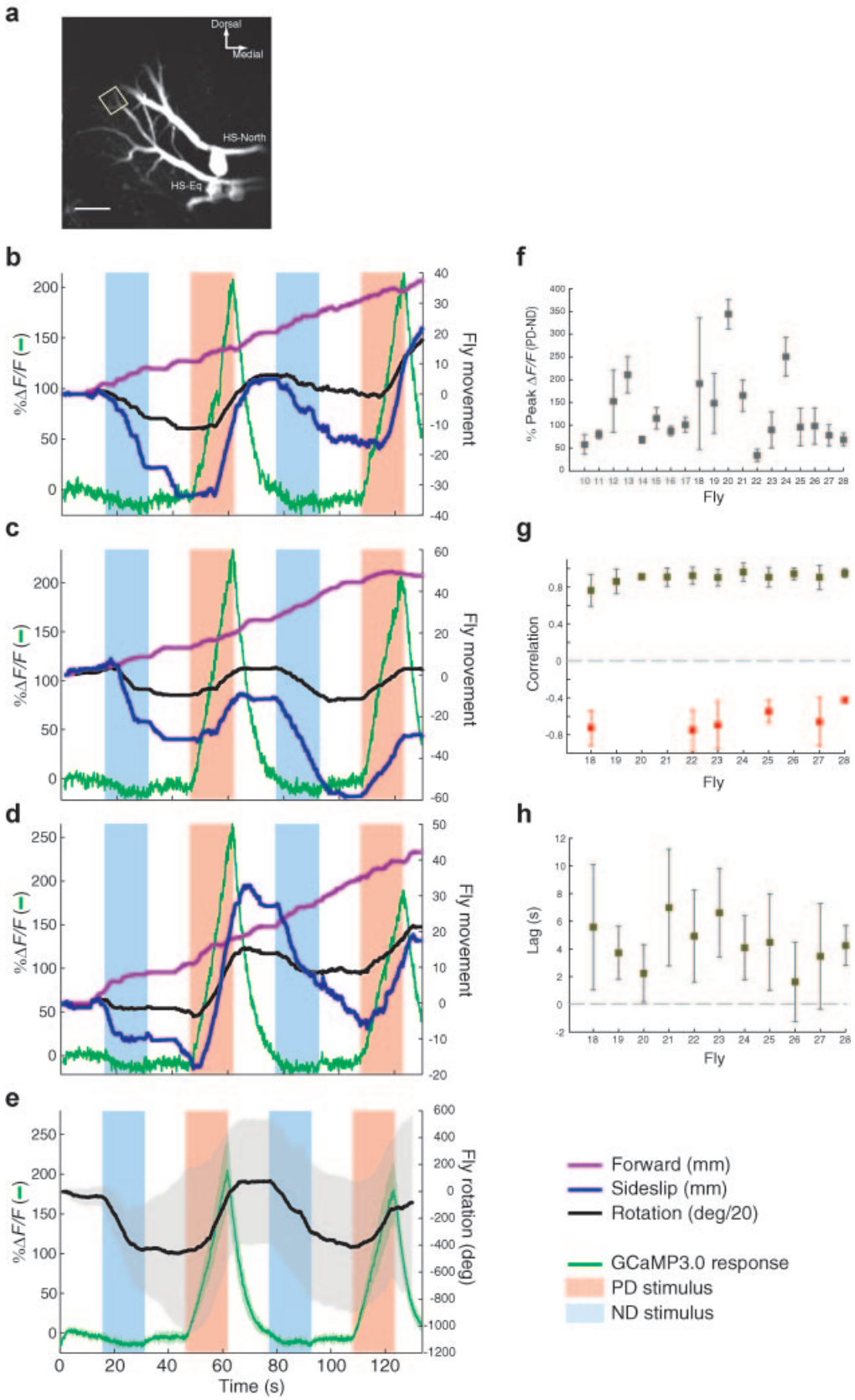


Figure 2. Individual antennal lobe projection neurons derived from the same ganglion mother cell. The fluorescent labels were generated in randomly chosen ganglion-mother-cell progeny during a mitotic recombination event initiated by heat-shock-induced production of FLP recombinase. Each panel represents the final adult morphology of one of the progeny from 2 single ganglion mother cell. Reprinted with permission from Yu et al. (2010).

compared to point-scanning implementations, however, owing to light scattering effects and the loss of incoherence arising from the simultaneous use of multiple pinholes. Spinning-disk confocal microscopes are powerful tools when sensitivity, photo-bleaching, or acquisition speed are greater concerns than spatial resolution and image contrast (Keller and Stelzer, 2008).

A key improvement in in vivo imaging technology has been the introduction of two-photon microscopy (Denk et al., 1990; Denk and Svoboda, 1997; Niell and Smith, 2004). Two-photon microscopes employ lasers producing light of approximately twice the wavelength usually used for conventional one-photon excitation of fluorophores. Essentially the fluorophore absorbs two photons within a very short period of time, each of which provides half of the necessary excitation energy. The magnitude of this two-photon effect is proportional to the square of the intensity of the excitation laser, so the probability of such an event occurring drops off sharply away from the focal volume. By rapidly point scanning with a strongly focused laser beam, this property inherently isolates a single focal plane and, just as in confocal microscopy, a three-dimensional image is built by moving the focal plane to acquire images of different sections of the specimen. One advantage of this technique for in vivo imaging is that the excitation wavelength is in the near infrared, which penetrates more deeply into biological tissue than does visible light. Also, because the excitation is predominantly restricted to the focal volume, photo-damage, and photo-bleaching outside the focal volume are significantly

Figure 3. Optical imaging of dendrites of a motion-sensitive neuron during tethered walking. **a**: Projected image of typical motion-sensitive neurons in the horizontal system expressing the genetically encoded calcium indicator GCaMP3.0. **b**–**d**: Examples of three trials for a single animal. The tethered fly was presented with moving visual stimuli that simulated movement in the neuron's preferred direction or in the opposite null direction. The fly was able to walk on a freely rotating ball while the calcium concentration of the horizontal-system neurons was imaged. The purple line shows the fly's behavioral response of walking forward, the blue shows movement to the side and the black shows rotation. The green line shows an indirect measure of neural activity provided by changes in GCaMP3.0 fluorescence caused by changes in intracellular calcium concentration. These changes in fluorescence ($\Delta F/F$) were imaged while the fly was behaving and responding to visual stimuli. Visual movement in the horizontal-system neuron's preferred direction were accompanied by increases in neural activity and a behavioral response of rotation in the direction of the visual movement. Combined data from 7 trials with the same fly are shown in (**e**). Reprinted from Seelig et al. (2010), with permission from Macmillan Publishers Ltd.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PD stimulus
ND stimulus

Figure 3.

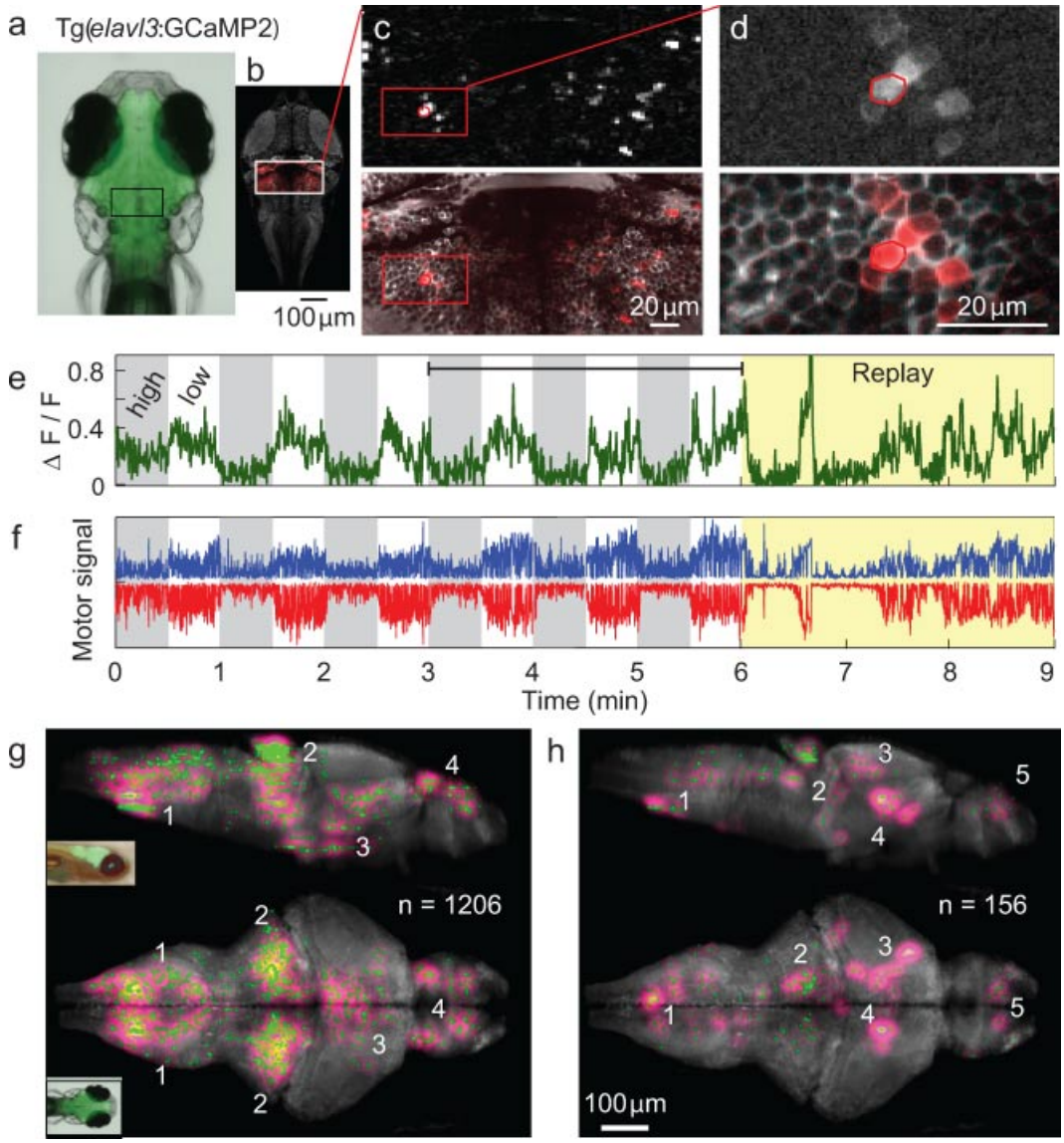

Figure 4. Optical imaging of whole-brain activity in larval zebrafish. The location of the neuron of interest in the zebrafish brain (**a**–**d**). Paralyzed fish were presented with whole-field visual stimuli that simulated being swept backward in a stream. The fish responded by producing fictive swimming motor patterns. The fish were trained (**e**,**f**) in a closed-loop scenario (gray and white bars), where a fictive motor response caused the visual stimulus to move in the appropriate direction. When the visual feedback was high (simulating a strong virtual fish), a small amount of fictive swimming produced a large response in the visual stimulus. When visual feedback was low (simulating a weak virtual fish), a large amount of fictive swimming was required to produce the same visual stimulus. Activity of the example neuron shown in (d) correlated with changes from high-feedback gain to low-feedback gain (e). The fish's motor response quickly adapted to periodic changes in the feedback gain as it adjusted its motor output to match the visual input (f). In an open-loop scenario (yellow bar), fictive motor patterns and activity of the example neuron were much more variable. Neuronal activity that was strongly correlated with motor output (**g**) occurred in brain regions that were distinct from those that were strongly correlated to visual stimulation alone (**h**). Scale bars, as indicated. Reprinted from Ahrens et al. (2012), with permission from Macmillan Publishers Ltd.

reduced. Yet, because this is a point scanning technique, similar to confocal microscopy, image acquisition is slow and the practical, achievable signal-to-noise ratio is very limited.

Despite the many advantages afforded by each of these imaging technologies, there is clearly a need for an imaging technology that provides for fast image acquisition, a high signal to noise ratio, low photo-bleaching and photo-toxicity, and excellent three-dimensional resolution. Here we introduce light-sheet microscopy, a technology that combines these attributes in a system designed for live imaging of developmental processes.

LIGHT-SHEET MICROSCOPY AND ITS APPLICATION TO NEUROSCIENCE

In vivo imaging applications typically require carefully balancing conflicting parameters, including high imaging speed, low photo-bleaching and photo-toxicity, good three-dimensional resolution, high signal-to-noise ratio, and excellent physical coverage. Physical coverage is particularly difficult to attain for large biological specimens since limitations in depth of optical penetration usually make it impossible to extract comprehensive information of the sample from a single optical view.

In order to address this fundamental requirement, new light-sheet fluorescence microscopy techniques have been developed over the course of the past decade. Such microscopes combine intrinsic optical-sectioning capability with high imaging speeds, good signal-to-noise ratio, and low-light exposure of the specimen (Huisken and Stainier, 2009; Keller and Dodt, 2011; Mertz, 2011; Santi, 2011; Tomer et al., 2011).

Conventional and confocal epi-fluorescence microscopes employ the same lens for fluorescence excitation and detection. In contrast, light-sheet microscopes rely on the principle of sample illumination with a thin sheet of laser light perpendicular to the axis of fluorescence detection (Siedentopf and Zsigmondy, 1903; Voie et al., 1993; Stelzer and Lindek, 1994) (Fig. 5A). The light sheet is coplanar with the focal plane of the fluorescence detection system. This approach directly provides optical sectioning: Fluorophores are only excited in the illuminated plane, thus photo-bleaching and other photo-damage are avoided outside the thin volume of interest. This circumstance gives light-sheet microscopes a decisive advantage in the fast imaging of sensitive biological specimens as well as in in vivo imaging over long periods of time (Huisken et al., 2004; Keller et al., 2008a,b, 2010; Scherz et al., 2008; Planchon et al., 2011; Truong et al., 2011; Tomer et al., 2012).

Light-sheet microscopes are furthermore particularly well-suited for multi-view imaging, that is observing the same specimen from multiple different directions. Thereby, parts of the specimen become visible that would otherwise be hidden or obscured when observed from a single direction, enhancing physical coverage for large biological specimens (Swoger et al., 2007; Preibisch et al., 2010; Tomer et al., 2012). In the following section, we provide a qualitative comparison of light exposure, spatial resolution, signal-to-noise ratio, and imaging speeds in light-sheet microscopes and widely-used confocal fluorescence microscopes.

Comparison of Light-Sheet Microscopy to Conventional Approaches

A distinct advantage of light-sheet microscopy is that only the observed plane is illuminated. As discussed above, this approach is in direct contrast to confocal fluorescence microscopes, in which illumination is not confined to the focal plane. Depending on the number of optical sections acquired in a volumetric imaging experiment, rates of photo-bleaching and photo-damage are typically several orders of magnitude higher in confocal fluorescence microscopy compared to light-sheet microscopy (Keller et al., 2008b).

The spatial resolution of light-sheet microscopes and confocal fluorescence microscopes is defined by the wavelength, the numerical aperture of the lenses, and the signal-to-noise ratio in the image. In theory, confocal fluorescence microscopy provides a factor of the square-root-of-2-better lateral resolution than light-sheet microscopy. In practice, however, the signal-to-noise ratio of confocal fluorescence microscopes is often insufficient to take advantage of this improved lateral performance (Stelzer, 1998; Keller and Stelzer, 2008). The situation is different for axial resolution. Light-sheet microscopy performs optical sectioning with a diffraction-limited illumination profile and provides multi-view capability, that is, the option to record multiple data sets of a specimen from different directions. In single-view experiments with lenses of low numerical apertures, axial resolution in the light-sheet microscope is mainly determined by the thickness of the light sheet (Engelbrecht and Stelzer, 2006; Keller and Stelzer, 2008). Exceptionally high axial resolution can be achieved by using Bessel beams for light-sheet microscopy (Planchon et al., 2011; Gao et al., 2012). In multi-view experiments, the axial resolution can be improved by fusing the information obtained along different recording angles. For highly transparent specimens, the fusion of a few angles already leads to almost isotropic, three-dimensional resolution, that is, the low axial resolution becomes equal to the considerably better lateral resolution (Swoger et al., 2007).

At high imaging speeds, confocal fluorescence microscopes provide images with a signal-to-noise ratio that is typically about one to two orders of magnitude lower than in light-sheet microscopes, owing to the short pixel dwell times associated with two-dimensional point scanning. Light-sheet microscopy employs camera-based detection, and thereby benefits from the high sensitivity and dynamic range of state-of-the-art CCD, sCMOS, and other semiconductor based cameras to achieve exceptionally high imaging speeds. For example, SiMView light-sheet microscopy, which performs simultaneous image acquisition with sCMOS cameras from multiple views (Tomer et al., 2012) (Fig. 5B), is capable of recording data at a continuous rate of more than 175 million voxels per second. This level of parallelization in light-sheet microscopes leads to a dramatic speed advantage over point-scanning confocal fluorescence microscopes, which employ two-dimensional scanning of the specimen, that is, recording fluorescence data voxel per voxel at maximum rates of about 1–10 million voxels per second. Depending on imaging speed requirements, volumetric imaging is typically performed in one of two ways in light-sheet microscopy. For low imaging speeds and large specimens, the light sheet and the detection objectives are held stationary and the sample is moved step-wise through the focal plane. For high imaging speeds, on the other hand, the specimen can be held stationary while the light sheet is scanned through it, and the detection objectives are moved in parallel to keep the light sheet and focal plane coplanar (Ahrens et al., 2013).

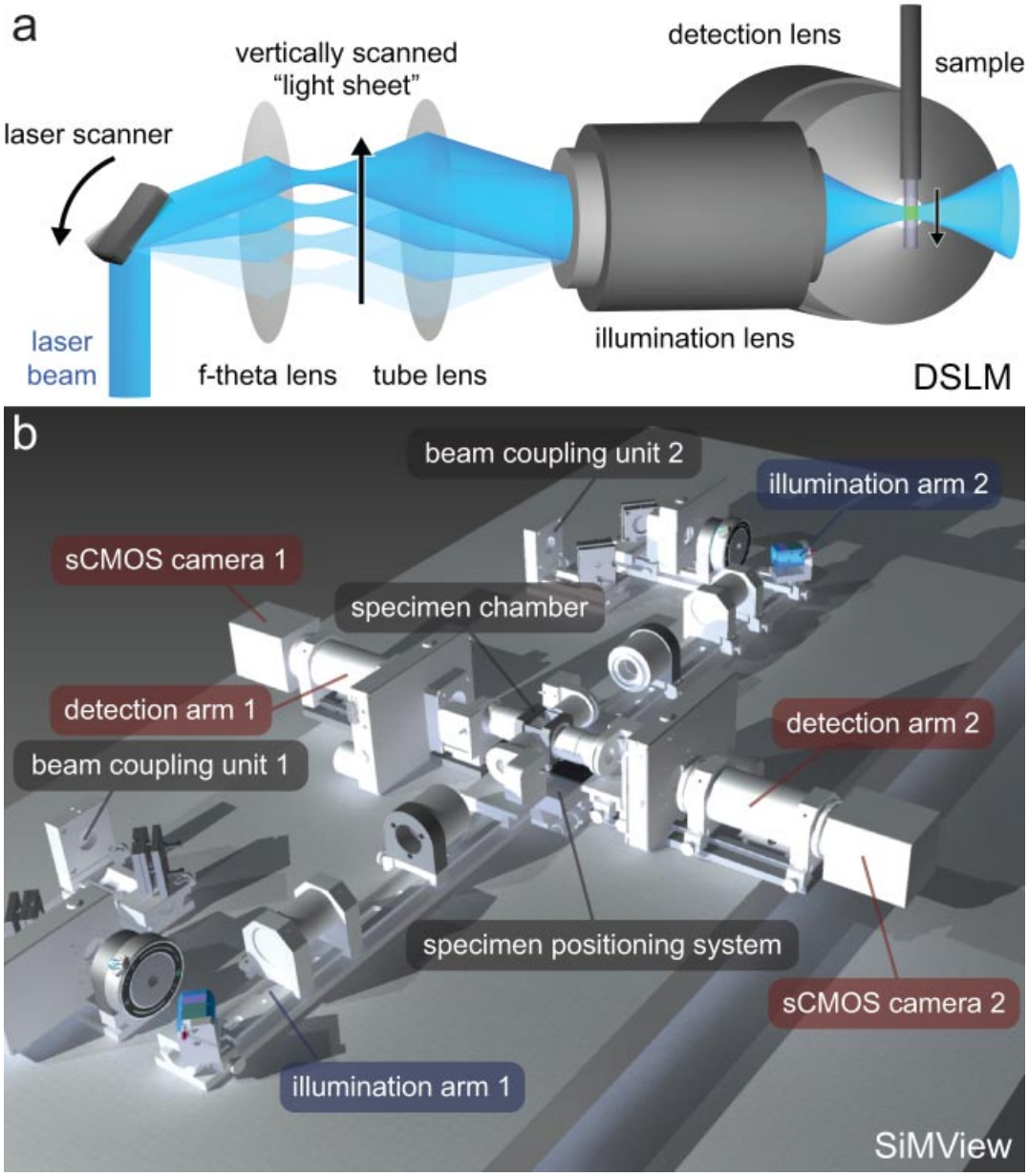

Figure 5. Scanned light-sheet fluorescence microscopy. **a**: The principle behind Digital Scanned Laser Light-Sheet Fluorescence Microscopy (Keller et al., 2008b). The f-theta lens converts the tilting movement of the scan mirror into a vertical displacement of the laser beam. The tube lens and the illumination objective focus the laser beam into the specimen, which is positioned in front of the detection lens. The laser beam thus illuminates the specimen from the side and excites fluorophores along a single line. Rapid scanning of a thin volume and fluorescence detection at a right angle to the illumination axis provides an optically sectioned image. **b**: Computer model of the opto-mechanical implementation of a light-sheet microscope for simultaneous multi-view imaging. The opto-mechanical modules of the instrument consist of two illumination arms for fluorescence excitation with scanned light sheets (blue), two fluorescence detection arms equipped with sCMOS cameras (red), as well as beam-coupling modules, specimen chamber, and specimen positioning system (gray). Panel (a) was reprinted from Keller et al. (2008b), with permission from the American Association for the Advancement of Science. Panel (b) was reprinted from Tomer et al. (2012), with permission from Macmillan Publishers Ltd.

Applying Light-Sheet Microscopy to Developmental and Functional Neurobiology

The high imaging speed and the reduced light load of the light-sheet microscope make this technique compatible with quantitative, live imaging of biological specimens under physiological conditions. The low-light load reduces photo-bleaching of fluorescent markers and reduces photo-toxicity, allowing continuous imaging for long periods of

time. These qualities make light-sheet microscopy an excellent method to study many areas of embryonic development, and do not limit its utility to imaging neural development (Keller, 2013a). As an example, it is possible to perform volumetric imaging of entire developing Drosophila embryos at 30-sec intervals for 24 hr (Tomer et al., 2012) and to reconstruct the movements and divisions of individual cells from such whole-embryo recordings (Fig. 6). Likewise, whole zebrafish embryos have been imaged with sufficient spatial and temporal resolution to allow comprehensive reconstruction of cell movements in the early embryo (Fig. 7) (Keller et al., 2008b; Keller, 2013b). The high speed of image acquisition with the light-sheet microscope also provides excellent temporal resolution. Currently available sCMOS cameras allow for acquisition of 4–5 megapixel images at sustained speeds of up to 100 frames per second. By integrating this detector technology into light-sheet microscopes equipped with an electronic control framework that eliminates overhead in the image-acquisition workflow and piezo-electronics suitable for fast optical scanning of the light-sheet through the specimen, volumetric imaging can be sped up by at least another factor of ten (Ahrens et al., 2013) compared to the previous state-of-the-art in light-sheet microscopy.

Complementing the imaging of developmental processes, light-sheet microscopy can also be combined with new generations of genetically encoded indicators of neuronal activity, such as calcium indicators and voltage indicators (Akerboom et al., 2012; Looger and Griesbeck, 2012), to provide a systems-level appraisal of nervous system activity. Light-sheet microscopy thus has the capability of recording system-level functional images with good three-dimensional resolution and temporal resolution of substantially less than 1 sec. It has been shown that even neuronal networks as large as the brain of larval zebrafish, which consists of ~100,000 neurons, can be imaged at a rate of 0.8 Hz while achieving single-cell resolution for more than 80% of all neurons (Ahrens et al., 2013).

CHALLENGES IN LIVE IMAGING EXPERIMENTS USING LIGHT-SHEET MICROSCOPY

One frequently encountered challenge of long-term, time-lapse recordings with the light-sheet microscope is the development of a suitable sample preparation strategy. Specimens need to be immobilized in front of the microscope objectives in a manner that is compatible with both acquiring good images and with normal development of the animal. In practice this typically means placing the specimen in a small, transparent plastic compartment (Keller et al., 2007) or embedding the specimen in a biological matrix, such as agarose gel (Huisken et al., 2004), with the lowest density that still provides the necessary mechanical support. For example, the upright specimen holder design of the SiMView light-sheet microscopy platform allows using agarose gels at concentrations as low as 0.3–0.4% (Tomer et al., 2012). A thin layer of agarose, which has a refractive index near that of water, becomes virtually transparent when it is submerged in water, and is suitable for acquiring good images, provided that the agarose is firm enough to hold the specimen motionless.

Once the specimen is gently immobilized, the challenge becomes providing the best culture conditions to keep the specimen alive for prolonged imaging experiments. Light-sheet microscopes typically use water-dipping detection objectives and a fluid-filled specimen chamber so the specimen can be completely immersed in the appropriate culture medium. The chamber can also be equipped with a perfusion system to continuously replace the medium. Drosophila and zebrafish embryos develop normally submerged in tap water or fish water, respectively, and do not require any perfusion. They can easily be grown in the microscope for a day or longer and imaged completely non-destructively. Mouse embryos, on the other hand, are very sensitive to light exposure and small changes in their environment. These embryos must be kept in a nutritive, isotonic medium at all times and maintained at a stable temperature of 37°C. Long-term, live imaging of mouse embryos strictly requires a minimization of laser light exposure and very gentle handling throughout the imaging experiment.

FLUORESCENT MARKER STRATEGIES AND OPTOGENETICS

Like confocal microscopy and wide-field fluorescence microscopy, light-sheet microscopes depend on the availability of high-quality fluorescent markers (Shaner et al., 2004; Chudakov et al., 2010; Progatzky et al., 2013). With live imaging, these markers are almost always genetically encoded fluorescent proteins expressed in one or more populations of cells. Imaging of live animals requires fluorescent markers that are compatible with both the imaging method and the requirements of the developing animal; as developing animals are intolerant of high intensity illumination, laser power must be kept to a minimum. This necessarily reduces the signal-to-noise ratio, which, in other types of imaging experiments, can be compensated by adding fluorophore-tagged antibodies. As a result, the selection of fluorescent protein is vitally important for the success of live-imaging experiments.

One approach that we use for live imaging is to employ a ubiquitous nuclear marker to visualize general morphological features in the developing embryo and a different color membrane or nuclear label of a specific class of neurons that we follow through development. The color of these fluorescent proteins affects their utility. Fluorophores with excitation and emission maxima at longer wavelengths can be used to image deeper tissues in a non-transparent specimen. Longer wavelengths of red light are scattered less as they pass through tissue, so more of the excitation energy reaches the fluorophores in the focal plane, and emitted red light is scattered less on the way out of the specimen. For this reason we prefer to use red fluorescent proteins for deep imaging whenever possible (Chudakov et al., 2010). A technological advance that would facilitate

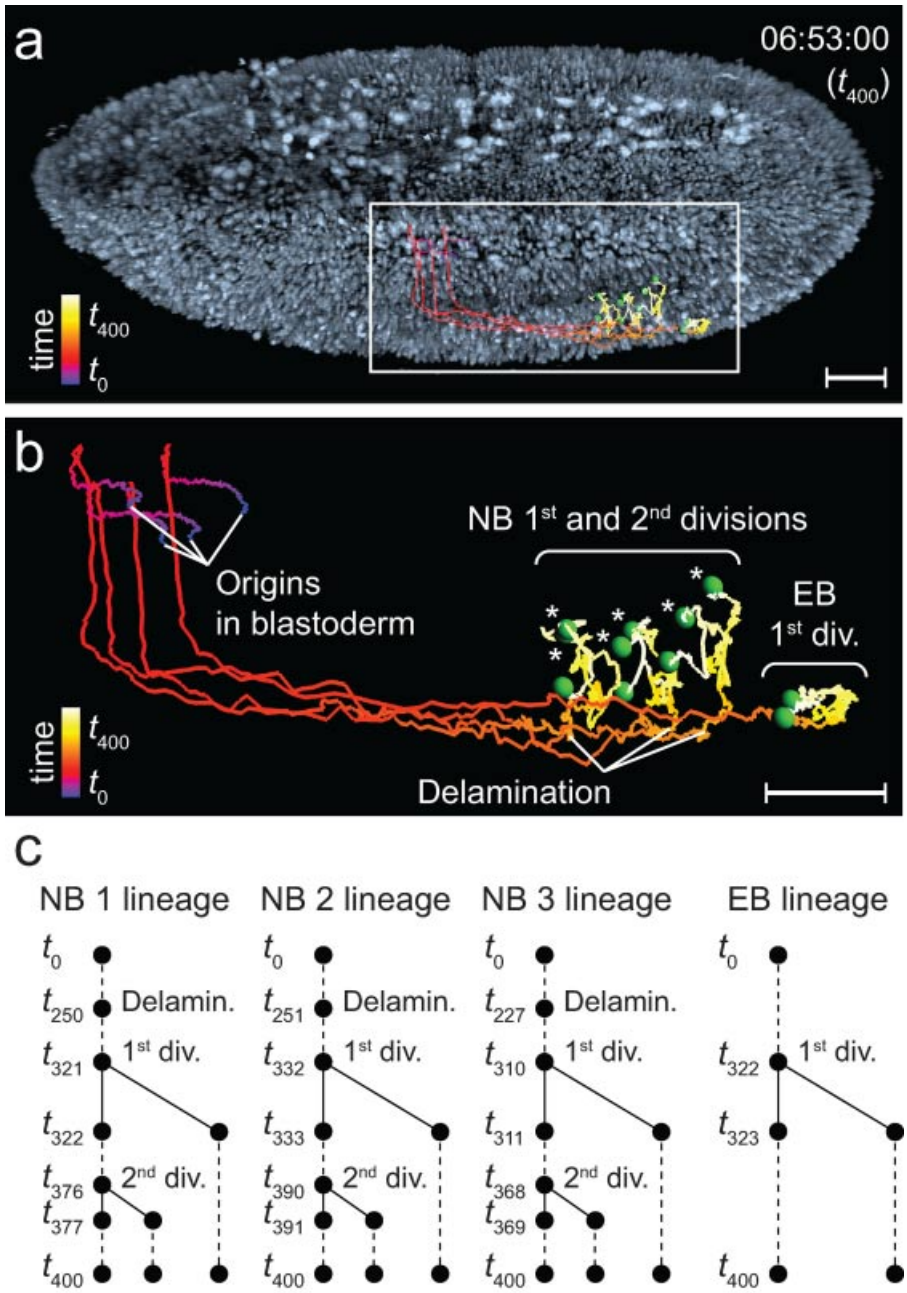

Figure 6. Reconstructing embryonic development with simultaneous multi-view light-sheet microscopy. **a**: SiMView recording of a histone-labeled Drosophila embryo superimposed with manually reconstructed lineages of three neuroblasts and one epidermoblast for 120–353 min after fertilization (time points 0–400). Track color encodes time. **b**: Enlarged view of tracks highlighted in (a). Green spheres show cell locations at time point 400. Asterisks mark six ganglion mother cells produced in two rounds of neuroblast division. NB, neuroblast; EB, epidermoblast. **c**: Lineage trees for the neuroblast/epidermoblast lineage reconstructions visualized in (a). Four blastoderm cells and their respective daughter cells were manually tracked from time point 0 to 400 (120–353 min post fertilization, 35-sec temporal resolution), using Imaris (Bitplane) and ImageJ (http://rsbweb.nih.gov/ij/). Tracks start in the blastoderm (time point 0). The neuroblasts delaminate between time points 227 and 251, and subsequently produce ganglion mother cells in two division cycles (first cycle between time points 310 and 332, second cycle between time points 368 and 390). The epidermoblast remains in the outer cell layer and divides once at time point 313. Manual tracking was performed until time point 400 for all cells. Delamin. = delamination, 1st div. = first division, 2nd div. = second division. Scale-bars, 30 μm. Reprinted from Tomer et al. (2012), with permission from Macmillan Publishers Ltd.

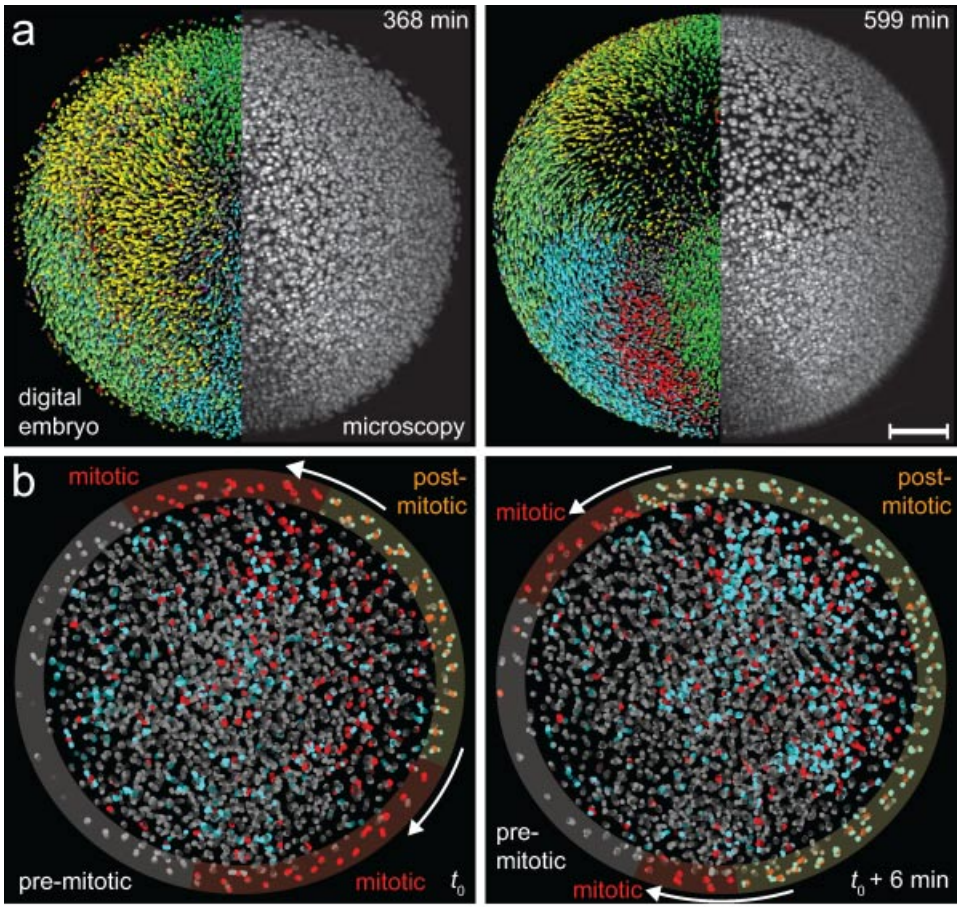

Figure 7. Tracking of cell movements and divisions in a digital reconstruction of the developing zebrafish embryo. **a**: Light-sheet microscopy data (right half of embryo, maximum projection) and a digital reconstruction of the embryo (left half of embryo) with color-coded indicators of the direction of migration. Color code: dorsal migration (cyan), ventral migration (green), toward or away from body axis (red or yellow), toward yolk (pink). **b**: A digital reconstruction of the developing zebrafish embryo with color-coded indicators of cell division. Color code: dividing cells (red) and their daughter cells (blue). Yellow, red, and gray overlays surrounding the image of the digital embryo indicate progression of the peripheral cell division waves during division cycle 12 (arrows show direction of peripheral waves; $t_0 = 216$ min post fertilization). Reprinted from Keller et al. (2008b), with permission from the American Association for the Advancement of Science.

live imaging of exceptionally deep biological tissues is the introduction of photo-stable and bright fluorescent proteins with excitation and emission spectra with peaks in far-red and near-infrared wavelengths, where scattering would be even less of an issue (Shcherbo et al., 2010; Lecoq and Schnitzer, 2011; Shemiakina et al., 2012). Likewise, the particular fluorophores being considered should have excitation and emission spectra that minimally overlap with the absorption spectra of biological structures (e.g., yolk, pigment) that will interfere with image acquisition.

Improvements in genetically encoded indicators of neural activity would also be a welcome advance for live imaging of neural development (Looger and Griesbeck, 2012; Tian et al., 2012). There is a need for improvements in the temporal response of neural activity indicators and the signal strength (Chen et al., 2013). The ideal activity indicator would have a time course that allows it to respond to single action potentials with a signal bright enough to detect in real time. It would also exhibit a large enough change in signal strength in response to neural activity ($\Delta F/F$) to resolve spikes in a single neuron or a single axon. Many research groups are now working to produce calcium and voltage indicators with a variety of response characteristics and with a variety of excitation and emission spectra. Currently, the vast majority of useful, genetically encoded calcium indicators fluoresce in the green region of the spectrum. Two-color imaging, for example of presynaptic and postsynaptic neurons, will be practical with a good, red-fluorescing activity indicator.

Another extension of in vivo imaging that is helping to improve our understanding of the functional development of the nervous system is the addition of optogenetic methods to non-invasively manipulate neural activity. Light-gated bacterial rhodopsins, such as channelrhodopsin and

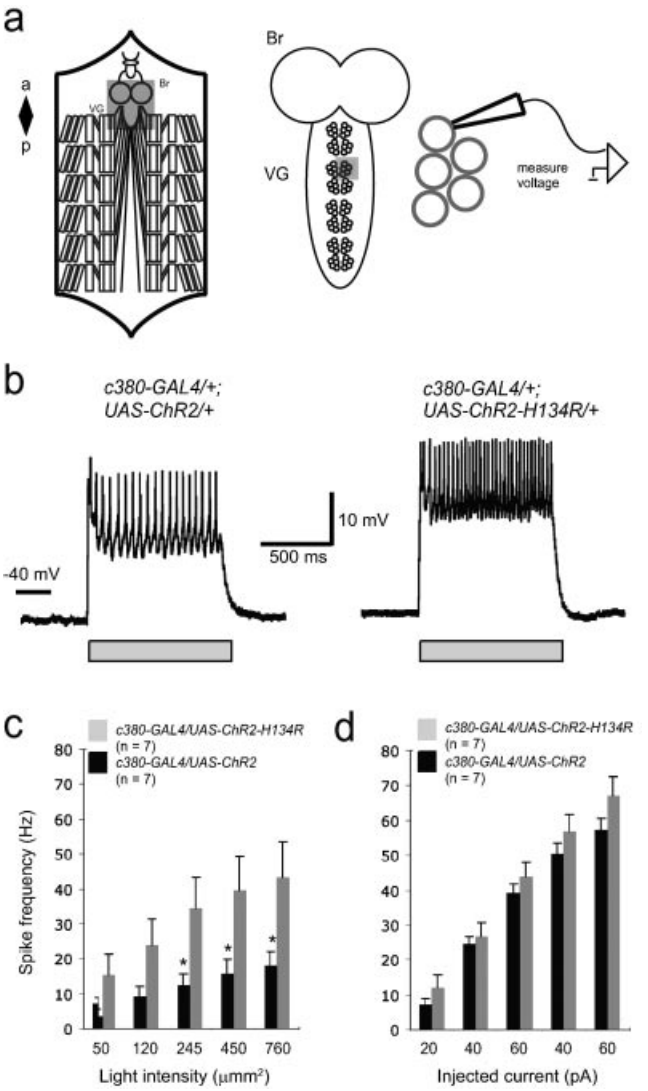

Figure 8. Whole-cell patch recordings of larval Drosophila motor neurons expressing two versions of Channelrhodopsin 2 (ChR2) while the central nervous system was exposed to blue light. **a**: Schematic of the regions stimulated. **b**: Membrane potential was measured while 1-sec pulses of 470-nm light triggered depolarization of the motor neurons. **c**: Higher light intensity evoked a higher spike frequency in animals expressing two different versions of ChR2. **d**: The responses to blue-light stimulation were similar to the effects of direct injection of depolarizing current. Reprinted from Pulver et al. (2009), with permission from The American Physiological Society.

archearhodopsin (Berndt et al., 2011; Han, 2012), expressed in neurons of interest can be used to quickly change the membrane potential when exposed to the appropriate wavelength of light (Fig. 8). By titrating light intensity and duration, the neurons can be induced to produce a range of activity, from single spikes to prolonged trains of action potentials (Pulver et al., 2009). By combining this technique with calcium imaging using GCaMP, the activity of one neuron or group of neurons can be optically controlled while the activity of another group of neurons is optically monitored (Prigge et al., 2012). Functional connections between the two populations of neurons can be reliably determined less-invasively in an intact nervous system without the use of electrodes. Ultimately, these types of studies can be combined with imaging of embryonic development to observe when neural circuits become functional in a normally developing animal.

CONCLUSIONS

Light-sheet microscopy provides a powerful combination of capabilities that render it particularly useful for the systems-level study of biological processes in vivo. Using light-sheet microscopy, dynamic events can be investigated in a completely non-invasive manner, even for relatively large, living biological systems. In this article, we discussed the enormous potential of this emerging technology in advancing our understanding of the nervous system, by providing access to its development and function at the whole-system level, and with unprecedented spatiotemporal resolution.

Importantly, progress in the development of light-sheet microscopy is faster than ever, and its performance has not yet reached a plateau. In particular, further improvements in imaging speed and spatial resolution can be expected in the near future. These capabilities will directly synergize with the rapid progress in related fields, such as the development of advanced fluorescent reporter strategies and automated approaches to the computational analysis of image data, opening up exciting new opportunities for microscopy-based research in the life sciences.

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REFERENCES

Ahrens MB, Li JM, Orger MB, Robson DN, Schier AF, Engert F, Portugues R. 2012. Brain-wide neuronal dynamics during motor adaptation in zebrafish. Nature 485:471–477.

Ahrens MB, Orger MB, Robson DN, Li JM, Keller PJ. 2013. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. Nat Methods 10:413–420.

Akerboom J, Chen T-W, Wardill TJ, Tian L, Marvin JS, Mutlu S, Calderón NC, Esposti F, Borghuis BG, Sun XR, Gordus A, Orger MB, Portugues R, Engert F, Macklin JJ, Filosa A, Aggarwal A, Kerr RA, Takagi R, Kracun S, Shigetomi E, Khakh BS, Baier H, Lagnado L, Wang SS-H, Bargmann CI, Kimmel BE, Jayaraman V, Svoboda K, Kim DS, Schreiter ER, Looger LL. 2012. Optimization of a GCaMP calcium indicator for neural activity imaging. J Neurosci 32:13819–13840.

Berndt A, Schoenenberger P, Mattis J, Tye KM, Deisseroth K, Hegemann P, Oertner TG. 2011. High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc Natl Acad Sci USA 108:7595–7600.

Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS. 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499:295–300.

Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA. 2010. Fluorescent proteins and their applications in imaging living cells and tissues. Physiol Rev 90:1103–1163.

Denk W, Svoboda K. 1997. Photon upmanship: Why multiphoton imaging is more than a gimmick. Neuron 18:351–357.

Denk W, Strickler JH, Webb WW. 1990. Two-photon laser scanning fluorescence microscopy. Science 248:73–76.

Engelbrecht CJ, Stelzer EHK. 2006. Resolution enhancement in a light-sheet-based microscope (SPIM). Opt Lett 31:1477–1479.

Gao L, Shao L, Higgins CD, Poulton JS, Peifer M, Davidson MW, Wu X, Goldstein B, Betzig E. 2012. Noninvasive imaging beyond the diffraction limit of 3D dynamics in thickly fluorescent specimens. Cell 151:1370–1385.

Graf R, Rietdorf J, Zimmermann T. 2005. Live cell spinning disk microscopy. Adv Biochem Eng Biotechnol 95:57–75.

Han X. 2012. In vivo application of optogenetics for neural circuit analysis. ACS Chem Neurosci 3:577–584.

Hockendorf B, Thumberger T, Wittbrodt J. 2012. Quantitative analysis of embryogenesis: A perspective for light sheet microscopy. Dev Cell 23:1111–1120.

Huisken J, Stainier DY. 2009. Selective plane illumination microscopy techniques in developmental biology. Development 136: 1963–1975.

Huisken J, Swoger J, Del Bene F, Wittbrodt J, Stelzer EHK. 2004. Optical sectioning deep inside live embryos by selective plane illumination microscopy. Science 305:1007–1009.

Keller PJ. 2013a. Imaging morphogenesis: Technological advances and biological insights. Science 340:1234168.

Keller PJ. 2013b. In vivo imaging of zebrafish embryogenesis. Methods 62:268–278.

Keller PJ, Dodt HU. 2011. Light sheet microscopy of living or cleared specimens. Curr Opin Neurobiol 22:138–143.

Keller PJ, Stelzer EH. 2008. Quantitative in vivo imaging of entire embryos with digital scanned laser light sheet fluorescence microscopy. Curr Opin Neurobiol 18:624–632.

Keller PJ, Pampaloni F, Stelzer EH. 2007. Three-dimensional preparation and imaging reveal intrinsic microtubule properties. Nat Methods 4:843–846.

Keller PJ, Pampaloni F, Lattanzi G, Stelzer EHK. 2008a. Three-dimensional microtubule behavior in Xenopus egg extracts reveals four dynamic states and state-dependent elastic properties. Biophys J 95:1474–1486.

Keller PJ, Schmidt AD, Wittbrodt J, Stelzer EHK. 2008b. Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. Science 322:1065–1069.

Keller PJ, Schmidt AD, Santella A, Khairy K, Bao Z, Wittbrodt J, Stelzer EH. 2010. Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. Nat Methods 7:637–642.

Khairy K, Keller PJ. 2011. Reconstructing embryonic development. Genesis 49:488–513.

Kunz T, Kraft KF, Technau GM, Urbach R. 2012. Origin of Drosophila mushroom body neuroblasts and generation of divergent embryonic lineages. Development 139:2510–2522.

Lecoq J, Schnitzer MJ. 2011. An infrared fluorescent protein for deeper imaging. Nat Biotechnol 29:715–716.

Looger LL, Griesbeck O. 2012. Genetically encoded neural activity indicators. Curr Opin Neurobiol 22:18–23.

Mertz J. 2011. Optical sectioning microscopy with planar or structured illumination. Nat Methods 8:811–819.

Niell CM, Smith SJ. 2004. Live optical imaging of nervous system development. Annu Rev Physiol 66:771–798.

Pawley JB. 2006. Handbook of biological confocal microscopy. New York: Springer.

Planchon TA, Gao L, Milkie DE, Davidson MW, Galbraith JA, Galbraith CG, Betzig E. 2011. Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination. Nat Methods 8:417–423.

Preibisch S, Saalfeld S, Schindelin J, Tomancak P. 2010. Software for bead-based registration of selective plane illumination microscopy data. Nat Methods 7:418–419.

Prigge M, Schneider F, Tsunoda SP, Shilyansky C, Wietek J, Deisseroth K, Hegemann P. 2012. Color-tuned channelrhodopsins for multiwavelength optogenetics. J Biol Chem 287: 31804–31812.

Progatzky F, Dallman MJ, Lo Celso C. 2013. From seeing to believing: Labelling strategies for in vivo cell-tracking experiments. Interface Focus 3:20130001.

Pulver SR, Pashkovski SL, Hornstein NJ, Garrity PA, Griffith LC. 2009. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae. J Neurophysiol 101:3075–3088.

Santi PA. 2011. Light sheet fluorescence microscopy: A review. J Histochem Cytochem 59:129–138.

Scherz PJ, Huisken J, Sahai-Hernandez P, Stainier DY. 2008. High-speed imaging of developing heart valves reveals interplay of morphogenesis and function. Development 135:1179–1187.

Seelig JD, Chiappe ME, Lott GK, Dutta A, Osborne JE, Reiser MB, Jayaraman V. 2010. Two-photon calcium imaging from head-fixed Drosophila during optomotor walking behavior. Nat Methods 7:535–540.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 22:1567–1572.

Shcherbo D, Shemiakina II, Ryabova AV, Luker KE, Schmidt BT, Souslova EA, Gorodnicheva TV, Strukova L, Shidlovskiy KM, Britanova OV, Zaraisky AG, Lukyanov KA, Loschenov VB, Luker GD, Chudakov DM. 2010. Near-infrared fluorescent proteins. Nat Methods 7:827–829.

Shemiakina II, Ermakova GV, Cranfill PJ, Baird MA, Evans RA, Souslova EA, Staroverov DB, Gorokhovatsky AY, Putintseva EV, Gorodnicheva TV, Chepurnykh TV, Strukova L, Lukyanov S, Zaraisky AG, Davidson MW, Chudakov DM, Shcherbo D. 2012. A monomeric red fluorescent protein with low cytotoxicity. Nat Commun 3:1204.

Siedentopf H, Zsigmondy R. 1903. Éber Sichtbarmachung und Gröåenbestimmung ultramikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser. Annalen der Physik 315:1–39.

Stelzer EHK. 1998. Contrast, resolution and the signal to noise ratio in fluorescence microscopy. J Microsc 1:15–24.

Stelzer EHK, Lindek S. 1994. Fundamental reduction of the observation volume in far-field light microscopy by detection orthogonal to the illumination axis: Confocal theta microscopy. Opt Commun 111:536–547.

Swoger J, Verveer P, Greger K, Huisken J, Stelzer EH. 2007. Multi-view image fusion improves resolution in three-dimensional microscopy. Opt Express 15:8029–8042.

Tian L, Akerboom J, Schreiter ER, Looger LL. 2012. Neural activity imaging with genetically encoded calcium indicators. Prog Brain Res 196:79–94.

Tomer R, Khairy K, Keller PJ. 2011. Shedding light on the system: Studying embryonic development with light sheet microscopy. Curr Opin Genet Dev 21:558–565.

Tomer R, Khairy K, Amat F, Keller PJ. 2012. Quantitative high-speed imaging of entire developing embryos with simultaneous multiview light-sheet microscopy. Nat Methods 9:755–763.

Truong TV, Supatto W, Koos DS, Choi JM, Fraser SE. 2011. Deep and fast live imaging with two-photon scanned light-sheet microscopy. Nat Methods 8:757–760.

Tsien RY, Ernst L, Waggoner A. 2006. Fluorophores for confocal microscopy: Photophysics and photochemistry. In: Pawley JB, editor. Handbook of biological confocal microscopy. New York: Springer.

Voie AH, Burns DH, Spelman FA. 1993. Orthogonal-plane fluorescence optical sectioning: Three-dimensional imaging of macroscopic biological specimens. J Microsc 170:229–236.

Yang JS, Awasaki T, Yu HH, He Y, Ding P, Kao JC, Lee T. 2013. Diverse neuronal lineages make stereotyped contributions to the Drosophila locomotor control center, the central complex. J Comp Neurol 521:2645–2662.

Yu HH, Kao CF, He Y, Ding P, Kao JC, Lee T. 2010. A complete developmental sequence of a Drosophila neuronal lineage as revealed by twin-spot MARCM. PLoS Biology 8:e1000461.

Yu HH, Awasaki T, Schroeder MD, Long F, Yang JS, He Y, Ding P, Kao JC, Wu GY, Peng H, Myers G, Lee T. 2013. Clonal development and organization of the adult Drosophila central brain. Curr Biol 23:633–643.

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