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Light-Sheet Microscopy and Its Potential for Understanding Developmental Processes

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Abstract

The ability to visualize and quantitatively measure dynamic biological processes in vivo and at high spatiotemporal resolution is of fundamental importance to experimental investigations in developmental biology. Light-sheet microscopy is particularly well suited to providing such data, since it offers exceptionally high imaging speed and good spatial resolution while minimizing light-induced damage to the specimen. We review core principles and recent advances in light-sheet microscopy, with a focus on concepts and implementations relevant for applications in developmental biology. We discuss how light-sheet microcopy has helped advance our understanding of developmental processes from single-molecule to whole-organism studies, assess the potential for synergies with other state-of-the-art technologies, and introduce methods for computational image and data analysis. Finally, we explore the future trajectory of light-sheet microscopy, discuss key efforts to disseminate new light-sheet technology, and identify exciting opportunities for further advances.

Contents

WHAT IS LIGHT-SHEET MICROSCOPY?

The biological processes governing the development and function of an organism are highly dynamic by nature—from subcellular phenomena, such as gene expression, protein localization and transport, and behavior of the cytoskeleton and organelles; to cellular processes, such as cell movements, cell divisions, and cell shape changes; to phenomena at the tissue and whole-organism level, such as large-scale morphogenetic changes and the assembly of organs or even entire embryos. The ability to visualize and quantitatively measure such dynamic processes in living organisms, at multiple scales and with high spatiotemporal resolution, is of critical importance for research in the life sciences. A key approach to implementing such measurements is the use of fluorescence microscopy for live imaging.

Fluorescence microscopy is a powerful form of light microscopy that excites fluorescent molecules with light of one wavelength and forms an image of the fluorescence light emitted by these molecules at a different wavelength. This mechanism provides an opportunity for selectively visualizing specific types of molecules or structures of interest labeled by genetically encoded reporters, interrogating dynamic processes with functional fluorescent indicators, and examining spatiotemporal relationships between multiple types of molecules or structures through multicolor imaging. State-of-the-art fluorescence light microscopes offer volumetric imaging capabilities with a spatial resolution of tens to hundreds of nanometers and a temporal resolution in the

range of milliseconds to seconds. In transparent specimens with minimal light scattering, absorption, and optical aberrations, light can penetrate up to several hundreds of micrometers to several millimeters of tissue before image contrast and spatial resolution degrade significantly.

Understanding the opportunities for and practical limitations of specific types of fluorescence microscopes requires consideration of not only the design principles underlying the microscope but also the so-called photon budget of the specimen under investigation. The latter term refers to the fact that each specimen contains a limited (potentially nonrenewable) number of fluorescent molecules that produce a statistically limited number of photons before transitioning to a nonfluorescing triplet state (also known as bleaching). Moreover, in live imaging, each biological specimen has a limited phototolerance, which, depending on the imaging technique and specimen, may be reached even before depleting the photon budget: Since fluorescence microscopes rely on excitation of molecules to a higher-energy state, these molecules in turn become more reactive than when they are in their ground state. By creating reactive oxygen species and free radicals, light exposure of the specimen can thus cause damage to molecules and impact the health of cells, which in turn can lead to cell death and ultimately damage to or death of the entire organism.

Multiple performance-related parameters are competing for the photon budget and for excitation light levels compatible with physiological conditions: the temporal resolution, the spatial resolution, the signal-to-noise ratio, and the length of the imaging experiment. High-resolution imaging of developmental processes can be particularly challenging in this regard, since all four parameters are often simultaneously important. Importantly, however, different fluorescence imaging techniques differ not only in their technical capabilities, e.g., by utilizing mechanisms that offer intrinsically higher speed or resolution, but also in their efficiency in using the available photon budget. In other words, some methods are better than others at converting this budget into actual performance. In the realm of biological live imaging, the most widespread fluorescence microscopy methods include conventional widefield microscopy, confocal microscopy, and two-photon microscopy.

Widefield fluorescence microscopy illuminates the specimen relatively uniformly, collects fluorescence light, and forms an image by using a camera. Since neither illumination nor detection is spatially selective, conventional widefield microscopes lack an intrinsic ability to resolve 3D structures (optical sectioning); however, the principle of camera-based detection provides high imaging speed, since state-of-the-art, scientific-grade cameras acquire multimegapixel frames at rates of hundreds of frames per second.

By contrast, confocal microscopes focus a laser beam into a small focal spot that is 3D scanned through the specimen while an image of the volume is formed voxel by voxel, using a detection pinhole that rejects fluorescence light originating from outside the focal volume. Most of the light is rejected in this manner, which translates to poor use of the photon budget and high rates of photobleaching, and the sequential acquisition process fundamentally limits imaging speed and requires high laser power to produce an adequate signal. However, the use of a pinhole for confocal detection offers optical sectioning and produces 3D images with good contrast.

Two-photon microscopy mitigates some of these disadvantages and further improves depth penetration by using a high-power, pulsed infrared light beam for fluorescence excitation. Fluorescence is almost exclusively generated within the focal volume, where power densities are high enough to enable the concurrent absorption of two photons by fluorescent molecules, and thus optical sectioning in the two-photon microscope does not require a pinhole for rejecting out-of-focus fluorescence light. Two-photon microscopy reduces photobleaching substantially compared to confocal microscopy but typically also negatively affects signal-to-noise ratio at a comparable imaging speed due to reduced rates of signal generation.

In conclusion, traditional imaging approaches are constrained by a fundamental performance trade-off, offering high speed at the expense of 3D imaging capability or realizing 3D imaging at the expense of speed, signal-to-noise ratio, and/or photobleaching rate.

Light-sheet microscopy was developed to address these limitations and enable gentle optical sectioning at high imaging speed. The conception of imaging using light sheets dates back more than 100 years (Siedentopf & Zsigmondy 1903), but this method found its way into the realm of biological live imaging only during the past 15 years. In light-sheet microscopy, the processes of sample illumination and fluorescence detection are decoupled, which is most commonly achieved by using one objective to illuminate the sample with a thin sheet of laser light and a second, perpendicular objective to collect fluorescence light emitted from within the illuminated plane. A camera then acquires an image of the entire illuminated plane in a single snapshot. This has three important implications: (*a*) The use of a light sheet for fluorescence excitation immediately confers optical sectioning capability, (*b*) laser light is confined to the plane of interest and no out-of-focus structures are illuminated, and (*c*) high imaging speeds are achieved by using a camera for image formation. Moreover, rapid volumetric imaging is possible simply by moving the light sheet through the sample along the detection axis and acquiring a sequence of images (Keller & Ahrens 2015, Winter & Shroff 2014). Owing to this unique combination of strengths, light-sheet microscopes offer long-term imaging of live specimens with high spatiotemporal resolution and enable a wide spectrum of powerful, new imaging experiments in cell and developmental biology (Keller 2013, Power & Huisken 2017).

HOW DID LIGHT-SHEET MICROSCOPY EVOLVE INTO A VALUABLE TOOL FOR DEVELOPMENTAL BIOLOGY?

Due to the strength of modern light-sheet microscopy in live imaging of biological specimens with minimal light exposure, the primary applications of this technique initially resided almost exclusively in the realm of developmental biology, as shown in early studies (Huisken et al. 2004, Keller et al. 2008). In the decade following these studies, light-sheet microscopy evolved rapidly, spurring the development of new implementations with vastly improved speed, spatial resolution, and image quality, as well as new conceptual strategies for improving depth penetration and imaging large, multicellular organisms. We provide a brief overview of those advances most relevant for developmental biology (Berthet & Maizel 2016, de Medeiros et al. 2016, Keller 2013, Liu & Keller 2016) (**Figure 1**) and discuss the complementary strengths of state-of-the-art implementations with respect to specimen size, spatial resolution, and temporal resolution (**Figure 2**).

Modern light-sheet microscopy owes much to the Ultramicroscope, the first light-sheet microscope, which was developed by Siedentopf & Zsigmondy (1903) to study the properties of nanoparticles in colloids. While the original Ultramicroscope utilized sunlight for illumination and relied on the eyes of the investigator to detect light scattered by the specimen, modern light-sheet microscopy developed in the 1990s for biological applications improved on this design by replacing sunlight with laser light and the human observer with a CCD camera (Voie et al. 1993). A decade later, the potential of this technique for visualizing dynamic processes in live specimens was recognized, and light-sheet imaging was utilized to record the beating heart of medaka embryos, as well as the early embryonic development of *Drosophila* (Huisken et al. 2004) and zebrafish (Keller et al. 2008). These studies also mark the introduction of different branches of light-sheet microscopy with characteristic differences in the mechanisms of light-sheet formation. Up to this point, light-sheet microscopes typically used cylindrical lenses to create a static light sheet, which illuminates an entire planar section of the specimen simultaneously [often referred to as selective

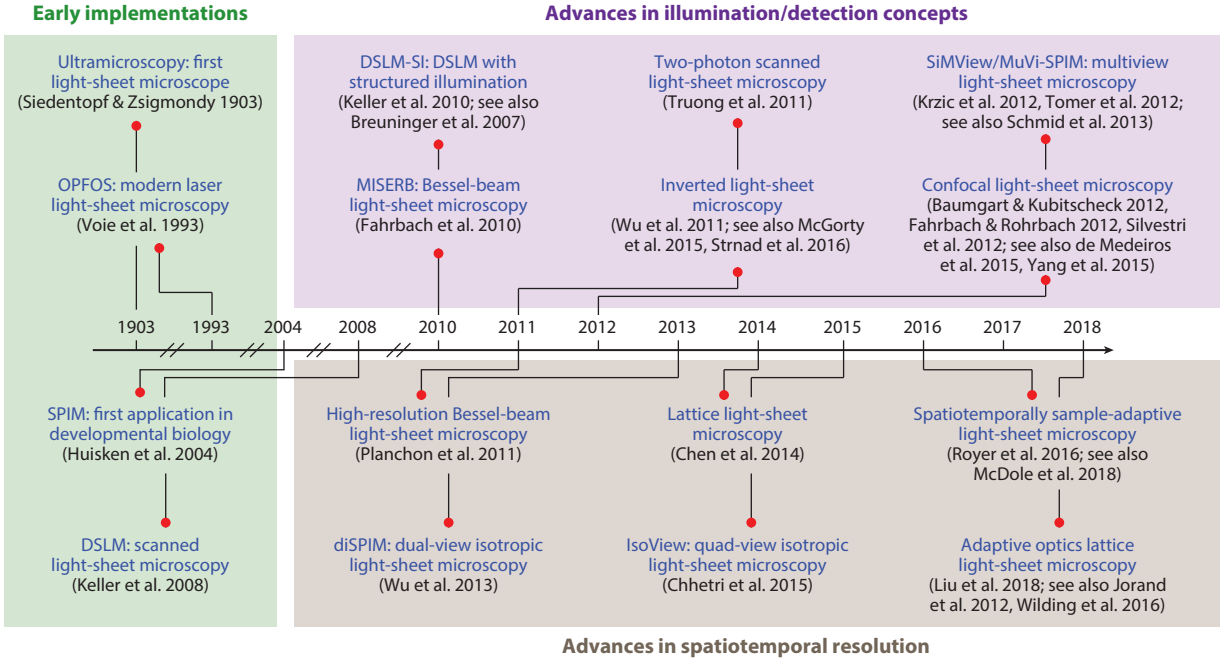


Figure 1

Timeline of advances in light-sheet microscopy with particular relevance for developmental biology. This timeline provides an overview of technical advances in spatiotemporal resolution and in strategies used for illumination and detection systems in light-sheet microscopy, focusing on implementations designed for imaging applications in developmental biology.

plane illumination microscopy (SPIM)] (Huisken et al. 2004, Voie et al. 1993). As an alternative, a new branch of light-sheet microscopy was developed that replaced this concept with 1D laser scanning, creating optical sections by rapidly moving a thin laser beam across a plane [digitally scanned laser light-sheet fluorescence microscopy (DSLM)] (Keller et al. 2008). Although both techniques expose the sample to the same amount of total light energy, static light sheets minimize peak laser power density, which can be helpful when one is imaging very sensitive biological specimens. However, imaging with a static light sheet often suffers from striping/shadowing artifacts (Rohrbach 2009), since the specimen perturbs the light sheet and different parts of the light sheet interfere with each other due to the coherent illumination. The spatially incoherent illumination of DSLM mitigates such artifacts and furthermore enabled the development of advanced forms of light-sheet microscopy based on structured illumination, multiphoton excitation, and beam shaping for creating thinner light sheets.

When one is imaging large, multicellular organisms with light microscopy, image quality can suffer from several limiting factors, including light scattering and tissue penetration by light at physiological wavelengths. These issues compromise image quality beyond a certain depth in basic SPIM or DSLM light-sheet microscopes. Two variants of the DSLM technique were thus developed to counteract the loss of image contrast resulting from light scattering and to increase depth penetration in live tissue. By using structured illumination patterns (Breuninger et al. 2007) created by modulating laser power during scanning (DSLM-SI) (Keller et al. 2010), multiple images of the same plane can be recorded with different spatial phase shifts in the pattern. A new image can then be computationally reconstructed from these images, in which the contribution of scattered

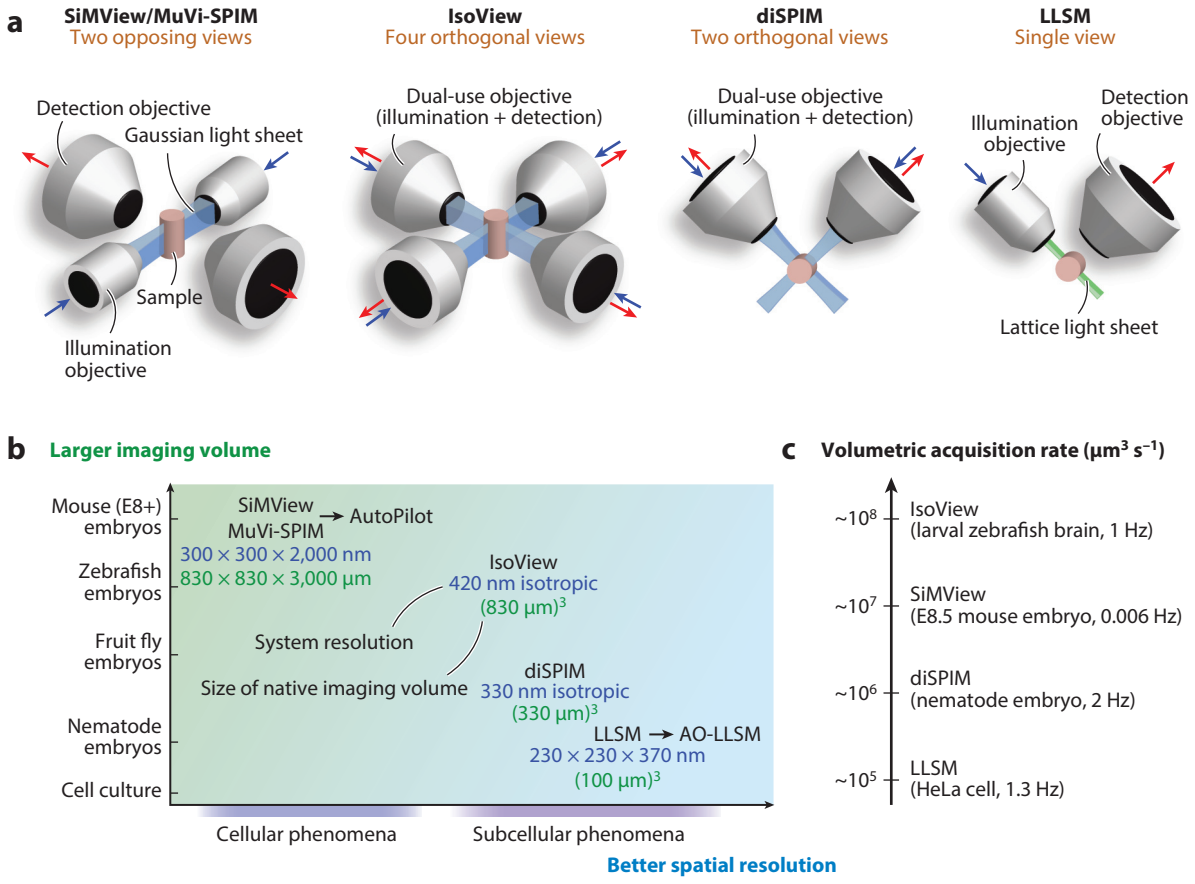


Figure 2

State-of-the-art light-sheet microscopy techniques used for developmental imaging. (*a*) Schematic illustration of state-of-the-art light-sheet microscopy techniques capable of imaging multicellular organisms with high spatial resolution, high temporal resolution, and/or optical access to large specimens along multiple views. Red and blue arrows indicate that an objective is used for fluorescence detection and laser illumination, respectively. SiMView, MuVi-SPIM, IsoView, and diSPIM light-sheet microscopes typically use Gaussian light sheets for fluorescence excitation (shown in *blue*), whereas lattice light-sheet microscopes typically employ a dithered optical lattice (shown in *green*). (*b*) Size of the native imaging volume versus system resolution for the techniques shown in panel *a*. Adaptive imaging techniques (AutoPilot) or adaptive optics (AO-LLSM) can help mitigate aberration-induced degradation of resolution. (*c*) Volumetric acquisition rates in typical imaging experiments performed with the techniques shown in panel *a*. Abbreviations: AO-LLSM, adaptive optics lattice light-sheet microscopy; diSPIM, dual-view inverted selective plane illumination microscopy; LLSM, lattice light-sheet microscopy; MuVi-SPIM, multiview selective plane illumination microscopy; SiMView, simultaneous multiview; SPIM, selective plane illumination microscopy.

fluorescent light is eliminated. By using this concept, a marked improvement in image contrast was demonstrated in both *Drosophila* and zebrafish embryos (Keller et al. 2010). The second variant of DSLM replaced the continuous-wave laser light source with a pulsed infrared laser for two-photon excitation in light-sheet microscopy (Truong et al. 2011). Since infrared light suffers less from scattering and absorption inside biological specimens than does the shorter-wavelength light required for one-photon excitation, depth penetration is improved. Efficient two-photon excitation (with high signal rates at low laser power) is feasible as long as light sheets are designed to cover a sufficiently narrow field of view. Creating long light sheets requires low numerical

apertures, and the probability of two-photon excitation is proportional to the fourth power of this numerical aperture. In practice, up to 100–200-μm-long light sheets have been successfully used for two-photon imaging in *Caenorhabditis elegans*, *Drosophila*, and zebrafish (Palero et al. 2010, Truong et al. 2011).

However, even with the help of structured illumination or multiphoton excitation, many biological specimens, such as *Drosophila*, zebrafish, and mouse embryos, are still too large to be imaged in high resolution in their entirety. Moreover, both concepts slow down imaging and can thus preclude imaging of fast dynamic processes. To address these limitations and increase physical coverage substantially, multiview light-sheet imaging was developed. Image quality is typically lowest in the parts of a specimen furthest away from the illumination and detection objectives, and by rotating the specimen relative to these objectives, complementary views of the specimen that capture those regions in higher quality can be recorded. In principle, the microscope can be kept stationary and the sample rotated mechanically to acquire multiple views (Huisken et al. 2004, Voie et al. 1993); however, this sequential scheme (plus the delay arising from mechanical specimen rotation) slows down imaging. A more effective solution well suited to imaging fast processes is the concept of simultaneous multiview imaging [simultaneous multiview (SiMView) and multiview selective plane illumination microscopy (MuVi-SPIM)] (Krzic et al. 2012, Tomer et al. 2012). In this design, the microscope is equipped with two illumination arms and two detection arms, which simultaneously illuminate the specimen with light sheets from two opposite sides and acquire images from two opposing views (**Figure 2*a***). This approach increases the volume imaged in high quality fourfold relative to conventional single-view imaging, without decreasing imaging speed. Key applications in developmental biology that benefit from such capabilities include, e.g., systematic cell tracking across entire developing *Drosophila* and zebrafish embryos (Amat et al. 2014).

In parallel to developing these strategies for fast, high-contrast imaging of large specimens, complementary efforts focused on improving spatial resolution in light-sheet microscopy. One such approach uses beam shaping to create thinner beams in a DSLM-type implementation, replacing the Gaussian beam in conventional DSLM with a Bessel beam (Fahrbach et al. 2010, Planchon et al. 2011). The Bessel beam has a thinner core than does the Gaussian beam but is also surrounded by a concentric ring system. To effectively improve resolution with Bessel beams, the contribution of this ring system to fluorescence excitation or image formation must be suppressed, e.g., by using two-photon excitation or structured illumination. However, light-sheet imaging with Bessel beams introduces a high peak power density, which increases the rate of photobleaching and photodamage and thus decreases the length of observation under physiological conditions. Lattice light-sheet microscopy (LLSM) addresses this limitation by replacing the Bessel beam with an optical lattice (a periodic pattern of thin beams) that is quickly dithered to illuminate an entire plane inside the sample (Chen et al. 2014) (**Figure 2*a***). A larger fraction of the plane is thereby illuminated instantaneously, which in turn reduces the local peak power density required to obtain images of comparable brightness. Such lattices can be used very effectively for imaging a small field of view on the order of 100 μm. Creating thin lattices or Bessel beams that span a larger field of view, however, is complicated by the fact that power requirements increase substantially. The resulting increase in light exposure quickly becomes prohibitive for live imaging. Thus, a different solution is needed for imaging large specimens, such as entire developing vertebrate or higher invertebrate embryos, with high spatiotemporal resolution.

Interestingly, this problem can be solved by further extending the concept of multiview imaging discussed above. In this context, it is important to note that lateral resolution in a light-sheet microscope is determined primarily by the numerical aperture of the detection objective, whereas

axial resolution is additionally affected by the thickness of the light sheet. This means that lateral resolution is not fundamentally different in, e.g., LLSM and a SiMView light-sheet microscope equipped with a large-aperture objective. Rather, the main difference concerns axial resolution, which is almost as high as lateral resolution in LLSM but is not nearly as high in the SiMView light-sheet microscope, due to the thicker light sheet. However, when one is acquiring two orthogonal views of the sample, e.g., by rotating the sample by 90°, these two views can be computationally combined to produce uniformly high resolution along all three dimensions (Swoger et al. 2007). This isotropic, 3D resolution is governed by the high lateral resolution of the microscope and is thus comparable to 3D spatial resolution in LLSM. The most effective implementation of this idea avoids physical rotation of the sample and instead acquires orthogonal views through an all-optical scheme. The two primary designs to this end use either two orthogonal objectives for illumination and detection in an alternating scheme [dual-view inverted selective plane illumination microscopy (diSPIM)] (Wu et al. 2013) or four orthogonal objectives, each of which is used simultaneously for light-sheet illumination and fluorescence detection (IsoView) (Chhetri et al. 2015) (**Figure 2*a***). The former approach has been successfully applied to imaging subcellular processes in individual cells as well as whole-embryo development in *C. elegans* with a system resolution of 330 nm (Wu et al. 2013). The latter approach enabled spatially isotropic imaging of *Drosophila* embryonic development and whole-brain/whole-CNS functional imaging in larval *Drosophila* and zebrafish with a system resolution of 420 nm (Chhetri et al. 2015). The simultaneous acquisition of four orthogonal views without signal cross talk in IsoView is possible through line-confocal detection (Baumgart & Kubitscheck 2012, de Medeiros et al. 2015, Silvestri et al. 2012), using a scheme that slightly offsets the scanned laser beams producing the four light sheets along the scan axis (Chhetri et al. 2015).

The imaging methods described above are designed to provide high spatial resolution and high temporal resolution while enabling the imaging of relatively large, multicellular organisms (**Figure 2*b*,*c***). In practice, however, most living specimens introduce optical aberrations that degrade the spatial resolution offered by the microscope under optimal conditions. Two complementary approaches have been developed to address this problem for large and small fields of view: sample-adaptive light-sheet imaging (the AutoPilot framework) (McDole et al. 2018, Royer et al. 2016) and lattice light-sheet microscopy with adaptive pupil corrections [adaptive optics lattice light-sheet microscopy (AO-LLSM)] (Liu et al. 2018), respectively. When imaging with a small field of view of several tens of micrometers (as is the case for AO-LLSM), e.g., to follow subcellular processes in a local domain of a multicellular organism (Liu et al. 2018), the wavefront distortion caused by the specimen can typically be considered to be approximately constant across this field of view. High resolution can thus be restored by measuring this wavefront distortion with a so-called guide star (a focused infrared beam for generating two-photon fluorescence) and by applying the opposite distortion to the wavefront with a deformable mirror. When imaging with a large field of view of hundreds of micrometers up to a millimeter (as is the case for the AutoPilot framework), e.g., to follow cellular dynamics across entire *Drosophila*, zebrafish (Royer et al. 2016), or mouse (McDole et al. 2018) embryos, wavefront distortion typically varies across this field of view, and the situation becomes more complex. Importantly, the low-order terms of the distortion, specifically the offsets and angles between light sheets and detection focal planes, are relatively uniform within one image plane and can be computationally analyzed without using the slow and expensive infrastructure associated with a guide star. By determining and correcting for these mismatches between light sheets and detection focal planes as a function of space and time (Royer et al. 2018), high spatial resolution can typically be recovered in many parts of the specimen with minimal disruption of time-lapse acquisition and minimal added light exposure (2–3% of the total light load).

HOW DOES LIGHT-SHEET MICROSCOPY ADVANCE OUR UNDERSTANDING OF DEVELOPMENTAL PROCESSES?

Deciphering development and developmental processes requires close examination on a range of scales—from the subcellular localization of transcription factors binding to DNA, to the behavior of organelles and the actin cytoskeleton, to the organization of whole tissues and eventually the entire organism. Light-sheet microscopy, although a relatively new technique in developmental biology, has proven invaluable for visualizing and quantitatively measuring these dynamic processes (**Figure 3**). Living cells, tissues, and developing embryos are extraordinarily sensitive to light exposure and are often optically challenging by way of thick cell layers, highly opaque and scattering tissue, and absorptive blood and capillary vessels. Embryos develop rapidly, can change dramatically in size and shape, and often require specialized culture conditions. Given these complexities in both size and scale, there is no one-size-fits-all microscope solution, and different methods and strategies must be used to approach questions from the subcellular level to the organismal level. In this section we discuss different light-sheet strategies that have been employed to address some of these questions and where these methods might be used or improved to address new problems or challenges.

Subcellular Imaging

Visualizing a living cell at the subcellular level requires a technique that is gentle enough not to damage the cell or bleach fluorescent reporters, that is fast enough to capture the rapid movements of single molecules, and that provides a high enough signal-to-noise ratio to discern structures on a nanoscale level. LLSM has been employed with great success to address these challenges and, when combined with adaptive optics and advanced computational reconstruction and deconvolution algorithms, has provided some of the most detailed looks inside the dynamic world of a cell. LLSM has been used to quantify the binding dynamics of the transcription factor Sox2 in live embryonic stem cells and to examine the complex, 3D architecture of the nucleus during different developmental states (Liu et al. 2014). Employing this method to examine transcription factor binding in live embryos, Mir and colleagues demonstrated the ability of LLSM to measure binding and diffusion dynamics in both *Drosophila* embryos and live preimplantation mouse embryos (Mir et al. 2018). Another light-sheet microscopy method uses a tilted light sheet and point-spread-function engineering to image through thick cells and reconstruct the entire nuclear lamina and mitochondria in 3D with super-resolution (Gustavsson et al. 2018).

By using a combination of LLSM and confocal microscopy, 4D multispectral visualizations and time lapses have been generated for organelles from the endoplasmic reticulum to the Golgi complex to lipid droplets and mitochondria with high temporal resolution and with unprecedented detail (Valm et al. 2017). Coupling these microscopes with wavefront-correcting techniques and image-processing algorithms allows for highly detailed reconstructions of subcellular processes, such as microtubule growth, membrane dynamics during mitosis, and migrating axons in the developing zebrafish spinal cord (Aguet et al. 2016, Liu et al. 2018, Yamashita et al. 2015).

While these methods provide incredibly detailed views into the internal workings of a cell, their application to large specimens must be tempered with one caveat in mind: With high resolution comes a narrow field of view. Many high- or super-resolution live-imaging techniques are furthermore limited to a depth of 100 μm or less in live tissue and rely heavily on nearly transparent or very low scattering samples to achieve good contrast. To tile such a small field of view across a large specimen would mean decreasing temporal resolution and increasing light load on the sample significantly, limiting the usefulness of such methods when applied to larger or more

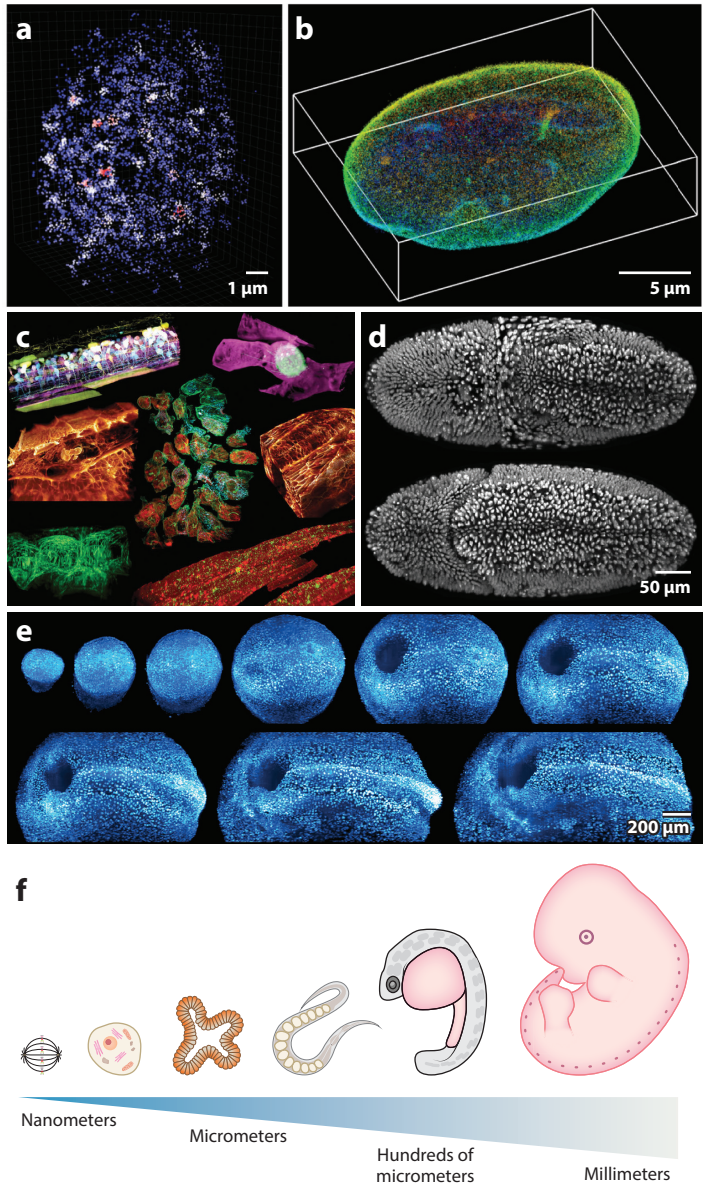


Figure 3

Imaging developmental processes with light-sheet microscopy. From the subcellular level (*a*), to the cellular level (*b*), to whole tissues or organs (*c*), to the entire embryo (*d*,*e*), light-sheet microscopy can be used to achieve high-resolution imaging across a broad range of scales. Examples shown are (*a*) stable Sox2 binding sites in embryonic stem cell nucleus; (*b*) nuclear lamina in a HeLa cell; (*c*) spinal cord axons, cancer cell metastasis, collective cellular motion, endocytosis, microtubule displacements, immune cell migration, and organelle dynamics; (*d*) *Drosophila* embryogenesis; and (*e*) postimplantation mouse development. Developmental processes typically concern spatial scales from nanometers to millimeters (*f*) and require specialized light-sheet techniques and mounting strategies dependent on the required field of view and optical accessibility/environmental requirements of the specimen. Panel *a* adapted with permission from Liu et al. (2014). Panel *b* adapted with permission from Gustavsson et al. (2018). Panel *c* adapted with permission from Liu et al. (2018). Panel *d* adapted with permission from Amat et al. (2014). Panel *e* adapted with permission from McDole et al. (2018).

opaque developing specimens. Therefore, when one is using light-sheet techniques to investigate larger tissues, organs, or whole organisms, different approaches are required.

Tissue and Organ Imaging

In addition to scale, imaging whole tissues or organs presents several new challenges, as samples can no longer be easily mounted on a coverslip, and light-sheet illumination and imaging are required up to depths of hundreds of micrometers or even millimeters. Heart development has been a particular area of focus for light-sheet microscopy, and the zebrafish embryo has proven to be an ideal model organism for this study, with a nearly transparent, optimally placed, and relatively small sized organ (Weber & Huisken 2015, Weber et al. 2017). Two-photon light-sheet microscopy improves the depth at which the heart can be reconstructed (Trivedi et al. 2015), and optical pacing or gating strategies coupled with light-sheet microscopy have enabled the 4D reconstruction of heart development without the motion artifacts arising from heartbeats (Ma et al. 2016). Many of these methods rely heavily on custom-built systems; however, commercial systems such as the ZEISS Lightsheet Z.1 have been successfully employed to study yolk sac development in the early mouse embryo (Udan et al. 2014). Spheroids, organoids, and other tissue mimics, which are often too large for standard confocal systems, are typically still small and transparent enough to be highly amenable to a standard light-sheet configuration and do not require particularly challenging mounting or growth conditions (Andilla et al. 2017, Swoger et al. 2014). Organoids can be embedded in hydrogel cylinders, and different fluorescent reporters or even calcium dynamics and cell death can be visualized (Gualda et al. 2014, Held et al. 2018). While not a cell culture or organoid system, the preimplantation mouse embryo provides many of the same advantages in terms of size and accessibility while providing an intact look at early embryo development and de novo cell biology. Inverted light-sheet microscopy has been used with great success to study the development of the mouse embryo over the first 4 days after fertilization (Strnad et al. 2016) and to more closely examine spindle formation and the first cell divisions that often lead to aneuploidy and death in mammalian embryos (Reichmann et al. 2018).

Whole-Animal Imaging

Imaging the developing embryo in toto not only adds to the culture challenges previously posed by individual cells, tissues, or explants but also presents a whole new set of problems. While small tissues and cells do well with more standard culture conditions, entire organisms not only have more complex requirements but also develop, grow, move, and behave. An organism that not only undergoes dramatic morphological changes but also develops to the point at which it will eat its way out of its mounting matrix and crawl away poses a particular challenge. Other organisms such as the mouse embryo are intolerant to mechanical constraints and furthermore demand very precise environmental conditions. Finally, whole organisms are quite simply large. Instead of imaging on a scale of tens of micrometers, we are faced with scales of up to millimeters, often eclipsing the available field of view in the detection system. Point-scanning systems simply cannot deal with specimens of this size in a timely fashion, in addition to the often-toxic light load that would result from attempting to obtain full coverage of a sample with these methods. Moreover, optical properties can change dramatically over time; a limb or a beating heart may appear where previously there was none, and the image quality in a microscope left in its original configuration would quickly deteriorate. Light-sheet systems for whole-animal imaging or very large explants thus need to be designed around accommodating the growing or changing organism. *C. elegans* embryos provided one of the first views into a developing embryo by using

light-sheet microscopy, as their relatively small size and ease of embedding and tolerance to a wide range of environmental conditions make them ideal for light-sheet systems. Light-sheet imaging of *C. elegans* embryos has enabled continuous visualization of the dynamics of nuclear movement during embryogenesis, as well as cell body migration and neuronal wiring in the developing brain (Fu et al. 2016; Wu et al. 2011, 2013). *Drosophila* embryos share many of these same features, with their relatively small size, ease of embedding, amenability to normal lab environmental conditions, and the ability to develop to the point at which they eat through agarose and escape the imaging chamber (Tomer et al. 2012). By imaging developing *Drosophila* embryos with SiMView light-sheet microscopy, the lineages of neuroblasts in early embryonic nervous system development were systematically reconstructed (Amat et al. 2014). Zebrafish embryos are very well suited for light-sheet imaging because of their optical transparency and external development. Light-sheet imaging of early zebrafish embryonic development enabled comprehensive reconstructions of cell positions, cell divisions, and migratory tracks, from which stereotypic patterns in early symmetry breaking and endoderm development were identified (Keller et al. 2008, Schmid et al. 2013).

The challenges that, e.g., *C. elegans*, *Drosophila*, and zebrafish embryos present by moving and behaving are also a testament to the gentleness of light-sheet microscopy: Such embryos can develop normally in the presence of almost continuous imaging. Clearly, then, to track normal development in its entirety, the microscope must be able to adapt to the movement, growth, and changing optical properties of the sample. The open-source software framework AutoPilot was implemented for the SiMView microscope platform, which continuously optimizes the various optical degrees of freedom of the microscope and keeps the embryo centered and in focus and expands the imaging volume as needed (McDole et al. 2018, Royer et al. 2016). These capabilities are especially critical for the in toto imaging and reconstruction of the postimplantation mouse embryo, which dramatically changes in shape, size, and optical properties over the course of 48 h (Ichikawa et al. 2014, McDole et al. 2018, Udan et al. 2014).

While zebrafish, *Drosophila*, and mouse embryos are perhaps more conventionally recognized model organisms, light-sheet microscopy has also proven quite useful for the study of other model organisms that previously have not been closely examined, largely due to the difficulty of imaging them by using standard confocal methods. The arthropod crustacean *Parhyale* has a Swiss army knife–like collection of limbs, each developing from specific regions of the embryo, and has proven to be both optically accessible and highly tolerant to light-sheet imaging, yielding interesting insights into limb development (Wolff et al. 2018). Additionally, chick and quail embryos are of interest to developmental biologists, as they develop morphologically similarly to human embryos, can be genetically modified, and (when used with an inverted light-sheet setup) provide stunning views of neurulation and primitive-streak formation (Gomez-Gaviro et al. 2017, Rozbicki et al. 2015).

Plant Imaging

While much of this section provides a very animal kingdom–centric view of the benefits of light-sheet microscopy in studying development, this method has also been successfully applied to the study of plant development. Many of the same challenges that confront the study of animal development, e.g., samples that move, grow, and have optically challenging and changing properties, are also faced by the plant community. It could be argued that carefully controlling light exposure is even more critical to plant development, yet this has not stopped the successful application of light-sheet microscopy to studying a wide spectrum of plant biology from whole *Arabidopsis* roots to subcellular structures (Maizel et al. 2011, Ovecka et al. 2018, Sena et al. 2011).

HOW CAN LIGHT-SHEET MICROSCOPY SYNERGIZE WITH OTHER STATE-OF-THE-ART TECHNOLOGIES?

Light-sheet imaging does not facilitate developmental studies solely by itself. Over the years, powerful synergies between light-sheet microscopy and other state-of-the-art technologies served to not only improve imaging performance (e.g., spatiotemporal resolution, signal-to-noise ratio) but also substantially broaden the scope of developmental processes that can be investigated, as well as providing means for perturbing developing systems with high spatiotemporal precision to gain a mechanistic understanding.

Our ability to visualize cellular processes has benefited substantially from the development of bright, photostable, nontoxic fluorescent reporters (Cranfill et al. 2016, Dean & Palmer 2014). For live-cell imaging, Costantini and colleagues developed a set of bright fluorescent proteins (FPs) optimized for oxidizing cellular environments and membranes (Costantini et al. 2015). Utilizing such FPs for high-resolution light-sheet microscopy promises powerful, quantitative cellular imaging experiments in minimally perturbed environments. For deep-tissue imaging, far-red fluorescent reporters reduce autofluorescence and mitigate image quality degradation by light scattering. A range of useful new FPs, including iRFP (Filonov et al. 2011), Wi-Phy (Auldridge et al. 2012), mIFP (Yu et al. 2015), and miRFP (Shcherbakova et al. 2016), have emerged in this context. In particular, the study of developing mammalian embryos or tissues will benefit from the combination of far-red or near-infrared reporters with multiview light-sheet microscopy. Bioorthogonal chemistry enabled modular tagging strategies using self-labeling [e.g., HaloTag (Los et al. 2008) and SNAP-tag (Keppler et al. 2003)] or enzyme labeling [e.g., biotin tag (Howarth et al. 2005)] such that fluorescent dyes or quantum dots can be specifically targeted to a protein of interest. Since organic dyes are small molecules that can be chemically modified through organic synthesis, they typically provide better photostability relative to FPs and can be flexibly tailored to the cellular environment or method of fluorescence excitation. The recent development of the Janelia Fluor dye series substantially improved quantum yield and photostability of HaloTag and SNAP-tag ligands (Grimm et al. 2017a) and provided a wide range of spectral options from blue to far red (Grimm et al. 2017b).

Recent advances in the fluorescent toolkit have reached far beyond bright and stable labeling of proteins. Live-cell nucleic acid probes enabled monitoring of RNA (Nelles et al. 2016, O'Connell et al. 2014, Paige et al. 2011, Tyagi 2009) and DNA (Chen et al. 2013, Ma et al. 2017, Qin et al. 2017) and have the potential to provide single-cell, single-molecule sensitivity. Moreover, fluorescent biosensors have been designed for studying the dynamics of metabolites, signaling molecules, and ions (Newman et al. 2011, Okumoto et al. 2012). In the context of zebrafish embryonic development, for example, transgenic reporter lines visualizing Wnt (Moro et al. 2012), BMP (Collery & Link 2011), TGFβ (Casari et al. 2014), Shh (Schwend et al. 2010), and Notch (Parsons et al. 2009) signaling can be combined with in toto light-sheet imaging to investigate how cell-intrinsic information is integrated with external signals to control cell behaviors—a paramount question in developmental biology.

Light-sheet microscopy, like all forms of light microscopy, is constrained by light scattering to relatively transparent samples or surface regions. Recent advances in tissue clearing techniques have enabled cellular analyses for whole organs or the whole body when combined with optical imaging and image analysis (Richardson & Lichtman 2015, Tainaka et al. 2016). Expansion microscopy (F. Chen et al. 2015) builds upon tissue permeabilization techniques and uses a swellable polymer network to achieve physical magnification of a specimen. Rapid imaging of expanded samples with high-resolution light-sheet microscopy (e.g., LLSM, diSPIM, and IsoView microscopy) has the potential to reveal rich structural details at the subcellular level and is helpful

for correlative studies between light and electron microscopy. Compared to point-scanning confocal or two-photon microscopy, light-sheet microscopy can substantially speed up image acquisition of large specimens while minimizing photobleaching (Keller & Dodt 2011). Although such techniques can be applied only to fixed specimens, the multiplexing capability of modern single-cell genomics has the potential to yield transcriptional profiles at an unprecedented level of detail, thus aiding investigations in developmental systems biology (Liu & Keller 2016). Developmental gene expression maps have been reconstructed for early zebrafish (Farrell et al. 2018), mouse (Visel et al. 2004), and human (Xue et al. 2013) embryonic development. Although the technical bottleneck in achieving truly single-cell resolution lies in our ability to recover anatomical information from the sequenced cells in their native environment, various RNA fluorescence in situ hybridization (FISH)-based imaging methods (K.H. Chen et al. 2015, Frieda et al. 2017, Moffitt et al. 2016) and single-cell isolation techniques (Islam et al. 2014, Junker et al. 2014, Redmond et al. 2014, Streets et al. 2014) have been developed to partially overcome this limitation. Combining systems-level imaging with a gene expression atlas will help reveal novel molecular fingerprints as well as molecular pathways driving differentiation, proliferation, migration, and morphological changes at the single-cell level during development.

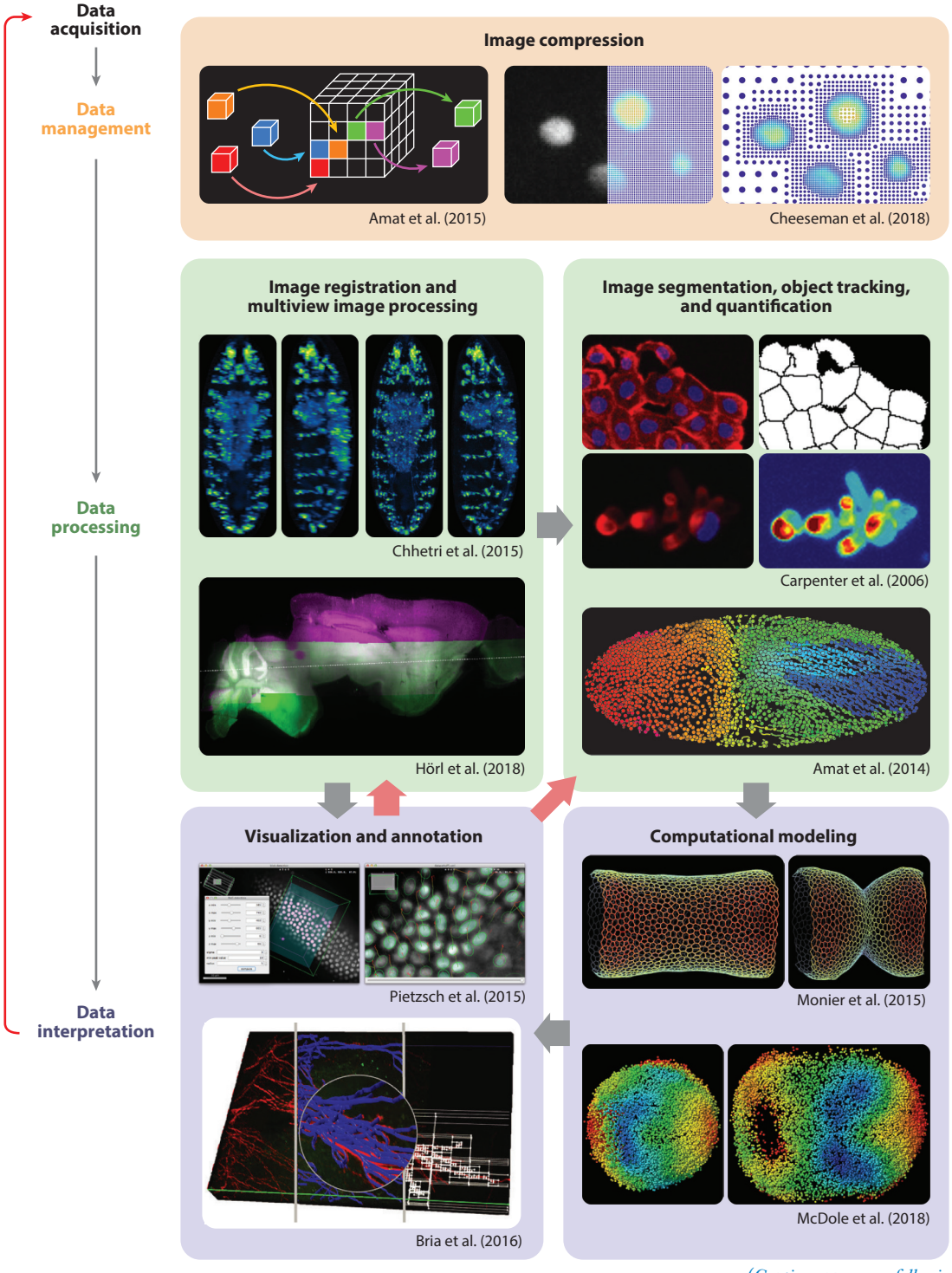
Finally, advances in optogenetic tools have allowed us to visualize selectively labeled cells and structures of interest while controlling and manipulating developmental processes with high spatiotemporal precision—a powerful step toward gaining mechanistic understanding. The use of photoconvertible FPs, such as tdEOS (McKinney et al. 2009) and mEOS (Paez-Segala et al. 2015), allows us to precisely mark cells of interest by optical means. The behavior of the marked cells can subsequently be monitored by live imaging. Photosensitizers such as KillerRed (Bulina et al. 2006) take advantage of the photochemical generation of reactive oxygen species and can be used for targeted cell ablation. Optogenetic tools can further be used to target and modify cell signaling cascades (Tischer & Weiner 2014). Combining optogenetic tools with live imaging and live-cell activity reporters will further our understanding of the spatiotemporal control of dynamic developmental programs.

HOW CAN WE EXTRACT USEFUL INFORMATION FROM LIGHT-SHEET MICROSCOPY IMAGES?

Computational data analysis is both a key challenge and a major opportunity in light-sheet imaging. The investigator can be caught by surprise with the amount of data generated after their first light-sheet imaging experiment: For example, a typical SiMView imaging experiment of *Drosophila* embryonic development performed in 30-s intervals over 1 day leads to ~1 million multimegapixel images, totaling ~10 TB of data (Tomer et al. 2012); high-speed light-sheet microscopes, such as the IsoView microscope, acquire data at rates of up to 10 TB per hour (Chhetri et al. 2015). However, this should not and does not have to result in a data deluge: A wide range of powerful computational image analysis methods are readily available to help investigators extract biologically meaningful information (**Figure 4**). In this section, we review a series of methods suitable for light-sheet microscopy. We first discuss recent advances in tools for data management, image processing, and visualization and then highlight some analysis methods tailored to specific types of biological questions.

Image Compression

Image compression is usually the first requirement if large amounts of data are captured on a daily basis. An efficient and effective compression method saves storage cost and reduces overhead



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Computational analysis of light-sheet image data of developmental processes. A typical framework for computational analysis of light-sheet microscopy images includes solutions for management of the raw data (image compression), image processing for creating intermediate levels of data representation (e.g., image registration and segmentation), and data interpretation (including image visualization and modeling). Example methods are included for each category to illustrate the corresponding step. Gray arrows represent the typical workflow for processing multiview, time-lapse light-sheet datasets. Red arrows indicate steps that involve user feedback or real-time user intervention. Panels are adapted from the references shown below each image. Panels from non–open access publications are adapted with permission.

during data transfer and subsequent processing. The KLB (Keller Lab Block) file format (Amat et al. 2015) first partitions the image data in small 5D (xyzct) data blocks and then losslessly compresses all blocks in parallel on multicore CPU architectures by using the bzip2 algorithm. B^3D is a recently developed compression library that relies on GPU processing for maximizing compression speed, focusing on applications that benefit from data compression directly during image acquisition (Balazs et al. 2017). If a further reduction in image size is intended beyond the algorithmic entropy of the data, a lossy compression algorithm is needed. A good lossy compression algorithm for biological images should aim to optimally represent the quantitative information contained therein. Amat and colleagues implemented a simple approach to lossy compression of light-sheet data by detecting background regions in the image data and storing only the image foreground, using KLB compression (Amat et al. 2015). Cheeseman and colleagues developed a content-based representation of the microscopy images that replaces pixels with particles of adaptive size positioned according to image content (Cheeseman et al. 2018). If useful information is available only on the surface of the biological specimen, image compression can even be achieved by dimensionality reduction, e.g., by projecting surface regions in the 3D image volumes to 2D "world maps" (Heemskerk & Streichan 2015).

Image Registration

Image registration becomes a recurrent requirement with the increasing need to spatially match images acquired from multiple views, to correct for sample drift over time, and to jointly analyze multiple specimens recorded in different experiments using a common framework. Several open-source software packages provide powerful solutions for linear/affine registration and multiview fusion/deconvolution of 3D image stacks, including methods for interest point–based image registration (Preibisch et al. 2010) and stitching (Hörl et al. 2018, Preibisch et al. 2009), content-based image fusion (Amat et al. 2015, Tomer et al. 2012), and 3D multiview deconvolution that results in images of isotropic resolution (Chhetri et al. 2015, Preibisch et al. 2014). Other complementary software packages address nonlinear distortions between images and can be applied to images of biological specimens with dramatically different geometry (Guignard et al. 2014, McDole et al. 2018).

Image Visualization

Image visualization is an obvious next step after the image data have been preprocessed and registered within a common coordinate system. Several commercial software packages (e.g., Imaris, Amira, and Volocity) and open-source software platforms [e.g., Fiji (Schindelin et al. 2012), Vaa3D (Peng et al. 2010), BioImageXD (Kankaanpää et al. 2012), Icy (de Chaumont et al. 2012), and others] are available as interactive 3D data visualization and annotation tools. Despite the interactive design and broad range of the image-processing tasks available in these software packages, there remain two major challenges in data visualization for large-scale light-sheet image datasets: (*a*) to

view 5D data interactively without excessive requirements regarding computer memory and (*b*) to deliver fast, real-time data visualization during image acquisition. Regarding the first challenge, Big Data Viewer (BDV) (Pietzsch et al. 2015), available as a Fiji plug-in, renders arbitrarily oriented virtual slices of 3D image volumes, while only a small fraction of the image data relevant to the virtual slice is loaded into memory. The infrastructure of BDV also supports other nonimage metadata being introduced into the visualization framework. BDV integrates seamlessly with Fiji plug-ins for multiview SPIM image registration (Preibisch et al. 2010) and with cell tracks/cell lineage data generated by the Massive Multiview Tracker (MaMuT) (Wolff et al. 2018). MaMuT combines the BDV and TrackMate plug-ins in Fiji and allows for browsing, annotating, and curating of large volumetric images and tracking data. Tracking or annotating can be done in a completely manual or a semiautomated fashion. MaMuT is also compatible with a number of external programs such as the TGMM framework for automated, large-scale cell tracking (Amat et al. 2014, McDole et al. 2018) and supports exporting cell tracks to Imaris via ImarisXT. Bria and colleagues developed TeraFly, an open-source software for interactive 3D visualization, with a focus on rapid access to and interaction with image volumes at multiple resolution levels based on user input via the computer mouse (Bria et al. 2016). Regarding the second challenge, ClearVolume has been developed as a GPU-accelerated open-source package to augment custom-built light-sheet microscopes with 5D real-time visualization (Royer et al. 2015).

Image Segmentation

Image segmentation refers to the process of partitioning images into meaningful regions (for example, foreground and background). It is often required as the first step of quantitative image analysis, providing an intermediate level of data representation that enables the investigator to subsequently perform quantitative measurements. Some recent image segmentation methods were designated for large-scale applications, including TWANG for cell segmentation based on nuclear markers (Stegmaier et al. 2014), TGMM and a graphical model framework for combined cell segmentation and tracking (Amat et al. 2014, Schiegg et al. 2014), and MARS and RACE for cell shape segmentation based on membrane markers (Fernandez et al. 2010, Stegmaier et al. 2016). For high-throughput cell image analysis, CellProfiler provides an open-source platform to identify and quantify cell phenotypes, including cell counts, cell size, cell/organelle shapes, and protein localization (Carpenter et al. 2006). For arbitrary image segmentation problems in bioimages, the Interactive Learning and Segmentation Toolkit (or ilastik) was developed to generate segmentation results interactively through active learning by using labels provided via a user interface (Sommer et al. 2011). In general, we expect synergies between human and machine to be essential for achieving near error-free segmentation/tracking results, despite the steady improvement in the accuracy of automated algorithms. Interactive data visualization and manual annotation can often be synergistically integrated with the individual steps of an automated image-processing workflow such that machine learning algorithms can take advantage of the user input to further improve the analysis result.

Computational Modeling

Computational modeling often constitutes the final step in the image analysis workflow and aims to generate biologically meaningful conclusions. One example of computational modeling is the construction of fate maps from large-scale cell lineaging data, where precursor cells are followed on their path to a destination tissue at more advanced developmental stages. Such analyses have been used, e.g., to map the fate of neuroblasts in *Drosophila* embryos at the single-cell level

(Amat et al. 2014). More recently, a statistical fate map of the postimplantation mouse embryo was built, using a multiembryo registration framework termed TARDIS, such that cell behaviors can be statically examined across individuals in space and time (McDole et al. 2018). Another example is biophysical modeling of morphogenesis (Gross et al. 2017, Keller 2013, LeGoff & Lecuit 2015). An elegant series of studies based on theoretical models inferred from live-imaging and laser ablation experiments revealed the physical mechanism of tissue spreading during zebrafish gastrulation (Behrndt et al. 2012, Campinho et al. 2013, Maitre et al. 2012, Morita et al. 2017). 3D reconstruction of *Drosophila* leg disc morphogenesis revealed the role of apoptotic forces in epithelium folding (Monier et al. 2015), and a continuum mechanics framework for modeling tissue invaginations observed in light-sheet image data provided insights into the impact of egg shape on *Drosophila* gastrulation (Khairy et al. 2018).

HOW IS NEW LIGHT-SHEET TECHNOLOGY DISSEMINATED?

The advancement of new imaging technologies and their application to an ever-increasing array of biological questions does little good if these tools and resources are inaccessible to the broader scientific community. Admittedly, many of the custom light-sheet systems discussed here require specialized instrumentation or software that is not easily ported to another facility. Commercial systems relieve some of the burden of building and maintaining a custom system but are often difficult to customize or adapt to the sometimes highly specific needs of the biological question at the time. Furthermore, once the data have been acquired, dissemination of those data quickly becomes another challenge. Light-sheet datasets often run into the multiterabyte range, and the variety of file formats and storage methods are a hurdle to quick and easy sharing. Despite these challenges, however, there are several ongoing efforts to unify and organize databases and computational tools, as well as to ensure access to the microscope systems themselves.

Microscope Accessibility

Projects and imaging centers that provide open access to light-sheet microscopy technology include the OpenSPIM project, the Janelia Advanced Imaging Center, and the Euro-BioImaging Consortium.

OpenSPIM. OpenSPIM takes the view that any lab with a minimal amount of technical skill can build a basic light-sheet system and that the open dissemination of plans and expertise is essential to advancing light-sheet technology and the field of biological imaging. The OpenSPIM project provides a curated parts list, open-source software, and a detailed guide for assembling your own light-sheet microscope (Pitrone et al. 2013). Each system can be customized to the needs of the user, with many of the parts easily purchased off the shelf from various retailers.

The Janelia Advanced Imaging Center. Supported by the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation, the Janelia Advanced Imaging Center hosts several custom, cutting-edge microscopes, from a lattice light-sheet microscope, to interferometric PALM, to a multifocus microscope, to the SiMView light-sheet microscope. These microscopes and the full support of a dedicated team of technicians are available to researchers free of charge after submitting a written proposal. The microscopes are reserved solely for the investigators for the duration of the visit and have a number of customizable environmental settings, available lasers, and illumination/detection settings.

Euro-BioImaging Consortium. The Euro-BioImaging Consortium aims to be Europe's open-access solution for a wide range of cutting-edge microscopes from light-sheet systems to medical imaging (such as PET and MRI). At the time of this writing, 29 nodes had been established in 11 different countries. The Euro-BioImaging and broader Global BioImaging communities envision providing access to microscopes and instrumentation and providing training in the use of imaging technologies and aim to have a global repository of image data and analysis tools.

Open-Access Tools

Obtaining access to these specialized microscope systems is only the start, and the question of what to do with the data once they are acquired is often left to the wayside until the user is suddenly confronted with several terabytes of image data. Even visualizing these large datasets can present a challenge, and many custom analysis tools were designed for a specific task in mind and are not easily adapted to large volumetric images. Commercial data visualization and analysis tools exist; however, they are often expensive, and the integration of new algorithms into proprietary software often cannot keep pace with the rapidly evolving field. Fortunately, several open-source efforts have emerged, and a collaborative effort to share data analysis tools and to make them as broadly applicable to users as possible has ameliorated many of the problems with visualizing and analyzing large datasets.

Fiji, or ImageJ, is an open-source package or suite of imaging tools supported by a global community of users and maintained by a dedicated team at the Laboratory for Optical and Computational Instrumentation at the University of Wisconsin, Madison. In addition to this resource having built-in image handling tools (such as visualization, editing, stack manipulation, and basic processing) and compatibility with a wide range of file formats, individual contributors can build and distribute freely downloadable plug-ins that offer a broad range of tools and manipulations. From image registration and movie making to tracking cells and tracing neurons, users can find just about anything to suit their needs on the small scale. For the large time-lapse datasets produced by many light-sheet microscopes, the ability of Fiji to cope is limited to the will of the contributors to design their plug-ins around such needs. Two plug-ins in particular, BDV and MaMuT, have emerged to make the visualization and annotation of large image datasets easier (see above for details).

Dataset Sharing

Even if the challenges of data visualization and storage are met, the dissemination of image datasets to the scientific community is a hurdle not only for researchers who want to make their data available but also for those who wish to download and analyze the data independently. Transferring massive multiterabyte datasets via FTP or shipping hard drives quickly becomes burdensome on the host lab and does not serve to make the data publicly available. While at the time of this writing the scientific community has not yet found a definitive solution to the open-access sharing of large-image datasets, the growing availability and use of light-sheet systems have made arriving at a solution a matter of pressing concern. Attempting to address this problem, the Image Data Resource (IDR) (**https://idr.openmicroscopy.org/about/**) and BioStudies (**https://www.ebi.ac.uk/biostudies/**) offer online, public data repositories that researchers can upload their datasets to, and in the case of the IDR, researchers are assigned a permanent DOI (digital object identifier) associated with the study. These opportunities for dataset sharing not only are essential for the generation of new tools and analysis but also can often yield entirely new insights and discoveries.

WHAT IS THE FUTURE TRAJECTORY OF LIGHT-SHEET MICROSCOPY?

What future advances do we expect to see in light-sheet microscopy? Which important observations and measurements are still out of reach, and how could light-sheet microscopy help us get there? In those cases in which we currently cannot yet see what we would ideally like to see, the key limitations generally fall into one of the following four categories: (*a*) We cannot look deep enough inside the biological specimen, (*b*) we lack spatial resolution, (*c*) we have insufficient temporal resolution, and (*d*) we harm or even kill the organism before being able to complete our measurements. In this section, we provide some thoughts regarding future opportunities for further improving light-sheet microscopy in these four directions.

The needs to improve deep-tissue imaging and spatial resolution are often related problems since existing methods already provide good spatial resolution under ideal optical conditions (e.g., at tissue surface) but fail to deliver images of comparable quality deep inside the specimen. Confounding factors are light scattering and optical aberrations. While the former remains a major challenge in light microscopy (and ultimately defines the limit of depth penetration), the latter can be addressed by mapping the wavefront distortions resulting from optical aberrations and by compensating for these distortions. Several powerful light-sheet methods to this end have emerged during the past few years, but further substantial improvements are still feasible. Traditionally, adaptive optics has been developed for techniques outside the field of light-sheet microscopy, and tight integration and tailoring of adaptive optics to the opportunities in and requirements for light-sheet microscopy will be essential for fully exploiting its potential. Achieving this goal especially requires the design of the computational core and optimization strategies underlying adaptive imaging as well as careful balancing of the quality of adaptive corrections and the impact of the required measurements on microscope performance, including temporal resolution and light exposure. A complementary direction for improving image quality deep inside live specimens is the use and further advancement of far-red and near-infrared fluorescent reporters. Substantial progress has been made in this area in recent years (Grimm et al. 2017b, Shcherbakova et al. 2016) and has enabled dramatic improvements in depth penetration, e.g., in the imaging of developing embryos (McDole et al. 2018).

Improvements in temporal resolution are conceivable in multiple, synergistic ways. One direction is the design of multiplexing strategies that parallelize light-sheet imaging of 3D volumes. A key advantage of this conceptual idea is that it does not necessitate an increase in local light power density and may thus offer increased speed without impacting sample health. By contrast, improving imaging speed by using faster cameras is technically straightforward and easily supported by continuous advances in camera technology but also requires a concurrent increase in light power density to avoid degradation of the signal-to-noise ratio. When imaging very large specimens, such as expanded tissues and organs (Chen et al. 2015a), imaging speed can also be constrained by the limited field of view of the microscope and the need for tiling-based image acquisition. Thus, another opportunity for improving imaging speed and tiling-free access to large specimens at high resolution is the development of advanced custom optics that combines high numerical apertures (and thus high spatial resolution) with a large field of view.

Imaging sensitive biological specimens rapidly and/or over long periods of time requires minimizing photodamage. Light-sheet microscopy is intrinsically well suited to meeting this challenge, but observation length and temporal sampling can ultimately still be limited by photodamage, in particular during high-resolution imaging. Advancing one-photon imaging assays that utilize state-of-the-art indicators in the (near-)infrared spectral domain is an effective strategy for addressing this limitation, since biological specimens are generally much less sensitive to exposure

to longer-wavelength light. In addition, the use and advancement of such indicators also improve depth penetration and image quality deep inside the sample, as discussed above.

Finally, important opportunities for further advancing light-sheet microscopy are also readily found beyond "just" improving raw performance, such as spatial or temporal resolution. New capabilities and experiments will become feasible by forming powerful synergies between different technologies, both within the realm of microscopy (such as by combining light-sheet microscopy and computational imaging) and across disciplines, e.g., by tight integration of real-time computational image analysis and light-sheet microscopy. Moreover, by enhancing these microscopes not only with automated computational algorithms for real-time image analysis but also with infrastructure for high-resolution optical manipulation, it will eventually be possible to image and precisely manipulate developmental processes in real time and in an entirely data-driven manner. We are currently still at the beginning of this process, and for the most part imaging and image analysis are independent, sequential steps in the experimental workflow. However, with the development of microscopes capable of, e.g., tracking cells, classifying their dynamic behaviors, or predicting their future fates in real time, followed by optical ablation or manipulation of gene expression in a specific subset of cells with certain dynamic fingerprints, one could design entirely new types of mechanistic investigations in developmental biology.

DISCLOSURE STATEMENT

P.J.K. filed provisional US patent application 62,354,384 for adaptive light-sheet microscopy on June 24, 2016, and US patent application 14/509,331 for orthogonal multiview light-sheet microscopy on October 8, 2014. P.J.K. furthermore holds US patent 9,404,869 (issued on August 2, 2016) for simultaneous multiview light-sheet microscopy.

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