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Review Article

Practical tips for imaging ascidian embryos

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Decades of studies on ascidian embryogenesis have culminated in deciphering the first gene regulatory “blueprint” for the generation of all major larval tissue types in chordates. However, the current gene regulatory network (GRN) is not well integrated with the morphogenetic and cellular processes that are also taking place during embryogenesis. Describing these processes represents a major on-going challenge, aided by recent advances in imaging and fluorescent protein (FP) technologies. In this report, we describe the application of these technologies to the developmental biology of ascidians and provide a detailed practical guide on the preparation of ascidian embryos for imaging.

Key words: ascidian, chordate, embryo, fluorescent protein, imaging.

Introduction

Ascidians are invertebrate chordates belonging to the subphylum Urochordata, the sister group of vertebrates. Their larval form is tadpole-like exhibiting a characteristic chordate body plan with an axial notochord and a dorsal neural tube. Ascidian embryogenesis proceeds with a much smaller number of cells compared to vertebrates (e.g. gastrulation starts when the embryo consists of 112 cells; Sherrard *et al.* 2010). During embryogenesis, cells divide with both spatially and temporally invariant patterns and follow highly reproducible cell lineages. These features are unique among chordate models, allowing description of developmental events with single cell resolution.

Ascidian embryogenesis has long been regarded as a typical case of mosaic development, with cytoplasmic factors partitioned during cleavage stages to specific cell lineages and specifying cell fates in a cell autonomous manner. However, it is now clear that cell interactions also play a critical role in ascidian development (reviewed in Lemaire 2009). Major efforts in ascidian developmental biology studies have focused on deciphering the gene regulatory mechanisms con-

trolling cell fate specification and have culminated in the first gene regulatory “blueprint” for the generation of major larval tissue types in chordates (Imai *et al.* 2006, 2009). In parallel to these efforts, some pioneering studies have been successfully applying imaging to quantitatively analyze cellular processes and intracellular dynamics observed during ascidian development. McDougall and Sardet (1995) studied the dynamics of repetitive Ca^{++} waves in the egg triggered upon fertilization, while Shiba *et al.* (2008) recently succeeded in imaging the dynamics of intracellular Ca^{++} increase in the sperm flagellum, which they find coincides with a change of the swimming direction. Sherrard *et al.* (2010) described the cell shape changes associated with gastrulation and, based on their observation, have successfully defined a set of parameters, with which the cell shape changes can be recapitulated by computer simulations. Prodon *et al.* (2010) analyzed mitotic spindle positioning during a series of unequal cell divisions, which result in segregation of the germline, with high spatiotemporal precision. Finally, Ogura *et al.* (2011) showed cell cycle lengthening of embryonic epidermal cells by insertion of G2 phase using a fluorescent ubiquitination-based cell cycle indicator, Fucci (Sakae-Sawano *et al.* 2008). In addition to these studies addressing cellular and subcellular events, fluorescent proteins (FPs) have also been used as lineage tracers, in particular, in neural lineages. Stolfi and Levine (2011) used neuronal precursor-specific promoters driving different FPs to visualize five neurons

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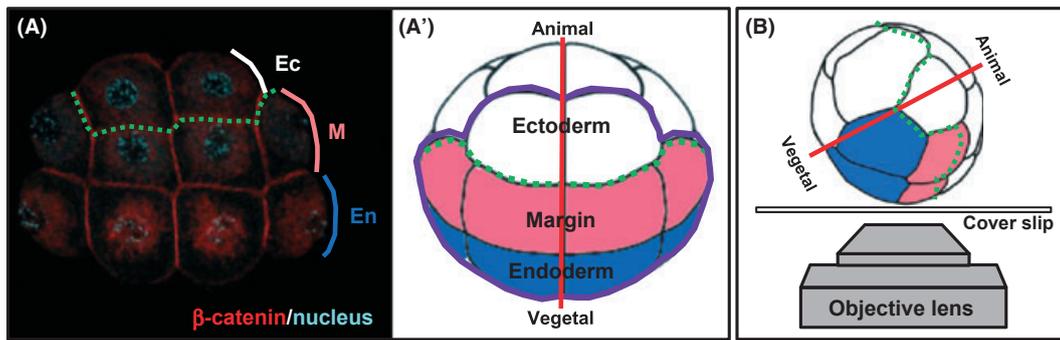


Fig. 1. (a) Confocal stack of an oriented late 32-cell stage embryo which was immunostained with anti- β -catenin antibodies and counterstained with 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI). The embryo was oriented as shown in (b) so that the dorsal side view encompassing three embryonic layers, endoderm (En: blue), margin (M: pink) and ectoderm (Ec: white), could be imaged. β -catenin exhibits nuclear localization in the endoderm precursors but not in the marginal cells, which is responsible for segregation of these two embryonic layers (Hudson *et al.* 2013). Green dotted lines highlight the frontier between the animal and vegetal hemispheres. (a') Schematic of the late 32-cell stage embryo in the dorsal side view with its animal-vegetal axis aligned as indicated (red line). Cells corresponding to those visible in (a) are surrounded in purple line. (b) Schematic showing how the embryo was oriented following the procedure described in the text in order to acquire the image shown in (a).

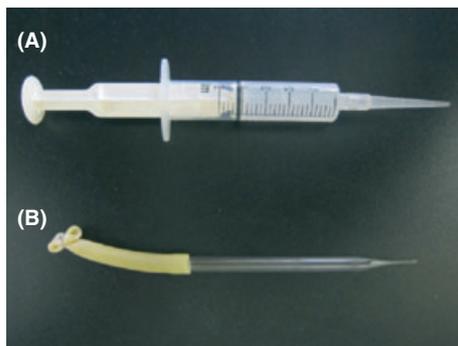


Fig. 2. (a) Syringe for depositing a thin line of silicone onto cover slips. (b) hand-made micropipette.

simultaneously in *Ciona* larvae. Horie *et al.* (2011) revealed the lineage origin of adult neurons by taking advantage of the unique nature of a photoactivatable fluorescent protein, Kaede, which undergoes irreversible photoconversion from green fluorescence to red fluorescence with the irradiation of ultraviolet light (Ando *et al.* 2002). In a few cases, gene regulatory networks have been linked to cellular processes. Christiaen *et al.* (2008) have identified a subcircuit responsible for cardiac precursor migration and placed it within the heart GRN. Another example concerns the notochord GRN: during the tubulogenesis of ascidian larval notochord, *ERM* (ezrin/radixin/moesin), a downstream target of *Brachyury*, has been shown to play an essential role in lumen formation of notochord cells (Dong *et al.* 2011).

In this report, we will describe detailed practical tips of how to mount and orient ascidian embryos for imaging, which are largely omitted in research papers.

We have also listed FP-fusion proteins used to visualize distinct subcellular structures/organelles in ascidian embryos (Table 1). We focus on two ascidian models, *Ciona intestinalis* and *Phallusia mammillata*, both of which belong to the same clade of ascidians, phleobranchia (Tsagkogeorga *et al.* 2009). The *Ciona* model is widely used due to its cosmopolitan distribution and is supported with a large resource of molecular tools. The eggs and embryos of *Ciona* are semi-transparent. In contrast, the distribution of *Phallusia mammillata* is limited to the European coastline, but the *Phallusia* model is more suitable for live imaging due to the optical transparency of its eggs/embryos and the robust translation of injected RNAs, allowing the detection of FP signals as early as the egg stage (Prodon *et al.* 2010). The following section is organized in two parts: (i) practical tips for imaging fixed embryos, and (ii) practical tips for live imaging, which are supplemented with two time-lapse movies as examples (Movie S1 and S2). While these practical tips are designed for the above-mentioned ascidian models, which are used in our laboratories, they should be applicable to other ascidian models and may provide a useful starting point for the imaging of other small invertebrate/marine embryos.

Technical tips for imaging ascidian embryos

Imaging fixed embryos

In this section, we will describe first (i) how to mount and orient embryos for microscopic observation and then; (ii) how to render embryos transparent using an

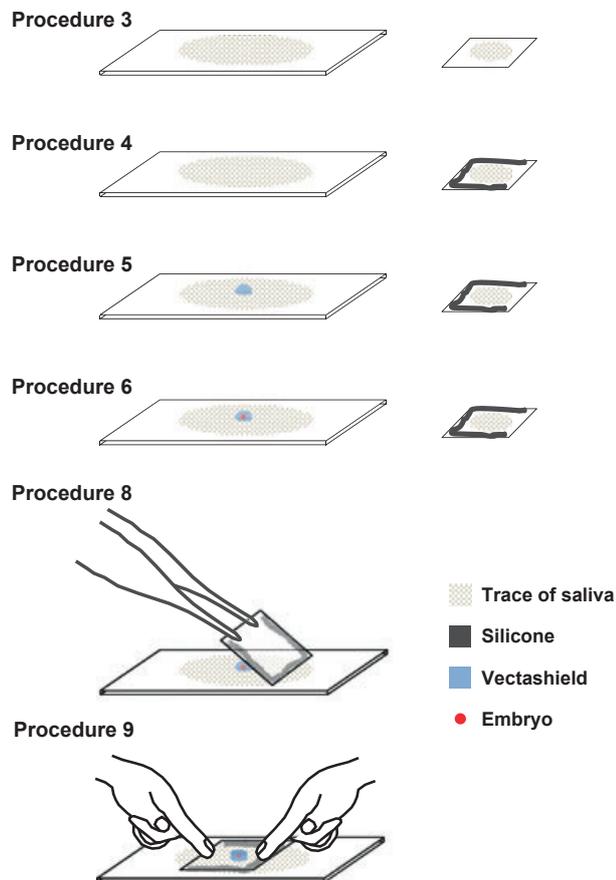


Fig. 3. Schematics showing procedures described in the section “Imaging fixed embryos: How to mount and orient embryos”. Procedure numbers correspond to those in the text.

optical clearing agent, benzylalcohol-benzyl-benzoate (BABB).

How to mount and orient embryos. One of the advantages of the ascidian model for the study of development biology is its invariant mode of embryonic cell divisions, which allows us to identify individual cells. However, in order to image them from the most adequate angles, it is imperative to orient the embryo so that cell(s) of interest face the objective. Ascidian early embryos are spherical and the sphericity changes dramatically during embryogenesis (Tassy *et al.* 2006). This is the case even during a single cell cycle. For example, the mid 32-cell embryo is spherical, while the late 32-cell embryo becomes flattened along the animal-vegetal axis. Flattened embryos tend to sit on their animal-pole or vegetal pole side in a non-viscous medium. When you want to observe cells located in the marginal zone of a flattened embryo, the embryo has to be oriented and maintained at a fixed position so that the cell of interest remains facing the cover slip during observation (Fig. 1).

Materials.

1. Cover slips (no. 0).
2. Standard glass slides.
3. VECTASHIELD mounting medium (Vector laboratories).
4. Silicone grease (Dow Corning high vacuum grease) filled in a plastic syringe equipped with a yellow tip (for pipetman). The yellow tip was trimmed with a scalpel blade so that the inner diameter of its tapered end is around 2 mm and the other end fits to the syringe’s open-end (Fig. 2).
5. Hand-made micro pipette (Fig. 2): Pull a glass tube (outer diameter 5 mm; inner diameter 3 mm) under flame so that one end becomes tapered to around 1 mm in diameter and, to the large end, attach a rubber tube of appropriate diameter with its other end stapled.
6. Tungsten needle (Brady 1965).
7. Depression slide: 4 mm thickness. Used only for selecting embryos prior to mounting on standard slides.

Method (Fig. 3).

1. Following immunofluorescence, remove most of the washing buffer and add about 50 μ L of VECTASHIELD to a 1.5 mL tube containing stained embryos.
2. Place embryos into a depression slide.
3. Lick the centre area of cover slip and glass slide and wipe with a Kimwipe. The “saliva coating” prevents embryos from sticking to the glassware.
4. Apply a thin line of silicone grease along three perimeters of the cover slip using the syringe (Fig. 2).
5. Place 2–3 μ L of VECTASHIELD on the centre of the glass slide.
6. Pick up a single embryo from the depression slide using a micro pipette (Fig. 2) under a stereomicroscope and place it in the drop of VECTASHIELD on the glass slide with a minimum carry-over of mounting medium.
7. Using a tungsten needle, orient the embryo with either the animal pole side up or down depending on the location of the cell of interest.
8. Using forceps, gently place the cover slip with a single motion.
9. Gently press the cover slip with fingers under a stereomicroscope until it is almost in direct contact with the embryo. Gently displace the cover slip in the X–Y dimension with your fingers so that the cell of interest faces you. This is the most difficult part of the entire process, which needs to be perfected by trial and error.

Figure 1 is an example of a 32-cell stage embryo oriented following this procedure. The image was acquired on a confocal microscope.

How to clear embryos in BABB

Tassy *et al.* (2006) have developed software named 3D Virtual Embryo, which allows quantification of the geometry and interactions of cells in interactive three-dimensional embryo models, and applied this approach to early ascidian embryos. In order to generate 3D digital replicas used in the quantifications in 3D Virtual Embryo, images are generated from confocal z-stacks of ascidian embryos labeled with phalloidin conjugated with fluorophore (for details of creating 3D digital replicas, please refer to Robin *et al.*, 2011). In order to acquire a confocal z-stack of the whole embryo, the embryo has to be rendered transparent using BABB. Here we describe how to clear ascidian embryos for confocal observation. This is particularly tricky as rendering the embryo transparent also renders it “invisible”.

Materials.

1. BABB: one part benzyl alcohol and two parts benzyl benzoate.
2. Dilution series of isopropanol: 25%, 50%, 75%, 100%.
3. Cover slips (no. 0).
4. Poly-L lysine coated glass slide: Place standard glass slides in staining rack. Soak in 2 N NaOH solution for 1–2 h. Rinse in H₂O in staining jar. Repeat five times. Soak in 0.01% poly-L-Lysine solution (w/v in water) for 1 h in staining jar. Rinse in H₂O. Rinse in 100% Ethanol. Air-dry the slides.
5. Thin sheet cut into pieces of narrow rectangular shape with the length of its longest perimeter roughly corresponding to that of the perimeter of cover slips. We find that the support sheet of Tough-Spots (Diversified Biotech) is perfect for this purpose.
6. Hand-made micro pipette (Fig. 2).
7. Tungsten needle (Brady 1965).
8. Depression slide: 4 mm thickness. Used only for selecting embryos prior to mounting on standard slides.

Method.

1. Following phalloidin staining, embryos are washed in PBT (PBS/0.1%Tween). Place embryos into a depression slide.

2. Pick up a single embryo from the depression slide using a micro pipette under a stereomicroscope and place it onto a poly-L-lysine coated standard glass slide under the stereomicroscope.
3. Quickly orient the embryo using a tungsten needle so that its animal-vegetal axis is placed roughly perpendicular to the surface. The embryo will soon adhere to the glass slide.
4. Repeat the process to adhere a few more embryos to the glass slide.
5. Remove the maximum amount of PBT using a micropipette.
6. Take the glass slide through an isopropanol series (25%, 50%, 75% and 2 × 100%), immersing it at each step for 1 min.
7. Immerse the glass slide in BABB (in glass container) for 1 min and repeat once.
8. Place two strips cut from the Tough-Spots support sheet onto the glass slide and then place a cover slip onto the strips. Wipe carefully and thoroughly residual BABB on the glass slide and seal the cover slip with nail polish.

Live imaging

In order to observe the dynamics of subcellular organelles/structures or proteins of interest during ascidian embryogenesis, there are two approaches to express fluorescent proteins (FPs) targeted to specific organelles or fused with a molecule of interest. One approach is based on injection of synthetic mRNA into unfertilized eggs. In the *Ciona* model, FP signals of proteins translated from injected RNA become detectable only around the onset of gastrulation, making this model unsuitable for observation of cellular/subcellular dynamics during cleavage stages. In contrast, *Phallusia* eggs and early embryos exhibit robust translation of injected RNA and FP signals become detectable in unfertilized eggs 3 h after RNA injection (Prodon *et al.* 2010). Detailed protocols for microinjection into ascidian eggs have been reported recently in Sardet *et al.* (2011). The second approach to express FP-fusion proteins in ascidian embryos is based on electroporation of plasmid DNAs consisting of a tissue- or cell type-specific promoter driving a FP-fused reporter gene (e.g. Roure *et al.* 2007; Dong *et al.* 2011). A number of tissue- and lineage-specific promoters have been characterized in the *Ciona* model, many of which (if not all) are functional in the *Phallusia* model (Rémi Dumollard and AM, unpubl. data, 2012). These promoters allow cell type- or lineage-specific observations. For a detailed protocol of elec-

Table 1. Fluorescent protein (FP)-fusion proteins used to visualize subcellular dynamics in ascidian embryos. We have listed FP-fusion proteins which have been reported to work in *Ciona intestinalis* (Ci) and *Phallusia mammillata* (Pm)

Organelle/Molecule	Species	Note	Reference
Microtubule			
Ensconsin (MAP7)-3xGFP	Ci	promoters (<i>FOG</i> , <i>brachyury</i> , <i>Mesp</i>)	Roure <i>et al.</i> (2007) Dong <i>et al.</i> (2011) Cooley <i>et al.</i> (2011) Prodon <i>et al.</i> (2010)
MAP7-EGFP	Pm	RNA injection	Prodon <i>et al.</i> (2010)
Actin			
mCherry-hActin	Ci	-human actin -promoter (<i>brachyury</i>)	Dong <i>et al.</i> (2011)
Lifeact-mEGFP	Ci	promoter (<i>brachyury</i>)	Dong <i>et al.</i> (2011)
mCherry-UtrCH	Ci	-calponin homology (CH) domain from utrophin -promoter (<i>brachyury</i>)	Dong <i>et al.</i> (2011)
Utrophin-GFP	Ci	promoter (<i>Mesp</i>)	Cooley <i>et al.</i> (2011)
ABD-venus	Pm	-actin-binding domain (ABD) from ABP-120 -RNA injection	Prodon <i>et al.</i> (2010)
Myosin			
mCherry-MLC	Ci	- <i>Ciona</i> myosin II light chain -promoter (<i>brachyury</i>)	Dong <i>et al.</i> (2011)
Chromosome			
H2B-venus	Ci	-histone H2B -promoter (<i>FOG</i>)	Roure <i>et al.</i> (2007)
H2B-RFP1	Pm	RNA injection	Prodon <i>et al.</i> (2010)
H2B-CFP	Ci	promoter (<i>Mesp</i>)	Stolfi <i>et al.</i> (2010)
H2B-mCherry	Ci	promoter (<i>Mesp</i>)	Stolfi <i>et al.</i> (2010)
Plasma membrane			
GAP43-venus	Ci	promoter (<i>FOG</i>)	Roure <i>et al.</i> (2007)
GAP43-CFP	Ci	promoter (<i>Epi1</i>)	Ogura <i>et al.</i> (2011)
PH-EGFP	Pm	-PIP ₂ binding domain from human PLC δ 1 -RNA injection	Prodon <i>et al.</i> (2010)
Kaede-CAAX	Ci	promoter (<i>Epi1</i>)	Ogura <i>et al.</i> (2011)
Venus-CAAX	Ci	promoter (<i>Epi1</i>)	Ogura <i>et al.</i> (2011)
mCherry-CAAX	Ci	promoter (<i>Epi1</i>)	Ogura <i>et al.</i> (2011)
membrane-GFP	Ci	-first 20aa of <i>Ciona</i> GAP-43 -promoter (<i>brachyury</i>)	Dong <i>et al.</i> (2009)
Dm-E-cadherin-mCherry	Ci	promoters (<i>FOG</i> , <i>brachyury</i>)	Roure <i>et al.</i> (2007) Dong <i>et al.</i> (2009)
Golgi			
ST-GFP	Ci	-membrane signal anchor sequence from rat sialyl transferase -promoter (<i>EpiB</i>)	Zeller <i>et al.</i> (2006)
Centrosome			
Aurora-Venus/mCherry	Pm	RNA injection	Hebras & McDougall (2012)
Midbody			
Aurora-Venus/mCherry	Pm	RNA injection	Hebras & McDougall (2012)
Venus-TPX2	Pm	RNA injection	Hebras & McDougall (2012)
Incenp-mCherry	Pm	RNA injection	Hebras & McDougall (2012)
Plk1-mCherry	Pm	-polo-like kinase -RNA injection	Hebras & McDougall (2012)

In the column “note”, the techniques used to express these proteins are mentioned together with some information concerning the molecules to which the FP is fused.

troportion, please refer to Christiaen *et al.* (2009) and Vierra & Irvine (2012).

In this section, we will describe (i) how to mount and orient live embryos for time-lapse imaging, and (ii) how to conduct time-lapse imaging. It should be noted that these technical tips are for observing embryos developing without chorion. We also sum-

marize in Table 1 the FP-fusion constructs that have been successfully used to visualize subcellular components in ascidian embryos. Finally, as supplementary data, we provide two movies that show the dynamics of microtubules in the epidermal lineage of *Ciona* tailbud stages and during early cleavage stages of *Phallusia* embryos.

*How to mount and orient live embryos**Materials.*

1. Cover slips (no. 0).
2. Glass slides.
3. 1.5% methylcellulose in artificial sea water (ASW): heat up ASW in a 15 mL tube to 80°C and then add methylcellulose powder. Vigorously shake the tube and then let it cool down to room temperature. Stick the tube to a vortex machine with sticky tape and shake it O/N. Once methylcellulose is dissolved, aliquot the solution into 1.5 mL tubes and store them at -20°C. Before use, thaw a tube and centrifuge it for 5 min at the maximum speed in order to remove residual air bubbles in the solution.
4. Silicone grease (Dow Corning high vacuum grease) filled in a plastic syringe equipped with a yellow tip (for Pipetman). The yellow tip was trimmed with a scalpel blade so that the inner diameter of its tapered end is around 2 mm and the other end fits to the syringe's open-end (Fig. 2).
5. Hand-made micro pipette (Fig. 2).
6. Tungsten needle (Brady 1965).

Method (basically the same procedure as schematized in Fig. 3).

1. Lick the centre area of the cover slip and glass slide and wipe with a Kimwipe. The "saliva coating" prevents embryos from sticking to the glassware.
2. Place 3 μ L of 1.5% methylcellulose/ASW at the centre of glass slide.
3. Apply a thin line of silicone grease along three perimeters of the cover slip.
4. Under a fluorescent stereomicroscope, identify an embryo with the desired levels of fluorescent signals. Make sure that this is done very quickly to avoid bleaching of the sample.
5. Pick up the embryo with micropipette with a minimum amount of carry-over ASW and place it in the methylcellulose drop on the glass slide.
6. Using a tungsten needle, gently stir the methylcellulose solution around the embryo in order to homogenize the methylcellulose solution and the carry-over ASW. Note that rapid movements of the tungsten needle will distort the shape of embryos.
7. Move the tungsten needle gently in the solution to push the embryo towards the surface of the glass slide (without directly touching the embryo). At the same time, orient it roughly to your desired position.

8. Using forceps, gently place the cover slip with a single motion.
9. Gently press the cover slip with your fingers under a stereomicroscope until it is almost in direct contact with the embryo. Gently displace the cover slip in the X-Y dimensions with fingers to orient the embryo. Again, it is important to be gentle in order not to distort or destroy the embryo. As mentioned previously, this is the most difficult part of the procedure and will require some practice.
10. Seal the remaining perimeter of the cover slip by depositing silicone grease gently along the perimeter. Take care that the tip of the syringe does not touch the cover slip during the process.
11. Put two small dots on the cover slip with a fine tip marker pen either side of the embryo so that you can find it more easily on the microscope.

Time-lapse imaging with a confocal microscope

Microscope. Leica SP5 confocal microscope (inverted).

Example of settings used. Movie S1 represents an example of time-lapse imaging of *Ciona* embryos with a confocal microscope. The settings used to acquire the movie are described below as a guide, although the exact settings would need to be modified to obtain the best imaging for each experiment and confocal microscope used. In order to label microtubules and chromosomes, unfertilized eggs of *Ciona intestinalis* were injected with a mixture of synthetic mRNAs encoding *ensconsin::3xVenus* (conc. in needle: 2.0 μ g/ μ L) and *H2B::mCherry* (1.5 μ g/ μ L) and fertilized. 4D images were captured with 40 \times /1.3NA objective lens using bidirectional mode (400 Hz) with line average of 2 at a resolution of 512 \times 512 pixels. AOTF settings were 12% of 488 line in an Argon laser for Venus imaging (collection between 506 and 533 nm) and 13% of 543 line of a HeNe laser for mCherry imaging (collection between 590 and 647 nm). Both channels were simultaneously scanned. One image stack consists of 25 z-sections with a 3 μ m optical slice thickness. Image stacks are collected every 1 min for 3 h. Under these settings, the image acquisition did not result in perturbation of embryonic development. Each frame of the movie corresponds to a maximum intensity projection of selected z-sections. The image acquisition was carried out at 18°C in a temperature-controlled room.

Time-lapse imaging with an epifluorescence microscope

Microscope. Olympus IX70 inverted epifluorescence microscope equipped with a cooled CCD camera (Micromax, Sony Interline chip, Princeton Instruments), a Xenon lamp and a Piezo (Physik Instrumente) and operated using MetaMorph software (Molecular Devices).

Example of settings used. Movie S2 represents an example of time-lapse imaging of *Phallusia* embryos with an epifluorescence microscope. As above, we provide below the details of the image acquisition settings as a guide, which should be adjusted depending on the precise experiment and equipment. Unfertilized eggs of *Phallusia mammillata* were injected with a mixture of synthetic mRNAs encoding MAP7::GFP (microtubules) and H2B::RFP (chromosomes) and fertilized. We used 200 ms exposures at each wavelength at 10 focal planes using a Piezo to position the objective lens coupled with a motorized stage (x–y) to follow several embryos. The image acquisition was carried out at 18°C in a temperature-controlled room. Such image stacks can be collected every 2 min for up to 10 h without perturbing embryonic development under these settings. The movie shown is a superposition of three z planes (from a total of 10).

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Movie S1. Microtubule dynamics in the epidermal lineage of a developing *Ciona* embryo. The sequence covers from the neurula to early tailbud stages. See text for the details.

Movie S2. Microtubule dynamics during early cleavage stages of a *Phallusia* embryo. See text for the details.

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