Protocol

Imaging of Fixed *Ciona* Embryos for Creating 3D Digital Replicas

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 presents a method for imaging embryonic development with cellular resolution in fixed ascidian embryos. A large fraction of the ascidian community primarily studies the development of the cosmopolitan ascidian *Ciona intestinalis*. Because the embryos of this species are insufficiently transparent and show significant autofluorescence, live imaging is difficult. Thus, whole embryos are fixed and optically cleared. They are then stained and imaged on a regular or two-photon confocal microscope. The resulting image stacks can subsequently be digitalized and segmented to produce 3D embryo replicas that can be interfaced to a model organism database and used to quantify cell shapes.

RELATED INFORMATION

Protocols are also available for **Time-Lapse Imaging of Live *Phallusia* Embryos for Creating 3D Digital Replicas** (Robin et al. 2011a) and **Creating 3D Digital Replicas of Ascidian Embryos from Stacks of Confocal Images** (Robin et al. 2011b).

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.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Artificial seawater-HEPES (ASWH) <R>

Dechorionation mixture <R>

Embryo fix solution (if embryo clearing is required; see Step 5) <R>

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Formaldehyde (EM grade) (if embryo clearing is not required; see Step 5)

Isopropanol (70%, 85%, 95%, 100%) (for clearing embryos; see Step 9)

Murray's clear (benzyl benzoate:benzyl alcohol, 2:1) (for clearing embryos; see Step 9)

Nail polish (clear)

PBS(A) <R>

PBT (0.1% Triton X-100 in PBS[A])

Phalloidin conjugated with Alexa-546

The samples can also be stained with Hoechst stain (Invitrogen H1399).

Tris (1 M, pH 9.5)

Equipment

Coverslips

Culture plates, six-well

Glass-bottom dish, 35-mm-diameter plastic dish (WillCo Wells GWSt-3522) (for embryos that are not cleared; see Step 9)

Treat the dish with gelatin/formaldehyde (each 0.1%) and rinse thoroughly to prevent embryos from sticking. A cover glass (#1, rectangular, VWR International) may be used instead of the glass-bottom dish. Treat the cover glass with gelatin/formaldehyde (each 0.1%) and rinse thoroughly to prevent embryos from sticking. Use a #0 cover glass as a cover.

Hand-driven centrifuge and 15-mL glass tubes

Imaging system

- Two-photon inverted microscope (Zeiss LSM 510 NLO)
- 63× objective (C-Apochromat 1.2 W Corr; Carl Zeiss, Inc.) illuminated with a Mai-Tai laser (Spectra-Physics) at a wavelength of 1020 nm in a nondescanned detection mode
- Cooling stage: Open perfusion microincubator (PDMI-2; Harvard Apparatus) with a temperature controller (TC-202A; Harvard Apparatus)
- Hardware and software as described in **Creating 3D Digital Replicas of Ascidian Embryos from Stacks of Confocal Images** (Robin et al. 2011b)

Nail polish

Pasteur pipettes (glass)

Petri dish (coated with 1% agarose)

Silicone isolators (peel-and-stick; Sigma-Aldrich S3185)

Slides (coated with 0.1% polylysine) (for clearing embryos; see Step 9)

METHOD

1. Dissect gravid *C. intestinalis* and collect eggs and sperm separately.
2. Keep the chorionated eggs in a six-well plate containing ~5 mL of ASWH.
3. Activate the sperm by adding 20 µL of concentrated sperm to 1 mL of ASWH containing 50 µL of 1 M Tris (pH 9.5).
4. Prepare eggs:
   i. Fertilize the embryos by adding 50 µL of activated sperm.
   ii. After 10 min, using a glass Pasteur pipette, transfer the embryos to a 15-mL glass hand-driven centrifuge tube. Centrifuge at full speed for 20 sec to collect the eggs at the bottom of the tube.

iii. Remove most of the ASWH and add 4 mL of dechorionation mixture. Pipette up and down until the chorions detach.

iv. When ~20% of the eggs are dechorionated, add ASWH to give a final volume of 15 mL, and centrifuge very gently.

v. Remove the supernatant and rinse three times with ASWH.

vi. Transfer the eggs to an agarose-coated Petri dish.

5. When embryos are at the appropriate stage for imaging, fix them in either ASWH containing 4% formaldehyde (if clearing is not required) or embryo fix solution (if clearing is required).

6. Rinse three times in PBT.

7. Transfer to PBT containing 5 µg/mL of phalloidin conjugated with Alexa-546. Incubate for 1 h at room temperature or overnight at 4°C.

8. Wash the embryos three times in PBS(A).

9. Treat the embryos in one of the following ways:

The clearing process may be omitted if a two-photon microscope is used, but the resulting images tend to have a lower resolution on the side of the embryo furthest from the objective.

To clear the embryos

i. Attach the embryos to polylysine-coated slides fitted with firmly adhered silicone isolators (to prevent compression of the embryos when the coverslip is applied).

ii. Take the embryos through an isopropanol series (70%, 85%, 95%, and 2 × 100%), immersing at each step for 45 sec.

iii. Wash three times in Murray's clear.

iv. Apply a coverslip, dry thoroughly, and seal with nail polish.

For embryos that are not cleared

i. Transfer the embryos directly to a glass-bottom dish (pretreated with 0.1% gelatin/formaldehyde). Alternatively, spread embryos on a #1 cover glass (also pretreated with 0.1% gelatin/formaldehyde and fitted with firmly adhered silicone isolators), cover with a #0 cover glass, and seal with nail polish.

10. Image embryos on an upright confocal microscope at 546 nm or on a two-photon microscope at 1000 nm.

As for live imaging, settings for imaging should be adjusted according to the size of the reconstructed structure, stage, etc. Because embryos are fixed, imaging the specimen can take as long as one wishes, and therefore image stacks from fixed specimens are generally of higher resolution than stacks obtained from live embryos, especially along the z-axis, along which images can be taken every 0.5–1.0 µm.

RECIPES

Artificial seawater-HEPES (ASWH)

Reagent	Amount to add for 1 L	Final concentration
NaCl	24.55 g	420 mM
KCl	0.67 g	9 mM
$CaCl_2 \cdot 2H_2O$	1.47 g	10 mM
$MgCl_2 \cdot 6H_2O$	4.48 g	24.5 mM
$MgSO_4 \cdot 7H_2O$	6.29 g	25.5 mM
$NaHCO_3$	0.18 g	2.15 mM
HEPES (1 M, pH 8.0)	5 mL	5 mM

Dechorionation mixture

ASWH <R>
1% sodium thioglycolate
0.05% pronase (Sigma-Aldrich)

Embryo fix solution

4% formaldehyde (EM grade)
50 mM EGTA
100 mM PIPES
400 mM sucrose
Adjust pH to 6.9.

PBS (A)

Reagent	Amount to add for 1 L	Final concentration
NaCl	8.0 g	137.0 mM
KCl	0.2 g	2.7 mM
KH_2PO_4	0.2 g	1.5 mM
NaH_2PO_4	1.14 g	8.0 mM

Adjust pH to 7.2 with HCl (~4–6 droplets of 6 M HCl).

REFERENCES

Robin FB, Dauga D, Tassy O, Sobral D, Daian F, Lemaire P. 2011a. Time-lapse imaging of live *Phallusia* embryos for creating 3D digital replicas. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot065847.

Robin FB, Dauga D, Tassy O, Sobral D, Daian F, Lemaire P. 2011b. Creating 3D digital replicas of ascidian embryos from stacks of confocal images. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot065862.

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