Protocol

Creating 3D Digital Replicas of Ascidian Embryos from Stacks of Confocal Images

François B. Robin, Delphine Dauga, Olivier Tassy, Daniel Sobral, Fabrice Daian, and Patrick Lemaire

During embryonic development, cell behaviors that are tightly coordinated both spatially and temporally integrate at the tissue level and drive embryonic morphogenesis. Over the past 20 years, advances in imaging techniques, in particular, the development of confocal imaging, have opened a new world in biology, not only giving us access to a wealth of information, but also creating new challenges. It is sometimes difficult to make the best use of the recordings of the complex, inherently three-dimensional (3D) processes we now can observe. In particular, these data are often not directly suitable for even simple but conceptually fundamental quantifications. This article describes a process whereby image stacks gathered from live or fixed ascidian embryos are digitalized and segmented to produce 3D embryo replicas. These replicas can then be interfaced via a 3D Virtual Embryo module to a model organism database (Aniseed) that allows one to relate the geometrical properties of cells and cell contacts to additional parameters such as cell lineage, cell fates, or the underlying genetic program. Such an integrated system can serve several general purposes. First, it makes it possible to quantify and better understand the dynamics of cell behaviors during embryonic development, including, for instance, the automatic detection of asymmetric cell divisions or the evolution of cell contacts. Second, the 3D Virtual Embryo software proposes a panel of mathematical shape descriptors to precisely quantify cellular geometries and generate a 3D identity card for each embryonic cell. Such reconstructions open the door to a detailed 3D simulation of morphogenesis.

RELATED INFORMATION

Protocols are also available for **Time-Lapse Imaging of Live *Phallusia* Embryos for Creating 3D Digital Replicas** (Robin et al. 2011a) and Imaging of Fixed *Ciona* Embryos for Creating 3D Digital Replicas (Robin et al. 2011b). The basic steps of embryo reconstruction are outlined in Figure 1.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Equipment

Apple computer running OS X 10.4 or later

Amira (v4.0 or later, including Avizo) http://www.vsg3d.com/vsg_prod_avizo_overview.php

This program is used for reconstruction. This protocol describes a particular use of the software to design 3D reconstructions. Licenses can be purchased on http://www.amiravis.com/.

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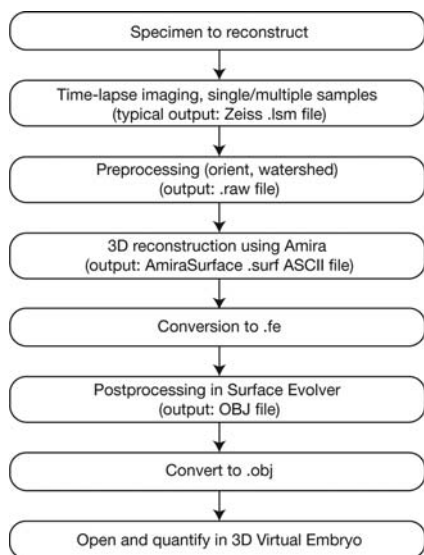**FIGURE 1.** Reconstruction flowchart. The chart provides an overview of the basic steps of embryo reconstruction.

ImageJ (http://rsbweb.nih.gov/ij/)

Watershed ImageJ plugin (http://bigwww.epfl.ch/sage/soft/watershed/)

> *Download the plugin to ImageJ/Plugins and restart ImageJ. The plugin is then available in the Plugins/ Watershed menu. This is a useful plugin for ImageJ for intensity-based image segmentation.*

jEdit (http://www.jedit.org/)

> *This text editor is useful for editing the small applets surf2fe.pl and fe2obj.php and the .surf, .fe, and .obj files.*

Surface Evolver (http://www.susqu.edu/brakke/evolver/evolver.html)

> *This is a very nice software program for computing minimal energy surfaces. In this protocol, we do not use it to search the minimal energy surface of the reconstructed object but to remove artifacts and improve the quality of the reconstruction.*

Local php server (http://www.entropy.ch/software/macosx/php/)

> *This is a PHP module for the Apache web server included in Mac OS X. We use it to load the fe2obj.php applet.*

Converters: surf2fe.pl and fe2obj.php

> *These small programs convert files from .surf to .fe format and from .fe to .obj, respectively. Go to http:// aniseed-ibdm.univ-mrs.fr/ for more information.*

METHOD

Prelude to the Reconstruction: Data Pretreatment and Data File Management

This section of the protocol presents the different techniques required to prepare the segmentation process.

File Format

1. To export the data file from the microscope, choose the output file format, which will depend on the microscope used. We use Zeiss .lsm format.

    i. If the raw data do not need preprocessing (see below), load the stack directly in the segmentation software (Amira), which reads a fair number of formats.

    ii. If Amira does not recognize your file format or if the data are pretreated (see below), open the image stack with ImageJ, make the necessary changes, and export in raw format. Amira

reads raw files, although you may have to manually indicate the voxel size as well as the file size.

> *Amira does not handle the time-lapse files efficiently. We recommend splitting the file into individual z-stacks and handling each z-stack separately.*

Data Pretreatment

2. Two circumstances may lead you to preprocess your files before reconstruction: The embryo is not properly oriented and/or the quality of the data is sufficient to perform partial automatic segmentation. When needed, both preprocessing steps are performed using the ImageJ software.

    i. Reorientation of embryos. The reconstruction process is easier if the embryos are properly oriented, that is, with their equator parallel to the imaging planes and their left–right planes of symmetry vertical on the screen. If this is not the case on the raw stack, import your stack into the ImageJ software to reorient the embryo. If the embryo was tilted during imaging, reorient it by first reslicing the stack from top (/Image/Stack/Reslice [/], which results in a frontal section), adjusting the tilt (/Image/Rotate/Arbitrary), and then re-slicing from top (which restores the initial configuration) (Fig. 2). If the embryo lies flat but the left–right axis need to be rotated, use /Image/Rotate/Arbitrary. Every rotation step is associated with interpolation of the data and therefore image quality loss, hence the importance of careful selection of the imaged embryos.

    ii. Automatic segmentation of each image using the ImageJ Watershed filter. In *Phallusia* embryos, the signal/noise ratio is excellent. This allows the reconstruction process to be accelerated using a preliminary automatic two-dimensional (2D) segmentation of the data. Note that the major limitation in automatic reconstruction is that in-plane membranes tend to be very dim compared with membranes running perpendicular to the imaging plane (see white arrows in Fig. 2, image 1). This preliminary segmentation will thus need to be manually edited for optimal results (see next section). We routinely use the ImageJ Watershed segmentation plug-in for this task. This algorithm segments the image by flooding a gray-level image and finding the watershed.
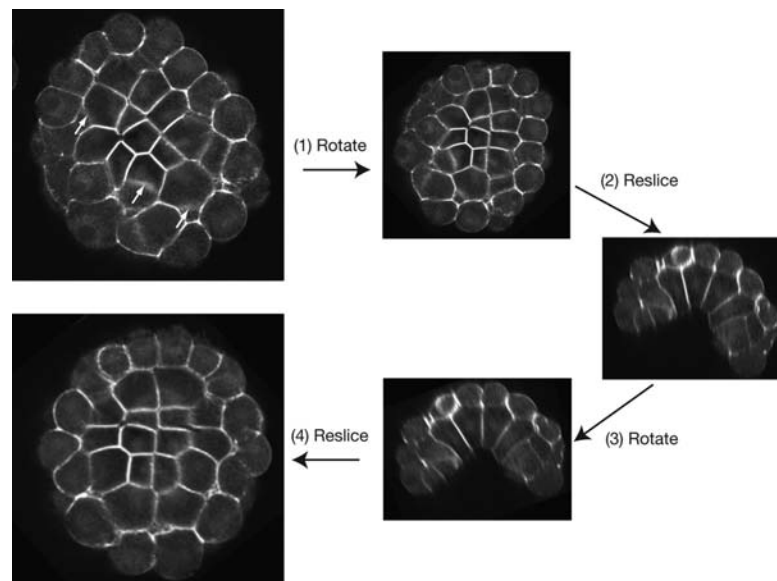

FIGURE 2. Adjusting orientation. Reorienting the image simplifies the reconstruction process. The data file is loaded in ImageJ, rotated horizontally (1), resliced from the top (2), tilt-adjusted (3), and then resliced again (4) from the top to achieve a symmetric orientation. All these steps are optional and ideally avoided, if the data set permits, so as to limit image degradation. In the first image, arrowheads denote membranes close to the imaging plane, which are faintly detected.

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3. Launch the Watershed plug-in. A prompt menu appears. Use it to smooth the image (to remove local catchment basins) and start the watershed.

4. To superimpose the initial image on the result of the segmentation, open the two stacks (result and initial data) and use RGB Merge (Image/Color/RGB Merge…) with the segmentation result as input for Red Green and the initial data as input for Blue. Convert the result of RGB merge into 8-bit (Image/Type/8-bit). As this segmentation result is binary, the magic wand of the Amira segmentation editor will allow the segmented areas to be separated easily. You can then navigate through the slices of your samples in Amira, select the regions you segmented automatically, and add them to new material. The overlay of the initial and segmented data permits quality control of the reconstruction.

Amira Segmentation

This section briefly presents the backbone of manual image segmentation using the Amira 3D Image Segmentation editor.

Loading the Image Stack

5. Use the "image properties" or "information" function of ImageJ, LSM Viewer, or Volocity to determine the properties of your data set (Fig. 3A).

    i. Dimensions. The dimension data define how your data are constructed, namely the number of rows and columns of your pictures and the number of *z* steps.

    ii. Resolution. The resolution defines the size of the sampled volume. The information may be encoded in two ways: the size of a volumetric pixel or the size of the whole sampled volume (bounding box). For the voxel size, *x* and *y* define pixel dimensions in the plane of the optical slices. The *z* value corresponds to the *z* step of your acquisition (the distance between two consecutive slices).

    When loading your data, you will need to define the dimensions of your stack. Without these, the software cannot read your data. You will be probed for the dimensions of the voxel, which you may subsequently change (Fig. 3A,B).

Starting Segmentation of a Stack

The segmentation process assigns to each voxel of the stack a label describing to which region or "material" the voxel belongs. The segmentation is stored in a separate data object called a LabelField.

6. Right click on the green icon and choose LabelField from the Labeling section (Fig. 3C, left). A new green icon appears, named LabelField, which will hold the segmentation results.

7. Click on the image segmentation icon: The image segmentation editor is displayed. By default, the segmentation editor operates in four-viewer mode. In this mode, three 2D viewers showing *x–y*, *x–z*, and *y–z* sections across the stack and an additional 3D viewer are displayed (Fig. 3C, right).

Image Segmentation: Defining the "Outside" and "Inside" of the Embryo

8. Segmentation proceeds by assessing an identity to regions of the stack. The very first step will define two identities—or materials: The "inside" material will correspond to the region that will be segmented (usually the embryo); the outside is the complementary region of the stack, which will remain untouched throughout the segmentation process. One can usually define this region by intensity threshold segmentation of the image, as we further describe in this protocol.

    i. Use the magic wand to select on your data the region without signal, which corresponds to the outside of your embryo. Settings of the magic wand should be tuned differently for each acquisition. Select as close as possible to the limit between inside and outside of the embryo (Fig. 4A).
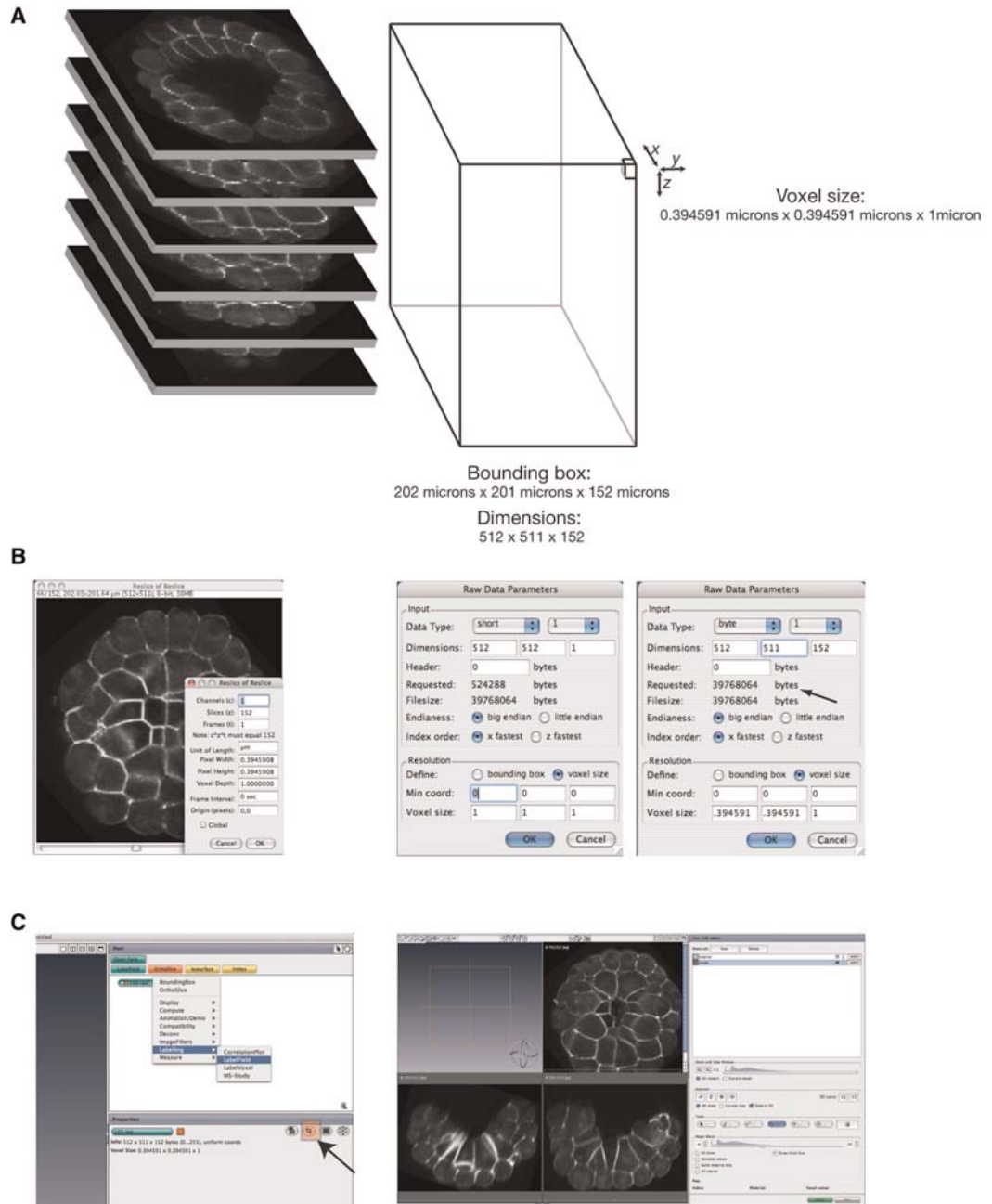
FIGURE 3. Loading data in Amira. (*A*) Definition of voxel, bounding box, and dimensions. These parameters are used by Amira to define the spatial dimensions of your image stack. (*B*) Defining voxel size in Amira. Interactive menus allow you to define the spatial properties of the image. In the example shown, the voxel size and dimensions are adjusted to the data set properties (voxel 0.394591 μm × 0.394591 μm × 1 μm; dimensions 512 × 511 × 152; and format of the initial data is byte). Note that requested bytes and file size should be identical (black arrow). (*C*) Entering the reconstruction interface. Left image shows the path to enter the reconstruction interface; right image shows the reconstruction interface. In the left image, the red box denotes the Crop editor that permits constant access to and correction of the spatial properties of the image.
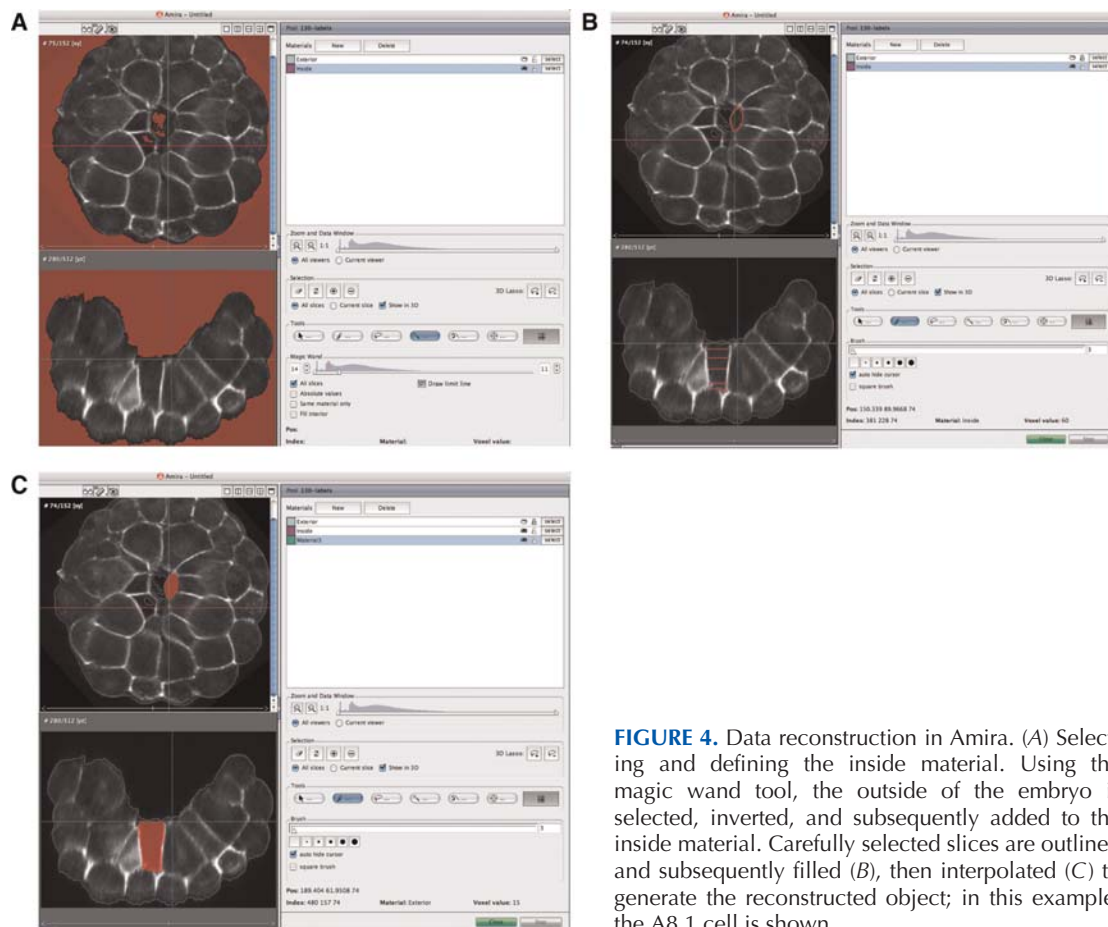
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FIGURE 4. Data reconstruction in Amira. (*A*) Selecting and defining the inside material. Using the magic wand tool, the outside of the embryo is selected, inverted, and subsequently added to the inside material. Carefully selected slices are outlined and subsequently filled (*B*), then interpolated (*C*) to generate the reconstructed object; in this example, the A8.1 cell is shown.

ii. Invert the selection (/selection/invert/all_slices) and smooth it (/selection/smooth/all_slices). Add the selection to the material called "Interior" by clicking on the + button. This procedure allows you to select the region in which your signal is located.

iii. Right click on "Exterior" and select "Lock Material" to lock the outside. It will not be modified until the last steps.

> *Depending on the settings used for the magic wand, some regions inside the embryo might have been initially defined as outside. Make sure these regions are redefined as inside by going through all slices and removing potential cavities.*

> *See Troubleshooting.*

Image Segmentation: Reconstructing the Embryo

9. Each cell of the embryo will now be segmented into a distinct object, called "material" by Amira.

   i. Click into the material list and choose *New material* from the right button menu.

   ii. Choose the first slice in which the object you want to reconstruct is well delimited on the *x–y* window of the 2D viewer.

   iii. Mark the cell by delimiting its contour with the brush and then filling this contour (Command-F). Select the new material in the material list and assign the voxels to that structure by clicking on the + button. The slice appears on the 3D viewer panel of the editor (Fig. 4B).

   iv. Slice through three slices or more and mark the same cell. Repeat until the end of the cell is reached.

    v. Choose from the menu bar: *Selection/Interpolate* (or Command-I). Intermediate slices are interpolated and included in the selection (Fig. 4C). Note that this method only relies on the selection input and not on the image data. It is therefore important to select the manually delineated slices carefully.

    vi. Select the material in the list, then press + to assign the selection to the material you created.

    vii. Check the contour of the cells, slice by slice (*x–y* viewer), and manually correct the imperfections with the brush, adding or removing material to best fit the actual data.

    viii. Repeat the procedure for all cells.

> *It is easier to start the reconstruction with cells that are either on the top or at the bottom of the data set. Save the segmentation results frequently while working.*

Generating the 3D Surface

10. Generate a triangulated surface grid corresponding to the previously segmented objects.

    i. Connect a *SurfaceGen* module (right click *SurfGen*) to the *object.Labels* data.

    ii. Press the *Apply* button (bottom right). A new data object *object.surf* is generated.

> *The generated mesh may generally be simplified without losing information. We usually decrease the number of triangles by 10–100 (option simplifier when you edit your object.surf). The exported file size must be <10 MB (~100,000 faces), otherwise the conversion process will take a very long time.*

11. Export in .surf (ascii) format (>File/Save As…, select HxSurface ascii [.surf] in the list of available formats). From now on, the data are no longer represented by a series of slices but by a set of points in space clustered in triangles and assembled as separate objects (Fig. 5A).

Object Editing

Postprocessing Using Surface Evolver

This procedure is to remove artifacts and improve the quality of the reconstruction (Brakke 1992).

12. Edit the names of the data files in the surf2fe.pl file (supplementary file opened as a text file, with Jedit for example), on lines 3 and 4 of the program (highlighted below):

```
use warnings;
use strict;
my $origin_file_name = "tata.surf";
my $dest_file_name = "tata.fe";
```
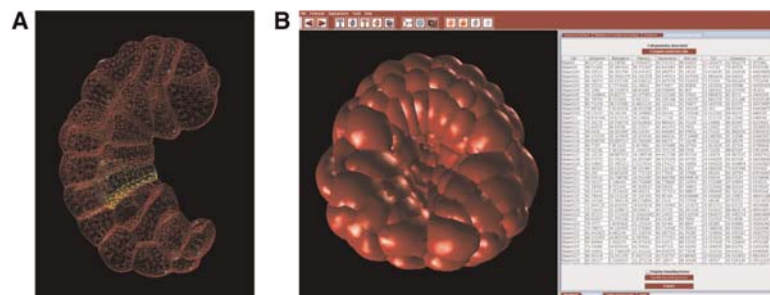

FIGURE 5. Shape analysis in the 3D Virtual Embryo software (3dve). (*A*) Embryonic cells are stored as individual elements (B8.1 is labeled in yellow). Cells are made of a mesh of triangles all oriented in the same direction. (*B*) Tools available in 3dve to quantify morphometric parameters include surface and volume measure, distance, cell geometry descriptors (measuring seven dimensionless parameters such as elongation, flatness, or sphericity), and neighbor and surface of contact.

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13. Convert into Surface Evolver format (.fe) on a Mac OS X.

  i. In a terminal window, browse the corresponding folder.

  ii. Launch the perl routine:

```
perl surf2fe.pl
```

  The routine creates the output file in the same folder.

14. Manually edit the exported file to remove the "exterior" material.

  i. Load the file in a text editor (jEdit or Text Edit).

  ii. Go to the last section of the file, which starts with the header bodies. Each cell (or body of material) is a list of triangles (the following numbers). The number at the beginning of each line is the number of the cell.

  iii. Remove the exterior material (the shell of the embryo). It corresponds usually to the longest object as it has most triangles.

  In jEdit, the width of the window adjusts to the length of the line, so it is easy to spot the longest line.

  iv. Replace the deleted cell by the last cell, so that the numbering of the cells remains consistent.

  v. Save the modified .fe file.

15. Postprocess the embryo.

  i. Load the modified .fe file in the program Evolver.

  ii. Postprocess the embryo using the following sequence of commands:

```
s
q
N
{ u ; V 2 } 20
g
```

  This sequence of commands corrects reconstruction artifacts and cleans the reconstruction outlook. More precisely, "s" starts the graphic mode and opens the graphic window so that you can see your embryo. "q" returns to the main prompt. "N" normalizes the volume for subsequent smoothing of the embryo. "u" and "V" equiangulate the triangles and average the vertices, resulting in a homogeneous mesh.

  iii. Export the file in .fe.dmp format using the *dump* command:

```
d
Enter
```

16. Manually edit the exported file:

  i. Back slashes and carriage return characters ending the object line and spaces starting the new line hamper proper file format conversion. Remove these from the .fe.dmp file using a text search function (from TextEdit or jEdit). For example:

```
21113 21114 21115 21116 21117 21118 21119 21120 21121 21122 \
21123 21124 −21175 −21176 −21177 −21178 −21179 −21180 −21181 −21182 \
```

  should be changed into:

```
21113 21114 21115 21116 21117 21118 21119 21120 21121 21122 21123 21124
−21175 −21176 −21177 −21178 −21179 −21180 −21181 −21182
```

  ii. Save as .fe.

  Steps 15 and 16 are dispensable. As postprocessing steps, they correct some imperfections of the reconstruction, such as staircase effects, but should be handled with care, and one should ensure that in a given type of reconstruction, these are not denaturing the shapes of the reconstructed structures, for example, by checking that the total difference in shape descriptors (as measured by 3dve) remains small for all considered objects and does not introduce artifactual modification of the replica.

File Conversion and Opening in the 3D Virtual Embryo Software

17. Convert file from .fe (Surface Evolver) to .obj (Wavefront) format using the php program fe2obj.php.

    i. Enable php on your computer.

    ii. Browse Users/yourname/Sites folder and delete index.html file.

    iii. Copy the fe2obj.php and your .fe file in Users/yourname/Sites.

    iv. Edit lines 31 and 32 of fe2obj.php to match input file name:
    ```
    $filename="tata.fe";
    $filetmpname="tata.fe";
    ```

    v. In System preferences/Sharing, check personal web sharing and click on your personal website address at the bottom of the page. This will open your default browser and take you to your personal web page. Click on fe2obj.php. This will actually start the conversion process. The output .obj file will appear in the loading window. Wait until the conversion is finished, then copy the text on the page and paste into a new text document using jEdit or TextEdit (raw text format) and save the file with the .obj file extension.

18. Rename objects in the .obj file: For each object, replace its current name with its actual name. The name of the object appears twice for each object, before the point list and before the triangle list. Blastomeres should be named according to Conklin nomenclature and later stages according to Aniseed anatomical ontology (go to http://aniseed-ibdm.univ-mrs.fr/ for more information).

19. Load in 3D virtual embryo and start exploring (Fig. 5B). To benefit from the linkage of the 3D virtual embryo software with the ascidian organism database (Aniseed), embryos should be staged and inserted in the embryo list (3dve/embryo/embryo-list.txt).

    3D digital reconstruction provides an efficient way to quantify cell morphology in simple cellular contexts, but in its current state it is not transposable for complex systems. Reconstruction takes ~5–15 min per cell, depending on the experience of the operator and the complexity of the cell, which limits reconstruction of even early stages of zebrafish development and clearly prohibits reconstruction of later stages (there are as many as 18,000 cells in a 18-h-old zebrafish). As it is, this technique requires around a full week of work to segment a 110-cell stage embryo stack, plus at least another week to postprocess the reconstruction data if the embryos are to be included in the database. The most critical factor hampering automatic cell membrane reconstruction is the poor signal from in-plane membranes (Fig. 2A). To circumvent this issue, alternate imaging techniques (e.g., multiview techniques such as digital scanned laser light sheet fluorescence microscopy [DSLM]), may prove adequate and should be coordinated with the development of appropriate automatic segmentation algorithms. As the reconstruction process is long and tedious, we recommend saving frequently and keeping a clear history of intermediate files as backups (see Table 1 for intermediate files list and format).

TROUBLESHOOTING

Problem (Step 8): The reconstructed cells do not completely fill the region defined as inside, leaving a large unreconstructed region surrounding the whole embryo.

Solution: Remove the regions initially defined as inside that were not included in cell reconstructions.

1. Select the "exterior" material and choose *Invert* volume from the editor's selection menu. Here you can use the *Shrink/Volume* and the *Smooth Labels* options to clean the surface.

2. Create a new material and add your selection to this material (the new inside).

3. Select the "inside" material and add the selection to the "exterior" material.

4. Select the material you have created (the new inside) and add it to the "inside material."

5. Delete the material you have created.

Problem (Step 8): The cells still do not cover the inside material perfectly, leaving relatively small unreconstructed regions depending on the outside material.

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TABLE 1. History of files used in the reconstruction process

Description	File format
Original confocal stack	.lsm
Preprocessed file	.raw
Original reconstruction (Amira label file)	.am
Export file for postprocessing	.surf binary
Postprocessed file	.fe
Final file	.obj

These files should be conserved and carefully named and annotated so as to keep track of the various reconstruction steps and potentially back off to the appropriate step if a mistake is spotted. After reconstruction is complete, it is imperative that three files be conserved: the initial imaged file, the original reconstruction file, and the final reconstruction file.

Solution: Consider the following strategies.

1. If there are few large gaps, edit the problematic regions manually.

2. If there are many small gaps:

   i. Apply *Segmentation/Smooth Label* with 3D volume option ticked. This should remove most of the small gaps.

   ii. Remove what is left from the inside by either assigning it to a cell or to the exterior material and proceed to a last smoothing step using *Segmentation/Smooth Label*.

DISCUSSION

The techniques we describe here permit the generation of 3D replicas of embryos from stacks of confocal images through ascidian embryos and their conversion to a format that can be read by the 3D Virtual Embryo software (Tassy et al. 2006). Generation of a 3D mesh in Amira is the temporal bottleneck of the procedure, each cell taking 10–15 min to reconstruct. The major limiting step for the automatic reconstruction of these embryos is the fact that membranes located in, or close to, the x–y imaging planes are either missed or imaged with very low intensity. The recent development of DSLM, which allows imaging of embryos from several directions (Keller et al. 2008), should greatly alleviate this difficulty and allow efficient application of automatic 3D segmentation algorithms (e.g., Zanella et al. 2007).

Once these replicas are imported in 3D Virtual Embryo (Tassy et al. 2006) individual cells or subpopulations of cells can be analyzed and compared at a given developmental stage or between stages. A panel of morphometric tools is available to quantify cellular morphology and cell–cell interactions (Fig. 5) (e.g., to track the volume of a given cell through time in a single embryo or measure changes in individual or aggregated shape descriptor values). Such descriptions constitute a necessary, although not sufficient, first step to understand the mechanical basis of morphogenesis. They may serve as starting points for surface-tension-based simulations of morphogenesis (as can be performed with Surface Evolver) comparing in silico and in vivo morphogenesis. These replicas also provide a quantitative framework for developing hypotheses regarding the range of inductive cell–cell interactions. In the past, such analyses have suggested that ascidian neural induction occurs at very short range and revealed that the precise measure of the area of contact between competent and inducing cells is a key determinant of the fate decision taken by competent cells (Tassy et al. 2006).

Finally, 3D Virtual Embryo is interfaced to the Aniseed model organism database for ascidians. Each reconstructed cell is thereby linked to embryological parameters such as its lineage or fate via sophisticated anatomical ontologies (Tassy et al. 2010). Most importantly, a link is also established with the underlying genetic program and it is possible, for instance, to search for genes whose expression profiles distinguish cells with distinct geometrical properties.

Ultimately, the 3D embryo replica described here and software such as 3D Virtual Embryo constitute some of the many building blocks necessary to construct genuine "virtual developing embryos"

that will integrate a careful description of cellular morphogenesis, physical modeling, and lineage-specific gene regulatory networks to simulate how unfolding of the genetic program coordinates cell-fate specification with embryo morphogenesis.

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