Emerging Imaging and Genomic Tools for Developmental Systems Biology

Zhe Liu^{1,*} and Philipp J. Keller^{1,*}

¹Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147, USA *Correspondence: liuz11@janelia.hhmi.org (Z.L.), kellerp@janelia.hhmi.org (P.J.K.) http://dx.doi.org/10.1016/j.devcel.2016.02.016

Animal development is a complex and dynamic process orchestrated by exquisitely timed cell lineage commitment, divisions, migration, and morphological changes at the single-cell level. In the past decade, extensive genetic, stem cell, and genomic studies provided crucial insights into molecular underpinnings and the functional importance of genetic pathways governing various cellular differentiation processes. However, it is still largely unknown how the precise coordination of these pathways is achieved at the whole-organism level and how the highly regulated spatiotemporal choreography of development is established in turn. Here, we discuss the latest technological advances in imaging and single-cell genomics that hold great promise for advancing our understanding of this intricate process. We propose an integrated approach that combines such methods to quantitatively decipher in vivo cellular dynamic behaviors and their underlying molecular mechanisms at the systems level with single-cell, single-molecule resolution.

Introduction

Animal development involves coordinated and dynamic cellular events that are exquisitely regulated at the molecular level with high spatiotemporal precision. In past decades, a combination of classical biochemical, cell biological, genomic, and genetic approaches provided a comprehensive view of molecular pathways important for various developmental processes. However, many of these classical studies use cell-population-based endpoint assays and thus provide little information about the kinetics, dynamics, and 3D architecture of cell-type specific molecular systems as they operate in living cells and give rise to the finely balanced lineage commitment and morphogenetic events underlying development. Moreover, it is remarkably challenging to quantify transient, dynamic morphogenetic states (e.g., migration, division, cell shape) and cell lineage commitment events during development at the whole-embryo level with high spatiotemporal resolution.

Our current ability to quantitatively measure complex in vivo cellular and molecular dynamics has been greatly advanced by the recent implementation of new non-invasive live-cell imaging and labeling techniques (Keller, 2013; Liu et al., 2015a). Here, we discuss state-of-art whole-embryo imaging and computational approaches enabling large-scale quantification of cell dynamics in developing embryos with high spatiotemporal resolution. Rapid advances in super-resolution in vivo imaging furthermore open the door to studying molecular structures and dynamics driving specific cellular behaviors. In parallel, the development of new single-cell genomics techniques bears great potential for highthroughput discovery of cell-type specific molecular systems. Together, these emerging technological advances outline a next-generation effort toward the comprehensive delineation of complex developmental processes at the systems level with single-cell, single-molecule resolution.

Whole-Embryo Imaging with Subcellular Resolution

In recent years, there has been rapid progress in the development of live imaging methods for studying developmental processes. Generally, technical advances in this domain are concerned with one or more of the following five key aspects of imaging performance: spatial resolution, temporal resolution, physical coverage of the system under observation (in particular for large and not entirely transparent biological specimens), long-term imaging capability, and photo-damage and phototoxicity (Keller, 2013; Pantazis and Supatto, 2014). High resolution in all spatial dimensions is crucial for discerning neighboring cells in multi-cellular organisms and for resolving subcellular structures and morphological details. High temporal resolution is needed to capture fast dynamic processes, such as cell migration and rapid cell shape changes. Full spatial coverage is needed for investigations at the systems level, such as quantitative analyses of whole-tissue morphogenesis or development of entire embryos. Long-term imaging capability is crucial when following dynamic processes over developmental timescales, such as the formation of organs from small pools of progenitor cells. Finally, light-based interrogation of the biological specimen should be as non-invasive as possible, so as not to perturb the system under observation. All of these parameters are competing for the same resource, specifically the total amount of photons that can be extracted from the specimen under physiological conditions. Depending on the requirements of the imaging experiment, this photon budget can be spent in different ways, e.g., to balance overall performance or to maximize spatial or temporal resolution by sacrificing performance in other areas. In many instances, it would of course be desirable not to have to sacrifice performance in one area versus another, in particular when imaging assay and resulting data are at risk of being constrained by a technical compromise. Working toward such capabilities, however, is only possible with an imaging method that makes optimal use of the photon budget in the first place.

Importantly, improvements continuously occur at all five fronts, but only very recently it has become possible to develop light microscopes that excel in all five areas simultaneously. These new developments relate to recent advances in light-sheet microscopy and are particularly important for

	Lattice Light-Sheet Microscopy (Chen et al., 2014a)	Dual-View Light-Sheet Microscopy (diSPIM) (Wu et al., 2013)	Isotropic Multi-View Light-Sheet Microscopy (IsoView) (Chhetri et al., 2015)
Technical concept	improving axial resolution using a thin light sheet constructed from an optical lattice	improving axial resolution and resolution isotropy using orthogonal two-view imaging	improving axial resolution, resolution isotropy and depth penetration using orthogonal four-view imaging
System resolution	$230 \times 230 \times 370 \text{ nm}^3$ (dithered mode); 150 × 230 × 280 nm ³ (structured illumination mode)	$330 \times 330 \times 330 \text{ nm}^3$	410 × 420 × 450 nm ³
Maximum reported imaging volume	$100 \times 50 \times 50 \ \mu m^3$	$78 \times 70 \times 50 \ \mu m^3$	$830 \times 400 \times 400 \ \mu m^3$
Maximum reported volumetric imaging speed	83,000 μm ³ /s	540,000 μm ³ /s	130,000,000 μm ³ /s
Application focus	high-speed, high-resolution imaging of small embryos (<i>C. elegans</i>) and individual, cultured cells		high-speed, high-resolution imaging of large embryos (<i>D. melanogaster</i> , <i>D. rerio</i>)
Volumetric imaging rates in IsoView and diSPIM experiments are limited by camera speed, whereas in lattice light-sheet experiments the primary			

Table 1. Methods for High-Speed Whole-Embryo Imaging with Subcellular Resolution

Volumetric imaging rates in IsoView and diSPIM experiments are limited by camera speed, whereas in lattice light-sheet experiments the primary bottleneck is typically signal-to-noise ratio rather than camera speed. Taking full advantage of IsoView system resolution with state-of-the-art sCMOS cameras with a pixel pitch of 6.5 µm requires a magnification setting of at least 30× in the microscope's detection arms (yielding a pixel size of 0.2 µm in sample space).

developmental systems biology, as they enable, for the first time, the study of developmental processes at the whole-embryo level with high spatial and temporal resolution. Below, we introduce these new imaging approaches for high-resolution, whole-embryo imaging.

Conceptually, light-sheet microscopy is a very old technique (Siedentopf and Zsigmondy, 1903)-it is about twice as old as confocal microscopy-but it emerged as a powerful technique for systems-level imaging in the life sciences only over the course of the past decade. The key technical concept in all light-sheet microscopes is the illumination of a thin volume section with a "sheet" of light (Fuchs et al., 2002; Huisken et al., 2004; Siedentopf and Zsigmondy, 1903; Voie et al., 1993) or with a rapidly scanned "pencil beam" (such as a weakly focused Gaussian laser beam) (Keller et al., 2008) entering the specimen from the side. To form an image, fluorescence light emitted by this illuminated section is then captured with a conventional wide-field detection system. By moving the illuminated plane step-by-step relative to the specimen, it is possible to rapidly acquire a 3D image dataset of the entire specimen volume. And by simply iterating this 3D acquisition procedure, this volume can be imaged as a function of time.

New developments in light-sheet microscopy over the course of the past decade have led to a wide range of advances, from functional imaging of entire nervous systems to capturing whole-animal development at the single-cell level (Keller and Ahrens, 2015; Stelzer, 2014; Winter and Shroff, 2014). The strength of light-sheet microscopy in such applications arises from its unique ability to combine high imaging speed with low light exposure and, thus, low photo-damage. This in turn enables rapid imaging of large specimens over extended periods of time, without perturbing specimen physiology, normal development and function of the system under study. Fairly high speed, signal-to-noise ratio, and long-term imaging capability were already realized in very early implementations of this technique (Holekamp et al., 2008; Huisken et al., 2004; Keller et al., 2007, 2008). However, spatial resolution has been a limiting factor in many experiments until very recently. The reason for this limitation is the fundamental tradeoff between spatial resolution and the size of the field of view in light-sheet microscopy, a relationship that is intrinsic to the physics of Gaussian beams: the size of the field of view is proportional to the square of the light-sheet width. As a result, spatial resolution in conventional light-sheet microscopy (and in most other light microscopes as well) is typically high (on the order of a few hundred nanometers) within the plane defined by the light sheet, but relatively low (on the order of several micrometers) perpendicular to this plane. In other words, spatial resolution is highly anisotropic, which makes it difficult to discern neighboring cells as distinct structures when imaging entire embryos or to resolve subcellular processes when imaging individual cells.

Several strategies have been devised over the course of the past 3 years to overcome this limitation (Chen et al., 2014a; Chhetri et al., 2015; Wu et al., 2013). These approaches focus on different spatial scales and exhibit different strengths (Table 1). Generally, they can be broken down into methods for (1) high-resolution imaging of individual cells and small multicellular organisms at a scale up to Caenorhabditis elegans embryos (Chen et al., 2014a; Wu et al., 2013), and (2) high-resolution imaging of vertebrate and higher invertebrate embryos, such as entire Danio rerio or Drosophila melanogaster embryos (Chhetri et al., 2015). The former category involves spatial scales of typically up to 50 \times 30 \times 30 μ m³ (C. elegans embryo), whereas the latter category is concerned with spatial scales up to 500 × 200 \times 200 μ m³ (Drosophila melanogaster embryo) or 700 \times 700 \times 700 μ m³ (Danio rerio embryo). This \sim 7,000-fold difference in specimen size implies that different technical challenges have to be considered and addressed in order to achieve high spatial resolution and good physical coverage without sacrificing temporal resolution. Differences in the underlying optical challenges also need to be taken into consideration, in particular the lower relative transparency and higher light

scattering and stronger aberrations encountered in large multicellular organisms. In the next two sections, we discuss these two scenarios and recent breakthroughs in methodological capabilities in more detail.

Imaging Small Multi-cellular Organisms with High Spatiotemporal Resolution

Spatial resolution in small, transparent specimens can be improved substantially by creating thin light sheets and thereby acquiring optical sections that are considerably thinner than those produced by conventional light-sheet microscopy. Thin light sheets can be constructed, for example, by sweeping a Bessel beam across the image plane (Planchon et al., 2011) or by dithering an optical lattice (Chen et al., 2014a), yielding an axial system resolution on the order of 300-370 nm. Bessel beams consist of a system of concentric rings (Durnin et al., 1987; Fahrbach et al., 2010) with a central peak that can be made thinner than the profile of corresponding Gaussian beams. Thus, when suppressing the contribution of the outer rings of the Bessel beam to image formation (which is possible, e.g., by using two-photon excitation or structured illumination approaches), the resulting images exhibit better axial resolution than those obtained with conventional Gaussian beams. The main drawbacks of the Bessel beam approach are the fairly high power densities and the illumination of out-of-focus regions of the specimen with the outer rings in the Bessel beam's structure, thus leading to considerably higher rates of photo-damage and photo-bleaching than light-sheet imaging with Gaussian beams (Planchon et al., 2011; Wu et al., 2013). More recently, this issue has been addressed with the introduction of lattice light-sheet microscopy, in which thin light sheets are instead created from periodic light interference patterns that are rapidly dithered to produce uniformly illuminated image planes (Chen et al., 2014a). The interference patterns required for creating the lattice light sheet can be formed with high precision in sufficiently transparent specimens, and are thus well suited for imaging small multi-cellular organisms, such as C. elegans embryos, at high spatial resolution. For example, using lattice light-sheet microscopy, subcellular AIR-2 protein localization and the distribution of actin in developing C. elegans embryos were imaged at a temporal resolution of 2.6-4.8 s across volumes of 53 \times 43 \times 33 μ m³ and 60 \times 37 \times 15 μ m³, respectively (Figures 1A and 1B).

An alternative state-of-the-art strategy to high-resolution imaging is based on the use of multi-view imaging (Swoger et al., 2007). Instead of improving axial resolution directly in the raw image data acquired by the light-sheet microscope, at least two orthogonal views of the sample are acquired (each still suffering from anisotropic resolution) and high spatial resolution is subsequently achieved by combining the image content of these views. In orthogonal views, the directions along which resolution is low (axially) and high (laterally), respectively, are permuted. By combining high-resolution information along all three spatial dimensions through a computational process called multi-view deconvolution, a single representation of the 3D image data of the sample is reconstructed from all contributing raw views with almost perfectly isotropic spatial resolution (Keller et al., 2006; Swoger et al., 2007). Such multi-view imaging experiments used to be limited to fixed specimens, since early light-sheet microscopes had to rotate the specimen mechanically for the acquisition of complementary views (Huisken et al., 2004; Keller et al., 2008, 2010); this process is typically so slow that concurrent dynamic changes in the sample, such as fast cell movements or cell shape changes, make it very challenging or even impossible to correctly register and combine multiple sequentially acquired views of the specimen. A recent, fast implementation of orthogonal two-view imaging, termed diSPIM microscopy (Wu et al., 2013), addressed this limitation very effectively by keeping the specimen stationary and instead alternating illumination and detection along two orthogonal axes, using two orthogonally oriented objectives that are both used for lightsheet illumination and fluorescence detection. After multi-view deconvolution of the diSPIM image data, this approach achieved a system resolution of 330 nm. Using diSPIM, it was possible to perform high-resolution imaging of C. elegans embryogenesis with a temporal sampling of 0.51 s for a volume of 78 \times 70 \times 50 μm³ (Figure 1C).

Imaging Entire Vertebrate and Higher Invertebrate Embryos with High Spatiotemporal Resolution

In order to obtain live imaging data of larger specimens with high spatiotemporal resolution, several additional challenges need to be addressed. Owing to the limited depth penetration of biological tissues with light at typical imaging wavelengths, neither lattice light-sheet microscopy nor orthogonal two-view imaging can produce high-resolution images of specimens as large as entire Drosophila or zebrafish embryos. During illumination and fluorescence detection, light is scattered and absorbed and aberrations further degrade image quality, such that it becomes impossible to acquire high-resolution image data beyond a certain depth into the tissue. For example, using lattice lightsheet microscopy, high-resolution imaging in Drosophila embryos has been demonstrated for depths up to 20 µm (Chen et al., 2014a), corresponding to about 1/10th of the total diameter of the embryo. One also needs to consider that it is technically challenging to create thin light sheets across a field of view significantly larger than 100 µm and that, depending on the temporal resolution required for a given experiment, it may in fact not be desirable to create an exceptionally thin light sheet in the first place: reducing light-sheet thickness means that more images need to be acquired to cover the same sample volume, since spatial sampling requirements dictate that neighboring image planes should ideally be offset by a distance not larger than half of the light sheet's width. Maximum temporal resolution is thus reduced when using imaging approaches employing thin light sheets.

These limitations can be effectively overcome by further advancing multi-view imaging based on relatively thick light sheets; since fewer images need to be recorded when using thicker light sheets, temporal resolution in a multi-view microscope can be very high as long as there are means to fully parallelize the acquisition of the multiple views. At the same time, multi-view imaging including both orthogonal and opposing views can also address constraints arising from limited depth penetration, since the acquisition of opposing views of the sample effectively doubles penetration depth. In older generations of light-sheet microscopy, the ability to acquire multiple views of the sample was used very successfully to improve physical



Figure 1. Imaging Developing C. elegans Embryos with High Spatiotemporal Resolution

(A) Illustration of the basic imaging arrangement in conventional light-sheet microscopy (first panel to the left) as well as in two advanced light-sheet-based imaging strategies for high-resolution imaging. High spatial resolution in all three dimensions can be achieved by using exceptionally thin light sheets (second panel to the left), such as in lattice light-sheet microscopy, or by performing simultaneous or near-simultaneous multi-view imaging (panel to the right), such as in lsoView and diSPIM light-sheet microscopy, respectively.

(B) High-resolution, whole-embryo imaging of *C. elegans* with lattice light-sheet microscopy. Maximum-intensity projections show the distribution of chromosomal passenger protein GFP-AIR-2 (green) relative to plasma membranes and histones (red) at the three different stages in early embryogenesis. (C) Lattice light-sheet imaging of Lifeact in a *C. elegans* embryo during pseudocleavage ingression (left) in maintenance phase just before the first division (center)

and during the first division (right).

(D) High-resolution, whole-embryo imaging of *C. elegans* with diSPIM. Left: maximum-intensity projections show GFP-labeled histones at selected time points during embryogenesis (developmental time is provided in hours and minutes). Right: the lower two panels show enlarged views of the boxed regions highlighted in the top two panels. YX and YZ views of the embryo are orthogonal with respect to each other.

(B) and (C) adapted from Chen et al. (2014a) with permission from AAAS. (D) adapted from Wu et al. (2013) with permission from Nature Publishing Group. Scale bars, 5 µm (B, C), 10 µm (D, left and top right), 3 µm (D, bottom right).

coverage in large, partially opaque living specimens (Keller et al., 2008, 2010; Krzic et al., 2012; Lemon et al., 2015; Schmid et al., 2013; Tomer et al., 2012). However, the acquisition of orthogonal views for improving not only physical coverage but also spatial resolution required mechanical rotation of the sample in these earlier microscope designs. Depending on how much acceleration and rotation motion the investigator is willing to subject the specimen to, this rotation takes at least several seconds to several tens of seconds. Over such long periods of time, cell positions and cell shapes in the living specimen change very significantly at scales above the resolution limit of the microscope, and these changes are locally highly variable, non-linear, and generally unpredictable (Tomer et al., 2012). In model systems exhibiting very fast cellular dynamics, such as early-stage Drosophila embryos, it is thus impossible to accurately register and combine information from orthogonal views with the precision needed to match the system resolution of the microscope (Tomer et al., 2012). For this reason, conventional light-sheet microscopes that are not capable of (near-)simultaneous orthogonal multi-view imaging are generally only suitable for highresolution imaging of chemically fixed or very slowly developing live specimens, such as mouse embryos, and cannot improve spatial resolution in highly dynamic samples without a risk of introducing artifacts during image processing.

To overcome these limitations, a new live imaging technique, termed IsoView microscopy, has recently been developed and shown to enable rapid high-resolution imaging of large multicellular organisms (Chhetri et al., 2015). IsoView microscopy acquires four orthogonal views of the specimen simultaneously (Figure 2A) by using scanned Gaussian beams for sample illumination and introducing a small spatial offset in beams along orthogonal axes. By combining this staggered beam-scanning approach with cameras capable of confocal line detection (Baumgart and Kubitscheck, 2012; Silvestri et al., 2012), crosstalk between orthogonal views can be completely avoided and the acquisition of all four views can be performed simultaneously without sacrificing image quality. Furthermore, IsoView microscopy takes advantage of custom-built objectives that enable four-view imaging of volumes as large as 800 × 800 × 800 µm³ with high numerical aperture (and thus high spatial resolution). Similar to diSPIM microscopy, raw multi-view image data acquired by IsoView microscopy are converted into a high-resolution representation of the specimen by multi-view deconvolution. IsoView microscopy thereby produces 3D image data with an isotropic spatial resolution down to 420 nm (Figure 2B). Importantly, high-resolution imaging with IsoView microscopy is applicable to relatively large, partially light-scattering samples, such as entire Drosophila and zebrafish embryos; for example, IsoView microscopy has been shown to provide an average, isotropic spatial resolution of 1.6 µm and a temporal resolution of 0.5 s when imaging entire stage 17 Drosophila embryos, improving 3D resolution at least 7-fold compared with conventional light-sheet microscopy. IsoView long-term, multi-color imaging has been used in gastrulating Drosophila embryos to capture both nuclei and cell shape dynamics over a period of several hours (Figure 2C). In addition, the high temporal resolution of ≥ 1 Hz enables functional imaging experiments with calcium indicators, and thus opens the door to integrated studies of development and emergence of function, for example, in the developing nervous system. As for diSPIM, imaging speed in IsoView microscopy is only limited by camera performance and the amount of fluorescent signal a living specimen can produce under physiological conditions. Since this physiological limit has not yet been reached in current experiments, we expect further increases in volumetric imaging rates with upcoming new generations of sCMOS camera technology.

Although multi-view imaging in light-sheet microscopy can substantially improve physical coverage of large non-transparent specimens, point-scanning two-photon microscopy represents the gold standard for deep-tissue imaging (Denk et al., 1990). Thus, for live imaging experiments in which deep-tissue penetration is a more important factor than imaging speed or resolution isotropy, this latter approach can be the method of choice. Two-photon excitation can be utilized in the context of light-sheet microscopy as well (Mahou et al., 2014; Tomer et al., 2012; Truong et al., 2011) but does not offer the same quantitative advantage in improving imaging depth when compared with point-scanning two-photon microscopy. The reason for this difference in performance is that light-sheet microscopes form an image directly from the fluorescence emitted by the specimen, and the quality of this image thus degrades rapidly in the presence of light scattering and optical aberrations. In contrast, point-scanning two-photon microscopes do not rely on direct image formation but rather assemble an image pixel-by-pixel by measuring a signal proportional to the number of photons emitted from each respective location in the specimen. This latter process is less sensitive to scattering and aberrations; the trajectory of photons can deviate to some extent from an undisturbed ballistic detection path without degrading final image quality, as long as these photons are still detected and accounted for. Powerful multi-color two-photon microscopes (Economo et al., 2016; Mahou et al., 2014) and adaptive imaging techniques (Ji et al., 2010; Wang et al., 2014) have been presented in recent years and complement the imaging capabilities enabled by recent developments in the light-sheet microscopy field.

Availability of Light-Sheet Microscopes for Imaging at High Spatiotemporal Resolution

As light-sheet microscopy is being more widely adapted, it has become possible to acquire such instruments through commercial channels. Like in many other areas of methods development, commercially available technology falls behind the state-of-theart methodology used in optical research laboratories by at least several years, which translates into several microscope generations in the rapidly evolving field of light-sheet microscopy. At the time of writing of this review, commercially available systems are generally incapable of imaging at the high spatiotemporal resolution discussed in the previous sections, with one notable exception: the high-resolution diSPIM method is already available as a commercial product (Applied Scientific Instrumentation). The commercialization of other methods is underway and many of the approaches discussed in this review will likely be available as commercial products in the future.

Although commercial distribution may not always be an option, we note that some high-resolution imaging techniques described here were published following the open-access



Figure 2. Imaging Developing Drosophila Embryos with High Spatiotemporal Resolution

(A) Isotropic multiview (IsoView) light-sheet microscopy facilitates imaging of large biological specimens with high spatiotemporal resolution using light-sheet illumination and fluorescence detection along four orthogonal directions.

(B) Isotropic resolution in IsoView microscopy is achieved by combining the image information encoded in the four complementary raw views. Spatial resolution is anisotropic in each raw view, i.e., high along the lateral dimensions and low along the axial dimension (see schematics of elongated point-spread functions (PSFs A-D) shown from a top view) but combining this information with multi-view deconvolution provides high spatial resolution in all three dimensions (post-fusion point-spread function).

(C) Multicolor imaging of *Drosophila* whole-embryo development with isotropic spatial resolution. Dorso-ventral maximum-intensity projections of multiview deconvolved IsoView image data of a gastrulating embryo are shown for four selected time points, highlighting fluorescently labeled nuclei (His2Av-mRFP1) and membranes (Spider-GFP). Colors are inverted for better visibility. An enlarged view of the region highlighted by the white box is shown in the top row of (D).

(D) Enlarged views of two regions in the wholeembryo image data, showing ventral furrow formation from a ventral view and cephalic furrow formation from a lateral view. Note that resolution is high in both orthogonal views of the embryo. Time is measured relative to the time points of egg laying.

(A)–(D) adapted from Chhetri et al. (2015). Scale bars, 50 μ m (C), 20 μ m (D).

a custom optics provider (Special Optics) and can also be substituted with standard components if a compromise with respect to spatial resolution or size of field of view is acceptable.

Finally, if cutting-edge performance and high spatiotemporal resolution are not needed, it is also possible to build basic low-cost systems using the extensive online resources made available by several open-access projects. Online resources for SPIM-type (Huisken et al., 2004) and DSLM-type (Keller et al., 2008) light-sheet microscopes are available, for example, through the openSPIM (http://openspim.org) and OpenSpinMi-

concept (Chhetri et al., 2015; Kumar et al., 2014). The publication of the IsoView microscope, for example, comprises detailed technical information enabling the reproduction of this system, including a computer model of the microscope, technical drawings of all of the microscope's custom-built components, models of the microscope's optical components, open-source software packages for IsoView image processing, and step-by-step instructions for setting up and aligning the microscope (Chhetri et al., 2015). The custom-built optical components used in the original IsoView design are available from croscopy (https://sites.google.com/site/openspinmicroscopy/) projects.

Computational Image Analysis for Developmental Systems Biology

Emerging systems-level imaging approaches capture developmental dynamics at the cellular and subcellular levels and are capable of producing massive amounts of information-rich image data, encoding, for example, the movements and cell shape changes of the tens of thousands of cells in developing

602 Developmental Cell 36, March 21, 2016 ©2016 Elsevier Inc.

Drosophila or zebrafish embryos. High-speed light-sheet microscopes, such as IsoView microscopy, acquire high-resolution image data already at rates of up to 10 TB per hour, and ongoing improvements in camera technology will inevitably lead to further performance advances over the course of the next few years. Data rates on the order of 100 TB per hour/microscope should in principle become feasible within 1-2 years, providing access to fast subcellular processes at the scale of entire embryos with unprecedented spatiotemporal resolution. The complexity and sheer size of typical whole-embryo recordings thus makes it generally impossible to extract biologically meaningful information exclusively by manual data inspection. In particular, quantitative analyses of large-scale cellular dynamics require efficient, automated image processing and computer vision approaches. However, rapid advances in high-performance light microscopy have outpaced the development of computational methods suitable for interpreting image data at such scales. The need for new computational approaches that scale with size and complexity of state-of-the-art image datasets has been recognized by many laboratories and complementary computational methods are now starting to catch up with state-of-the-art imaging methods. In the following, we provide a brief overview of recently developed methods that address typical challenges encountered in systems-level imaging of developmental processes.

The spectrum of computational challenges is broad and often starts already at the level of image acquisition itself. In particular, approaches to rapid (and ideally real-time) data visualization (Peng et al., 2010; Pietzsch et al., 2015; Royer et al., 2015) and high-throughput data handling and image compression (Amat et al., 2015) are needed in order to work with next-generation imaging methods in a routine setting. Regarding the former, a powerful method termed ClearVolume has been developed for real-time data visualization at the runtime of high-speed imaging experiments (Royer et al., 2015). Vaa3D and BigDataViewer (as well as a number of other methods) are available as interactive 3D data visualization and annotation frameworks (Peng et al., 2014; Pietzsch et al., 2015). Regarding data handling, largescale image datasets can be rapidly compressed without loss of information using, for example, 3D JPEG or KLB compression. The recently developed KLB file format (Amat et al., 2015) has the key advantage of providing exceptionally high read/write speeds on the order of 500-1,000 MB/s when used on computers equipped with modern multi-core processors and stores multi-dimensional image data following a block-based scheme that provides efficient access to subregions of the image data.

The next step in the image processing workflow frequently involves image deconvolution (which is indispensable for multiview imaging approaches such as diSPIM and IsoView), a procedure that enhances image contrast and spatial resolution. Modern implementations of the commonly used Lucy-Richardson algorithm (Richardson, 1972) for 3D image deconvolution take full advantage of high-end graphics cards to achieve high data throughput in a cost-effective setting, without sacrificing image quality (Chhetri et al., 2015; Preibisch et al., 2014). Following these initial image processing and data management tasks, computational methods are then needed for the actual image analysis and extraction of meaningful biological information from the image data. Typical requirements to this end range from 3D cell shape segmentation to cell tracking and reconstruction of developmental lineages. Some recent methods in this domain were designed with large-scale applications in mind, including the TWANG and RACE frameworks for 3D nucleus shape and 3D cell shape segmentation, respectively (Stegmaier et al., 2014, 2016), the TGMM framework for automated cell tracking and cell lineage reconstructions (Amat et al., 2014), and a graphical-model-based approach for joint segmentation and cell tracking (Schiegg et al., 2014). Additional computational strategies have been devised for the construction of developmental atlases from systems-level image data, providing a reference scaffold for data annotation at the cellular level, quantifying stereotypy of the developmental building plan at a given developmental stage and systematically mapping gene expression information across entire tissues (Du et al., 2014; Fowlkes et al., 2008; Heckscher et al., 2014; Long et al., 2009).

Integrating Imaging and Image Analysis

While many ongoing computational efforts focus on solutions to so-called offline data analysis, that is, the processing and analysis of image data after the imaging experiment has already been conducted, a powerful next step in this field will be the development of methods that are not only capable of extracting useful information from image data in a robust and automated manner but also do so at a speed that matches or surpasses the speed of image acquisition itself. This level of performance would push computational capabilities into the realm of online analyses, enabling new types of experiments in a closed-loop manner. For example, with the ability to perform automated, real-time cell segmentation, cell tracking, cell lineage reconstructions and assessment of protein localization in individual cells, this information could be evaluated by a software layer deployed on the microscope control computer itself, evaluated in real-time and then utilized to instruct an optical manipulation system to perturb individual cells or entire anatomical regions in the developing organism in a spatiotemporally well-controlled and welldefined manner. Depending on the level of sophistication introduced in this software layer, it is in principle even possible to automatically identify cell types from live imaging data using information about cell positions, movements and morphogenetic changes at the cellular and tissue level (Amat et al., 2014), classify new cellular dynamic behaviors, and identify transient cellular differentiation states. By targeting cells identified and classified with such approaches, using laser ablation or light-mediated activation/repression of gene expression, one could devise precise functional perturbation experiments that automatically and directly test mechanistic hypotheses about developmental models in situ and at the whole-embryo level. As discussed above, high-throughput image analysis methods already exist and some of these approaches are in fact capable of keeping up with the speed of 3D image acquisition at the scale of entire developing vertebrate and higher invertebrate embryos (Stegmaier et al., 2016). Thus, as a next step, it will now be important to integrate such methods with cutting-edge microscopes to unlock the full combined potential of these methodologies.

Single-Cell Genomics and Imaging Techniques

Quantitatively deciphering developmental programs requires comprehensive understanding of regulatory mechanisms at both

cellular and molecular levels. Emerging high-resolution, wholeembryo imaging techniques have the capacity to quantitatively unmask detailed information on transient dynamic morphogenesis and cell lineage commitment events orchestrating embryogenesis (Keller, 2013). However, the rapid delineation of cellular dynamics atlases of development has outpaced our current understanding of the underlying molecular control mechanisms. Specifically, it is largely unclear how the highly regulated spatiotemporal choreography of development is encoded in the genome and how cell-type-specific behaviors such as cell-fate determination, cell shape changes, and migration are regulated by elaborate molecular interactions in live cells with high spatiotemporal precision.

Bridging these knowledge gaps likely requires improvements in two critical areas. First, we need to devise efficient and systematic strategies to discover cell-type-specific molecular systems at the whole-embryo level with single-cell resolution. Correlating cellular dynamics with a gene expression atlas will shed light on cell-type specific genetic fingerprints and molecular pathways driving distinct cellular dynamics. Second, we need to decode how particular molecular systems come into play in live cells and regulate complex cellular dynamics in real time. Rapidly advancing high-throughput sequencing-based transcriptome profiling methods and high-resolution live-cell imaging techniques have recently reached single-cell, single-molecule sensitivity (reviewed in Liu et al., 2015a; Macaulay and Voet, 2014) and thus open unique opportunities to address these problems. Here, we discuss current technological advances and the challenges of applying new single-cell genomics and complementary imaging methods to better understand the developmental fate map at the molecular level.

Mapping Gene Expression Atlas at Single-Cell Resolution

High-resolution whole-embryo imaging and imaging analysis techniques dissect complex cellular behaviors at the single-cell level in the context of the whole embryo. However, current developmental gene expression maps in higher model organisms have not yet reached single-cell resolution (Visel et al., 2004). Without comparable resolution in mapping cellular dynamics and gene expression, it is challenging to discover new gene pathways driving cell-type specific behaviors. Although a range of high-throughput sequencing genomics and RNA-fluorescence in situ hybridization (FISH)-based imaging techniques have been developed to probe gene expression programs in single cells (Crosetto et al., 2015; Macaulay and Voet, 2014), each technique has its own trade-offs and limitations when applied to developmental biology (Figure 3).

Single-cell transcriptome analysis is currently routinely applied to cell culture systems or early-stage embryos (Kolodziejczyk et al., 2015; Xue et al., 2013). However, the main technical barrier preventing us from applying single-cell genomics techniques to studying gene regulation in a complex specimen (e.g. large developing embryos or organs) is lack of efficient methods for isolating distinct cell populations with high spatiotemporal precision. Currently, most single-cell isolation techniques such as fluorescence-activated cell sorting (FACS) are optimized for large quantities (millions of cells) of homogenized cell suspension, and therefore are not suitable for isolating small amounts of cells from a developing embryo. Another apparent challenge

604 Developmental Cell 36, March 21, 2016 ©2016 Elsevier Inc.

Developmental Cell Review

is tracing the spatial origin of the isolated cells in the context of the whole embryo's anatomy. This step is important, because information obtained from single-cell genomics subsequently needs to be correlated with whole-embryo developmental imaging datasets for extracting molecular pathways underlying distinct cellular behaviors. Recently, several methods have been developed for single-cell isolation from complex samples. Here we briefly discuss the pros and cons of each technique and explain potential challenges for registering information obtained from these techniques to cellular dynamics maps generated by whole-embryo imaging.

Micromanipulation is a technique for isolating individual cells by manual pipetting (Hashimshony et al., 2012; Henry et al., 2015; Sugino et al., 2006; Yan et al., 2013) (Figures 3A-3C). One advantage of this technique is that there are effectively no lower bounds with respect to the amount of required starting materials. Usually, the tissue of interest (such as a particular brain region or a section of an embryo) is dissected out based on anatomical markers and is then dissociated into single cells. Cells are first visualized using fluorescence microscopy and then isolated manually by pipetting. Recently, computer-aided semiautomatic robotic systems have been developed to increase detection sensitivity, reduce labor, and ensure reproducibility. Using precise cell-type-specific Gal4 driver lines, a small number of neuron progenitor cells can be extracted from a fly embryo (Liu et al., 2015b). Despite its great sensitivity, trade-offs of micromanipulation include low throughput and vulnerability to human errors. The spatial information of extracted cells is lost due to single-cell dissociation. However, when using precise genetic tools such as knock-in fluorescent markers and cell-type-specific enhancer driven reporters, one could trace the cells back to specific regions of the embryo according to the expression pattern of the reporter and the selection of the dissection area. This strategy is still not ideal since pre-selected genetic markers will sometimes label more than one cell type at a time, and there is no opportunity to utilize information obtained during the course of the imaging experiment to guide the cell selection and labeling. An attractive solution to these problems is the use of photoswitchable fluorescent proteins, such as the tdEOS and mEOS series (McKinney et al., 2009; Paez-Segala et al., 2015), for cell labeling. During whole-embryo imaging experiments, one could then use precise optical manipulation methods to photo-convert and mark cell populations exhibiting distinct behaviors. These cells could subsequently be isolated by micromanipulation for a subsequent genomic experiment. This strategy bypasses limitations in genetic labeling and enables characterization of new transient differentiation states mainly based on cellular behaviors measured in the imaging experiment.

Laser capture microdissection (LCM) uses focused UV and infrared laser beams to cut out and then capture single cells from fixed or frozen tissue sections (Pang et al., 2014; Redmond et al., 2014; Ribes et al., 2010) (Figure 3D). Compared with microfluidics, LCM suffers from limited throughput. However, this technique can directly provide spatial information for isolated cells in the context of the embryo's anatomy. More importantly, in conjunction with photo-conversion during an imaging experiment, it would be possible to precisely isolate cells with distinct dynamic behaviors observed during a whole-embryo imaging experiment. It is worth noting that a cryo-sectioning-based



Figure 3. Strategies for Constructing a High-Resolution Gene Expression Atlas of Animal Development

(A) A region of interest in an embryo containing diverse cell types (highlighted by different colors).

(B) Spatial information and spatial context are typically lost during the sample preparation process involved in micromanipulation and microfluidics.

(C) Single-cell genomic techniques provide extensive information about molecular complexity underlying cell-type diversity.

(D) Laser capture microdissection enables isolation of individual cells from tissue sections, providing spatial information for extracted cells. The white boxes highlight positions from which cells are isolated.

(E) Whole-embryo imaging-based techniques such as RNA-FISH measure single-cell expression patterns across the embryo with spatial context of gene expression preserved.

(F) A combination of genomics and imaging approaches yields a high-resolution genome-wide gene expression atlas of development.

method (tomo-seq) has recently been developed to map spatiotemporal gene expression during embryo development (Junker et al., 2014). In tomo-seq, fixed samples (such as embryos or tissue fragments) are serially dissected into small sections along each Cartesian axis, and RNA is extracted from each section. Barcoding enables pooling of RNA from multiple sections before RNA sequencing. Computer tomography algorithms are utilized for the inference and generation of 3D transcriptional maps based on the sectioning transcriptomics dataset. Although single-cell resolution is challenging to achieve through sectioningbased methods, this approach provides a universal technique for high-throughput assembly of developmental gene expression maps at high spatial resolution.

Microfluidics couples real-time imaging analysis with an automatic microfluidic chip device to separate individual cells (Islam et al., 2014; Marcus et al., 2006; Streets et al., 2014) (Figures 3A-3C). These techniques require much fewer cells than FACS and are more efficient and quantitative than micromanipulation. The possibility of incorporating downstream enzymatic reactions into the chip has the potential to increase the throughput of the library preparation process. Recently, a droplet-based microfluidic strategy (Drop-seq) has been developed to generate hundreds and thousands of single-cell RNA-seq libraries in parallel, at relatively low cost. In Drop-seq, samples (such as tissues or cultured cells) are dissociated to form single-cell suspensions. A droplet microfluidics device is used to encapsulate individual cells in single microparticles that deliver barcoded primers in nanoliter-scale aqueous compartments formed by precisely combining aqueous and oil flows. Each droplet is used as an enzymatic chamber for cell lysis and mRNA capture. After RNA hybridization, microparticles are pooled for reverse transcription and RNA-seq library generation. Due to the highly parallelized single-cell mRNA barcoding, it was possible to prepare ~450,000 single-cell RNA-seq libraries in a single experiment (Klein et al., 2015; Macosko et al., 2015). With this new method, the cell-type diversity in heterogeneous stem cell populations (Klein et al., 2015) or complex tissues, such as the retina (Macosko et al., 2015), can be characterized extensively at the single-cell level. Clearly, Drop-seq can be applied to efficiently identify and classify new cell types during development. However, several issues need to be considered before implementing this method in the context of developmental biology. First, to ensure single-cell and single-microparticle encapsulation, only about 2%-5% of the input cells are captured and eventually give rise to single-cell RNA-seq libraries. Thus, a large amount of starting cell materials (millions of cells) is needed to achieve sufficient sampling coverage. To this end, multiple embryos at the same developmental stage may need to be pooled to perform such experiments. Secondly, both mRNA capture and DNA conversion rates are about \sim 10%. This means that most low-abundance genes are not detected in Drop-seq experiments. Another apparent limitation is that it is impossible to directly register the massive amounts of single-cell expression data generated by Drop-seq to specific, spatially defined cell populations identified in the course of an embryo imaging experiment. Such a registration likely requires the aid of the whole-embryo imaging-based methods described below.

High-Resolution Gene Expression Pattern in Whole Embryos

Whole-embryo imaging techniques (such as RNA-FISH or live gene expression kinetics assays) measure gene expression patterns and kinetics in intact embryos at the single-cell level (Figure 3E). Traditionally, RNA-FISH techniques can only be used to study the expression of a few genes at a time (Lecuyer et al., 2007; Little et al., 2013). It is worth noting that previous large-scale RNA-FISH efforts enabled the systematic mapping of gene expression patterns during fruit fly development (Lecuyer et al., 2007). However, this strategy requires the availability of large quantities of embryos for a parallel hybridization experiment. Due to the limited availability of embryos in higher organisms such as mouse, this strategy quickly becomes impractical.



Figure 4. Imaging Molecular Structure and Dynamics Underlying Complex Cellular Behaviors

(A) Actin-tdEos speckle image of an entire PtK1 cell with a corresponding fluorescent speckle microscopy flow map and an enlarged view of the lead-ing-edge flow. LM, lamella; LP, lamellipodium. Vector colors reflect flow speed (color bar). Scale bar, 10 μ m.

(B) Diffusivity map of a glycine receptor construct in a mouse hippocampal neuron from single-molecule tracking of an anti-GFP antibody coupled to Atto 647N. Top: 9,453 trajectories of the receptor construct superimposed with an ensemble (GFP) fluorescence receptor image. The color code distinguishes different trajectories. Bottom: diffusivity map superimposed with the corresponding Voronoi tessellation. Scale bars, 5 μ m.

(C) 3D imaging of heterochromatin (green; HP1-GFP) and transcription factor binding site organization (yellow dots; JF549-Halo-Sox2) in live embryonic stem cells by lattice light-sheet single-molecule imaging. 3D JF549-HaloTag-Sox2 single molecule imaging is performed with iterative Z scanning (300-nm steps) by lattice light-sheet microscopy. 7,000 stable Sox2 binding sites (yellow dots; residence time >6 s) are shown in the reconstructed image. Scale bar, 2 µm.

(D) Two-color extended-resolution SIM imaging of actin dynamics in a COS-7 cell. Skylan-NS-Lifeact is shown in green (PA NL-SIM) and mCherry- α -actinin in purple (TIRF-SIM). Scale bar, 5 μ m.

(A) adapted from Burnette et al. (2011) with permissiong from Nature Publishing Group; (B) adapted from El Beheiry et al. (2015) with permission from Nature Publishing Group; (C) adapted from Liu et al. (2014); (D) adapted from Li et al. (2015) with permission from AAAS.

With recent advances in barcoding imaging techniques and single-molecule imaging modalities, it is possible to measure the expression of hundreds of genes in individual cultured cells (Chen et al., 2015; Lubeck et al., 2014). Recently, the fluorescent in situ RNA sequencing (FISSEQ) technique has been developed to perform RNA sequencing for thousands of genes (Lee et al., 2014) in single cells. The FISSEQ workflow begins with fixing cells on a glass slide and performing reverse transcription in situ. After reverse transcription, cDNA fragments are circularized and amplified into diffraction-limited clusters containing multiple copies of the original cDNA sequence. For sequencing, randomized sequencing primers are hybridized to individual clusters, followed by ligation of fluorescent oligonucleotides. After imaging, the fluorophores are cleaved from the ligation complex and can be primed for the next round of sequencing. Currently, one technical limitation for FISSEQ is the lack of rRNA depletion. In regular cells, the rRNA reads comprise ${\sim}40\%\text{--}80\%$ of total detections and thus limit the current detection threshold for FISSEQ to about ${\sim}200\text{--}400$ mRNA molecules per cell. This means that probably only the most abundant cell-type-specific genes can be reliably detected. However, if technical challenges such as rRNA depletion, sample clarification, correction of sample-induced aberrations, background reduction, and more efficient fluorescent signal amplification can be overcome, it would be possible to use high-throughput RNA-FISH or FISSEQ methods to systematically register single-cell genomics data to generate a high-resolution, spatiotemporal gene expression atlas of development (Figure 3F). In addition, fluorescent reporter or single mRNA imaging systems have been deployed to study live-cell gene expression kinetics in vivo (Bothma et al., 2014; Garcia et al., 2013; Klochendler et al., 2012). Although only one or two genes can be measured at a time, these methods provide direct means to studying regulatory links between cellular dynamics and gene expression kinetics.

In summary, single-cell genomics techniques provide us with unbiased genome-wide gene expression information but generally require dissociation of the embryo into single cells, making it hard to trace the spatial origin of cells in the anatomical context of the embryo. Whole-embryo RNA-FISH-based gene expression measurements provide us with a relatively comprehensive view of spatial gene expression patterns across the embryo with single-cell resolution but currently can only be applied to study the expression of a few genes at a time. Thus, both genomics and imaging-based methods may need to be combined to gain a comprehensive and high-resolution gene expression atlas of development (Figure 3).

Imaging Molecular Dynamics in Single Cells

Cell-type-specific molecular systems encoded in animal genomes represent the foundational programs for directing cellfate commitment and maintenance of cell identity. Significant



Figure 5. An Integrated Approach toward Understanding Animal Development at the Single-Cell Single-Molecule Level

For next-generation developmental systems biology, multiple methodological approaches including genomics, whole-embryo imaging, and super-resolution imaging need to be combined to gain a comprehensive view of developmental processes and underlying mechanisms.

Top left: the transcription dynamics of a single reporter gene (hb) in a developing embryo were monitored by using 24 MS2 stem loops system at the single-cell, single-molecule level. The 24 MS2 stem loops in the mRNA are labeled with the MCP-GFP protein. One frame of the typical imaging field of view is shown at the bottom. Nuclei, red; transcription sites, yellow dots. Scale bar, 10 µm.

Top right: Fast, automated lineage-tracking and reconstruction of zebrafish embryogenesis using a SiMView light-sheet microscopy recording (Tomer et al., 2012) of a nuclei-labeled (H2B-eGFP) zebrafish embryo. A radially-symmetric spatial color code was assigned at the beginning of epiboly and then propagated in time by cell tracking to indicate the developmental origin of cells at later time points in development.

Bottom left: Differentially expressed genes across 39 retinal cell populations were systematically detected by Drop-seq at the single-cell level. In this heatmap, rows correspond to individual genes found to be selectively upregulated in individual clusters (p < 0.01, Bonferroni corrected); columns correspond to individual cells, ordered by cluster (1–39). Clusters with >1,000 cells were downsampled to 1,000 cells to prevent them from dominating the plot.

Bottom right: An example of 3D single-molecule tracking of HaloTag-Sox2 expressed in embryonic stem cells and labeled with tetramethylrhodamine using multifocus microscopy. Volume rendering of a Sox2 single-molecule image (purple) superimposed with single-molecule trajectories. Color bar shows the corresponding frame number. Scale bar, 2 µm.

Top-left panel adapted from Garcia et al. (2013), with permission from Elsevier. Top-right panel adapted from Amat et al. (2014). Bottom-left panel adapted from Macosko et al. (2015), with permission from Elsevier. Bottom-right panel adapted from Chen et al. (2014b).

insights into static snapshots of molecular composition and organization in the cell have been gained by using a combination of genomics, biochemistry, and structural biology. However, these techniques will not be able to provide detailed information about the kinetics and dynamics of molecular machinery as it operates in living cells. Observation of molecular structure and dynamics inside living cells is essential for a quantitative understanding of how precise spatiotemporal cellular dynamics are generated at the molecular level during animal development. With recent advances in molecular imaging and chemical dyes (reviewed in Liu et al., 2015a), it has become possible to perform single particle tracking (SPT) of individual protein molecules in single live cells (Abrahamsson et al., 2013; Chen et al., 2014b; Elf et al., 2007; Gebhardt et al., 2013; Grimm et al., 2015; Hager et al., 2009; Izeddin et al., 2014; Mazza et al., 2012; Mueller et al., 2013; Normanno et al., 2015). These fast high-resolution imaging methods provide means for visualizing and measuring the in vivo behavior of dynamically regulated transcription factor binding events at cis-regulatory DNA targets such as enhancers and core promoters. Once a DNAbinding protein reaches its target site, we can estimate how long the protein stays bound in order to produce the desired outcome such as transcriptional activation, chromatin remodeling, and even genome editing (Chen et al., 2014b; Knight et al., 2015; Normanno et al., 2015; Voss et al., 2011). Furthermore, it has become possible to perform non-invasive imaging of molecular structures in live specimens with high spatiotemporal resolution (Figure 4). For example, detailed maps elucidating the local diffusion pattern in highly heterogeneous, subcellular environments can be generated by live-cell SPT-based imaging and computational methods (Burnette et al., 2011; El Beheiry et al., 2015) (Figures 4A and 4B). Mechanical tension in the cell membrane can be imaged by FRET-based force biosensors (Leerberg et al., 2014; Morimatsu et al., 2013). Live-cell 3D molecular interaction maps can be obtained by an SPT strategy based on lattice light-sheet microscopy (Figure 4C) (Liu et al., 2014). Multi-color live-cell super-resolution imaging of actin dynamics can be achieved by a combination of total internal reflection fluorescence (TIRF)-structured illumination microscopy (SIM) and nonlinear-SIM (Figure 4D) (Li et al., 2015). These emerging high-speed molecular imaging platforms open up a unique opportunity for revealing the control logic and dynamics of molecular systems driving distinct cellular behaviors, and thus hold great promise for bridging currently missing links between molecular, cell, and developmental biology.

Outlook

In the future, combining whole-embryo imaging techniques with real-time image analysis and optical manipulation strategies would enable automated detection of cell populations with distinct dynamic characteristics and optical perturbation or labeling of particular cell populations for subsequent functional observation or genomics experiments. Correlating high-resolution cellular dynamics and gene expression atlases of development will systematically unmask candidate genetic pathways governing the spatiotemporal regulation of developmental dynamics. Super-resolution in vivo imaging of structural features and dynamics associated with a particular genetic pathway would first confirm the function of the pathway and then guide us toward an understanding of molecular control mechanisms and kinetics driving complex cellular behaviors.

Overall, emerging imaging and genomic techniques have paved the road for developmental biologists to elucidate dynamic and complex developmental processes with unprecedented detail at single-cell, single-molecule resolution (Figure 5). However, despite recent technological improvements in imaging and genomics, multiple technical challenges still lie ahead. First, automated and robust computational methods need to be developed for real-time analysis of cellular dynamics in large-scale image data generated by state-of-the-art systems-level imaging approaches. Second, more efficient strategies are needed for isolating specific cell populations discovered and tagged in live imaging experiments for downstream genomic analyses. Finally, next-generation deep-tissue imaging methods will be needed to investigate molecular and cellular dynamics in advanced developmental stages and perhaps even in adult tissues.

REFERENCES

Abrahamsson, S., Chen, J., Hajj, B., Stallinga, S., Katsov, A.Y., Wisniewski, J., Mizuguchi, G., Soule, P., Mueller, F., Dugast Darzacq, C., et al. (2013). Fast multicolor 3D imaging using aberration-corrected multifocus microscopy. Nat. Methods *10*, 60–63.

Amat, F., Lemon, W., Mossing, D.P., McDole, K., Wan, Y., Branson, K., Myers, E.W., and Keller, P.J. (2014). Fast, accurate reconstruction of cell lineages from large-scale fluorescence microscopy data. Nat. Methods *11*, 951–958.

Amat, F., Hockendorf, B., Wan, Y., Lemon, W.C., McDole, K., and Keller, P.J. (2015). Efficient processing and analysis of large-scale light-sheet microscopy data. Nat. Protoc. *10*, 1679–1696.

Baumgart, E., and Kubitscheck, U. (2012). Scanned light sheet microscopy with confocal slit detection. Opt. Express 20, 21805–21814.

Bothma, J.P., Garcia, H.G., Esposito, E., Schlissel, G., Gregor, T., and Levine, M. (2014). Dynamic regulation of eve stripe 2 expression reveals transcriptional bursts in living *Drosophila* embryos. Proc. Natl. Acad. Sci. USA *111*, 10598–10603.

Burnette, D.T., Manley, S., Sengupta, P., Sougrat, R., Davidson, M.W., Kachar, B., and Lippincott-Schwartz, J. (2011). A role for actin arcs in the leading-edge advance of migrating cells. Nat. Cell Biol. *13*, 371–381.

Chen, B.C., Legant, W.R., Wang, K., Shao, L., Milkie, D.E., Davidson, M.W., Janetopoulos, C., Wu, X.S., Hammer, J.A., 3rd, Liu, Z., et al. (2014a). Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. Science 346, 1257998.

Chen, J., Zhang, Z., Li, L., Chen, B.C., Revyakin, A., Hajj, B., Legant, W., Dahan, M., Lionnet, T., Betzig, E., et al. (2014b). Single-molecule dynamics of enhanceosome assembly in embryonic stem cells. Cell *156*, 1274–1285.

Chen, K.H., Boettiger, A.N., Moffitt, J.R., Wang, S., and Zhuang, X. (2015). RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science *348*, aaa6090.

Developmental Cell Review

Chhetri, R.K., Amat, F., Wan, Y., Hockendorf, B., Lemon, W.C., and Keller, P.J. (2015). Whole-animal functional and developmental imaging with isotropic spatial resolution. Nat. Methods *12*, 1171–1178.

Crosetto, N., Bienko, M., and van Oudenaarden, A. (2015). Spatially resolved transcriptomics and beyond. Nat. Rev. Genet. *16*, 57–66.

Denk, W., Strickler, J.H., and Webb, W.W. (1990). Two-photon laser scanning fluorescence microscopy. Science 248, 73–76.

Du, Z., Santella, A., He, F., Tiongson, M., and Bao, Z. (2014). De novo inference of systems-level mechanistic models of development from live-imaging-based phenotype analysis. Cell *156*, 359–372.

Durnin, J., Miceli, J., Jr., and Eberly, J.H. (1987). Diffraction-free beams. Phys. Rev. Lett. 58, 1499–1501.

Economo, M.N., Clack, N.G., Lavis, L.D., Gerfen, C.R., Svoboda, K., Myers, E.W., and Chandrashekar, J. (2016). A platform for brain-wide imaging and reconstruction of individual neurons. eLife 5, e10566.

El Beheiry, M., Dahan, M., and Masson, J.B. (2015). InferenceMAP: mapping of single-molecule dynamics with Bayesian inference. Nat. Methods *12*, 594–595.

Elf, J., Li, G.W., and Xie, X.S. (2007). Probing transcription factor dynamics at the single-molecule level in a living cell. Science 316, 1191–1194.

Fahrbach, F.O., Simon, P., and Rohrbach, A. (2010). Microscopy with self-reconstructing beams. Nat. Photon. *4*, 780–785.

Fowlkes, C.C., Hendriks, C.L., Keranen, S.V., Weber, G.H., Rubel, O., Huang, M.Y., Chatoor, S., DePace, A.H., Simirenko, L., Henriquez, C., et al. (2008). A quantitative spatiotemporal atlas of gene expression in the *Drosophila* blastoderm. Cell *133*, 364–374.

Fuchs, E., Jaffe, J., Long, R., and Azam, F. (2002). Thin laser light sheet microscope for microbial oceanography. Opt. Express *10*, 145–154.

Garcia, H.G., Tikhonov, M., Lin, A., and Gregor, T. (2013). Quantitative imaging of transcription in living *Drosophila* embryos links polymerase activity to patterning. Curr. Biol. *23*, 2140–2145.

Gebhardt, J.C., Suter, D.M., Roy, R., Zhao, Z.W., Chapman, A.R., Basu, S., Maniatis, T., and Xie, X.S. (2013). Single-molecule imaging of transcription factor binding to DNA in live mammalian cells. Nat. Methods *10*, 421–426.

Grimm, J.B., English, B.P., Chen, J., Slaughter, J.P., Zhang, Z., Revyakin, A., Patel, R., Macklin, J.J., Normanno, D., Singer, R.H., et al. (2015). A general method to improve fluorophores for live-cell and single-molecule microscopy. Nat. Methods *12*, 244–250.

Hager, G.L., McNally, J.G., and Misteli, T. (2009). Transcription dynamics. Mol. Cell *35*, 741–753.

Hashimshony, T., Wagner, F., Sher, N., and Yanai, I. (2012). CEL-Seq: singlecell RNA-Seq by multiplexed linear amplification. Cell Rep. 2, 666–673.

Heckscher, E.S., Long, F., Layden, M.J., Chuang, C.H., Manning, L., Richart, J., Pearson, J.C., Crews, S.T., Peng, H., Myers, E., et al. (2014). Atlas-builder software and the eNeuro atlas: resources for developmental biology and neuroscience. Development *141*, 2524–2532.

Henry, F.E., Sugino, K., Tozer, A., Branco, T., and Sternson, S.M. (2015). Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. Elife *4*, http://dx.doi.org/10.7554/eLife.09800.

Holekamp, T.F., Turaga, D., and Holy, T.E. (2008). Fast three-dimensional fluorescence imaging of activity in neural populations by objective-coupled planar illumination microscopy. Neuron 57, 661–672.

Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., and Stelzer, E.H.K. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. Science *305*, 1007–1009.

Islam, S., Zeisel, A., Joost, S., La Manno, G., Zajac, P., Kasper, M., Lonnerberg, P., and Linnarsson, S. (2014). Quantitative single-cell RNA-seq with unique molecular identifiers. Nat. Methods *11*, 163–166.

608 Developmental Cell 36, March 21, 2016 ©2016 Elsevier Inc.

Izeddin, I., Recamier, V., Bosanac, L., Cisse, I.I., Boudarene, L., Dugast-Darzacq, C., Proux, F., Benichou, O., Voituriez, R., Bensaude, O., et al. (2014). Single-molecule tracking in live cells reveals distinct target-search strategies of transcription factors in the nucleus. Elife 3, http://dx.doi.org/10.7554/eLife. 02230.

Ji, N., Milkie, D.E., and Betzig, E. (2010). Adaptive optics via pupil segmentation for high-resolution imaging in biological tissues. Nat. Methods 7, 141–147.

Junker, J.P., Noel, E.S., Guryev, V., Peterson, K.A., Shah, G., Huisken, J., McMahon, A.P., Berezikov, E., Bakkers, J., and van Oudenaarden, A. (2014). Genome-wide RNA tomography in the zebrafish embryo. Cell *159*, 662–675.

Keller, P.J. (2013). Imaging morphogenesis: technological advances and biological insights. Science 340, 1234168.

Keller, P.J., and Ahrens, M.B. (2015). Visualizing whole-brain activity and development at the single-cell level using light-sheet microscopy. Neuron 85, 462–483.

Keller, P.J., Pampaloni, F., and Stelzer, E.H.K. (2006). Life sciences require the third dimension. Curr. Opin. Cell Biol. *18*, 117–124.

Keller, P.J., Pampaloni, F., and Stelzer, E.H. (2007). Three-dimensional preparation and imaging reveal intrinsic microtubule properties. Nat. Methods *4*, 843–846.

Keller, P.J., Schmidt, A.D., Wittbrodt, J., and Stelzer, E.H.K. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. Science *322*, 1065–1069.

Keller, P.J., Schmidt, A.D., Santella, A., Khairy, K., Bao, Z., Wittbrodt, J., and Stelzer, E.H. (2010). Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. Nat. Methods 7, 637–642.

Klein, A.M., Mazutis, L., Akartuna, I., Tallapragada, N., Veres, A., Li, V., Peshkin, L., Weitz, D.A., and Kirschner, M.W. (2015). Droplet barcoding for singlecell transcriptomics applied to embryonic stem cells. Cell *161*, 1187–1201.

Klochendler, A., Weinberg-Corem, N., Moran, M., Swisa, A., Pochet, N., Savova, V., Vikesa, J., Van de Peer, Y., Brandeis, M., Regev, A., et al. (2012). A transgenic mouse marking live replicating cells reveals in vivo transcriptional program of proliferation. Dev. Cell 23, 681–690.

Knight, S.C., Xie, L., Deng, W., Guglielmi, B., Witkowsky, L.B., Bosanac, L., Zhang, E.T., El Beheiry, M., Masson, J.B., Dahan, M., et al. (2015). Dynamics of CRISPR-Cas9 genome interrogation in living cells. Science *350*, 823–826.

Kolodziejczyk, A.A., Kim, J.K., Tsang, J.C., Ilicic, T., Henriksson, J., Natarajan, K.N., Tuck, A.C., Gao, X., Buhler, M., Liu, P., et al. (2015). Single cell RNAsequencing of pluripotent states unlocks modular transcriptional variation. Cell Stem Cell *17*, 471–485.

Krzic, U., Gunther, S., Saunders, T.E., Streichan, S.J., and Hufnagel, L. (2012). Multiview light-sheet microscope for rapid in toto imaging. Nat. Methods *9*, 730–733.

Kumar, A., Wu, Y., Christensen, R., Chandris, P., Gandler, W., McCreedy, E., Bokinsky, A., Colon-Ramos, D.A., Bao, Z., McAuliffe, M., et al. (2014). Dualview plane illumination microscopy for rapid and spatially isotropic imaging. Nat. Protoc. 9, 2555–2573.

Lecuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., and Krause, H.M. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell *131*, 174–187.

Lee, J.H., Daugharthy, E.R., Scheiman, J., Kalhor, R., Yang, J.L., Ferrante, T.C., Terry, R., Jeanty, S.S., Li, C., Amamoto, R., et al. (2014). Highly multiplexed subcellular RNA sequencing in situ. Science *343*, 1360–1363.

Leerberg, J.M., Gomez, G.A., Verma, S., Moussa, E.J., Wu, S.K., Priya, R., Hoffman, B.D., Grashoff, C., Schwartz, M.A., and Yap, A.S. (2014). Tensionsensitive actin assembly supports contractility at the epithelial zonula adherens. Curr. Biol. *24*, 1689–1699.

Lemon, W.C., Pulver, S.R., Hockendorf, B., McDole, K., Branson, K., Freeman, J., and Keller, P.J. (2015). Whole-central nervous system functional imaging in larval *Drosophila*. Nat. Commun. *6*, 7924.

Li, D., Shao, L., Chen, B.C., Zhang, X., Zhang, M., Moses, B., Milkie, D.E., Beach, J.R., Hammer, J.A., 3rd, Pasham, M., et al. (2015). ADVANCED

IMAGING. Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics. Science 349, aab3500.

Little, S.C., Tikhonov, M., and Gregor, T. (2013). Precise developmental gene expression arises from globally stochastic transcriptional activity. Cell *154*, 789–800.

Liu, Z., Legant, W.R., Chen, B.C., Li, L., Grimm, J.B., Lavis, L.D., Betzig, E., and Tjian, R. (2014). 3D imaging of Sox2 enhancer clusters in embryonic stem cells. Elife 3, e04236.

Liu, Z., Lavis, L.D., and Betzig, E. (2015a). Imaging live-cell dynamics and structure at the single-molecule level. Mol. Cell 58, 644–659.

Liu, Z., Yang, C.P., Sugino, K., Fu, C.C., Liu, L.Y., Yao, X., Lee, L.P., and Lee, T. (2015b). Opposing intrinsic temporal gradients guide neural stem cell production of varied neuronal fates. Science *350*, 317–320.

Long, F., Peng, H., Liu, X., Kim, S.K., and Myers, E. (2009). A 3D digital atlas of *C. elegans* and its application to single-cell analyses. Nat. Methods 6, 667–672.

Lubeck, E., Coskun, A.F., Zhiyentayev, T., Ahmad, M., and Cai, L. (2014). Single-cell in situ RNA profiling by sequential hybridization. Nat. Methods *11*, 360–361.

Macaulay, I.C., and Voet, T. (2014). Single cell genomics: advances and future perspectives. PLoS Genet. *10*, e1004126.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell *161*, 1202–1214.

Mahou, P., Vermot, J., Beaurepaire, E., and Supatto, W. (2014). Multicolor twophoton light-sheet microscopy. Nat. Methods *11*, 600–601.

Marcus, J.S., Anderson, W.F., and Quake, S.R. (2006). Microfluidic single-cell mRNA isolation and analysis. Anal. Chem. 78, 3084–3089.

Mazza, D., Abernathy, A., Golob, N., Morisaki, T., and McNally, J.G. (2012). A benchmark for chromatin binding measurements in live cells. Nucleic Acids Res. *40*, e119.

McKinney, S.A., Murphy, C.S., Hazelwood, K.L., Davidson, M.W., and Looger, L.L. (2009). A bright and photostable photoconvertible fluorescent protein. Nat. Methods *6*, 131–133.

Morimatsu, M., Mekhdjian, A.H., Adhikari, A.S., and Dunn, A.R. (2013). Molecular tension sensors report forces generated by single integrin molecules in living cells. Nano Lett. *13*, 3985–3989.

Mueller, F., Stasevich, T.J., Mazza, D., and McNally, J.G. (2013). Quantifying transcription factor kinetics: at work or at play? Crit. Rev. Biochem. Mol. Biol. 48, 492–514.

Normanno, D., Boudarene, L., Dugast-Darzacq, C., Chen, J., Richter, C., Proux, F., Benichou, O., Voituriez, R., Darzacq, X., and Dahan, M. (2015). Probing the target search of DNA-binding proteins in mammalian cells using TetR as model searcher. Nat. Commun. *6*, 7357.

Paez-Segala, M.G., Sun, M.G., Shtengel, G., Viswanathan, S., Baird, M.A., Macklin, J.J., Patel, R., Allen, J.R., Howe, E.S., Piszczek, G., et al. (2015). Fixation-resistant photoactivatable fluorescent proteins for CLEM. Nat. Methods *12*, 215–218.

Pang, X., Hogan, E.M., Casserly, A., Gao, G., Gardner, P.D., and Tapper, A.R. (2014). Dicer expression is essential for adult midbrain dopaminergic neuron maintenance and survival. Mol. Cell Neurosci. *58*, 22–28.

Pantazis, P., and Supatto, W. (2014). Advances in whole-embryo imaging: a quantitative transition is underway. Nat. Rev. Mol. Cell Biol. *15*, 327–339.

Peng, H., Ruan, Z., Long, F., Simpson, J.H., and Myers, E.W. (2010). V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. Nat. Biotechnol. 28, 348–353.

Peng, H., Bria, A., Zhou, Z., Iannello, G., and Long, F. (2014). Extensible visualization and analysis for multidimensional images using Vaa3D. Nat. Protoc. 9, 193–208.

Developmental Cell 36, March 21, 2016 ©2016 Elsevier Inc. 609

Pietzsch, T., Saalfeld, S., Preibisch, S., and Tomancak, P. (2015). BigData-Viewer: visualization and processing for large image data sets. Nat. Methods *12*, 481–483.

Planchon, T.A., Gao, L., Milkie, D.E., Davidson, M.W., Galbraith, J.A., Galbraith, C.G., and Betzig, E. (2011). Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination. Nat. Methods *8*, 417–423.

Preibisch, S., Amat, F., Stamataki, E., Sarov, M., Singer, R.H., Myers, E., and Tomancak, P. (2014). Efficient Bayesian-based multiview deconvolution. Nat. Methods *11*, 645–648.

Redmond, L.C., Pang, C.J., Dumur, C., Haar, J.L., and Lloyd, J.A. (2014). Laser capture microdissection of embryonic cells and preparation of RNA for microarray assays. Methods Mol. Biol. *1092*, 43–60.

Ribes, V., Balaskas, N., Sasai, N., Cruz, C., Dessaud, E., Cayuso, J., Tozer, S., Yang, L.L., Novitch, B., Marti, E., et al. (2010). Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube. Genes Dev. *24*, 1186–1200.

Richardson, W.H. (1972). Bayesian-based iterative method of image restoration. J. Opt. Soc. Am. 62, 55–59.

Royer, L.A., Weigert, M., Gunther, U., Maghelli, N., Jug, F., Sbalzarini, I.F., and Myers, E.W. (2015). ClearVolume: open-source live 3D visualization for light-sheet microscopy. Nat. Methods *12*, 480–481.

Schiegg, M., Hanslovsky, P., Haubold, C., Koethe, U., Hufnagel, L., and Hamprecht, F.A. (2014). Graphical model for joint segmentation and tracking of multiple dividing cells. Bioinformatics *31*, 948–956.

Schmid, B., Shah, G., Scherf, N., Weber, M., Thierbach, K., Campos, C.P., Roeder, I., Aanstad, P., and Huisken, J. (2013). High-speed panoramic light-sheet microscopy reveals global endodermal cell dynamics. Nat. Commun. *4*, 2207.

Siedentopf, H., and Zsigmondy, R. (1903). Über Sichtbarmachung und Grö-Benbestimmung ultramikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser. Ann. Phys. *315*, 1–39.

Silvestri, L., Bria, A., Sacconi, L., Iannello, G., and Pavone, F.S. (2012). Confocal light sheet microscopy: micron-scale neuroanatomy of the entire mouse brain. Opt. Express 20, 20582–20598.

Stegmaier, J., Otte, J.C., Kobitski, A., Bartschat, A., Garcia, A., Nienhaus, G.U., Strähle, U., and Mikut, R. (2014). Fast segmentation of stained nuclei in terabyte-scale, time resolved 3D microscopy image stacks. PLoS One *9*, e90036.

Stegmaier, J., Amat, F., Lemon, W.C., McDole, K., Wan, Y., Teodoro, G., Mikut, R., and Keller, P.J. (2016). Real-time three-dimensional cell segmentation in large-scale microscopy data of developing embryos. Dev. Cell 36, 225–240. Stelzer, E.H. (2014). Light-sheet fluorescence microscopy for quantitative biology. Nat. Methods *12*, 23–26.

Streets, A.M., Zhang, X., Cao, C., Pang, Y., Wu, X., Xiong, L., Yang, L., Fu, Y., Zhao, L., Tang, F., et al. (2014). Microfluidic single-cell whole-transcriptome sequencing. Proc. Natl. Acad. Sci. USA *111*, 7048–7053.

Sugino, K., Hempel, C.M., Miller, M.N., Hattox, A.M., Shapiro, P., Wu, C., Huang, Z.J., and Nelson, S.B. (2006). Molecular taxonomy of major neuronal classes in the adult mouse forebrain. Nat. Neurosci. *9*, 99–107.

Swoger, J., Verveer, P., Greger, K., Huisken, J., and Stelzer, E.H. (2007). Multiview image fusion improves resolution in three-dimensional microscopy. Opt. Express *15*, 8029–8042.

Tomer, R., Khairy, K., Amat, F., and Keller, P.J. (2012). Quantitative high-speed imaging of entire developing embryos with simultaneous multiview light-sheet microscopy. Nat. Methods *9*, 755–763.

Truong, T.V., Supatto, W., Koos, D.S., Choi, J.M., and Fraser, S.E. (2011). Deep and fast live imaging with two-photon scanned light-sheet microscopy. Nat. Methods *8*, 757–760.

Visel, A., Thaller, C., and Eichele, G. (2004). GenePaint.org: an atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res. 32, D552–D556.

Voie, A.H., Burns, D.H., and Spelman, F.A. (1993). Orthogonal-plane fluorescence optical sectioning: three-dimensional imaging of macroscopic biological specimens. J. Microsc. *170*, 229–236.

Voss, T.C., Schiltz, R.L., Sung, M.H., Yen, P.M., Stamatoyannopoulos, J.A., Biddie, S.C., Johnson, T.A., Miranda, T.B., John, S., and Hager, G.L. (2011). Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. Cell *146*, 544–554.

Wang, K., Milkie, D.E., Saxena, A., Engerer, P., Misgeld, T., Bronner, M.E., Mumm, J., and Betzig, E. (2014). Rapid adaptive optical recovery of optimal resolution over large volumes. Nat. Methods *11*, 625–628.

Winter, P.W., and Shroff, H. (2014). Faster fluorescence microscopy: advances in high speed biological imaging. Curr. Opin. Chem. Biol. 20C, 46–53.

Wu, Y., Wawrzusin, P., Senseney, J., Fischer, R.S., Christensen, R., Santella, A., York, A.G., Winter, P.W., Waterman, C.M., Bao, Z., et al. (2013). Spatially isotropic four-dimensional imaging with dual-view plane illumination microscopy. Nat. Biotechnol. 31, 1032–1038.

Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C.Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y.E., et al. (2013). Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500, 593–597.

Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., Liu, P., Lian, Y., Zheng, X., Yan, J., et al. (2013). Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat. Struct. Mol. Biol. 20, 1131–1139.