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REVIEW

# Toward High-Content/High-Throughput Imaging and Analysis of Embryonic Morphogenesis

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**Summary: In vivo study of embryonic morphogenesis tremendously benefits from recent advances in live microscopy and computational analyses. Quantitative and automated investigation of morphogenetic processes opens the field to high-content and high-throughput strategies. Following experimental workflow currently developed in cell biology, we identify the key challenges for applying such strategies in developmental biology. We review the recent progress in embryo preparation and manipulation, live imaging, data registration, image segmentation, feature computation, and data mining dedicated to the study of embryonic morphogenesis. We discuss a selection of pioneering studies that tackled the current methodological bottlenecks and illustrated the investigation of morphogenetic processes in vivo using quantitative and automated imaging and analysis of hundreds or thousands of cells simultaneously, paving the way for high-content/high-throughput strategies and systems analysis of embryonic morphogenesis. genesis 49:555–569, 2011. © 2011 Wiley-Liss, Inc.**

**Key words:** live microscopy; data registration; cell segmentation; cell tracking; data mining; methods; developmental biology

## INTRODUCTION

Developmental biology is undergoing two important simultaneous transitions: from qualitative to quantitative science and from manual to automated experimental or analytical investigation. These transitions greatly benefit from recent technological advances in live imaging and computational analysis, as well as from strategies developed in other disciplines, such as in cell biology. The specific study of cellular processes shaping an embryo and the control of embryonic morphogenesis can be investigated in vivo with higher spatial and temporal resolution, from the subcellular scale to the level of entire tissue or organism, and with richer quantitative measurements. As in other domains of biology, automated and quantitative experimental approaches open the field to high-content and high-throughput investigations. The in vivo study of embryonic morphogenesis, recording large multidimensional imaging data and computing multiple relevant features for each embryo (high-content approach), and repeating this experiment on many embryos in various experimental conditions (high-throughput approach), will permit multiscale, systematic and statistical experimental investigations. Such approaches should improve our understanding of morphogenesis by investigating fundamental questions related to emergence, self-organization, stochasticity, plasticity, and robustness during embryonic development. It should allow researchers to study in details not only the average behaviors but also the variability between cell populations or embryos, and events or phenotypes that are rare, subtle, transient or unsynchronized. It also forces developmental biologists to define standards that are crucial for communication

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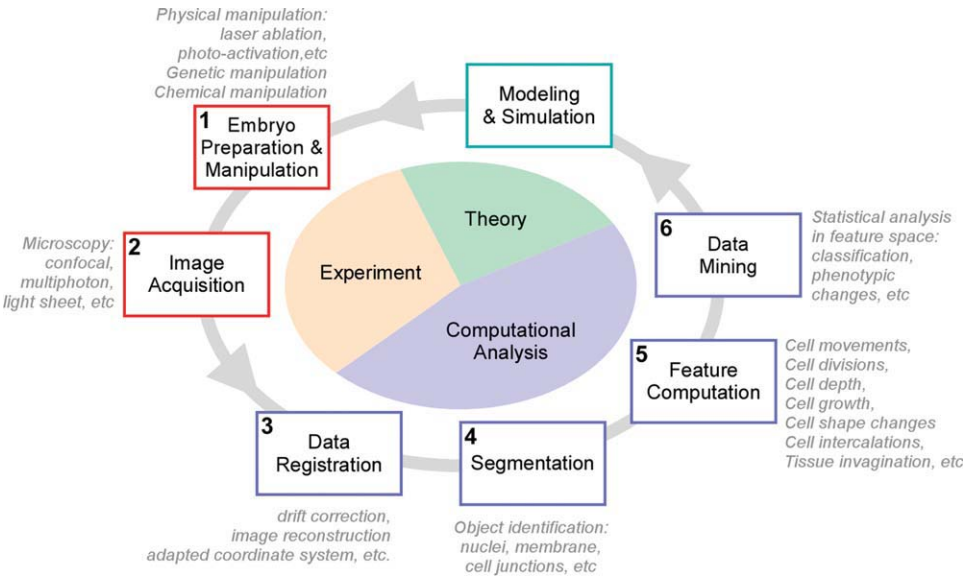


**FIG. 1.** Experimental workflow for high-content/high-throughput imaging and analysis of embryonic morphogenesis.

between disciplines and for proper interdisciplinary investigation. Finally, high-content/high-throughput approaches, such as the emerging strategy of in toto imaging of embryonic development (Megason and Fraser, 2003; Megason and Fraser, 2007) will provide a framework for integrative or systems biology. This review specifically focuses on in vivo and dynamic investigation of morphogenetic processes during embryonic development. We discuss the key challenges faced by high-content/high-throughput imaging and analysis of embryonic morphogenesis compared with its application in cell biology. Inspired by current similar approaches in the cell biology field, we decompose the typical experimental strategy into six steps: embryo preparation and manipulation, live imaging, data registration, image segmentation, feature computation, and data mining. We discuss a selection of pioneering studies tackling the current methodological bottlenecks and illustrating the investigation of morphogenetic processes in vivo using quantitative, automated imaging and analysis of hundreds or thousands of cells simultaneously, paving the way for high-content/high-throughput strategies.

## Overall Workflow of High-Content/High-Throughput Imaging and Analysis To Investigate Embryonic Morphogenesis

High-throughput imaging strategies have been recently developed in cell biology (Pepperkok and Ellenberg, 2006). In addition, many studies report imaging of millions of cells, computing of thousands of features and extracting biologically useful information using data classification within the multidimensional space of these features (see (Carpenter *et al.*, 2006; Held *et al.*, 2010; Loo *et al.*, 2007; Perlman *et al.*, 2004), for instance). The investigation of therapeutic effects of drugs based on large scale screens using automated imaging and analysis presented in (Loo *et al.*, 2007) is a striking illustration. In this study, the authors reported the automated identification of cell cycle phases after imaging millions of fluorescently labeled cells by measuring two features (nuclear surface and intensity of the fluorescence signal from the nuclei) and by classifying into several groups the positions of the cells within the 2D space of these features [see Supplementary Fig. 3 in (Loo *et al.*, 2007)]. The application of similar automated strategies in developmental biology would allow to study the behaviors of large cell populations within an embryo and to investigate morphogenetic mechanisms in a systematic and statistical manner. However, the transfer of methods from cell to developmental biology is not straightforward and raises specific challenges that will be discussed in this review. A few recent studies report large scale in vivo imaging and automated quantitative analysis of embryonic morphogenesis that partially or fully follow an experimental workflow inspired by what is currently done in cell biology. For clarity purpose, each step of this workflow (see Fig. 1) is briefly defined here and illustrated using the specific case of *Drosophila* mesoderm spreading (DMS) study presented in (McMahon *et al.*, 2008; Supatto *et al.*, 2009). Further discussion will be developed in the following sections of this review. This workflow involves six major steps (see Fig. 1):

### Step 1: Embryo Preparation and Manipulation

This step includes the choice of model system and of cell labeling, specific genetic or physical manipulations

and mounting procedure for imaging. In the DMS case, *Drosophila* embryos with nuclear labeling using ubiquitous transgenic expression of Histone2A-GFP were used to follow cell trajectories and divisions during mesoderm spreading. The *klarsicht* mutant was used to improve optical properties and imaging depth penetration. The specific morphogenetic phenotype of FGF receptor mutant (*heartless*) was compared with wild-type behavior. Embryos were mounted using standard procedure permitting stable imaging over 3-hour periods.

### Step 2: Image Acquisition

In vivo imaging of embryos in 2D or 3D is carried out with suitable and optimized microscopy technique or techniques with sufficient signal to noise ratio (SNR), spatial and temporal resolution, field of view, and depth penetration required for proper image analysis, in a manner that does not compromise normal biology and embryo viability. In the DMS study, embryos were imaged using 2-photon excited fluorescence microscopy. Each imaging dataset was made of 1 billion voxels, with up to 2000 cells imaged within the field of view during 2–3 hours of development, with subcellular spatial resolution and 45 second time resolution.

### Step 3: Data Registration

Data registration corresponds to any transformation applied to the experimental data permitting the comparison of one experiment to the other. In the DMS study, data registration corresponded to correction of embryo drift during image acquisition, temporal synchronization of sequences based on the onset of specific morphogenetic processes, and definition of specific coordinate system adapted to the embryo's body plan.

### Step 4: Image Segmentation

Segmentation is an image processing step aiming at identifying specific biologically relevant objects within the acquired images, such as nuclei, cell membrane, cell junctions, organelle, cytoplasm, etc. As in many recent quantitative studies of morphogenetic processes, the DMS investigation uses nuclear segmentation to identify the spatial distribution of cell centers in each successive 3D-image dataset of a sequence. In this study, up to 100,000 cell positions were segmented per embryo.

### Step 5: Feature Measurement

The features or descriptors are quantitative values that can be manually or computationally calculated to characterize the segmented objects. Although thousands of features can be computed in cell biology, study of embryo development involves many features that are specific to morphogenetic processes. In the DMS study, the features computed from 3D-nuclear segmentation data were cell trajectories (based on 3D-cell tracking), cell intercalation rate, and cell division rate, orientation and spatio-temporal patterns.

### Step 6: Data Mining

Data mining allows extracting meaningful information by performing statistical analysis in the multidimensional space of the features. For instance, to characterize wild-type and mutant phenotypes, the DMS study reported a basic step of classification based on the angular displacement of cells. This analysis permits (i) characterizing the wild-type phenotype involving collective and reproducible cell migration, and (ii) identifying two distinct behaviors (normal and disrupted) within the cells from mutant embryos.

Beyond limitations and advantages that are specific to model systems or biological questions that are investigated, the study of embryonic morphogenesis using high-content/high-throughput strategies generally faces many challenges beyond those encountered by similar studies in cell biology. First, the dynamic and multidimensional nature of morphogenetic processes with multiple time and spatial scales strongly limits the transfer of methods from cell to developmental biology. For instance, while many studies in cell biology are limited to the investigation of fixed cells in 2D (see (Loo *et al.*, 2007), for instance), the investigation of embryonic morphogenesis often requires in vivo, 3D and dynamic analyses (Fig. 2 and Supplementary Movie S1). In practice, the inhomogeneous properties of 4D imaging datasets often prevent the use of image processing or analytical methods that work well in cell biology. In addition, each step of the workflow requires new tools that are specific to developmental biology: specific microscopy techniques, specific registration procedures, specific feature measurements that are not shared with cell biology (such as cell intercalation and tissue invagination) or specific data mining techniques. Because of these challenges, most current studies, such as the study of DMS, are limited in term of data throughput and automation. In the following sections, we will review a selection of recent publications tackling the current methodological bottlenecks for each step of the workflow.

## EMBRYO PREPARATION AND MANIPULATION

The first step of the workflow includes the choice of labeling, the embryo manipulation, and mounting procedure for imaging (step 1 in Fig. 1). The labeling of specific cellular components is required for the segmentation step: fluorescent labeling of nuclei or membrane is usually first used to develop automated quantitative strategies (see Table 1). The embryo manipulation can be genetic [use of mutant to study specific morphogenetic phenotypes (McMahon et al., 2008)], chemical

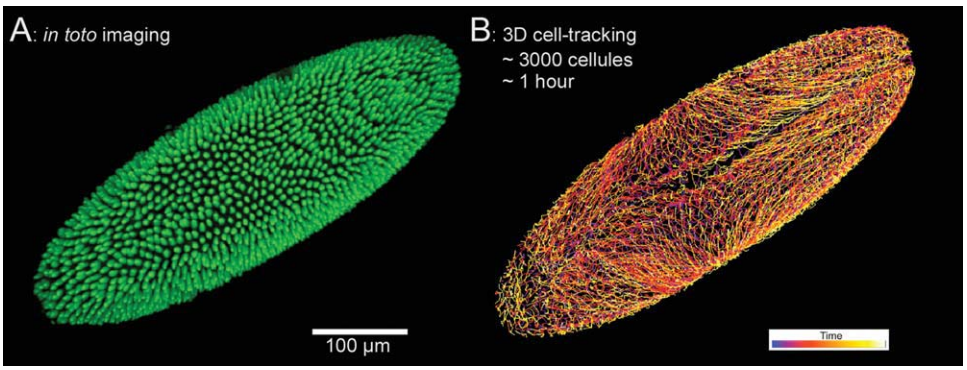


**FIG. 2.** In toto imaging of a *Drosophila* embryo during gastrulation. Commercial 2-photon excited fluorescence microscopy was used for 4D-imaging (**a**) of early *Drosophila* embryos labeled with Histone-GFP. The spatial sampling and 2 min-time interval between 3D-acquisitions were sufficient for 3D-segmentation and 3D-tracking of the nuclear positions (**b**). Supplementary Movie 1 provides animated version of this figure. Embryos were prepared using standard procedures (Supatto *et al*., 2009) and imaged using a TriMScope from LaVision BioTec, a Coherent femtosecond laser source at 940 nm wavelength and a 20x, 0.95NA water immersion Olympus objective. Imaris software from Bitplane was used for segmentation and tracking of nuclei.

[use of drugs as activators or inhibitors of specific signaling pathways (Lecaudey et al., 2008)], optical [fluorescence activation (Kicheva et al., 2007; Plachta et al., 2011), laser ablation (Hutson et al., 2003; Rauzi et al., 2008; Supatto et al., 2005), photo-induced release of caged compounds (Neveu et al., 2008; Ouyang et al., 2009)] or magnetic (Desprat et al., 2008). The mounting procedure critically depends on the model system and on the microscopy technique.

Although many tools already exist to improve this step of the workflow and to investigate specific questions about morphogenesis, the main challenge is how to integrate them into a high-throughput automated strategy. First, due to the often time-consuming nature of the embryo manipulation and mounting procedures, it is still difficult to image a large number of embryos in parallel or successively in an automated manner. However, recent studies reported high-throughput imaging and phenotyping of live embryos (Chung *et al*., 2008; Pardo-Martin *et al*., 2010). Pardo-Martin *et al*. (Pardo-Martin *et al*., 2010) demonstrated the automated mounting, orientation, optical manipulation, high-resolution imaging, and storage of zebrafish embryos using a fluidic system combined with a microscopy setup (see Fig. 3) enabling screening of up to 180 embryos per hour. In (Chung *et al*., 2008), worms were sorted at 900 worms per hour and innovative tools were developed to achieve efficient automated manipulation, such as the immobilization of worms without anesthetic drugs, using both mechanical immobilization with suction and brief cooling. These automated manipulations of embryos are promising for achieving rapid sequential imaging of several embryos. However, the investigation of morphogenesis often requires long term acquisition (from several minutes to several days) and sequential acquisition limit the number of embryos. Whereas the simultaneous imaging of embryos using several microscopes in parallel can be limited due to their cost, it is still possible in some cases to image several embryos in parallel using a single microscope. Specific embryo preparation and mounting procedures have been developed for this purpose (Megason, 2009; Yamagata *et al*., 2009). For instance, Yamagata *et al*. reported the simultaneous imaging of 168 early mouse embryos, imaged with 15 min time intervals between each 3D acquisition during 70 hours of development with a spinning disk confocal microscope and without apparent phototoxicity (Yamagata *et al*., 2009). They used superovulation and in vitro fertilization to prepare up to 200 embryos at once for imaging of preimplantation stages (Yamagata *et al*., 2009). However, this strategy requires embryos to be small and develop slowly to permit multiple acquisitions in parallel. Although early mouse embryos fulfill these requirements, it is hardly the case for older mouse embryos that are bigger or for other species that develop faster, such as *Drosophila*.

Even as the investigation of many embryos under different conditions is challenging and limits the experimental throughput, it is important to note that the high-content nature of the study of embryonic morphogenesis starts with the large number of cells imaged within each embryo during long periods of time. Pioneering studies in this field reports the study of only a few embryos; however, for each embryo hundreds to thousands of cells were followed simultaneously over a few or more hours of development (Tables 1 and 2), generating large image datasets encompassing hundreds to thousands of time points, each time point corresponding up to hundreds of millions of voxels. Handling and analyzing these large sets of data, coming from only a few embryo samples, going through the subsequent steps in the workflow depicted in Figure 1, is indeed already a huge challenge. For this reason, the first step of the workflow (embryo preparation, manipulation and

**Table 1**
*Imaging Parameters in Selected References Investigating Embryonic Morphogenesis Using Large Imaging Datasets*

| References | Model | Microscopy | Labeling | Size of imaging dataset (Go) | Field of view ($\mu m^3$) | Voxel size ($\mu m^3$) | Voxels per timepoint ($10^6$) | Time sampling (s) | Total time duration (h) | # of timepoints |
|---|---|---|---|---|---|---|---|---|---|---|
| (Bao *et al.*, 2006; Murray *et al.*, 2006) | *C. elegans* | Confocal | Histone-GFP | 4 | $64 \times 46 \times 30$ | 0.008 | 11 | 60 | 6 | 350 |
| (McMahon *et al.*, 2008; Supatto *et al.*, 2009) | *Drosophila* | TPLSM | Histone-GFP | 4 | $200 \times 200 \times 100$ | 0.25 | 16 | 45 | 3 | 250 |
| (Blanchard *et al.*, 2009) | *Drosophila* & zebrafish | Confocal | Cadherin-GFP | ? | ? | ? | ? | 30–120 | 0.8 | 100 |
| (England *et al.*, 2006) | Zebrafish | Confocal | Nuclear-GFP | ? | ? | ? | ? | 120 | 20 | 600 |
| (Olivier *et al.*, 2010) | Zebrafish | SHG and THG | Label-free | 4–10 | $900 \times 900 \times 440$ | 16 | 22 | 80 | 3 | 150 |
| (Keller *et al.*, 2008) | Zebrafish | DSLM | Histone-GFP | 3000 | $1000 \times 1000 \times 1000$ | 1.47 | 800 | 90 | 24 | 1000 |
| (Sato *et al.*, 2010) | Quail | Confocal and TPLSM | Histone-GFP | 20 | $1200 \times 1200 \times 10$ | ? | ? | 120–720 | 10 | 50–300 |

mounting) is presently adequate with respect to the rest of the workflow, and high-content investigation with automated and quantitative analysis starts with the study of a few embryos.

## EMBRYO IMAGING

As in cell biology, optical fluorescence microscopy appears as the method of choice for imaging live embryos with high spatial and temporal resolution and for both structural and functional imaging (step 2 in Fig. 1). However, properties of embryonic systems, such as speed of development, optical opacity, size, shape, and photo-sensitivity, set the limits of current microscopy techniques for live imaging of embryonic morphogenesis. So far, embryonic morphogenesis has been investigated in vivo using different microscopy techniques: standard or spinning disk confocal microscopy (Bao *et al.*, 2006; Blanchard *et al.*, 2009; Butler *et al.*, 2009; England *et al.*, 2006; Sato *et al.*, 2010), multiphoton microscopy (Helmchen and Denk, 2005; Olivier *et al.*, 2010; Sato *et al.*, 2010; Squirrell *et al.*, 1999; Supatto *et al.*, 2009) or light sheet microscopy (Huisken and Stainier, 2009; Keller and Stelzer, 2008). The improvement of in vivo imaging of large cell populations within embryos tremendously benefit from recent efforts to push microscopy to image deeper into scattering embryos (McMahon *et al.*, 2008), faster (Keller *et al.*, 2008), with a larger field of view (Keller *et al.*, 2008; Megason, 2009; Sato *et al.*, 2010), without fluorescent labeling (Olivier *et al.*, 2010) or with optimized excitation scheme (Olivier *et al.*, 2010). These improvements open the field to in toto imaging strategies (Megason and Fraser, 2003; Megason and Fraser, 2007) for investigating embryonic morphogenesis.

Beside the challenge of imaging deeper into scattering tissues or capturing larger field of view with fast dynamics, a specific challenge of high-content/high-throughput imaging of embryos compared with cell biology is the inhomogeneous quality of the imaging datasets. The properties of embryonic systems mentioned above determine image quality and are not only variable between species, but temporal and spatial variability exists within each embryo. For instance, the velocity of cells movements can be strongly variable depending on the organ or the developmental stage, requiring different time resolutions. Optical properties can also drastically change in time and space within a single embryo (Supatto *et al.*, 2009) resulting in inhomogeneous spatial resolution and signal levels. Hence, the challenge of high-content/high-throughput imaging of embryonic morphogenesis is not only to use or design a microscope that overcome the fundamental limitations but also to adapt the performance of the imaging technique depending on time and space, and to produce datasets with a quality as homogeneous as

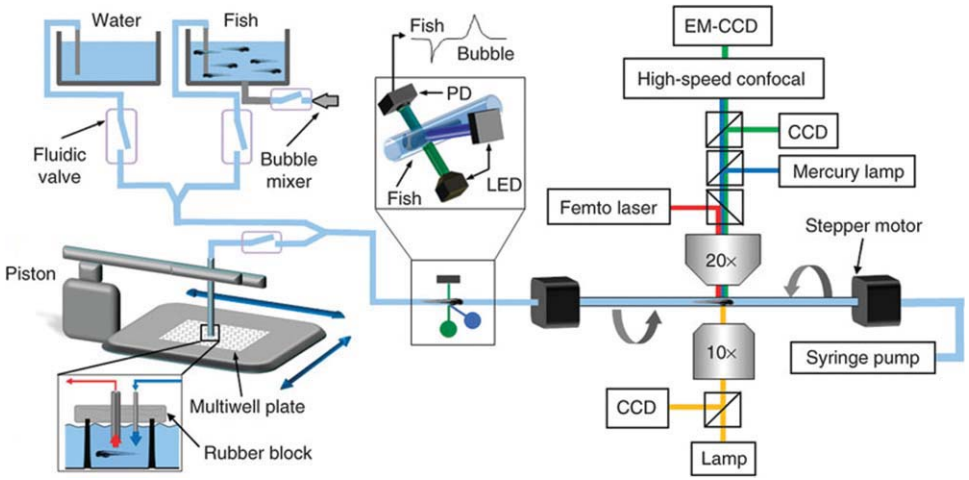


**FIG. 3.** High-throughput chemical and genetic screening of zebrafish larvae with a combined fluidic and microscopy platform. Larvae are automatically loaded onto the system for automated imaging and optical manipulation with confocal microscopy and femtosecond laser nanosurgery. More details on this high-throughput setup are in (Pardo-Martin *et al*., 2010). Reprinted by permission from Macmillan Publishers: Nature Methods, (Pardo-Martin *et al*., 2010), copyright (2010).

possible. Inhomogeneous image quality (such as spatial resolution, signal to noise ratio, or contrast) within multidimensional imaging datasets have drastic consequences on the accuracy and precision of subsequent image processing steps (see Fig. 1). For instance, anisotropic and inhomogeneous spatial resolution limits the accuracy and precision of segmentation and detection of specific 3D morphogenetic events. In this case, the performance of a basic algorithm designed to detect cell divisions would depend on where within the tissue and in which direction cells are dividing. For this purpose, specific efforts have been made to capture multidimensional imaging datasets of developing embryos with more homogeneous quality. Reduced photobleaching and homogeneous signal level were obtained using simple adjustment of laser power in time and space (Bao *et al*., 2006) or more sophisticated excitation scheme (Olivier *et al*., 2010). Olivier *et al*. adapted the scanning scheme of a mutiphoton microscope to the size and shape of the embryo: using slower scanning and longer acquisition in deep region of the embryos, they optimized the illumination to obtain homogeneous signal and reduced phototoxicity (Olivier *et al*., 2010). Preibish *et al*. reported isotropic spatial resolution obtained by acquiring imaging data with a light sheet microscope from multiple views and by reconstructing 3D datasets based on image registration (Preibisch *et al*., 2010). Multidirectional illumination and structured illumination have also been applied in light sheet microscopy to obtain even fluorescence excitation inside embryos (Huisken et al., 2007) and to homogenize signal contrast within the acquisition volume (Keller *et al*., 2010). Finally, the application of adaptive optics to correct for inhomogeneous optical aberrations within embryos is a promising direction for high-content/high-throughput imaging of embryonic morphogenesis (Booth, 2007; Jesacher *et al*., 2009; Ji *et al*., 2010; Rueckel *et al*., 2006). All of the aforementioned techniques still present limitations: for instance, multiview acquisition (Preibisch *et al*., 2010) or structured illumination (Keller *et al*., 2010) degrades the time resolution when they are applied to the entire embryo, even in places where they do not provide any improvement to image quality. As a consequence, future progress in high-content/high-throughput imaging of embryos will certainly benefit from the improved ability of microscope illumination and acquisition schemes to adapt to the changing properties of the developing embryos.

Photo-induced disruption of normal biology during imaging of embryonic morphogenesis is a key limiting factor that is usually underestimated or neglected, as illustrated by its poor representation in the literature. However, high-content imaging of embryos requires considerable amount of light irradiation and the potential perturbation mechanisms affecting normal development have not been fully described and understood. It is important to note that phototoxic effects depend on many parameters, such as laser power, wavelength, or excitation regime (continuous or pulsed). They can be related to various detrimental mechanisms such as heating, oxidative stress, photochemical reactions, or optical breakdown (Niemz, 2004). They can depend linearly or nonlinearly on the laser power (Hopt and Neher, 2001). Their manifestation can be obvious or subtle, from obvious cell death, tissue necrosis, or photobleaching, to subtle disruption of developmental dynamics, such as altered cell division patterns. Hence, better understanding of the nature of these phototoxic effects should allow optimizing illumination scheme and reducing photo-toxicity while maintaining constant

**Table 2**
*Analysis Parameters in Selected References Investigating Embryonic Morphogenesis Using Large Imaging Datasets*

| References | Model | Max # of cells | Segmentation (N: nuclei, M: membrane) | Features | Main biological question | 2D or 3D analysis? | Automated Segmentation? | Automated feature computation? |
|---|---|---|---|---|---|---|---|---|
| (Bao *et al.*, 2006; Murray *et al.*, 2006) | *C. elegans* | 350 | N | Cell movements<br>Cell divisions<br>Cell death | Cell lineage | 3D | Yes | Manual correction |
| (McMahon *et al.*, 2008; Supatto *et al.*, 2009) | *Drosophila* | 1000 | N | Cell movements<br>Cell divisions (position and orientation)<br>Cell intercalations | Role of FGF signaling pathway in mesoderm spreading | 3D | Manual correction | Manual correction |
| (Blanchard *et al.*, 2009) | *Drosophila* | 700 | M | Cell shape changes<br>Cell intercalations | Individual cell shape changes and cell integration in remodeling tissues | 2D | Yes | Yes |
| (England *et al.*, 2006) | Zebrafish | >100 | N | Cell movements<br>Tissue deformations | Forebrain morphogenesis and causes of cyclopia | 3D | ? | Yes |
| (Olivier *et al.*, 2010) | Zebrafish | 1000 | N and M | Cell movements<br>Cell divisions (position, duration, orientation)<br>Cell volume | Cell lineage and cell division pattern | 3D | Yes | Yes |
| (Keller *et al.*, 2008) | Zebrafish | 15000 | N | Cell movements<br>Cell divisions | Cell lineage and cell division pattern | 3D | Yes | Yes |
| (Sato *et al.*, 2010) | Quail | 1300 | N | Cell movements | Vascular morphogenesis | 3D | Manual correction | Manual correction |

signal, as demonstrated in the case of multiphoton microscopy (Ji *et al*., 2008). In general, phototoxicity and imaging invasiveness are difficult to investigate as their study requires interdisciplinary approaches. For instance, the noninvasiveness of novel microscopy techniques has been claimed and documented recently using embryo imaging datasets that actually exhibited abnormal development (Keller *et al*., 2010; Keller *et al*., 2008). In contrast, a fruitful interdisciplinary collaboration allowed successful observation of unanticipated disruptions of cell division waves during zebrafish early development due to the sample mounting procedure (Olivier *et al*., 2010). These examples illustrate that proper interdisciplinary investigation is critical to develop efficient high-content/high-throughput imaging and analysis of embryonic morphogenesis.

## DATA REGISTRATION

Data registration includes any transformation applied to the experimental data enabling researchers to compare one experiment to the other (step 3 in Fig. 1). This is a key step toward robust high-content/high-throughput approaches in developmental biology. Registration is crucial to make accurate quantitative measurements of features or to enable researchers to integrate the results from other laboratories. For instance, interspecimen registration to map cell location in *C. elegans* (Long *et al*., 2009) or gene expression pattern in *Drosophila* (Fowlkes *et al*., 2008) into canonical digital atlases despite significant variation in morphology or gene expression pattern between individuals illustrates the critical role played by data registration. For the specific study of morphogenetic processes, many experimental and analytical procedures refers to data registration: it includes image alignment (Liebling *et al*., 2005; Preibisch *et al*., 2009; Preibisch *et al*., 2010; Sato *et al*., 2010), drift correction (Supatto *et al*., 2009), temporal synchronization of image sequences (Blanchard *et al*., 2009; Supatto *et al*., 2009), definition of coordinate system adapted to the embryonic body plan or of dimensionless parameters describing morphogenetic processes (Bao *et al*., 2006; England *et al*., 2006; Quesada-Hernández *et al*., 2010; Supatto *et al*., 2009). Figure 4 illustrates experimental data analyses performed only after data registration: the reconstruction of the average spatial cell lineage on a prototypical embryo from multiple embryos (Fig. 4a,b) or the representation of cell division orientation distribution from multiple embryos on a single graph (Fig. 4c–e).

Temporal synchronization of imaging sequences is a critical step permitting the comparison of morphogenetic processes from one embryo to the other. In most cases, this temporal registration is based on the identification of morphogenetic events occurring at specific embryonic stage: for instance, the location of the cephalic furrow or of somites was used to synchronize image sequences in early *Drosophila* and zebrafish embryos, respectively (Blanchard *et al*., 2009). This registration can be automated and based on quantitative measurements: the onset germband extension in *Drosophila* was automatically detected based on 3D tracking of ectoderm cells and used as a time reference (Supatto *et al*., 2009).

Various techniques of spatial registration of imaging datasets have been applied in developmental biology to reconstruct 3D volumes from multiview acquisitions (Preibisch *et al*., 2010), to perform data stitching after tiled image acquisition of large fields of view (Megason, 2009; Preibisch *et al*., 2009; Sato *et al*., 2010) or to align datasets from several embryos using specific biological landmarks, such as the embryonic midline in early embryos (Blanchard *et al*., 2009; Supatto *et al*., 2009). Spatial registration is also required to correct for drift occurring during long-term imaging of developing embryos. For instance, *Drosophila* embryos that are glued to a coverslip to prevent drifting during long term imaging can still rotate within their vitelline membrane. This angular drift was corrected using spatial registration based on midline cells tracking (Supatto *et al*., 2009).

Importantly, data registration includes the definition of spatial positions or orientations within an embryo using a coordinate system adapted to the body plan or the tissue shape. It permits the identification of anterior-posterior or dorso-ventral axes of the embryo or the apico-basal and planar directions of epithelia. The use of appropriate coordinate system is specifically important for decomposing 3D movements of cells and revealing the spatial organization of morphogenetic processes in accordance to the body plan (McMahon *et al*., 2008; Supatto *et al*., 2009). A cylindrical coordinate system adapted to *Drosophila* body axes was obtained by fitting a cylinder on the ectoderm cell layer surrounding the embryo during gastrulation (Supatto *et al*., 2009). In *C. elegans*, cell lineage was defined depending on cell position within the body axes (Bao *et al*., 2006). In zebrafish, cell location was expressed relative to the embryonic surface (England *et al*., 2006). Figure 4c illustrates an example of such registration: the cell division angular orientations was defined in early zebrafish in respect to anterior-posterior and dorso-ventral axes (Quesada-Hernández *et al*., 2010). On the basis of appropriate coordinate system, the definition of dimensionless parameters that do not depend on embryonic size, such as angular positions or orientations (England *et al*., 2006; Quesada-Hernández *et al*., 2010; Supatto *et al*., 2009) is especially useful to compare between different embryos and study wild-type and mutant phenotypes (Fig. 4d,e).

From the practical point of view, data registration is usually applied directly to the imaging datasets (image-based registration, such as in (Liebling *et al*., 2005)) or landmark-based registration, such as using fluorescent bead in (Preibisch *et al*., 2010). However, data registra-

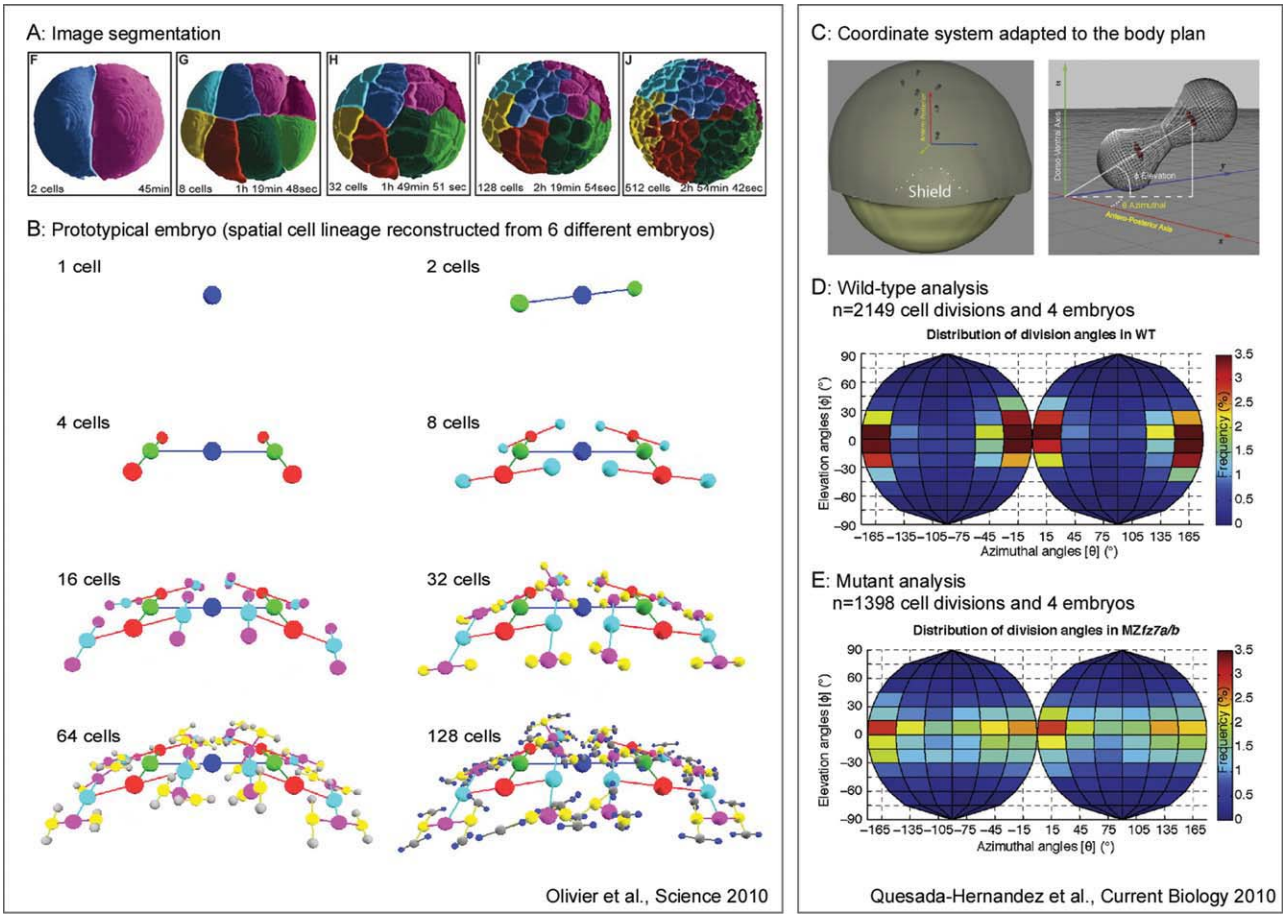


**FIG. 4.** Investigating cell lineage and cell division orientation in early zebrafish embryos. The early zebrafish embryo imaging using label-free nonlinear microscopy reported in (Olivier *et al*., 2010) allowed automated segmentation of cell shapes from 1 to 512-cell stage in (**a**) and automated reconstruction of a prototypical embryo and its spatial cell lineage from 6 different embryos (**b**). Using a coordinate system adapted to the body plan (**c**) (anterior-posterior and dorsal-ventral axes), the orientation distribution of thousands of cell division occurring during early zebrafish development are plotted for wild-type (**d**) and mutant embryos (**e**). Figures adapted from (Olivier *et al*., 2010) and (Quesada-Hernández *et al*., 2010).

tion can be performed during later steps of the workflow, based the segmented objects (step 4 in Fig. 1) or during feature computation (step 5 in Fig. 1). For instance, the position of specific cell populations, such as midline cells, can be used as biological landmarks for registration of cell trajectories (Supatto *et al*., 2009). The choice of references and of artificial or biological landmarks is critical in data registration. For instance, temporal synchronization of image sequences from wild-type and mutant embryos using the onset of specific morphogenetic processes requires these events not being disrupted in the mutant embryos. More generally, registration procedures are highly specific to imaging methods, model systems and biological questions and the comparison of analysis from different laboratories requires the choice of the same registration references. As a consequence, it is essential to explicitly describe these methods in publications and to define standard procedures to allow integration of data from different sources.

## SEGMENTATION

After image acquisition and data registration, the identification of relevant biological objects within images of embryos, such as nuclei, cell membrane, cell junction, or cytoplasm can be performed using image segmentation (step 4 in Fig. 1). Although specific morphogenetic features, such a tissue movements or deformation have been computed using techniques that are not based on image segmentation (Supatto *et al*., 2005; Zamir *et al*., 2006), segmentation appears necessary for any cell-based investigation of morphogenetic processes. The identification of cell as objects whose positions are tracked during development using nuclear or membrane segmentation constitute the primary step of most quantitative studies of morphogenesis using cell as a building-block of development (Table 2). The nucleus is probably the simplest structure to identify and segment within a cell. The center position of the segmented

nucleus can be used to define the spatial position of a cell for cell tracking and movement analysis. In addition, fluorescent labeling of nuclei provides a direct indicator for cell divisions. For these reasons, most large scale studies of embryonic morphogenesis currently rely on 3D segmentation and tracking of nuclei (Table 2). In various model systems, such as *C. elegans*, *Drosophila*, zebrafish or quail, hundreds to thousands of nuclei have been manually or automatically segmented and tracked in 3D to investigate embryonic morphogenesis (Table 2).

In a high-content imaging experiment, the identification of thousands of cells during thousands of time points in a single embryo means millions of objects need to be segmented per imaging dataset [5 million in (Keller *et al.*, 2008)]. In this context, fully manual segmentation is unrealistic and semi-automated segmentation (with automated processing and manual correction) strongly limits the number of embryos or the number of simultaneous cells that are investigated at once (McMahon *et al.*, 2008; Sato *et al.*, 2010; Supatto *et al.*, 2009). For this reason, high-content/high-throughput analysis of embryonic morphogenesis critically requires automated image segmentation.

Methods for automated segmentation are extensively developed in image processing laboratories, especially for 2D analyses in cell biology. The reader can find reviews comparing commercial and open-source software for segmenting (and tracking) of cells (Hand *et al.*, 2009) or describing cell segmentation methods including thresholding, template matching, watershed transform or model-evolution (Meijering *et al.*, 2009). Open-source tools, such as GoFigure (Gouaillad *et al.*, 2007; Megason, 2009), are specifically designed for segmenting cells in 3D during embryonic development. A few studies reported automated segmentation of nuclei in *C. elegans* (Bao *et al.*, 2006; Murray *et al.*, 2006) or of both cell nuclei and membrane in zebrafish (Olivier *et al.*, 2010; Zanella *et al.*, 2010). However, automated and efficient 3D image segmentation from large imaging datasets quickly reaches limitations, computationally and algorithmically, and remains challenging for several reasons. Beside the 3D nature of morphogenetic processes that usually prevents 2D approaches, the inhomogeneous nature of imaging datasets of embryos strongly limit the performance of segmentation algorithms. Although some inhomogeneities originate from labeling or imaging techniques (inhomogeneous labeling, SNR, spatial resolution, or signal contrast), others are intrinsic to embryonic development (variation in cell density, in cell movements, in cell shapes from one tissue to another, from one developmental stage to another, or between species). For instance, the size of nuclei can drastically vary throughout development, especially during early stages (see the variation in nuclear size in *C elegans* (Bao *et al.*, 2006)). Hence, accurate nuclear segmentation requires algorithms that are not sensitive to variation in nulear size. In general, it is crucial to characterize and improve the precision, accuracy (result comparison to a gold standard) and robustness (response to noise or inhomogeneities) of segmentation algorithms for applications in developmental biology. The evaluation of segmentation performance usually relies on the comparison with "gold standards" or "ground-truths". Often, these ground-truths were established by manual segmentation (Keller *et al.*, 2008), but this approach is applicable only for reasonably small datasets (where it is amenable to manual segmentation by a skilled worker). Thus, the efficient and robust establishment of ground-truths to facilitate the evaluation and improvement of segmentation algorithms is a current bottleneck in developmental biology, investigated by only a few studies (Zanella *et al.*, 2010). In parallel, the generation of more homogeneous imaging datasets using improved microscopy techniques (see Embryo Imaging section) should significantly help efficient automated segmentation for high-content/high-throughput analyses of embryonic morphogenesis.

## FEATURE COMPUTATION

Features are descriptors or quantitative values that can be manually or computationally estimated to characterize the segmented objects (step 5 in Fig. 1). Within the workflow, this step aims at automatically identify and quantify from large imaging datasets morphogenetic events occurring during development, such as cell trajectories or cell shape changes; rate or pattern of cell divisions; cell depth and growth; cell intercalations or tissue invagination. In cell biology, many different features have been computed from imaging data (Carpenter *et al.*, 2006) and can be used in developmental biology. However, some features, such as cell intercalation and tissue invagination are specific to morphogenetic processes and do not have equivalents in cell biology. Approaches for quantitative analyses of such features are still emerging in developmental biology.

Cell trajectories and cell divisions based on 3D nuclear segmentation and 3D-tracking are usually the first features that are computed from embryo image datasets (Fig. 2 and Table 2). Automated reconstruction of cell lineage has been obtained from such analysis in worm and zebrafish embryos (Bao *et al.*, 2006; Murray *et al.*, 2006; Olivier *et al.*, 2010). Interestingly, the comparison of cell lineage from different embryos in these studies enables the construction of average and prototypic embryonic development, as shown in Figure 4b. In addition, the recent literature is brimming with quantitative features describing morphogenetic processes that are manually measured or automatically computed (Table 3). At the cellular level, many features that are useful for investigating morphogenesis are common with those used in cell biology. For instance, (Tassy *et al.*, 2006)

**Table 3**
*Embryonic Morphogenetic Features Computed or Manually Measured from Imaging Datasets and Selection of Related Publications. This List of Morphogenetic Features and Publications is not Exhaustive*

| Morphogenetic features | Selected publications |
|---|---|
| Relative cell positions | (Campana *et al.*, 2010)[z,2] |
| Cell shape (geometric descriptors) | (Tassy *et al.*, 2006)[a,s,2] |
| Cell volume | (Olivier *et al.*, 2010)[z,2] |
| Cell death | (Bao *et al.*, 2006; Murray *et al.*, 2006)[c] |
| Cell topology (#of neighbors) | (Blankenship *et al.*, 2006; Zallen and Zallen, 2004)[d,1] |
| Planar cell polarity and polar order | (Aigouy *et al.*, 2010)[d,2] |
| *Cell divisions:* | |
| spatio-temporal pattern | (Bao *et al.*, 2006; Murray *et al.*, 2006)[w,2]<br>(McMahon *et al.*, 2008)[d]<br>(Keller *et al.*, 2008; Olivier *et al.*, 2010)[z,2] |
| Orientation | (McMahon *et al.*, 2008)[d]<br>(Gong *et al.*, 2004; Quesada-Hernández *et al.*, 2010)[z,1] |
| Asymmetry | (Bischoff *et al.*, 2008; Yamanaka *et al.*, 2010)[m,1] |
| cell cycle duration | (Olivier *et al.*, 2010)[z,2] |
| *Cell movements:* | |
| Trajectory | (Bao *et al.*, 2006; Murray *et al.*, 2006)[w,2]<br>(England *et al.*, 2006; Keller *et al.*, 2008; Olivier *et al.*, 2010)[z,2]<br>(Kulesa *et al.*, 2010; Sato *et al.*, 2010)[a]<br>(McMahon *et al.*, 2008)[d,2] |
| Random motion | (Benazeraf *et al.*, 2010)[a] |
| Flow velocity | (Aigouy *et al.*, 2010; Supatto *et al.*, 2005)[d,2]<br>(Zamir *et al.*, 2006)[a,2] |
| Planar cell intercalation | (Blanchard *et al.*, 2009)[z,d,2] |
| Radial cell intercalation | (McMahon *et al.*, 2008)[d,2] |
| Rosette formation | (Blankenship *et al.*, 2006)[d,2] |
| Tissue invagination / apical constriction | (Martin *et al.*, 2009)[d] |
| Tissue deformation | (Aigouy *et al.*, 2010; Supatto *et al.*, 2005)[d,2]<br>(England *et al.*, 2006, Blanchard, 2009 #476)[z,d,2]<br>(Sato *et al.*, 2010; Zamir *et al.*, 2006)[a] |

Model system: [d]drosophila, [z]zebrafish, [a]avian, [m]mouse, [w]c.elegans, [s]ascidia. [1]manual measurements.
[2]studies that specifically developed automated computation of features.

reported the use of geometric descriptors (cell elongation, sphericity, flatness, squareness, convexity, percentage of occupancy of the embryo) to quantitatively investigate the control of cell shapes during early ascidian embryogenesis. At the multicellular scale, features become more specific to developmental biology. For instance, the quantification of cell division spatio-temporal pattern in *Drosophila* (McMahon *et al*., 2008), asymmetry in mouse (Bischoff *et al*., 2008) or orientation in zebrafish (Quesada-Hernández *et al*., 2010) (Fig. 4c–e) enable developmental biologists to investigate key processes during embryonic morphogenesis. It is interesting to notice that such quantitative measurements are not only a validation of visual observations, but they can reveal patterns or organization that are not obvious without quantitative analysis: for instance, while spatial waves of divisions can be observed qualitatively by visualizing raw imaging data in early zebrafish (Keller *et al*., 2008; Olivier *et al*., 2010), only a quantitative analysis reveals such waves during mesoderm formation in *Drosophila* (McMahon *et al*., 2008) as they are almost impossible to detect visually. Importantly, simple measurements at cellular scales proved to be sufficient to investigate the state of a tissue at larger scales: (Zallen and Zallen, 2004) reported quantitative investigation of tissue ordering during *Drosophila* development based on basic cell topology quantification (the number of neighbors of each individual cell of the tissue); (Aigouy *et al*., 2010) investigated the mechanisms that couple planar cell polarity and tissue shape during *Drosophila* wing morphogenesis by defining quantitative features, such as nematic and polar orders. Basic measurements have been performed at the embryonic tissue level, such as cell population density (Campana *et al*., 2010) or tissue deformation (Supatto *et al*., 2005). The quantification of mesoscopic multicellular processes such as cell intercalation (Blanchard *et al*., 2009; McMahon *et al*., 2008), tissue invagination

(Martin *et al.*, 2009), or collective cell migration (Supatto *et al.*, 2009) are key to investigate fundamental morphogenetic events.

Among the quantitative features used to investigate morphogenetic processes (Table 3), many are still manually measured and are designed for specific model systems, developmental stages or biological questions. Hence, one of the main challenges of high-content/high-throughput analysis of embryonic morphogenesis is to design automated feature computation based on segmentation data and to define standard metrics that are shared within the entire community. Only a few methodological studies are dedicated to the design of feature computation algorithms applied in developmental biology: for instance, (Blanchard *et al.*, 2009) reported the quantification of cell shape changes, collective cell intercalation, tissue strain rates, and tissue rotation rate using automated computational tools applied to *Drosophila* and zebrafish embryos. It is important to develop algorithms quantifying stereotypical events of morphogenesis that are not specific to tissue, developmental stage or species. However, one specific challenge in developmental biology compared to cell biology for feature computation is the inhomogeneity in dynamics and geometry of morphogenetic processes throughout development and between species. For instance, cell intercalation is used by developmental biologists to describe morphogenetic processes occurring at many different time scales, spatial scales, and geometries (planar or radial intercalation) in different model systems, illustrating the difficulty in defining common and standard metrics. To improve the automated quantification of morphogenetic features, it could be useful to learn from other disciplines. For instance, statistical physics provides descriptors, such as order parameters, that could be used to describe ordering during tissue morphogenesis (Aigouy *et al.*, 2010; Pietak and Waldman, 2008); discrete quantifications of cell rearrangements could be inspired by recent work in continuous medium mechanics (Graner *et al.*, 2008).

## DATA MINING

Data mining is the final step of the high-content/high-throughput experimental workflow and aims at extracting biologically meaningful information from the large amount data generated (step 6 in Fig. 1). For instance, phenotypic classes of wild-type and mutant embryos can be automatically identified after computing specific morphogenetic features and performing a statistical analysis within the multidimensional space of these features. Although sophisticated data mining strategies have been developed in cell biology, such as using machine learning for feature classification (Held *et al.*, 2010; Loo *et al.*, 2007), development of similar analyses for the study of embryonic morphogenesis are still in its infancy. In most cases, qualitative or visual descriptions are used to extract specific biological insights from feature computation: for instance, in Figure 4d,e, the distribution of cell division orientation in wild-type and mutant zebrafish embryos are plotted in two different graphs and a color code helps the reader to visually classify the different patterns. Such visual classification was also provided in (McMahon *et al.*, 2008; Supatto *et al.*, 2009) to identify two different cell migration patterns, normal and disrupted, in *Drosophila* mutant embryos. Very few studies related to the investigation of embryonic morphogenesis report the use of automated data mining. For instance, (Campana *et al.*, 2010) presented automated cell type sorting based on spatial distribution features. They demonstrated the automated identification of epithelial enveloping layer cells from deep layer cells in early zebrafish embryos. This study provides a good example of accuracy and robustness testing of the automated analysis. It shows that accuracy depends on developmental stage and a robust algorithm is required for performing automated analysis during the entire developmental process.

Automated data mining will become critical to extracting biological insights from a large amount of computed features. Thus, the development of data mining specifically dedicated to embryonic morphogenesis is critical for future advance of high-content/high-throughput imaging and analysis strategies.

## CONCLUSIONS

The study of embryonic morphogenesis using high-content and high-throughput strategies is holding great promises. It will specifically permit large scale and systematic analysis of morphogenetic features in various experimental conditions, such as after genetic, chemical, mechanical or optical manipulations of the embryos. As reviewed here, many recent studies reported promising advances toward effective high-content/high-throughput imaging and analysis of embryonic morphogenesis. However, many aspects of the experimental workflow are still challenging and underdeveloped compared with cell biology and require improvement toward more quantitative and automated approaches. In addition, their current development depend on the biological model system: for instance, while many recent works illustrate the power of live imaging for investigating mouse embryonic morphogenesis (Kwon *et al.*, 2008; Morris *et al.*, 2010; Trichas *et al.*, 2011; Yamanaka *et al.*, 2010), most studies developing quantitative and automated strategies toward large scale analysis have been done using other model systems (Tables 2 and 3). The multidisciplinary nature of the experimental workflow presented in Figure 1, at the crossroad between microscopy, computer science and developmental biology, means that future progress relies on proper interdisciplinary investigations and synergistic interactions of large scientific networks. It is im-

portant to note that several approaches such as data registration and image segmentation have been discussed in this review from the developmental biology perspective, whereas a massive body of literature on these subjects from medical and computer science fields are available and should be investigated for an exhaustive interdisciplinary point of view. Finally, Figure 1 illustrates the next promising yet challenging step in the systems analysis of embryonic morphogenesis: the integration of high-content/high-throughput experimental and computed data into multicellular, multiscale modeling. This endeavor will take interdisciplinary cooperation and integration between biology, computer science, chemistry, and physics to a new level, and is beyond the scope of the current review. As demonstrated by Turing's classic paper on morphogenesis in the 1950s (Turing, 1952), the cross-discipline scientific interest and interdisciplinary nature of morphogenesis modeling have been recognized for some time, though key questions still remain in this field for the modern researchers, especially in the context of facilitating modeling with the high-content/high-throughput analyses discussed earlier. How to interface experimental results with modeling? How to feed the large amount of experimental and computed data generated by the workflow described in this review into modeling and simulations? Which modeling and simulation approaches would be most amenable? We believe the successful development of high-content and high-throughput strategies will begin to lay the foundation for addressing these questions, paving the way to a system level understanding of the control of embryonic morphogenesis.

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