Symmetry breaking in the early mammalian embryo: the case for quantitative single-cell imaging analysis

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**Abstract:** In recent years, advances in imaging probes, cutting-edge microscopy techniques and powerful bioinformatics image analysis have markedly expanded the imaging toolbox available to developmental biologists. Apart from traditional qualitative studies, embryonic development can now be investigated in vivo with improved spatiotemporal resolution, with more detailed quantitative analyses down to the single-cell level of the developing embryo. Such imaging tools can provide many benefits to investigate the emergence of the asymmetry in the early mammalian embryo. Quantitative single-cell imaging has provided a deeper knowledge of the dynamic processes of how and why apparently indistinguishable cells adopt separate fates that ensure proper lineage allocation and segregation. To advance our understanding of the mechanisms governing such cell fate decisions, we will need to address current limitations of fluorescent probes, while at the same time take on challenges in image processing and analysis. New discoveries and developments in quantitative, single-cell imaging analysis will ultimately enable a truly comprehensive, multi-dimensional and multi-scale investigation of the dynamic morphogenetic processes that work in concert to shape the embryo.

**Key words:** embryonic development / lineage tracing / single molecule dynamics / fluorescent probes / SHG nanoprobes / systems biology / pluripotency

**Introduction**

During preimplantation development, the zygote undergoes multiple cell divisions and transitions from the 2-, 4- and 8-cell blastomere embryo through the compacted 16- and 32-cell morula stages to the blastocyst. The blastocyst consists of two different cell populations: the inner cell mass (ICM), which gives rise to the embryo proper, and the trophectoderm (TE), which will develop into the placenta. The localization of the ICM on one site of the blastocyst cavity (the blastocoel) indicates the first appearance of the embryonic-abembryonic (Em-Ab) axis. In most non-mammalian species, asymmetry by maternal factors in the fertilized oocyte already determines somatic cell development (Grunert and St Johnston, 1996; van Eeden and St Johnston, 1999). In contrast, mammalian embryonic development is much more regulative and plastic upon experimental interference. Despite many studies examining the emergence of early developmental differences in the embryo, it remains debated whether the first asymmetry predicting lineage bias already occurs in blastomere embryos or whether cells are allocated depending on their position and polarity at the 16- to 32-cell stage (Dietrich and Hiiragi, 2007; Bischoff et al., 2008). Indeed, the removal of individual blastomeres or their rearrangement up to the 8-cell stage does not interfere with their potential to form a blastocyst, highlighting the developmental plasticity of these cells (Tarkowski, 1959, 1961; Hillman et al., 1972). While embryonic blastomeres seem to be identical cells, some studies suggest that the orientation of cleavage division in 2-, 4- or 8-cell stage embryos predisposes cells for developmental bias. Typically, research in the field of early developmental biology has mostly focused on gene expression levels, morphological differences or asymmetric localization of cell fate determinants, resulting in compelling but incomplete experimental evidence for a detailed understanding of the regulatory mechanisms in preimplantation embryos. Therefore, the exact timing and emergence of factors responsible for developmental differences predicting the contribution of cells to either the ICM or the TE still remains a major research question.

To get more insight into the dynamic processes responsible for early preimplantation cell fate decisions, there is a necessity to apply the most recent advancements in quantitative single-cell imaging analysis in experimental approaches with high accuracy in time and space. Historically, large systems have been studied by concentrating on smaller functional subunits rather than understanding the interplay between the hierarchies of biological organization. Focusing exclusively on an individual level of developmental organization can however provide only limited insight into the global understanding of the different levels of developmental complexity. Therefore, the quantitative analysis of large and complex systems like the developing embryo requires a systemic approach collecting quantitative biological information at different levels of complexity, and using this information to construct useful and insightful models that capture the observed behaviors of the developing embryo. Here,
In vivo lineage tracing to examine the correlation between cell lineage and cell fate

Tracking a cell and its progeny in space and time as it moves from an early to a more developed state provides a representation of the combination of events required for differentiation to a specified fate during development. Studying embryonic development in the very early phases up to a few cells can be quite demanding experimentally but its analysis is manageable given the low complexity of the preimplantation embryo. Individual, differentially labeled nuclei can easily be followed and their relative position and history can be assessed even by eye. More extensive studies of the complex cell dynamics involved in embryonic development up to later stages however require robust image segmentation and tracking algorithms that extract and follow cells with high spatial and temporal fidelity.

Proper lineage tracing calls for a technique that is capable of monitoring cell movements and morphological changes in precise locations in vivo. One category includes sparse cell labeling, involving either early stage dye and radioactive tracer injections or fluorescent protein expression driven by tissue-specific promoters (Kretzschmar and Watt, 2012). For example, when a mouse that expresses a Cre recombinase under a tissue-specific promoter is crossed with a mouse, which harbors a reporter protein flanked by a lox-STOP-lox DNA sequence, the reporter will only be activated in cells that express the promoter (Nowak et al., 2008). The experimenter should however take into account potential adverse effects on the development of the particularly sensitive early mouse embryo resulting from heat shock or drug inducible promoters and invasive dyes, respectively. Furthermore, although sparse labeling methods reduce the complexity of the lineage tracing analysis by focusing on the part of signal that is relevant (i.e. a subset of cells is fluoresently labeled), they lack information about neighbor cell conditions and overall cellular context.

To address these shortcomings, alternative methodologies for lineage tracing have been introduced, that capitalize on global cell labeling by expressing ubiquitously fluorescent proteins for the study of cell and population dynamics during various developmental processes. Indeed, nuclei or membranes labeled with non-modifiable fluorescent reporters have been employed in non-invasive lineage tracing studies to track lineage formation and the emergence of asymmetry in preimplantation embryos. For example, time-lapse imaging has been used in several reports to follow individual cells continuously expressing nuclear green fluorescent protein (GFP) from the 2-cell blastomere stage to the blastocyst (Kurotaki et al., 2007; Morris et al., 2010). While some experiments did suggest that the orientation of cleavage divisions in 2-cell or 4-cell blastomeres causes cells to have a certain propensity to contribute to embryonic or abembryonic regions (Bischoff et al., 2008), other studies reported that cell allocation appears to align with the ellipsoidal shape of the zona pellucida (ZP) (Kurotaki et al., 2007). A potential reason for this discrepancy could be due to the inherent limitation of global labeling techniques for lineage tracing: precise segmentation and tracking of individual cells is a challenging task given dense cell labeling.

This problem requires different labeling methods that minimize tracking uncertainties over prolonged time windows. The Brainbow multicolor labeling strategy addresses certain limitations of global cell labeling that arise when only one fluorescent protein is used. Here, three or four different fluorescent proteins, each flanked by two or three pairs of lox DNA sequences, are sequentially arranged in the transgene (Fig. 1A) (Livet et al., 2007). Accordingly, Cre recombination on pairs of lox sites results in the expression of multiple combinations of fluorescent proteins generating unique color labeling of individual cells when multiple copies of the Brainbow cassette are present (Fig. 1B) (Livet et al., 2007). The expression of transgenes under a ubiquitous or tissue-specific promoter enables continuous multicolor lineage tracing of individual progenitor cells to different tissues during development. In contrast to single color reporter systems, the Brainbow method can provide great insight in cell dynamics during development by enabling the visualization of the allocation of distinct cell populations derived from a common founder. The indelible labeling of the Brainbow method offers the advantage of tracking cells over an extended period of time during development. However, it lacks the ability to instantaneously label cells after Cre-lox recombination, making spatiotemporal cell tracking dependent on the maturation of the newly expressed fluorescent proteins. Still, in vivo imaging can be induced by crossing Brainbow animals with tamoxifen inducible CAG-CreER<sup>12</sup> mice allowing for the temporal control of Cre recombination. Using this technique in preimplantation mouse embryos, Tabansky and colleagues could visualize the contribution of individual blastomeres to different blastocyst lineages as well as post-implantation stages (Tabansky et al., 2013). They reported a significant bias in the contribution of labeled blastomeres to embryonic and extra-embryonic lineages for several embryos as early as the 4-cell stage (Tabansky et al., 2013).

An alternative labeling strategy to further minimize tracking uncertainties is the recently established PhOTO (photoconvertible optical tracking of) zebrafish. The PhOTO zebrafish method permits instantaneous, non-random cell labeling and tracking with high fidelity in space and time (Dempsey et al., 2012a, 2014). Here, sparse and global cell labeling techniques are combined by the constitutive expression of two distinct fluorescent proteins targeted to either the membrane or the nucleus with one of the two fluorescent proteins being the green-to-red photoconvertible Dendra2 (Fig. 1C) (Dempsey et al., 2012a). The non-invasive, instantaneous photoconversion of membrane- or nucleus-targeted Dendra2 allows for the precise tracking of converted red cells of interest during development. While the photoconverted red labeling of Dendra2 is non-permanent and offers the possibility of tracking the extent of cell division by the fluorescent intensity of individual cells, this temporary labeling does complicate long-term lineage tracing. Combining the strengths of both the Brainbow and the PhOTO methods could address this limitation. Together, these two techniques would enable precise and immediate labeling of individual founder cells paired with clonal multicolor labeled progeny tracking, ultimately providing a powerful tool for in vivo examination of the correlation between cell lineage and cell fate.

Regardless of the employed lineage tracing methodology, complete tracking of up to hundreds of cells over long periods of time in both sparse and global cell labeling techniques requires robust software for the visualization, annotation and semi-automated or automated analysis of the resulting large datasets. To address the need for powerful and reproducible analysis of complex spatiotemporal development, Amat and colleagues have developed an open-source framework for the...
segmentation and tracking of cell nuclei, that reached high accuracy and speed in the reconstruction of cell lineages in different organisms (fruit fly, zebrafish and mouse embryos) from different microscopy modalities (light-sheet and standard confocal microscopes) (Amat et al., 2014). The framework was tested against ground truth provided by the manual annotation of several experts resulting in high accuracy of segmentation and tracking. The development, continuous optimization and standardization of software for quantitative lineage tracing will further facilitate data analysis and reduce observation biases.

Quantitative imaging of transcription factor dynamics

Photoconvertible or photoactivatable proteins are powerful labeling probes for cell or protein tracking during in vivo imaging, since they offer the advantage of singling out labeled targets of interest by changing their fluorescence spectra. Of particular use is the fluorescence decay after photoactivation (FDAP) assay that enables the large-scale volume analysis of the diffusion behavior and turnover of a photoactivated or photoconverted population of molecules independently of newly synthesized proteins.

A recent study took advantage of applying the FDAP assay in vivo and investigated the kinetics of Oct4 (also known as Pou5f1), a key transcription factor (TF) controlling preimplantation development, fused to photoactivatable GFP (paGFP) (Patterson and Lippincott-Schwartz, 2002; Plachta et al., 2011). Selective photoactivation of Oct4—paGFP in nuclei of individual blastomeres at different stages of the developing mouse embryo was achieved by using femtosecond-pulsed, near-infrared laser (Pantazis and Gonzalez-Gaitan, 2007). Subsequently, photoactivated TF movement out of the nucleus was followed using time-lapse confocal imaging (Fig. 2A) (Plachta et al., 2011). The
Figure 2 Quantitative imaging methods to investigate molecule dynamics in single cells. (A) Schematic overview of the fluorescence decay after photoactivation (FDAP) assay. A defined fraction within one cell (e.g. the nucleus) is photoactivated or photoconverted followed by the analysis of fluorescent intensity in the nucleus (indicated by ‘region of interest’ (ROI)). The changes in fluorescent intensity represent the movement of the fluorescent proteins in and out of the nucleus, their degradation and their immobile fraction by time-lapse imaging. (B) Schematic overview of fluorescence correlation spectroscopy (FCS). Fluorescent molecules diffusing in and out of a limited focus volume over time are recorded (left graph). The detected signals are cross-correlated in a correlation curve (right graph). (C) Schematic overview of fluorescent recovery after photobleaching (FRAP). A defined region in the fluorescent nucleus of a cell is bleached followed by time-lapse imaging to investigate recovery of the bleached area (ROI) by neighboring fluorescent proteins.
quantitative analysis of Oct4–paGFP kinetics resulted in the discovery of two populations of cells with distinct Oct4 kinetics in early blastomeres before morphological (Kelly, 1977) or clear transcriptional differences are present (Guo et al., 2010): the population of cells with slow TF kinetics and a high immobile fraction were much more likely to become part of the eventual ICM of the developing embryo, and conversely, most cells that eventually make up the TE result from cells with fast TF kinetics and a low immobile fraction (Plachta et al., 2011).

Thus, the presence of TF kinetics heterogeneity as early as the 4-cell to 8-cell stage can serve as a predictive measure of developmental cell lineage allocation in early mammalian embryos (Pantazis and Bollenbach, 2012).

FDAP can also be used to study the kinetics of photoconvertible proteins. The key advantages of using photoconvertible proteins in developmental studies are: (i) targeted TFs can be visualized prior to photoconversion rendering them particularly amenable for high-throughput studies, and (ii) both the non-photoconverted as well as the photoconverted populations can be tracked in parallel, which could provide more refined FDAP kinetic analysis (e.g. potential differential nucleocytoplasmic distribution and degradation) than when using photoactivatable proteins. Yet, until recently, spatially confined photoconversion using high-power, pulsed laser illumination was extremely inefficient. Now, Dempsey and colleagues have reported a unique optical mechanism, termed primed conversion, where dual-wavelength continuous-wave illumination results in pronounced photoconversion of fluorescent proteins (Dempsey et al., 2015). Confined primed conversion can be implemented on a conventional confocal microscope and succeeds in precise targeting of single cells in complex 3D structures. Hence, this discovery opens the path toward new ways of imaging, facilitating accurate characterization of protein dynamics in vivo, such as spatially confined labeling of photoconverted TFs using primed conversion to study TF kinetics at different developmental stages over time.

A complementary small-scale volume method to study molecule dynamics in vivo is fluorescence correlation spectroscopy (FCS). FCS is a sensitive analytical method to study the diffusion of single molecules by measuring fluctuations in fluorescence in a stationary, single point measurement volume defined by the optical system (Fig. 2B) (Kim et al., 2007). In contrast to the large-scale volume FDAP analysis, FCS cannot provide any information on the immobile fraction of molecules. The obtained intensity values correspond to the average number of fluorescent particles diffusing over time in the defined volume. A recent study investigated the dynamics of individual Oct4-paGFP as well as other pluripotency-related TFs in vivo using FCS (Kaur et al., 2013). Interestingly, the results obtained from the sub-space observation of a limited amount of TF diffusion rates were consistent with the previously performed population analysis of TF kinetics using FDAP (Kaur et al., 2013).

To infer the global dynamic behavior of TFs from local FCS experiments in the mouse embryo, FCS will need to be combined with volumetric imaging approaches like light-sheet fluorescence microscopy. Applying such a methodology will allow parallel measurement of many volumes to estimate accurate diffusion coefficients and concentration maps of diffusing or interacting proteins in living biological samples at high temporal resolution (Wohland et al., 2010; Capoulade et al., 2011). The low phototoxicity of light-sheet illumination permits the observations to be performed over a long time and the frame rate of camera detectors define the maximum number of diffusion coefficients that can be measured.

**Visualization of chromatin dynamics in individual blastomeres**

Embryonic development is accompanied by many changes in nuclear architecture. For example, upon fertilization, the genomes of the gametes undergo intense chromatin remodeling to form a totipotent zygote (Hemberger et al., 2009). Furthermore, the differentiation of pluripotent cells, rich in euchromatin, to terminally differentiated cells coincides with a gradual establishment of repressive domains and chromatin compaction (Zhu et al., 2013). While it is generally assumed that chromatin organization and plasticity are correlated with cellular potency, their interdependence has not been experimentally addressed. Recently, two different imaging techniques, TALE-mediated genome visualization (TGV) and fluorescent recovery after photobleaching (FRAP), have been developed and these can provide more insight into the question by visualizing chromatin dynamics in vivo.

Genome organization is well coordinated in mammalian cells and affects the regulation of multiple genes. DNA fluorescent in situ hybridization (DNA-FISH) in fixed samples used to be the only method to study nuclear positioning of endogenous target sequences. To investigate changes in gene positioning and nuclear dynamics in vivo, Miyanari and colleagues have established a method called TALE-mediated genome visualization (TGV) (Miyanari et al., 2013). Here, transcription activator-like effectors (TALEs), DNA binding proteins that can be designed to bind specific target sequences, are fused to fluorescent proteins to visualize endogenous repetitive domains of interest in vivo. TGV can provide insight into the influence of genome architecture on gene regulation in vivo. However, the detection of single genomic loci would require more than one TALE per target sequence to gain high enough signal relative to background. Therefore, further optimization of this technique would be necessary to accomplish single gene resolution.

Another experimental procedure to study chromatin dynamics is fluorescent recovery after photobleaching (FRAP). FRAP is a method that involves bleaching of labeled molecules in a defined region of interest using high intensity laser illumination (Fig. 2C). The subsequent monitoring of the extent and speed of fluorescence recovery of the bleached region by the influx of non-bleached molecules from neighboring areas using time-lapse imaging provides information about the overall population dynamics (Axelrod et al., 1976). To visualize the mobility of core histones within single blastomeres in the preimplantation mouse embryo, Boskovic and colleagues injected mRNA encoding for GFP-tagged histones into zygotes and performed FRAP experiments in defined areas of individual nuclei at different embryonic stages (Boskovic et al., 2014). Their study revealed differences in chromatin mobility as development progresses: whereas high chromatin mobility is indicative of the totipotent 2-cell stage embryo, chromatin mobility decreased in later embryonic stages. Likewise, pluripotent cells in the ICM showed relatively higher chromatin mobility than TE cells at the blastocyst stage, signifying chromatin mobility as a measure of cellular potency (Boskovic et al., 2014).
In contrast to FDAP using photoactivation or photoconversion, FRAP analysis is limited to measuring relatively slow diffusion rates. In cases where the diffusion of a molecule of interest occurs during photobleaching, the bleached area will be expanded. The resulting deviation from the actual bleached geometry can pose a challenge to accurately measure kinetic parameters (Mueller et al., 2008). Both FRAP and FDAP measurements are typically performed in large volumes and can provide detailed information about population dynamics such as the overall immobile fraction. Still, care must be taken in interpreting FRAP results, as they are very sensitive to the selection of the model and to experimental assumptions (Mueller et al., 2008).

**Single molecule sensitivity with engineered nanoprobes**

The precise examination of individual molecule dynamics in single cells with excellent spatiotemporal resolution, with the goal to capture the complexity of the developing embryo, requires robust imaging probes to get more insight into the mechanisms underlying cell fate allocation. Currently used fluorescent probes for the characterization of biological targets in dynamic imaging experiments do however have some fundamental shortcomings. Single-photon excited fluorescence of fluorescent proteins often display a low signal-to-noise ratio (SNR) when expressed in the developing embryo (Fig. 3A). This observation is due to tissue autofluorescence: background emission of light caused by various cellular and tissue components when excited with visible light. Two-photon excited fluorescence, the emission of light by the simultaneous absorption of two photons of near-infrared light, can reduce the natural emission of light by biological structures, since it is less scattering and absorbing than visible light (Fig. 3B). Still, another issue that arises when performing fluorescence imaging is dye saturation; the fluorescence signal increases linearly with illumination intensity, but reaches an upper limit since the maximum number of photons that can be

![Figure 3](http://molehr.oxfordjournals.org/)

**Figure 3** Fluorescence excitation and emission of fluorescent proteins and signal generation of SHG nanoprobes. (A) In single-photon excited fluorescence, a photon with a frequency ω will excite a fluorescent protein resulting in emission of lower energy light (<ω) as fluorescence. (B) Two-photon microscopes can deliver two low-energy photons (both with frequency ω) to a fluorescent protein simultaneously after which higher energy fluorescent light is emitted, but still with lower energy than the two photons combined (<2ω). (C) In second harmonic generation (SHG), two photons (both with frequency ω) simultaneously strike a non-centrosymmetric nanocrystal, resulting in a virtual transition to a higher energy state followed by the emission of fluorescent light with twice the energy (2ω).
emitted by an excited fluorophore in a given time is restricted by its excited state lifetime that is typically only a few nanoseconds long. Therefore, dye saturation results in limited sensitivity of the fluorescent signal and reduced temporal resolution. In addition, perhaps the most significant limitation when applying fluorescent probes for long-term imaging is photobleaching. While photobleaching is used as a tool for FRAP experiments, the permanent loss of fluorescence signal limits the length of time that biological targets can be studied during conventional imaging. To overcome these deficiencies, various nanoprobes, whose photophysical properties are fundamentally different to genetically encoded fluorescent proteins, have been developed for \textit{in vivo} imaging. Some of the most prominent ones include quantum dots (Bruchez \textit{et al.}, 1998; Chan and Nie, 1998), upconverting nanoparticles (Chatterjee \textit{et al.}, 2008), fluorescent nanodiamonds (Igarashi \textit{et al.}, 2012) and second harmonic generation (SHG) nanoprobes (Pantazis \textit{et al.}, 2010), also reviewed in Pantazis and Supatto (2014).

Many of the nanoprobes mentioned above have been successfully applied \textit{in vitro} and \textit{in vivo}, but it is important to consider some limitations that need to be addressed to benefit their successful and reliable implementation. For example, quantum dots and upconverting nanoprobes require surface coating to avoid leakage of potentially toxic components to biological tissues that could have adverse effects on the well-being of the embryo. Furthermore, blinking, a large fluctuation in fluorescence of a probe under continuous excitation, is a recurring feature of quantum dots, which can complicate the detection and tracking of individual molecules over time. In addition, fluorescent nanodiamonds have wide emission spectra, limiting their combination with other fluorescent labels. In contrast, SHG nanoprobes do not display these limitations and could therefore provide a powerful alternative for long-term imaging (Pantazis \textit{et al.}, 2010). Still, a promising future application of most of these nanoprobes is dependent on their reliable production and precise targeting to biological molecules of interest to faithfully recapitulate endogenous single molecule dynamics.

SHG nanoprobes are non-centrosymmetric nanocrystals that lack an inversion symmetry (Pantazis \textit{et al.}, 2010). They can generate an appreciable SHG signal: a nonlinear optical process in which two photons with the frequency \( \omega \) interact with the nanocrystal and produce one photon of half the wavelength and twice the frequency \( 2\omega \) (Fig. 3C). Since SHG does not involve absorption of light (i.e. it only involves a virtual energy transition), they neither bleach nor blink, and the signal they generate does not saturate with increasing illumination intensity. The resulting contrast and detectability of SHG nanoprobes provide a unique alternative for fluorescence imaging \textit{in vivo} (reviewed by Dempsey \textit{et al.} (2012b)). For example, the use of SHG nanoprobes in second harmonic generation correlation spectroscopy (SHG-CS) would offer an effective method for the precise detection of single molecule dynamics over extended periods of time with up to a 100-fold lower detection limit than that of conventional FCS (Liu and Irudayaraj, 2013), whose analysis is often complicated by background fluorescence and photobleaching (Hansen and Harris, 1998; Thompson \textit{et al.}, 2011). Additionally, different SHG-capable materials have distinct spectral SHG signal intensity profiles and offer the possibility of detecting multiple SHG nanoprobe-labeled proteins of interest in parallel. Recent \textit{in vivo} studies demonstrated that barium titinate SHG nanoprobes are not toxic and produce efficient SHG signal in whole organism imaging (Pantazis \textit{et al.}, 2010; Culic-Viskota \textit{et al.}, 2012), making SHG nanoprobes promising candidates for long-term single molecule imaging and tracking of proteins \textit{in vivo}.

**Quantitative image analysis**

The diverse number of methods that have been developed for single molecule tracking and cell tracing require precise quantitative analysis to reveal a biologically relevant information. Even though the human eye still outperforms computer vision algorithms in complex cases, at least qualitatively, the sheer data size and collection rates of modern microscopes are turning the expanding palette of automated image analysis algorithms into an essential toolbox for every experimentalist. Besides being orders of magnitude faster in many circumstances, automated analysis is also intrinsically more quantitative and objective than the human eye, which is not very sensitive to small variations in intensity and shapes, while prone to subjectivity (Nattkemper \textit{et al.}, 2003), boredom and fatigue, and possible confirmation bias.

Image analysis starts with the extraction of objects of interest from the images by a process called segmentation (Gonzales and Woods, 2008; Qiang \textit{et al.}, 2010). Segmentation is the process of separating pixels belonging to the objects of interest (e.g. cells) from the irrelevant ones, such as those that belong to the image background or other parts of the signal that are of no interest for the analysis. In studies that involve following the evolution of objects over time (e.g. in cell lineage reconstruction), objects are linked throughout the complete series by tracking (Jaqaman \textit{et al.}, 2008). Global movements or deformations of the sample might require an alignment (or registration) step before individual features can be tracked (Wang \textit{et al.}, 2014). Furthermore, samples that are too large to fit in the field of view of the microscope might be imaged as a series of partially overlapping 3D tiles followed by algorithmic recombination by the process of mosaicing or stitching (Emmenlauer \textit{et al.}, 2009).

Once the objects of interest have been extracted from the images, calculation of large numbers of features for each of the objects allows for extended statistical analysis (Rines \textit{et al.}, 2008). Machine learning approaches can make use of the calculated feature vectors for object classification (e.g. for cell phenotyping, (Jones \textit{et al.}, 2009)), but also to make the segmentation of the objects more robust (Sommor \textit{et al.}, 2011).

An essential prerequisite for successful image analysis is that all steps in the workflow from sample preparation to image segmentation are optimized to collect the best possible images for the planned analysis (Ljosa and Carpenter, 2009; Roeder \textit{et al.}, 2012). In particular, the choice of the best microscope type and acquisition protocol is of paramount importance. Image preprocessing steps, such as filtering (Milanfar, 2013) or deconvolution (Sibirita, 2005; Goodwin, 2014), can make successive analysis even more robust by increasing resolution and SNR of the signal of interest, correcting aberrations (Van Der Voort and Strasters, 1995), and suppressing parts of the image that are of no value. Among the modern imaging technologies, light-sheet fluorescence microscopy has evolved to be the most promising technique to image complex 3D tissue structures at intermediate planar but good axial resolution (thus making it near isotropic) and at high speed (Steitzer, 2015). Thanks to the limited photodamage caused by the thin sheet of light at the focal plane, very long acquisitions are possible. To achieve isotropic resolution with good contrast even deep into large specimens like the developing embryo, several rotated 3D views can be acquired on the light-sheet microscope and merged using a multiview deconvolution algorithm post-acquisition to provide high information content in all regions of the specimen (Preibisch \textit{et al.}, 2014). Alternatively, near-isotropic resolution can
be achieved by two-photon scanned light-sheet microscopy (2P-SPIM) that achieves higher penetration depth than standard light-sheet microscopy at a comparable acquisition speed and photodamage level, by using ultrafast near-infrared laser pulses to create a two-photon excitation light sheet (Truong et al., 2011). Altogether, the light-sheet microscope is becoming the tool of choice for the study of embryo development. However, the high spatial and temporal resolution required to capture complex mechanistic processes during development can result in very large datasets that can easily reach several terabytes in size for a single acquisition.

In general, even simple visualization of large datasets requires dedicated software tools that intelligently load and display only the relevant part of the data at the required resolution level in real time. Optimized access to the image data can also enable visualization of data at arbitrary angles. Both commercial and open source data viewers such as Imaris (http://www.bitplane.com), Arivis (http://www.arivis.com) and Big-DataViewer (Pietzsch et al., 2015) exist and can handle such large datasets. The open-source live 3D visualization software ClearVolume (Royer et al., 2011) even allows for the real-time visualization and analysis of light-sheet data while it is being acquired on the microscope.

Manual analysis of very large embryos is unfeasible and robust software tools for automated segmentation and tracking are being developed for such tasks (Truong and Supatto, 2011; Amat et al., 2014). However, even the best algorithms do not reach perfect accuracy and manual interaction with automatic segmentation and tracking can curate and correct errors; for instance, wrong cell feature linking can be corrected by manually rearranging tracks with commercial and open source data viewers. Active learning software can use the corrections provided by its human operator to update its internal model and avoid repeating the same mistakes in future runs (Beaver, 2011). Alternatively, a classifier can learn how to segment a complex tissue from examples provided by experts on some training data and then be used to automatically process large numbers of images in a batch processing mode (Sommer et al., 2011).

Discussion and conclusion

Recent developments in genetic and genomic techniques have provided much insight into the molecular mechanisms regulating various biological systems. However, many of these molecular approaches are based on static whole cell population analysis and offer limited information about heterogeneity within cell populations or single molecule dynamics and 3D organization in vivo. The current progress of developing quantitative single-cell imaging techniques is poised to offer precise single-cell and individual protein dynamic data with unmatched spatiotemporal resolution, giving unprecedented insight into the role of single molecule transcription factors and chromatin dynamics for lineage allocation during early embryonic development. The increasing acquisition of knowledge from cutting-edge quantitative imaging combined with current advances in single-cell genomics heralds an exciting period that will further accelerate the quest for a detailed understanding of the molecular mechanisms regulating early cell fate decisions in the mammalian embryo.

Spatiotemporal quantitative imaging in vivo is not merely limited to early embryonic development but can also be exploited as a tool to study development and regeneration in whole organisms with potential benefits for future biomedical applications (Dempsey et al., 2012a, 2015). The additional information acquired using these quantitative imaging techniques is critical for our understanding of the regulation of developmental processes in healthy and diseased states such as cancer. Studying dynamics within single cells combined with lineage tracing in vivo will provide more insight into the molecular identity of, for example, adult stem cells in mammalian tissues and their progeny. Together, spatiotemporal quantitative imaging will result in a detailed understanding of the molecular mechanisms responsible for maintenance of the stem cell state in different mammalian tissues as well as those regulating their differentiation in more specified cell types. By analogy, this approach will enable the study of the mechanisms behind uncontrolled de-differentiation to cancer cells. Information obtained from quantitative single-cell imaging can contribute to the identification of markers that can be used for the isolation of specific stem cell types or potential drug targets important for future use in regenerative medicine.

Authors’ roles

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Conflict of interest

The authors declare competing financial interests. Aspects of the work mentioned in this review are the subject of granted patents and patent applications filed by the Swiss Federal Institute of Technology in Zurich (ETH Zurich), Zurich, Switzerland and the California Institute of Technology (Caltech), Pasadena, CA, USA.

References


Beaver WM. Machine learning and the quantitative analysis of confocal microscopy with an application to the embryogenesis of drosophila melanogaster. UCSD, 2011. https://escholarship.org/uc/item/3n1479g.


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