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Image analysis for understanding embryo development: a bridge from microscopy to biological insights

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The digital reconstruction of the embryogenesis of model organisms from 3D + time data is revolutionizing practices in quantitative and integrative Developmental Biology. A manual and fully supervised image analysis of the massive complex data acquired with new microscopy technologies is no longer an option and automated image processing methods are required to fully exploit the potential of imaging data for biological insights. Current developments and challenges in biological image processing include algorithms for microscopy multiview fusion, cell nucleus tracking for quasi-perfect lineage reconstruction, segmentation, and validation methodologies for cell membrane shape identification, single cell gene expression quantification from *in situ* hybridization data, and multidimensional image registration algorithms for the construction of prototypic models. These tools will be essential to ultimately produce the multilevel *in toto* reconstruction that combines the cell lineage tree, cells, and tissues structural information and quantitative gene expression data in its spatio-temporal context throughout development.

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Introduction

Understanding the processes underlying animal embryogenesis that convert a single cell (zygote) into a multicellular organism formed by a rich diversity of cells organized in time and space should come from the quantitative reconstruction of 3D + time observations at all scales. The recent advances in microscopy technologies, biological markers, and automated processing methods are making possible a complete revolution in Developmental Biology, leading to the achievement of the long-standing goal of reconstructing embryogenesis by integrating cellular and molecular dynamics [1]. Breakthroughs in microscopy observation to achieve quantitative measurements at all levels of organization with the best spatial and temporal resolution [1–4,5••,6,7] rely on recently developed photonic microscopy concepts (nonlinear optics (NLO), selective-plane illumination microscopy (SPIM), photo-activated localization microscopy (PALM), stimulated emission depletion microscopy (STED), fluorescence resonance energy transfer (FRET), etc.) [8–10,11••], coupled with the development of new biological markers (fluorescent proteins, photo-activable compounds, fluorescent nanoparticles such as quantum dots). Deriving biological insights from microscopy observations largely relied so far on manual and fully supervised image analysis. The development of mathematical methods that aim to digitalize embryogenesis of animal models from 3D + time data acquired with state-of-the-art microscopy technologies is expected to revolutionize practices and provide a quantum leap in quantitative and integrative biology [2]. Automated processing and analysis are *sine qua non* conditions: methods should be designed to deal with the massive complex new data, and their efficiency is crucial to fully exploit its potential for biological insights [3]. Achieving the automated reconstruction in space and time of the cell lineage tree annotated with quantitative information for cell shape is a major challenge. Then integrating cellular and molecular dynamics might be achieved by first reconstructing the spatio-temporal dynamics of gene expression [12••]. To achieve such goals, the development of sophisticated image processing methods is a major issue. Integrating these methods in data management workflows including new formal analysis methodologies and tools is then the next bottleneck. In this context, Developmental Biology is becoming a new interdisciplinary field where biologists' verbal descriptions are turned into more quantitative and formal descriptions amenable to automated quantitative analysis and comparison.

We propose for this emerging field the following equation:

$$\begin{aligned}\text{Further biological insights} &= f(\text{biological makers and probes} \\ &\times \text{microscopy technology} \\ &\times \text{image processing efficiency} \\ &\times \text{quantitative data mining and analysis efficiency})\end{aligned}$$

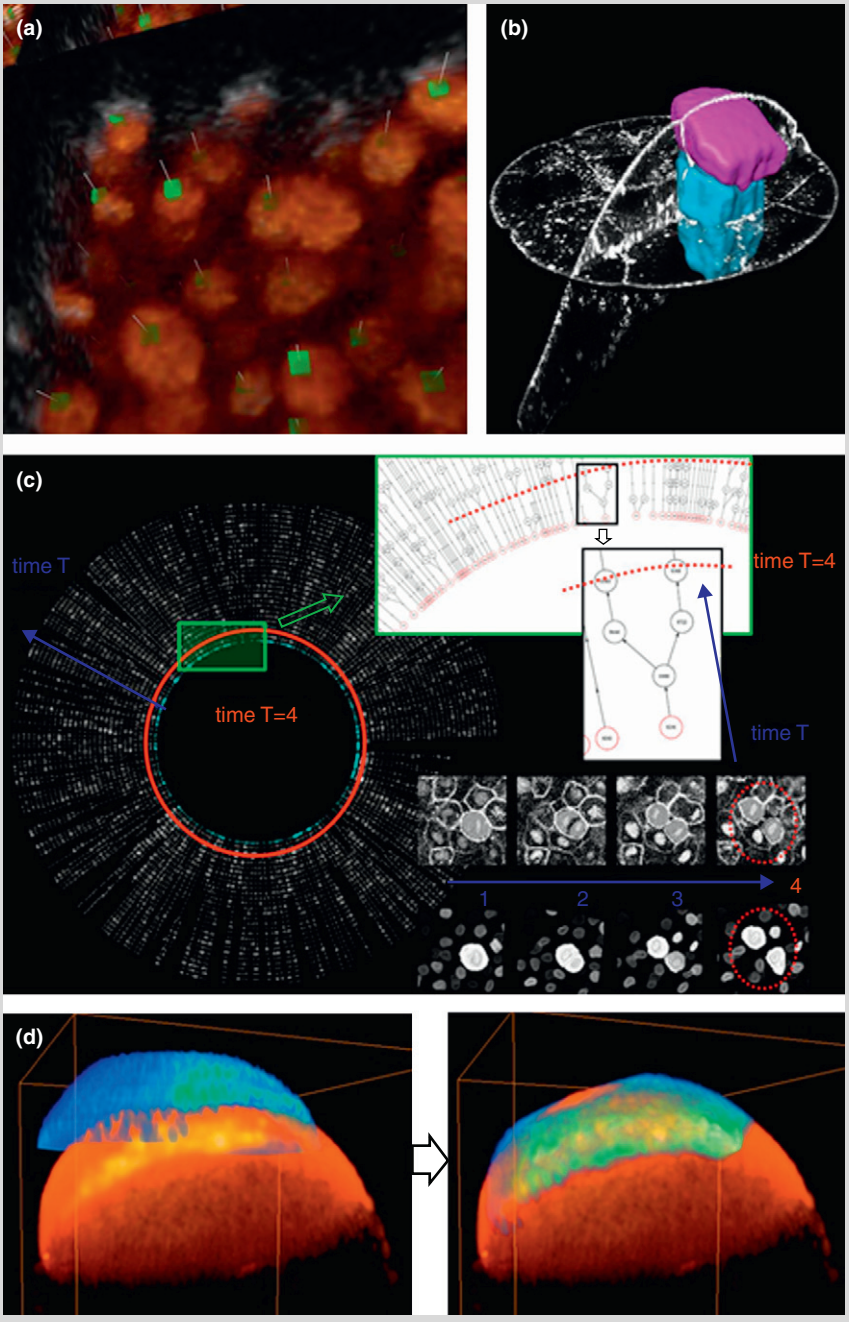
Box 1 Image processing methods for quantitative biology

(a) Cell detection: To identify the position of a cell. Usually this position corresponds to the gravity center of cell nuclei. In the figure, the position of identified cells is specified with a small green box.

(b) Cell segmentation: To identify the set of voxels that belong to one cell. Segmenting a cell from an image containing cell membranes allows extracting the shape. In the figure, the voxels corresponding to two different cells have been labeled into two colors.

(c) Cell tracking: To find the position of an individual cell in consecutive time steps. It allows reconstructing a graph with the cell lineage tree. It is usually performed by linking cell positions that have been previously detected for each time step of the sequence. In the figure, a lineage tree subgraph associated with a cell that divides in $T = 4$ has been highlighted.

(d) Gene expression registration: To find the geometrical transformation that allows aligning two different images based on a common element such as a gene expression pattern. In the figure, the gene expression rendered in blue has been aligned to the reference gene expression highlighted in orange.



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Within this framework, image processing methodologies are not yet standardized and depend on the type of images and biological questions to be answered. This implies the adaptation of generic image processing concepts such as segmentation or registration to specific biological problems (see Box 1 for a summary of the main image processing tools useful for quantitative biology). In this article, we outline the most relevant

Figure 1

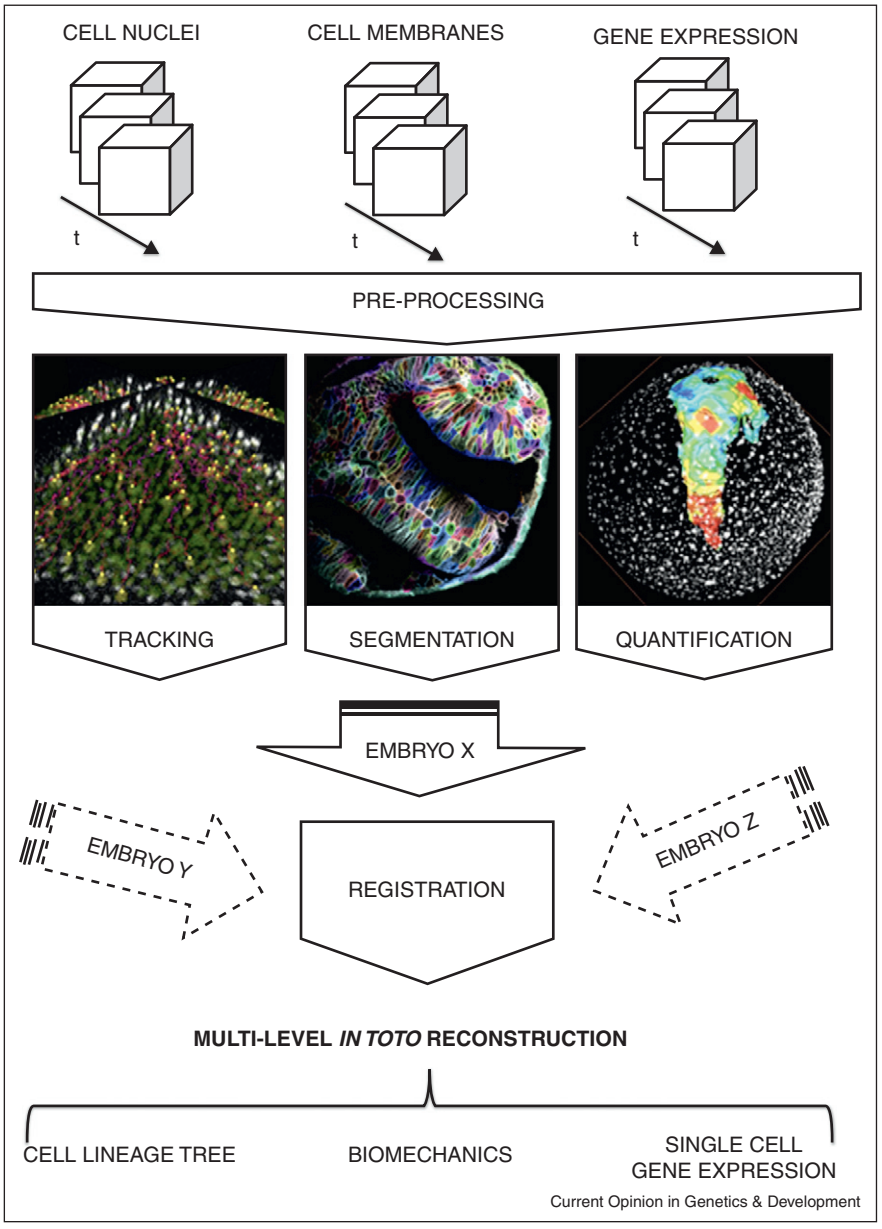

Image processing workflow for multilevel *in toto* embryo reconstruction. The 4D digital reconstruction of embryo development requires several image processing tools. We depict a general workflow for this task: a first preprocessing step that might include deconvolution, filtering, and multiview fusion. Cell detection and tracking algorithms to reconstruct the lineage tree, usually applied to an acquisition channel with cell nuclei. Image segmentation algorithms for cell shape extraction applied over an acquisition channel highlighting cell membranes (segmentation methods can be initialized using the cell positions identified in the lineage tree reconstruction). Signal aggregation methods for quantifying gene expression at the single cell level from a fluorescent transgenic line (measurements at the individual cell level are based on cell shape extracted by segmentation algorithms). Altogether, cell tracking, cell segmentation, and gene expression quantification at the single cell level produce an *in toto* reconstruction of embryo development. In order to build a 4D gene expression atlas from the combination of individuals from a cohort, image registration methods are used to align different embryos (based on common landmarks between individuals such as anatomical features or a reference gene expression pattern). In this way, a multilevel *in toto* analysis using the cell lineage, biomechanics, and genetic expression can be achieved.

steps of an image processing workflow for quantitative and integrative Developmental Biology as well as the main open challenges in the field.

In toto imaging modalities

The reconstruction of cell lineage and cell fate is a long-standing goal of embryology that might be achieved from *in toto* imaging for a number of model organisms throughout embryogenesis. However imaging a whole organism with subcellular resolution for extended periods of time is highly challenging for a number of reasons including tissues thickness and opacity. In addition, it is compulsory for further image analysis and cell tracking to face a compromise between information content in the deepest regions and imaging rate. Parallelized linear microscopy approaches such as light sheet based fluorescence microscopy (SPIM, dynamic light scattering microscopy (DLSM) and their variants) provide fast imaging but suffer from loss of information with depth [11••]. Point scanning two-photon laser microscopy (2P-LSM) provides deeper imaging but exhibits slower frame rates. Furthermore, the usual implementations of these two paradigms do not allow homogeneous illumination in nonplane (i.e. spherical) samples. This involves trading off the visibility of deep structures against the absence of illumination induced perturbation in superficial regions. The development of conformal scanning addresses this issue. Later trends point to improvements of spatial and temporal resolutions of SPIM/DLSM approaches, such as the use of structured illumination [10], two-photon excitation light-sheet techniques, that combines the advantages of both, better penetration as in 2P-LSM, and less photo-damage and higher frame rate (TV Truong, abstract in *SPIE Photonics West* 2011, 320), or self-reconstructing beam (MISERB) increasing both image quality and penetration depth of illumination beams in strongly scattering media [13].

Additionally, fluorescent proteins expression is generally weak and poorly localized at very early stages of embryonic development and the possibility to reveal subcellular structures with second and third harmonic generation signals (SHG and THG) in unstained specimens is very valuable [5••,14]. Latest trends would also allow combining light-sheet and second harmonic techniques (TV Truong, abstract in *SPIE Photonics West* 2011, 320).

Image reconstruction and enhancement: preprocessing techniques

Many different image processing techniques have been proposed to improve signal-to-noise as a preprocessing step. The choice of the filtering technique remains coupled to next stages of detection, tracking, and segmentations, because its validation depends on the final output. Nevertheless, contextual considerations about image content and work in artificial data may help this decision. Because the most dominant noise source in LSM imaging is modeled as a Poisson noise rather than a Gaussian noise, nonlinear filtering techniques are usually chosen for preprocessing, using partial differential equations (PDEs) and variational formulations [15,16•], mathematical morphology [17], diffusion filtering [18], or wavelet-domain thresholding techniques [19].

On the other hand the SPIM acquisition usually requires a multiview reconstruction scheme in order to generate a full volume from views acquired at different orientations, therefore fusing the areas with useful information from different views into a single volume [11]. This task has been approached extending the classic iterative deconvolution framework [20]. As point spread function (PSF) characterization could be challenging, non-PSF dependent methods have been proposed [21•], selecting the useful area of each view obtaining a proper weighting function, and obtaining a final volume as the weighted average of all the contributing views. Along with the multiview reconstruction another image processing problem has to be approached that is the prior alignment of all the different views. This step has been solved using the *a priori* knowledge of the acquisition orientation and an additional fine step using either pixel-based methods (U Kržič, PhD thesis, University of Heidelberg, 2009) or the detection and alignment of bead constellations [21•].

Lineage tree reconstruction: individual cell detection and tracking

The digitalization of the position of each cell along time, leading to the cell lineage tree sequencing is one of the core challenges of Developmental Biology. Most of the processing methodologies for lineage reconstruction rely on the same strategy: first detecting individual cells in each time frame and then linking the temporal information a posteriori. Cell nuclei detection [22] using image processing methods based on intensity maxima and shape and size constraints has successfully allowed the quantitative study of collective cell movements in Drosophila [23,24], zebrafish [25•] and quail [26]. More sophisticated methods for nuclei detection based on advection-diffusion equations [27] are more efficient with noisy images, but at the expense of higher computational cost. Generally, the main problem of cell identification arises when cells are very close to each other because of low optical resolution or anisotropic voxel size — so that processing methods have to be able to infer the cell frontiers inside cell clusters [28].

While global cell displacement can be analyzed without a precise lineage tree, the reconstruction of perfect lineages is a very sensitive task: small errors amplify along the lineage. In order to perform the lineage tree reconstruction, detected cells should be linked along time and the combinatorial problem of matching cells through time steps should be solved by deciding a strategy that balances between local and global optimization techniques

[29]. Adding some extra contextual information may be useful: for example, cell can die (apoptosis) or divide (mitosis), but a cell cannot have two parents except in the special case of fusion [30]. Thus, topological changes are limited and can be efficiently addressed by level sets approaches [31]. This contextual knowledge may also be used to correct the detection stage, when the addition or elimination of cells in certain positions provides more stable states in the linking process [32]. So far, accurate and complete lineaging of the whole organism has been only done for *Caenorhabditis elegans* [33•] until adulthood and for the very first developmental stages of the zebrafish [5]. The lineaging strategy followed in the later case consisted in designing an *ad hoc* processing pipeline that used contextual characteristics such as the number of cells in each cell cycle and symmetry of cell division. Furthermore, it proposed a system that validated each generation of the cell lineage tree before processing the following generation, so that errors do not propagate along the lineage.

Another alternative for lineage reconstruction is to process directly the 3D + time sequence as a unique 4D image. Particle tracking in a spatio-temporal volume through minimal paths has been applied to detect intermittent objects [34]. The evolution of the cell envelope during mitosis in 4D confocal images has been analyzed with a deformable surface model [35]. Zebrafish migrating cells have been modeled as hypertubes in the 3D + time space either using morphological operators [36] or PDE methodologies [37]. These techniques that require processing directly the whole spatio-temporal sequence will greatly benefit from progress in the mobilization of high computation power.

The validation step is one of the main bottlenecks of the cell lineage reconstruction strategies. Assessing tracking errors and correcting false negative and false positive errors by navigating in the 3D + time space remains a challenge. Future automated systems should provide a detection error rate tending to zero or point to possible errors and provide tools for an easy correction. We expect next breakthrough in 3D + time image quality to help reaching such a goal. We estimate that the complete zebrafish lineage up to 12 h of development could be quasi perfectly reconstructed with state-of-the-art processing methodologies when acquisition time for the whole volume drops down to less than 1 min with an isotropic voxel size of 1 μm^3, less than 0.5 μm optical axial resolution and optimal signal to noise ratio. In the next future, the availability of quasi-perfect cell lineages will open a new challenge: how to compare and measure similarities and differences between individuals? This question has been raised in species with stereotyped and small lineages such as *C. elegans* [38]. Investigating more complex and less deterministic lineages will open a completely new field that should adapt methodologies from information and graph theory [39,40].

Cell shape analysis: image segmentation algorithms

Image segmentation refers to the process of partitioning a digital image into multiple sets of pixels each one corresponding to a specific object of the image — that is locating the object boundaries. Several general methods for image segmentation have been adapted to cell biology problems [41]. When analyzing images of embryo development, depending on the imaging modality, it is possible to segment the cell nucleus shape, the cell membrane shape or both. Cell nucleus segmentation provides information about the cell progression through mitosis and has been used for the phenotypic profiling of hundreds of human genes [42]. However, given the typical spatial resolution when imaging a whole animal model such as zebrafish, the segmentation of cell nuclei does not give much more information than cell position identification. Segmentation of cell shapes directly from the acquisition channel with signal for cell membrane contours has been used to reconstruct the development of the apical meristem of *Arabidopsis thaliana* [43•] with a watershed-based segmentation method that inherently benefits from the fact that segmentation is done in parallel over the whole image and has low computational cost. In the context of vertebrate embryogenesis, the most coherent approach, given two acquisition channels with cell nucleus and cell membrane signals respectively, is to initialize membranes segmentation with the lineage reconstruction. Thus, a region growing method can be applied for the segmentation of each cell starting from the detected cell nucleus center. The viscous watershed extension provides more stable results than the standard watershed and can be used with multiharmonic imaging of zebrafish development [5••]. Cell membrane images from zebrafish development have been segmented using methods based on the numerical solution of PDEs [44]. Depending on the resolution and the developmental stage, it is also possible to segment suprastructures at the tissue level [45]. Whatever the strategy, the assessment of segmentation accuracy remains a major challenge, and very few studies compare segmentation results with a *gold standard* shape [16•]. The latter has to be produced by human experts using specialized software for manual 3D image segmentation [46].

Gene expression quantification: image registration techniques

Assessing gene expression patterns with resolution at the single cell level in the whole organism allows correlating the spatio-temporal gene expression with the control circuitry that specifies their occurrence and cell behaviors and cell shape changes underlying morphogenesis and differentiation. However, current techniques such as *in situ* hybridization (fluorescence *in situ* hybridization

(FISH)) [47] cannot label more than a few RNA species at a time. Matching a large number of gene expression patterns requires gathering data from different specimens into one common, canonical space where all the information can be simultaneously studied. To produce such 3D atlas of genetic expression requires the automated analysis of *in situ* data with the segmentation of individual cell shape and the quantification of gene expression assuming a linear relationship between the fluorescence intensity and the RNA concentration within a cell [48,49]. Image registration algorithms are then used to process datasets coming from different individuals differing in size and orientation to fit them into the same referential space. Most of the registration procedures described so far include an initialization algorithm that coarsely aligns anatomical landmarks such as body axes [12••,50]. Subsequent fine registration procedures include pixel-based alignment methods adapted from medical image analysis such as mutual information [51] or object-based registration [50,52]. This methodology has been applied for building single-cell resolution gene expression atlas in *C. elegans* [50], in Drosophila [12••] and at a smaller scale in zebrafish [51]. While the next generation of *in situ* hybridization techniques is expected to overcome the current limitation in the number of genes simultaneously analyzed [53], the most exciting challenge for image processing comes from the possibility of developing 4D atlas of gene expression through the use of transgenic animal lines. This will imply the development of registration strategies that allow aligning not only the geometrical features but also the developmental speed of the specimen. Working directly in the 3D + time domain would allow measuring the evolution of gene expression through time as well as its relationship with the cell lineage tree [54,55].

Perspectives

Image filtering, multiview fusion, nuclei detection, cell tracking, membranes segmentation, gene expression registration, etc., an increasing number of image processing tools allow combining the cell lineage, structural information, and quantitative gene expression data in their spatio-temporal context (see Figure 1). All these processing methodologies produce new opportunities for data analysis, process modeling, and thus biological insights. The availability of quasi-perfect cell lineages will allow the reconstruction of digital fate maps, highlighting the role of the cell lineage memory in stem cells properties and regenerative medicine. Accurate cell shape segmentation will help understanding the role of tissue deformation and mechanical forces during embryogenesis [1,4]. 4D image registration techniques will allow creating prototypes that integrate both cell lineage and gene expression data. Overall, efforts in generating spatio-temporal atlases of gene expression combined with the cell lineage tree data over the whole organism will lead to a multilevel *in toto* representation of the organism [56•] with each cell of the digital embryo represented by a set of points (x; y; z; t; s; g1; g2; g3;::: gn) where s corresponds to the cell shape and gi corresponds to the activity of a certain gene. In order to generate such a representation, several improvements with respect to the current state of the art should be done in image processing, with special attention to validation methodologies and visualization techniques [57]. It is important to remark that the use of the image processing methodologies outlined in this article requires a very close collaboration between biologists and specialists in image processing. Furthermore there is no silver bullet: each unique biological problem requires fine tuning a specific image processing system. Thus, probably the most crucial challenge is the integration of different perspectives at the crossroad of Biology, Engineering, Computer Science, Physics and Mathematics in order to propose the new methodologies and frameworks required by the post-genomic era.

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