Chapter 14

Embryological Methods in Ascidians: The Villefranche-sur-Mer Protocols

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Abstract

Ascidians (marine invertebrates: urochordates) are thought to be the closest sister groups of vertebrates. They are particularly attractive models because of their non-duplicated genome and the fast and synchronous development of large populations of eggs into simple tadpoles made of about 3,000 cells. As a result of stereotyped asymmetric cleavage patterns all blastomeres become fate restricted between the 16- and 110 cell stage through inheritance of maternal determinants and/or cellular interactions. These advantageous features have allowed advances in our understanding of the nature and role of maternal determinants, inductive interactions, and gene networks that are involved in cell lineage specification and differentiation of embryonic tissues. Ascidians have also contributed to our understanding of fertilization, cell cycle control, self-recognition, metamorphosis, and regeneration. In this chapter we provide basic protocols routinely used at the marine station in Villefranche-sur-Mer using the cosmopolitan species of reference *Ciona intestinalis* and the European species *Phallusia mammillata*. These two models present complementary advantages with regard to molecular, functional, and imaging approaches. We describe techniques for basic culture of embryos, micro-injection, in vivo labelling, micro-manipulations, fixation, and immuno-labelling. These methods allow analysis of calcium signals, reorganizations of cytoplasmic and cortical domains, meiotic and mitotic cell cycle and cleavages as well as the roles of specific genes and cellular interactions. Ascidians eggs and embryos are also an ideal material to isolate cortical fragments and to isolate and re-associate individual blastomeres. We detail the experimental manipulations which we have used to understand the structure and role of the egg cortex and of specific blastomeres during development.

Key words: Ascidians, eggs, embryos, isolated cortex, methods, micro-injections, in vivo labelling, imaging, micro-manipulations, immuno-labelling.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_14,

1. Introduction

The European tradition of studying tunicate embryos (ascidians and appendicularians) started with Kowalevsky in 1866 (1) and Fol in 1879 (2) who discovered that these marine invertebrates (**Fig. 14.1a**, **d**) developed from a simple tadpole larvae (**Fig. 14.1g**, **h**) which represented a greatly simplified chordate body plan. The first experimental manipulations separating blas-

Fig. 14.1. (continued) network (*red*) and incubated in DiO-C2(3) to label mitochondria (*green*). (**I**) Montage of the two fluorescent channels from an egg, spliced together along the animal, vegetal axes (confocal section). Note the subcortical layer rich in mitochondria (Mito) and poor in ER (*arrows*) in the vegetal hemisphere; from Prodon et al. (33). (**J**) 16 cell stage embryo, *arrows* show the myoplasm in the smaller posterior-most (P) blastomeres. (**K**) Ascidian egg fertilization calcium wave: the wave of elevated calcium (Ca^{2+}, *red*) starts from the point of sperm entry (*arrow*) and propagates through the fertilized egg. Confocal section of an egg injected with Calcium-Green dextran. (**L**) *Phallusia* egg injected with two synthetic mRNAs: one coding for a histone (RFP fusion, in *red*) and the other for a nuclear and kinetochore marker (Venus fusion, in *green*). The injected egg was then fertilized. The image shows a 4 h post-fertilization gastrula stage embryo with nuclear (*arrows*) and mitotic chromosomes plus kinetochores (*arrowheads*) labelling. (**M**) Ascidian embryo injected with mRNA encoding EGFP at the 1 cell stage (*upper* embryo) and at the 2 cell stage (one blastomere which gives rise to a half-labelled embryo, *lower* embryo). (**N**) *Phallusia* egg fixed with formaldehyde 5 min post-fertilization and labelled with rhodamine phalloidin (in *red*). Accumulations of actin microfilaments are observed in the vegetal (*v*) contraction pole (*arrowheads*) and at the animal (*a*) pole corresponding to the position of the first meiotic spindle (*arrow*). (**O**) *Phallusia* egg fixed with methanol 5 min post-fertilization and immuno-labelled for microtubules (MT, rhodamine-coupled secondary antibody, in *red*) and mitochondria (Mito, Cy5-coupled secondary antibody, in *magenta*). DNA is labelled with Hoechst (in *blue*). This view of the animal pole shows the meiotic spindle before polar body extrusion. (**P**) *Phallusia* 4 cell stage embryo fixed with methanol and immuno-labelled for aPKC and mitochondria. View of the CAB region (*arrowheads*) showing accumulation of aPKC at the posterior pole. Primary antibody against aPKC is used at 1/100 dilution and signal is amplified with biotin/streptavidin (coupled with fluorescein, in *green*). Mitochondria are labelled with a secondary antibody coupled with Cy5 (in *magenta*). (**Q**) *Phallusia* 4 cell stage embryo fixed with methanol and immuno-labelled for aPKC. *Posterior view* showing aPKC enrichment in the CAB (*arrowheads*). Primary antibody against aPKC is used at 1/500 dilution, and the signal is amplified with TSA (Alexa488, in *green*). (**R**) *Ciona* isolation/re-association of blastomeres: indicated cells were isolated or co-isolated from 8 cell stage embryos and cultured until control embryos reached the 110 cell stage. According to the cell lineage, at this developmental stage the A4.1 lineage generates four notochord, four neural, three endoderm and one trunk lateral precursors. In the A4.1-derived partial embryo (*left*), however, a notochord marker gene, *brachyury*, is expressed in eight cells (*arrow*), indicating an ectopic formation of notochord precursors. In contrast, when A4.1 was co-isolated with a4.2 (*right*), the derived partial embryo expresses *brachyury* in four cells (*arrow*), indicating that cellular interactions between A4.1 and a4.2 lineages repress the formation of ectopic notochord fates. This cell isolation experiment resulted in identification of an Ephrin ligand, which is expressed in a4.2-derived cells and acts as the signal to repress notochord fates. *See* Picco et al. (20). (**S**) *Phallusia* isolated cortices: low magnification view of a field of cortical fragments isolated from eggs and 8 cell stage embryos (*arrowheads*: CAB). ER is labelled with CM-DiI-C16(3) (in *red*). (**T**) *Phallusia* cortical fragment isolated from an egg (about 15 μm in diameter). The ER network (*arrowheads*) adhering to the plasma membrane is labelled with DiO-C6(3) after fixation (in *green*). (**U**) *Phallusia* cortex isolated from an 8 cell stage embryo. ER labelled with DiO-C6(3) after fixation (in *green*) accumulates in the CAB (*arrowheads*). (**V**) *Phallusia* cortex isolated from an 8 cell stage embryo. The cortex was prepared in presence of EGTA and taxol, then fixed and immuno-labelled for microtubules (MT, Cy5-coupled secondary antibody, in *magenta*) and aPKC (primary antibody used at 1/100 dilution). The aPKC signal was amplified with the biotin/streptavidin system (fluorescein-coupled, in *green*). MT and aPKC are retained in the CAB (*arrowheads*) after cortex isolation.

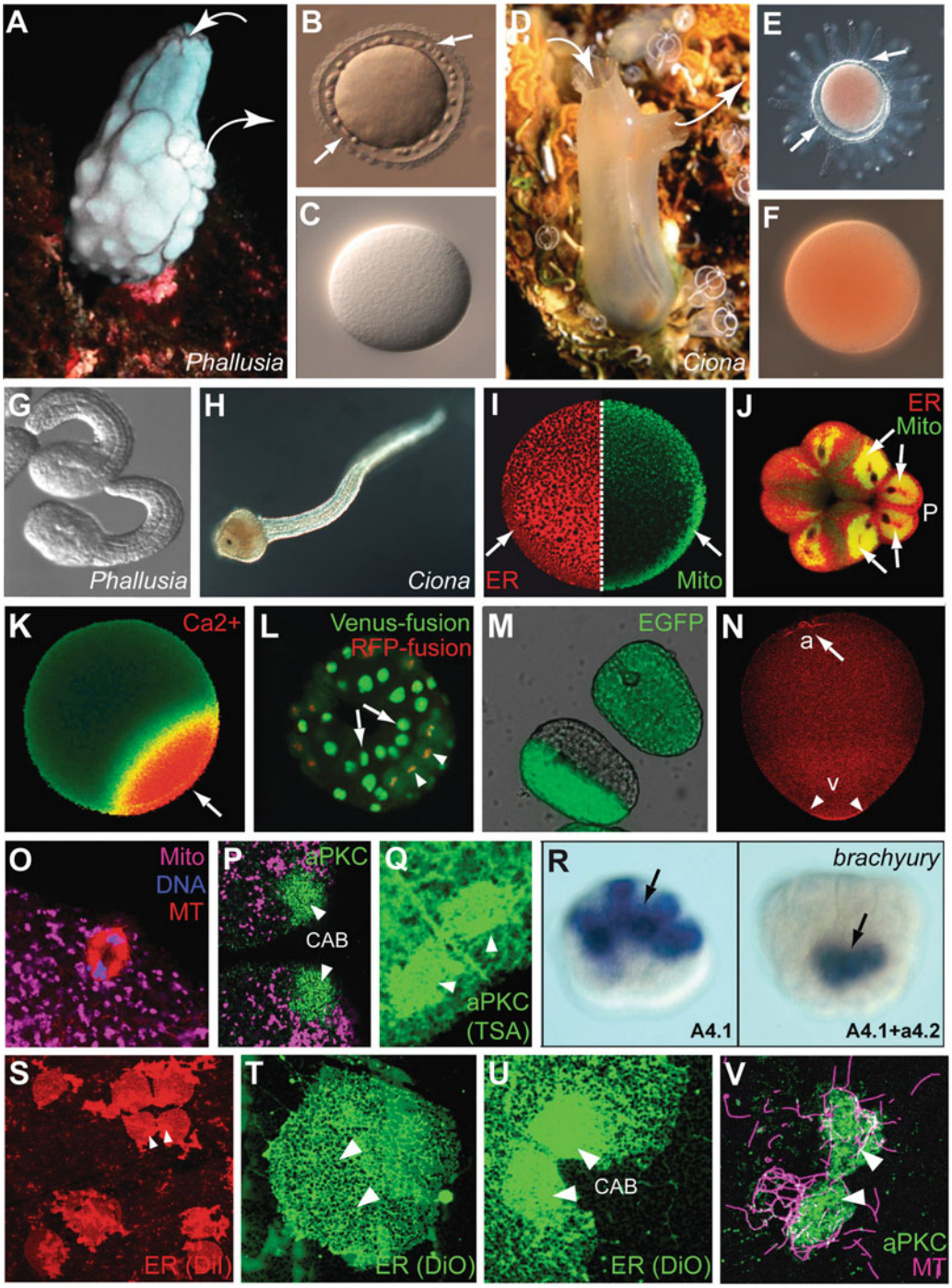

Fig. 14.1. Examples of labelled *Phallusia* and *Ciona* eggs and embryos. (**A, B, C**) *Phallusia mammillata*. (**A**) Adult animal with siphons (*arrows*); (**B**) unfertilized egg with chorion (*arrows*); (**C**) dechorionated egg (diameter is about 110–120 μm). (**D, E, F**) *Ciona intestinalis*. (**D**) Adult animal with siphons (*arrows*); (**E**) unfertilized egg with chorion (*arrows*); (**F**) dechorionated egg (diameter is about 120–130 μm). (**G**) Two *Phallusia* tadpoles whose development from fertilization has been filmed in time lapse (DIC optics) for 10 h in a micro-chamber (see whole sequence on BioMarCell web site, **Note 1**). (**H**) *Ciona* tadpole (16 h post-fertilization). (**I, J**) *Phallusia* eggs were injected with DiI-C16(3) to reveal the ER

tomeres of any embryos were performed by Chabry using the ascidian *Ascidiella aspersa* (3). More than a century ago, Conklin proposed that "organ forming substances" were located in peripheral regions of the ascidian egg and in particular that the "myoplasm", a sub-cortical domain (coloured yellow in *Styela partita*) gave rise to tail muscle cells (4). Fifty years later Italian biologists (5) and more recently Japanese investigators (6–8) showed through key ablation and transplantation experiments that determinants of axis establishment and muscle cell differentiation were situated in cortical and sub-cortical domains. Some of these determinants have now been identified as localized maternal mRNAs (9, 10) like in the fly *Drosophila*, the toad *Xenopus* and the jellyfish *Clytia* (11–13).

Recent molecular phylogeny studies suggest that ascidians (urochordates) are the closest sister groups of vertebrates (14). There is a sense that certain questions tackled on vertebrate models may be more easily addressed using the tadpole of ascidians, a relatively simple assemblage of approximately 3,000 cells whose lineages are well documented. A small set of precursor cells specified between the 16- and 110 cell stages generates the six tissues and a population of primordial germ cells making up the tadpole (8, 15, 16). Ascidians are particularly attractive to study the so-called mosaic type of development, maternal determinant segregation, and cell and tissue differentiation in a simple tadpole. They are also used to address questions of self-recognition, metamorphosis and regeneration (7, 17). In addition to partitioning maternal determinants, the stereotyped cell division patterns have enabled researchers to rapidly identify the inductive interactions that take place between blastomeres. The signalling pathways and gene networks involved in these cellular interactions are being unravelled using sophisticated micro-manipulation and gene-based strategies (18–20).

Five ascidian species *Ciona intestinalis, Ciona savignyi, Phallusia mammillata, Halocynthia roretzi, Botryllus schlosseri* and the larvacean *Oikopleura dioica* have become prominent models for research (21). A dynamic and growing scientific community (about 500 people), which meets every 2 years (International Tunicate Meetings), contributes to the development and propagation of the urochordate model systems (21, 22). Many tools and approaches have been developed for the cosmopolitan species of reference *C. intestinalis*: a sequenced genome which is small and non-duplicated (about 160 Mb and 15,000 genes) (23), micro-manipulations and injection of synthetic mRNAs (24), introduction of plasmids by electroporation (25), gene silencing using morpholino oligo-nucleotides (26), the recent mastering of culture, transgenesis as well as successes with RNAi approaches (27–31), and excellent databases (*see* **Note 1**). Some of these tools are becoming available for the other ascidian model species which present advantages complementary to those

of *C. intestinalis*. Eggs and embryos of *P. mammillata* are remarkably transparent and are well suited for live imaging and early expression of exogenous mRNA (32–35). Cell lineages and morphogenetic events can be easily observed from egg to tadpole using specific labelling methods, observation chambers and time-lapse imaging stations. The larger (280 μm) eggs and embryos of *H. roretzi* are the best suited for micro-manipulations (36). Finally *C. savignyi* is useful for comparison of gene regulatory sequences with *C. intestinalis* (37, 38). This diversity of available models is suited to evo-devo studies of genes and molecular and cellular mechanisms (19, 39).

There are several useful resources for those who may consider working with ascidians. Noriyuki Satoh's classical book "*Developmental Biology of Ascidians*" remains the reference (7). Recent advances in the ascidian field are presented in special issues (17), International Tunicate Meetings reports (32) or reviews (16, 40–42). The Tunicate portal web site regroups a large amount of information about leading laboratories and resources (*see* **Note 1**). Time tables of development, digitized representations, and videos of *C. intestinalis* embryos are available on the ANISEED and FABA sites (*see* **Note 1**). Videos of fertilization and development of *P. mammillata* can be downloaded from our BioMarCell and BioDev web sites (*see* **Note 1**). Concerning methods, a chapter by B.J. Swalla in *Methods in Cell Biology* gives a phylogenetic description of urochordates as well as basic methods of culture, fertilization, etc. (43) and a chapter by W. Smith in this issue of *Methods in Molecular Biology* covers genetic approaches in ascidians. A list of methods for labelling marine embryos can be found in the Center for Cell Dynamics (Friday Harbor, USA) web site (*see* **Note 1**). A recent Cold Sping Harbor protocole series also covers many aspects of experimentation with *Ciona*.

In this chapter, we present the basic protocols used at the Villefranche-sur-Mer marine station ("Observatoire Océanologique de Villefranche-sur-Mer") by members of the Developmental Biology research unit. Three groups in this department (McDougall, Sardet, Yasuo) work on ascidians as their main experimental models (*C. intestinalis* and *P. mammillata*). Here we detail most basic techniques of ascidian culture, embryology, and cell biology, except for in situ hybridization (*see* **Note 2**) which is described in a separate volume of *Methods in Molecular Biology* devoted to mRNA visualization (44).

2. Materials

2.1. Fertilization and Culture of Ascidian Embryos

1. Animals: ascidians are sessile marine animals easy to collect from docks. The ascidian *P. mammillata* (termed *Phallusia*,

Fig. 14.1a–c) can be obtained all year long on the Mediterranean and Atlantic coasts while *C. intestinalis* (termed *Ciona*, **Fig. 14.1d–f**) can be collected in many temperate regions of the world. Both species can be maintained in aquaria at appropriate temperatures (16–18°C for *Ciona*, 18–22°C for *Phallusia*). There are institutional suppliers such as the Ascidian Stock Center at UC Santa Barbara (*see* **Note 1**), USA, or the Station Biologique de Roscoff, France. *Ciona,* which has marked reproductive periods in the wild, has been successfully cultivated through several generations in Japan, USA and Europe (31, 45). *Phallusia* gives abundant gametes throughout the year but the quality of embryonic development is best in spring and fall. They can be kept gravid in aquaria for several months under constant light when fed artemia and micro-plankton.

2. Sea water (SW): natural SW is sterilized with a large volume 0.2 μm filter unit. Natural SW can be replaced by ASW and/or supplemented with TAPS buffer and/or BSA (see below).
3. Artificial sea water (ASW): 420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$ and 10 mM Hepes buffer, pH 8.0. Sterilize with a 0.2 μm filter and add 0.05 g/L kanamycin sulphate. It can be stored at 4°C for several days. Note that *Ciona* development is sensitive to the SW quality and may be better with ASW.
4. TAPS buffer stock solution: 500 mM *N*-tris (hydroxymethyl)methyl-3-aminopropanesulphonic acid, pH 8.2. Store at room temperature (RT).
5. TAPS-SW, EDTA-SW and BSA-SW: although filtered natural SW or ASW works well, adding TAPS buffer at a concentration of 10 mM final or/and 1 mM EDTA can sometimes increase the quality of embryonic development for certain batches. Some batches of embryos can also be very sticky, in which case adding bovine serum albumin (BSA) at a concentration of 0.1% is helpful (rinse with SW before fixation).
6. 10× trypsin stock solution: 1% trypsin in SW and 100 mM TAPS, pH 8.2. Store at –20°C in 1 mL aliquots.
7. 1× Pronase/thioglycolate solution: 0.05% pronase and 1% thioglycolate in SW. Can be kept at 4°C and used for 1 week. For longer storage, aliquots of 20× stock solution (1% pronase, 20% thioglycolate) can be kept at –20°C.
8. GF (gelatin/formaldehyde): dissolve gelatin and paraformaldehyde each at a concentration of 0.1% in

distilled water by heating them under a fume hood at 60°C for 1 h. Store in 50 mL aliquots at 4°C.

9. Non-sticky coated dishes and glassware for *Phallusia*. To coat plastic or glass surfaces, wet with a thin layer of GF, dry, and rinse well in distilled water. After use, GF-coated dishes can be rinsed with tap water, stored at RT and re-used several times. Pasteur pipettes or glass capillaries should be similarly coated by passing GF through them a few times, allowed to dry and then rinsed.
10. Non-sticky coated dishes and glassware for *Ciona*: GF is not as effective at preventing sticking of *Ciona* eggs and embryos. It is therefore best to use agarose-coated dishes. Heat 1% agarose in SW and pour in dishes to make a thin (2 mm) layer, leave to cool and rinse in SW. For storage (up to a week), add ASW to agarose dishes and store wet at 4°C. New glassware should be soaked in tap water for 1–2 days (no detergent) to reduce stickiness. With use, pipettes and tubes become coated with egg debris which also prevents dechorionated eggs from sticking.
11. Micro-pipettes for handling eggs: a glass tube (outer diameter 5 mm; inner diameter 3 mm) is pulled under flame, so that one end becomes tapered to around 1 mm in diameter. To the large end, attach a rubber tube with its other end stapled (**Fig. 14.2b**). For pipetting very small volumes, pull a coated capillary tube (10–50 μL) under a flame to the desired diameter (just over the width of an egg) and attach it to an adaptor (Fisher Scientific 4,356 M) 30 cm long tube fitted with a mouth piece (suction by mouth pipette) or to a stapled tube as above (suction by hand pressure). Coated plastic tips for mechanical pipettes (200 μL yellow tip, small opening >200 μm diameter) may also be used.
12. GF-coated glass slides and cover-slips: apply thin layer of GF solution (about 50 μL for a slide, 20 μL for a cover-slip), dry then wash in distilled water.
13. Vaseline (local supermarket) and silicone grease (Dow Corning high vacuum grease): fill a syringe equipped with a plastic yellow tip with the grease.
14. Paper frame chamber for live imaging: this method is best for long-term observation and time-lapse acquisitions; it ensures excellent exchange of gasses (O_2/CO_2) and can even sustain development through metamorphosis (3–5 days after fertilization). Prepare small paper frames (18 mm × 18 mm) to fit under cover-slips (22 × 22, #1). Use lens cleaning paper or tissue paper which when wet will reach a thickness close to that of eggs and embryos: 120–150 μm. Deposit a paper frame on a coated slide and

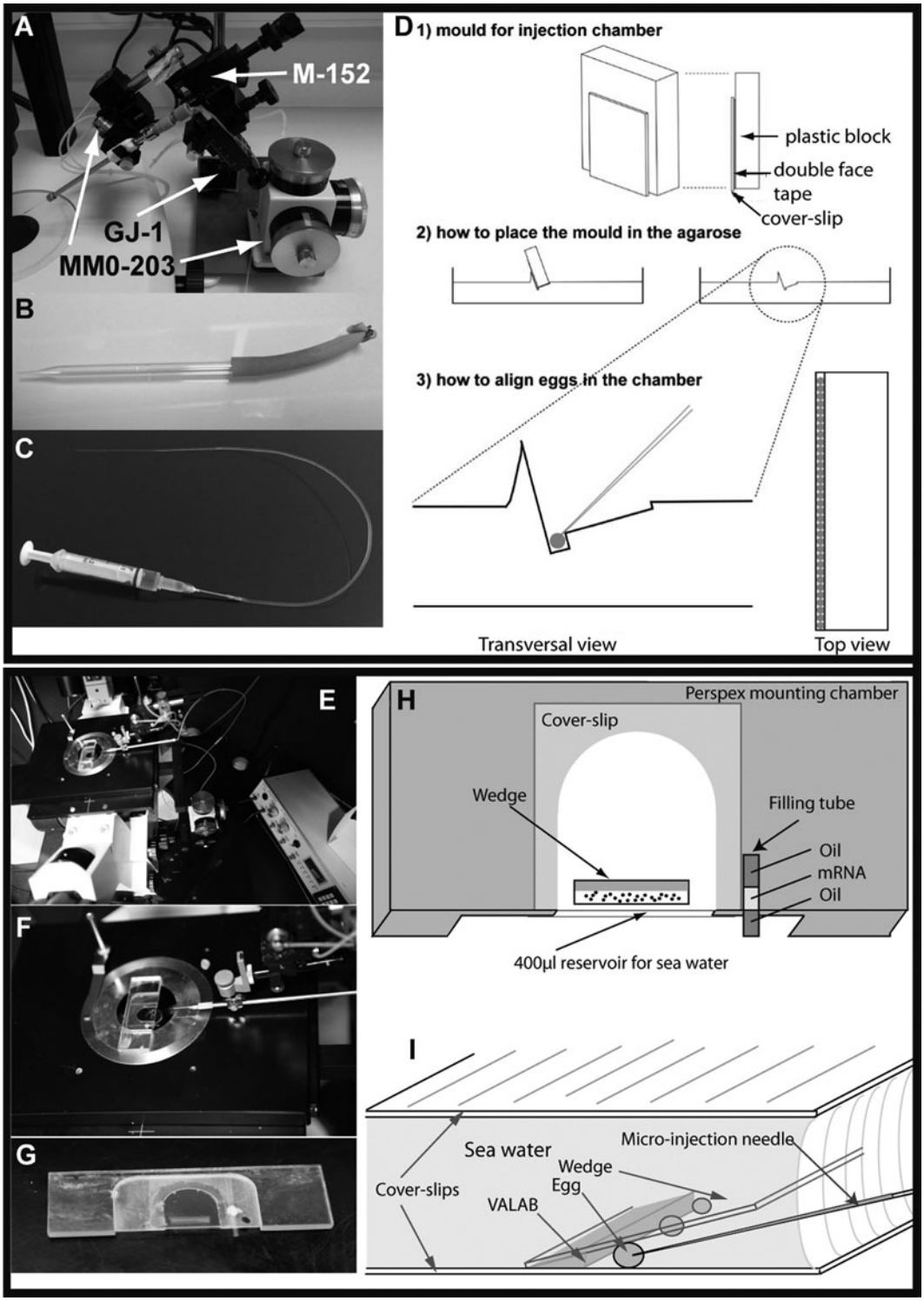

Fig. 14.2. Micro-injection setup. (**A–D**) Stereo-microscope setup/(**E–I**) inverted microscope setup. (**A**) Enlarged view of the manipulator assembly. The needle holder is connected to the glass syringe using the following Bio-Rad products: 1/16″ OD post-pump fittings and double Luer tubing adaptor. (**B**) Micro-pipette. (**C**) Mineral oil needle filler.

surround with a thin line of vaseline or silicone grease extruded from the syringe, drawn as a square (slightly smaller than the cover-slip). Deposit a tiny drop of SW with a few eggs or embryos in the centre of the frame. Pipette 30–50 μL SW onto paper frame. Cover with a coated cover-slip. Press delicately on the cover-slip to make sure there is a good seal all around and that eggs or embryos are just held in place in the micro-drop between coated slide and cover-slip. There should be an air space between the drop of SW and the moistened paper frame.

15. Vaseline/silicone chamber for live imaging: suitable for frequent or short-term observations (up to a few hours). Using the syringe, extrude two parallel lines of vaseline (or silicone) grease on a GF-coated slide. Place the embryos in a drop of SW (about 20 μL) on the slide. Cover with a GF-coated cover-slip, and gently flatten the grease lines with forceps until the embryo is slightly compressed such that it no longer moves when the cover-slip is tapped delicately. Fill the rest of the space between slide and cover-slip with SW placed on the side of the cover-slip, again checking that the embryos remain snug. This chamber can be perfused with solutions (for example, activated sperm to fertilize the eggs or a chemical inhibitor) or can be sealed with vaseline (or silicone) grease to prevent evaporation.

2.2. Injection of Eggs and Embryos

1. Set up on stereo-microscope/injecting from above: this is the most common way of injecting large numbers of eggs or embryos using a needle located above a line of eggs or embryos and approaching them at an angle (**Fig. 14.2a–d**). We use the following equipment: Leica S8APO stereo-microscope with Leica TL BFDF (brightfield–darkfield transmitted light base), Narishige MMO-203, three-axis oil hydraulic micro-manipulator, Narishige M-152 manipulator, Narishige GJ-1 magnetic stand, Narishige IMH1 injection holder assembly, glass syringe with a male Luer-Lock connection fitting, iron plate. For making injection chamber:

Fig. 14.2. (continued) A hand-pulled capillary is connected, via a Teflon tube, to a needle attached to a 2 mL plastic syringe. (**D**) Agarose injection chamber. Schematics showing: (**1**) how to make a mould for injection chamber; (**2**) how to place the mould; (**3**) how to align ascidian eggs in the chamber. (**E**) The wedge injection chamber mounted on the stage of an inverted IX70 Olympus microscope with micro-injection needle, three-way micro-manipulator and injection box shown. (**F**) Close up of the wedge injection chamber with micro-injection needle horizontal to the stage. (**G**) Close up of the wedge injection chamber with the wedge and filling tube visible. (**H**) Schematic showing the wedge injection chamber with the wedge and filling tube highlighted. In this example mRNA has been loaded into the filling tube. (**I**) Close up of the injection showing the needle filled with injection solution being inserted into an egg held in place in the wedge.

1.5% agarose in SW, plastic Petri dishes (5 cm diameter), cover-slip (#1), plastic block (about 15 mm × 15 mm × 5 mm), double face tape.

2. Setup on inverted microscope/horizontal "wedge" method: detailed articles have been published on this method (46) (**Fig. 14.2e–i**). We use the following equipment: an inverted Olympus IX70 microscope with stage control on the left to free the right hand for the micro-manipulator, MHO-103 three-axis oil micro-manipulator (Narishige, now the MMO series), type-A stage-side mounting system (mounted on the right-hand side) with a NR adaptor pillar, H1-7 type injection needle holder (IMH1 set with Teflon tubing and connector) connected to a B-8B ball joint connector, a silent air compressor (local supermarket) connected to a Narishige IM300 (we use about 60–70 psi air pressure from the compressor). Plexiglass stage mounting chambers are made by our workshop. For making wedge injection chamber, use GF-coated cover-slips (#1, 22 mm × 22 mm).
3. Morpholinos are purchased from Gene Tools. They are resuspended at 2 mM in distilled water and stored in aliquots at –80°C (note that the manufacturer recently recommended storing morpholino at RT).
4. Synthetic mRNAs are prepared as concentrated solutions (1–2 μg/μL) in distilled water and small aliquots are frozen at –80°C. We routinely use the vectors pRN3 (47) or its derivative pSPE3 (48) to synthesize mRNA for microinjection. For pRN3, the ORF of the gene of interest is PCR amplified such that it can be cloned directionally into the multi-cloning sequence at BglII, EcoRI and NotI restriction sites. For pSPE3, the ORF of interest is first cloned into a Gateway entry vector (pENTR/D-TOPO) and then inserted into the destination vector using recombination cloning technology (Invitrogen). Both pRN3 and pSPE3 constructs are linearized with SfiI restriction enzyme and used as a template for in vitro mRNA synthesis using T3 mMESSAGE MACHINE kit (Ambion). The vector pCS2+ (49) can also be used for mRNA synthesis, but the resulting mRNAs must be additionally polyadenylated using a Poly (A) Tailing Kit (Ambion) for efficient translation in ascidian embryos.
5. Fast Green (Sigma-Aldrich): 1 mg/mL in distilled water (2× stock).
6. Fluorescent dextrans, 10 kDa molecular weight (Texas-Red-coupled, fluorescein-coupled, rhodamine-coupled; Molecular Probes): 2 mM in distilled water (2× stock).

2.3. In Vivo Labelling of Eggs and Embryos

1. DiO-C2(3) (Molecular Probes): a 1,000× stock solution is made at 5 mg/mL in ethanol and kept at 4°C. Observe with fluorescein filter set.
2. MitoTracker (Molecular Probes). We have successfully used MitoTracker Red FM, MitoTracker Green FM and MitoTracker Red CM-H2XRos. For each of these, a 1,000× stock solution is made at 1 mM in DMSO and stored at –20°C.
3. Rhodamine 123 (Molecular Probes): a 1,000× stock solution is made at 1 mg/mL in ethanol and kept at 4°C. Observe with fluorescein filter set.
4. TMRM and TMRE (Molecular Probes): a 1,000× stock solution is made at 1 mM in ethanol and kept at 4°C. Observe with rhodamine filter set.
5. Hoechst 33342: powder is dissolved in distilled water at a concentration of 10 mg/mL and stored in aliquots at –20°C. An aliquot is diluted 1/50 (200 μg/mL) in distilled water and stored for several months at 4°C as a 400× stock solution for in vivo labelling of chromosomes. Observe with UV filter set.
6. Calcium-Green dextran, 10 kDa molecular weight (Molecular Probes): dissolve in injection buffer (180 mM KCl, 100 μM EGTA, 30 mM BES buffer, pH 7.1) at a concentration of 10 mM. Observe with fluorescein filter set.
7. Fura-2 dextran, 10 kDa molecular weight (Molecular Probes): dissolve as for Calcium-Green dextran. Observe with Fura-2 filter set (an excitation filter wheel is required).
8. DiI-C16(3) (1,1′-dioctadecyl-3,3,3;3′-tetramethylindocarbocyanine perchlorate; Molecular Probes). For injection and endoplasmic reticulum (ER) labelling, a saturated solution of DiI is made by mixing several crystals of DiI in 100 μL of soybean oil (Wesson oil). For plasma membrane labelling, make a 2.5 mg/mL stock solution in ethanol and store at –20°C. Observe with rhodamine filter set.
9. Succinylated concanavalin A, Alexa488 conjugate (Molecular Probes): a 1,000× stock solution is made at 5 mg/mL in 0.1 M sodium bicarbonate pH 8.3, aliquoted and stored at –20°C. Observe with fluorescein filter set.
10. FM 4-64 lipophilic dye (Molecular Probes): a stock solution is made at 10 mg/mL in DMSO, aliquoted and stored at –20°C. Observe with rhodamine filter set.
11. FITC: fluorescein isothiocyanate (Molecular Probes). Make 100× stock solution by dissolving FITC in DMSO at 10 mg/mL. Store at –20°C. Observe with fluorescein filter set.

12. Syto12 (Molecular Probes): 5 mM solution in DMSO. Store at –20°C. Observe with fluorescein filter set.
13. Nile Blue powder (Merck).

2.4. Blastomere Isolations and Ablations

1. Glass needle/knife: the tapered part of a Pasteur pipette is pulled under flame to create a very thin string of glass, which should be like a wool fibre. When first using a new glass embryo knife it is necessary to coat the knife to prevent sticking. This is done by smashing an egg or embryo and pulling the knife back and forth through the cellular debris.
2. 1.5% agarose-coated Petri dishes: make them like 1% agarose dishes (**Section 2.1**).
3. EMC (or calcium-free artificial sea water): 480 mM NaCl, 9.4 mM KCl and 23.6 mM EGTA. Equilibrate pH to 8.0 with NaOH, autoclave and store at RT.

2.5. Fixing and Immuno-labelling Eggs and Embryos

1. PBS solution: make a standard 10× PBS solution (1.37 M NaCl, 26.8 mM KCl, 100 mM Na_2HPO_4, 17.6 mM KH_2PO_4, pH 7.5), autoclave and store at RT. Dilute in distilled water to prepare 50 mL of 1× PBS solution (termed PBS) which can be stored for use at 4°C for 1–3 days.
2. 20% formaldehyde stock solution: dissolve 10 g of paraformaldehyde powder in 50 mL of distilled water and add 80 µL of 10 N NaOH. Keep at 50°C overnight (ON) and mix until completely dissolved. Pass through a 0.4 µm filter and store in aliquots at –20°C. To thaw, aliquots must be warmed at 50°C for 1 h. For convenience, formaldehyde solution can also be purchased commercially (32% stock solution without methanol, Electron Microscopy Sciences).
3. Formaldehyde fixative: dilute the formaldehyde stock solution to 4% in PBS, add NaCl to 0.5 M. Store at 4°C for 1 month maximum in 1.3 mL aliquots in screw cap tubes; for longer storage, keep at –20°C.
4. Methanol fixative: 90% methanol and 50 mM EGTA, pH 7.5. Store at –20°C in 1.3 mL aliquots in screw cap tubes.
5. PBS-Tween solution (PBS-Tw): add Tween20 to PBS at a final concentration of 0.1%. Store at 4°C for 1–3 days.
6. PBS-Triton solution (PBS-Tr): add TritonX100 to PBS at a final concentration of 0.1%. Store at 4°C for 1–3 days.
7. PBS-Tw-BSA: add Tween20 and BSA at final concentrations of 0.1 and 0.5%, respectively, in PBS.
8. PBS-BSA: dissolve BSA in PBS at a final concentration of 1%. Store at –20°C in aliquots.
9. Primary antibodies: antibodies are aliquoted and stored at –80°C. Once thawed, aliquots can be kept at 4°C for

months (*see* **Note 3**). In the example given in **Sections 3.5** and **3.6**, we use an antibody which labels aPKC (rabbit polyclonal sc216 from Santa Cruz Biotechnology) at a working dilution ranging from 1/100 (with fluorescently coupled secondary antibody or biotin/streptavidin amplification) to 1/500 (with TSA method), the NN18 antibody (mouse monoclonal N5264 from Sigma-Aldrich, *see* **Note 4**) which labels mitochondria at a working dilution of 1/400 and anti-tubulin antibody YL1/2 (rat monoclonal ab6160 from Abcam) at a working dilution of 1/500.

10. Secondary antibodies: fluorescently labelled secondary antibodies raised in goat against rabbit, mouse and rat immunoglobulin are purchased from Jackson Immuno Research laboratories. Antibodies against rabbit are pre-absorbed by the manufacturer against human serum proteins, whereas antibodies against mouse and rat are also pre-absorbed against related species (rat for antibody against mouse and inversely). All antibodies are reconstituted at the concentration recommended by the manufacturer. We add an equal volume of pure glycerol for cryo-protection and store the antibodies as small aliquots at –80°C. Once thawed, working aliquots can be kept at 4°C for several months (*see* **Note 3**). In the examples provided in the method (**Sections 3.5** and **3.6**) we use a Cy5-conjugated goat anti-mouse, and rhodamine or Cy5-conjugated goat anti-rat, each at a 1/100 dilution.
11. Biotin-conjugated antibodies and fluorescent streptavidin: purchased from Jackson Immuno Research laboratories and handled like fluorescently labelled secondary antibodies (see above). In the example provided in **Sections 3.5** and **3.6**, we used a biotin-conjugated goat anti-rabbit at 1/200 dilution and a fluorescein-conjugated streptavidin at 1/100 dilution.
12. HRP-coupled antibodies and TSA kits (Molecular Probes). In the example provided in **Section 3.5**, we use an HRP-conjugated goat anti-rabbit at 1/100 dilution. We recommend the Alexa488-TSA kit, which gives very strong green fluorescence labelling without background (for TSA kits using other fluorophores or haptens, *see* **Note 5**).
13. Fluorescent phalloidins (Sigma-Aldrich or Molecular Probes): reconstituted at a concentration of 50 μg/mL (approx. 35 μM, 1 unit/μL) in DMSO (*see* **Note 6**) and stored in aliquots at –20°C. If the fluorescent phalloidin is provided in methanol, the methanol must first be eliminated by evaporation (keep vial opened for a few days at RT in a dark chamber) before reconstituting in DMSO.

Once thawed, use phalloidin at a dilution of 1/100 and store the working aliquot at 4°C. In the examples provided (**Sections 3.5** and **3.6**), we used rhodamine–phalloidin.

14. Hoechst 33342: Use 200 μg/mL working aliquot as a 100× stock solution for chromosome labelling on fixed samples (**Section 2.3**).
15. Citifluor AF1 antifade mounting medium (Electron Microscopy Sciences). Store at –20°C in aliquots. Once thawed, store the working aliquot at 4°C.

2.6. Isolated Cortices: Preparation, Labelling and Imaging

1. Glass cover-slips (18 mm × 18 mm, #1) successively cleaned with 10% Tween20, distilled water and ethanol. Keep them dry on parafilm in closed Petri dishes.
2. EMC (**Section 2.4**).
3. Buffer X: 350 mM K-aspartate, 130 mM taurine, 170 mM betaine, 50 mM glycine, 19 mM $MgCl_2$ and 10 mM Hepes buffer. Equilibrate pH at 7.0 with KOH. Sterilize with 0.2 μm filter and store at –20°C in 10 mL aliquots.
4. CIM solution: 800 mM glucose, 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA and 10 mM MOPS buffer. Equilibrate pH at 7.0 with KOH. Sterilize with 0.2 μm filter and store at –20°C in 10 mL aliquots.
5. CIM fixative: add 3.7% formaldehyde (without methanol trace, **Section 2.5**) and 0.1% glutaraldehyde to CIM solution just before use.
6. CM-DiI-C16(3) ("Fixable DiI"; Molecular Probes). Add 20 μL ethanol to a vial of fixable DiI. This 2.5 mg/mL stock solution can be stored at 4°C for several days. Observe with a rhodamine filter set.
7. DiO-C6(3) (Molecular Probes). This dye labels all membranes including the ER network, the plasma membrane, and occasional vesicles and mitochondria in preparations of living or fixed isolated cortices. A stock solution at 0.2 mg/mL in ethanol is stable and can be stored at 4°C for several years. Observe with a fluorescein filter set.

3. Methods

3.1. Fertilization and Culture of Ascidian Embryos

3.1.1. Obtaining Gametes

1. Dissect the hermaphroditic animals by cutting through their tunics between the siphons and peel away the tunic (50).
2. Pierce the overlying oviduct and collect the eggs using plastic or glass pipettes, massaging the oviduct to empty all the eggs. Deposit the eggs in SW and store them at the appropriate temperature (16°C for *Ciona* and 18°C for *Phallusia*).

3. Then collect sperm from the underlying spermiduct using a pipette or a needle and syringe. Concentrated sperm can be stored several days at 4°C in small plastic tubes. *Phallusia* eggs can be efficiently fertilized by sperm from the same individual (therefore one should take care not to contaminate eggs with sperm while collecting gametes), whereas self-fertilization is inefficient in *Ciona*, so it is necessary to open at least two animals.
4. It is also possible to obtain immature oocytes from ovary tissue in order to study oocyte maturation and polarity. We have analysed this process for *Ciona* (50).

3.1.2. Egg Dechorionation

Ascidian eggs are surrounded by a chorion layer containing test cells and follicle cells (**Fig. 14.1b**, **e**) which should be removed for observation and subsequent experimental procedures (unless one wants to observe tadpoles, which emerge from the chorion by hatching). After dechorionation (**Fig. 14.1c**, **f**), eggs and embryos tend to stick, so they should be handled gently and transferred with large diameter plastic pipettes or coated glass pipettes into dishes which are specially coated. For transferring a small number of eggs or embryos, we use hand-held micro-pipettes.

1. For *Phallusia*: add 1 mL 10× trypsin stock solution to 9 mL chorionated eggs in SW, add 250 µL of 500 mM TAPS buffer and shake or rotate gently (20 rpm) for 2–4 h at 18–22°C (51). Eggs can also be treated briefly with pronase/thioglycolate (as for *Ciona*) if test cells are not completely removed.

   When most eggs have a smooth surface (**Fig. 14.1c**) and settle to the bottom of the dish, wash several times with SW by swirling eggs to the centre of the dish with a gentle wrist motion. The chorion debris, dead cells and chorionated eggs will float and can be removed with vacuum aspiration.

   Transfer dechorionated eggs to a GF-coated Petri dishes (5 or 10 cm diameter) at a low density, so that they are not touching.

   Eggs can be kept at 18°C for several hours or up to a day, awaiting fertilization, injection or fixation.
2. For *Ciona*: add 250 µL of 1 N NaOH to 10 mL of 1× pronase/thioglycolate solution and mix well. Then add 250 µL more of 1 N NaOH followed by mixing (pH will rise to around 10). Adding NaOH creates white precipitates which should vanish once the solution is mixed.

   Transfer chorionated eggs (about 5 mL) to a 15 mL glass test tube (with use these tubes become coated with debris which prevents sticking, so we simply rinse in tap water—no detergent—and re-use the same glass tubes).

Add 1 mL of the basic pronase/thioglycolate solution to the glass tube containing chorionated eggs. Mix well by gentle pipetting. Wait 5 min or so until eggs sediment to the bottom of the tube. Remove as much supernatant as possible.

Add remaining volume of the basic pronase/thioglycolate solution to the packed chorionated eggs and mix with gentle pipetting. Within 2–3 min, chorions should start to dissolve. Check occasionally under a stereo-microscope, and once the majority (2/3) eggs lose their chorion (**Fig. 14.1f**), gently add SW to fill the glass tube.

Wait for dechorionated eggs to settle to the bottom (dechorionated eggs sediment quickly) and remove supernatant. Gently add fresh SW and repeat the washing procedure three times.

Transfer eggs to agarose-coated Petri dishes (5 or 10 cm diameter) and place at 16°C.

Eggs can be kept for several hours or up to a day, awaiting fertilization, injection or fixation.

3.1.3. Sperm Activation

If one wishes to fertilize eggs rapidly and synchronously, it is best to first activate sperm and test the different sperm batches individually for their ability to fertilize (52). If synchrony is not important, one can simply add concentrated sperm to eggs in SW at a dilution of 1/1,000 and most eggs will become fertilized over a period of 10–30 min.

1. Activation by exposure to chorionated eggs: add 25 μL of concentrated sperm to 5 mL SW containing 100 μL chorionated eggs; shake gently (20 rpm) for 15–60 min. Let chorionated eggs sediment to the bottom of the tube; the activated sperm suspension can be kept at 4°C and used to fertilize for several hours.
2. Activation by alkaline SW (with NaOH): dilute 10 μL of concentrated sperm in 2 mL of SW and then add 4–12 μL of 1 N NaOH. Wait 1 min and add the activated sperm to eggs. You have to find the right concentration of NaOH (usually 8 μL work well). This activation works very well with *Phallusia* sperm but should be done each time, just before fertilization.
3. Activation by alkaline SW (with Tris–HCl buffer): this is the preferred method for *Ciona*. Add 25 μL of 1 M Tris–HCl buffer (pH 9.5) to 0.5 mL of SW. To this alkaline SW, add 2.5 μL of concentrated sperm; mix and wait 1 min before fertilizing.

3.1.4. Fertilization and Culture of Embryos

Similar methods are used to fertilize *Phallusia* and *Ciona* eggs.

1. For *Phallusia*: to fertilize eggs in a synchronous manner, add 2 mL of activated sperm to 10 mL of dechorionated eggs in SW. Mix well. You can judge if synchronous fertilization has been achieved by observing egg shape change: 80% of the eggs should lose their roundness and become pear shaped within 5 min of sperm addition.

   Once eggs are fertilized, wash extensively with SW and culture the embryos at 18–22°C, either spread in a monolayer (but not touching) in appropriately sized coated Petri dishes or keep as a suspension in a GF-coated beaker equipped with a paddle rotating at 50 rpm. For *Phallusia* maintained around 20°C, first cleavage takes place about 50 min after fertilization, gastrulation after 3 h and the embryo develops into a tadpole in 12 h. To see fertilization and development of *Phallusia* consult the BioMarCell film archive (*see* **Note 1**).

2. For *Ciona*: to fertilize eggs in a synchronous manner, add 500 μL of sperm activated with alkaline SW to 10 mL of dechorionated eggs in SW. Mix well. With an appropriate stereo-microscope, one can see active sperm moving around eggs. Eggs start to spin and, when fertilized, become pear shaped.

   After 10 min incubation, transfer eggs to another agarose-coated Petri dish containing SW and culture them at 16–18°C. Information on *Ciona* embryonic stages, morphology and cell lineages can be found on FABA and ANISEED databases (*see* **Note 1**).

3.1.5. Mounting Live Embryos for Imaging

Live ascidian eggs and embryos can be placed in glass bottom dishes (MatTek corporation) first coated with GF or cellular debris for observation with an inverted microscope. For improved optics on inverted or upright microscopes, we routinely mount live *Phallusia* eggs and embryos on coated slides using two types of observation chambers (paper frame chamber or vaseline chamber, **Section 2.1**). Live *Ciona* eggs and embryos can be similarly imaged for brief periods on GF-coated slides or on slides which have been licked to make them less sticky. During the time when slides are not being imaged, they should be kept in humid chambers made by placing wet paper towels inside appropriately sized plastic boxes or Petri dishes.

3.2. Injection of Eggs and Embryos

Two different set ups are routinely used in our laboratory and are somewhat complementary being more or less convenient for functional or imaging studies. One is based on a stereo-microscope with the needle at an angle, while the other is based on an inverted microscope with the needle horizontal (**Fig. 14.2**).

3.2.1. Injection Using the Stereo-microscope Setup

This injection method (**Fig. 14.2a–d**) is suitable for injecting large numbers of eggs and embryos, for instance, when they are to be used for functional studies followed by in situ hybridization or immuno-labelling.

1. Making the agarose injection chamber: a mould has to be prepared, a cover-slip (#1, thickness similar to the diameter of *Ciona* eggs: 120 μm) is stuck to a plastic block of about 15 mm × 15 mm × 5 mm on one of the largest surfaces using double face tape. One end of the cover-slip should protrude, ideally about 150 μm, from an edge of the plastic block. Carefully bring 1.5% agarose-containing SW just to a boil in a microwave oven. Pour the agarose SW into a plastic Petri dish (5 cm diameter) and float the Petri dish on ice-filled water.

   Place the mould in the centre of the dish as shown in **Fig. 14.2d**. Make sure that the cover glass does not touch the bottom of the dish. Let the agarose harden for about 2 min. Carefully pull out the mould and cover the agarose with SW.

   The agarose injection chamber can be stored at 4°C and be re-used for several injections.

   Using a micro-pipette, align dechorionated eggs in the well made with the protruding edge of the cover-slip of the mould.

2. Loading the needle: the injection holder assembly is connected to a glass syringe, via Teflon tubing which is filled with mineral oil (make sure that there are no air bubbles in any parts of this assembly).

   Needles are made from glass capillaries containing a thin glass filament (Harvard Apparatus GC100TF-10) using a needle puller (we use a Narishige PN-30).

   Needles are backfilled with about 0.5 μL of the solution to inject (morpholino, mRNA, etc.) by depositing the injection solution on the open large end of the needle, so that the solution will be transferred to the tip of needle along the filament by capillary action. Gently tap the needle while holding it upright to remove most of the air bubbles from the injection solution (injections solutions may be coloured using Fast Green).

   Once the injection solution is transferred to the tip of the needle, the needle should be filled completely with mineral oil using a hand-pulled capillary attached by tubing to a syringe (**Fig. 14.2c**).

   When the needle is inserted into the needle holder, special care should be taken not to introduce air bubbles. Apart from tiny air bubbles found in the injection solution, there should be no air bubbles in mineral oil from the needle to

the glass syringe. It is also important that the barrel and piston parts of the glass syringe move very smoothly.

3. To start injecting, proceed in the following way: break the tip of the needle by bringing it very slowly to a piece of cover-slip placed at a slant in the injection chamber. This is a critical step for successful injection. Make the opening of the tip as tiny as possible. The syringe piston should be pressed gently while the needle is brought towards the cover slip. This allows one to visualise when the needle is broken as small amounts of the coloured solution (Fast Green) in the injection needle can be seen leaking into the chamber.

   Place the tip of needle at the centre of egg and then apply brief suction by pulling on the syringe piston, so that a "hole" is made in the plasma membrane.

   Press the piston gently to deliver injection solution into the egg.

3.2.2. Injection Using the Inverted Microscope

We use this method (**Fig. 14.2e–i**) for mRNA injection into *Phallusia* eggs since it cuts down on the cost because 1 μL of mRNA can be used repeatedly for approx. 1 month (stored at 4°C in filling tubes, **Fig. 14.2e**). This method is also convenient for imaging live eggs and embryos under a light or confocal microscope while they are being injected or soon after (for example, to image calcium signals).

1. Making a wedge injection chamber: cut coated cover-slips into small pieces (approx. 4 mm ×10 mm) and attach to whole coated cover-slips with VALAB (composed of 1:1:1 vaseline, lanolin and bees wax) and a spacer to elevate one side of the wedge. The spacer is a cut piece of cover-slip (**Fig. 14.2h, i**).

   Heat on a hot plate until VALAB melts.

   Remove from hot plate and allow VALAB to cool (approx. 30 s or when it turns opaque).

   Carefully remove spacer with forceps (we keep spacers and re-use them); this creates the wedge, a slanted coated-glass sandwich.

   Attach the wedge to a plexiglass mounting chambers using silicone grease to seal on three sides and add dechorionated eggs or embryos using a mouth pipette (up to 100 eggs/embryos per wedge).

   Attach a second cover-slip to sandwich the wedge and fill the reservoir with approx. 400 μL SW (**Fig. 14.2h, i**) (*see* **Note 7** for alternative wedge).

2. Loading the needle: first, make a filling tube to hold the injection solution. Cut glass capillaries into pieces approx. 1 cm long. To one end add 0.5 μL mineral oil, then 0.5 μL injection solution and again 0.5 μL mineral oil.

The filling tube is fixed to the underside of the wedge using VALAB (**Fig. 14.2h**).

Needles are made from glass capillaries that do not contain a thin glass filament (Harvard Apparatus GC100T-10) using a needle puller (we use a Narishige PN-30).

The needle is inserted into the needle holder which is connected to an IM300 injection box via Teflon tubing and advanced towards the filling tube.

The needle is first broken carefully against the filling tube then front-filled with about 1 nL of injection solution (Morpholino, mRNA or fluorescent indicator) from a filling tube (**Fig. 14.2h**) by brief suction ("fill" function, approx. 30 psi). Needles that fill too fast are discarded and those that fill too slowly are broken again.

3. Injection: once the needle is filled with injection solution the balance pressure is adjusted, so that the meniscus moves slightly out towards the pipette tip.

   Needles are inserted into the centre of the egg (**Fig. 14.2i**) and suction is applied (Narishige "fill" function) to break the plasma membrane.

   Eggs are injected at approx. 5–10 psi air pressure using the foot pedal attached to the IM300.

   Filling tubes containing injection solutions can be stored at 4°C and re-used for several weeks.

3.2.3. Gene Knockdown with Morpholino

An aliquot of the morpholino to be injected is heated at 65°C for 10 min and made to the desired concentration with distilled water. A range (0.25–1.0 mM pipette concentration) is first injected to determine which concentration is best suited for the experiment. Prior to injection, the solution is spun in a micro-centrifuge at maximum speed for 5 min to sediment particles that might block the injection needle.

3.2.4. Injection of mRNAs and Plasmid DNA

Synthetic mRNAs are prepared as concentrated solutions (1–2 μg/μL) in distilled water and small aliquots are frozen at –80°C. Solutions are centrifuged in a micro-centrifuge at maximum speed for 5 min prior to loading the needle or filling tube, in order to sediment particles that might block the injection needle. For injection, different concentrations are tested after dilution in distilled water, ranging from 0.25 to 2.0 μg/μL in the pipette; generally using the lower concentrations for functional studies and the higher concentrations for visualization of fluorescent fusion proteins (**Fig. 14.1l**, **m**). For *Phallusia*, fluorescence from fluorescent protein constructs can be observed in an unfertilized egg a few hours after injection of concentrated mRNAs. Expression can be detected more rapidly in eggs that are fertilized, and fluorescent proteins continue to accumulate in dividing embryos.

For *Ciona*, unfertilized eggs do not translate exogenous mRNAs, but expression of fluorescent protein constructs can be observed few hours after fertilization (gastrula stage embryo) (35). Plasmid DNA is treated in a similar fashion for micro-injection, but at a lower concentration (generally 50 ng/μL).

3.2.5. Quantitation of Injection

The final concentration of the injected solution in the egg will be 1–10% of the pipette concentration depending on the injected volume. Injection volume is estimated by the diameter of clearing in the cytoplasm upon injection; this cleared space can be rendered more visible by the addition of an equal volume of Fast Green (0.5 mg/mL final) or fluorescent dextran (1 mM final) to the morpholino or mRNA injection solution. Centrifugation to clear the injection solution should be performed after the addition of these dyes. Typically, eggs are injected until 1/4 diameter of egg is filled with the coloured solution. The injected amount can be further quantified from the intensity of signal in the egg using a fluorescence microscope (for a precise method to quantify the amount of protein expressed from injected mRNA, *see* **Note 8**).

3.3. In Vivo Labelling of Eggs and Embryos

Dechorionated eggs of *Phallusia* can be labelled with vital fluorescent dyes to study the distribution of many organelles as well as to observe surface, cortical and cytoplasmic reorganizations (*see* **Note 9**). Dyes for mitochondria, chromosomes, plasma membrane and yolk platelets are cell permeable, whereas labelling with probes for calcium and ER requires injection.

3.3.1. Mitochondria

Incubate dechorionated eggs or embryos for 15–20 min in SW containing a 1/1,000 dilution of the stock solution of any one of the following mitochondrial dyes (**Section 2.3**): DiOC2(3), Mitotracker, TMRM, TMRE, Rhodamine123 (51, 53) (**Fig. 14.1i**, **j**). Wash once with SW before observation with the appropriate filter set.

3.3.2. Chromosomes

Incubate eggs or embryos in 0.5 μg/mL Hoechst 33342 in SW for 15 min and wash twice before observation (*see* **Note 10**). Alternatively the less soluble Hoechst 33258 (make a stock solution as for Hoechst 33342) can be injected to avoid background labelling of sperm if they cannot be washed away in time.

3.3.3. Plasma Membrane

Four different approaches can be used to label plasma membrane.

1. DiI-C16(3): add 1 μL DiI Ethanol stock solution to 1 mL SW. Pull the solution in a syringe and run through a small gauge needle several times to create micelles. Add to an equal volume of eggs or embryos suspended in SW. Incubate 2–5 min, during which some DiI micelles will fuse with the plasma membranes. Wash by transferring eggs or embryos

to a large volume of SW. Mount and observe soon after labelling.

2. FM 4-64: dilute FM 4-64 stock solution to 20 μg/mL in SW and mix this working solution 1:1 with the live embryos just prior to observation in a dish or directly on the GF-coated slide (35). Washing is not necessary (*see* **Note 11**).
3. FITC: under alkaline conditions, FITC binds to the plasma membrane and does not penetrate the egg. Incubate eggs in FITC SW pH 10 (add 10 μL FITC stock to 990 μL SW to make 0.1 mg/mL, add 50 μL NaOH 1 N) for 20 min. Wash with SW before observation.
4. Succinylated concanavalin A: centrifuge the stock solution before use to pellet insoluble particles. Add to eggs in SW at 5 μg/mL final concentration and incubate for 15 min, then wash twice with SW. Other lectins can be used (33) (*see* **Note 12**).

3.3.4. Yolk Platelets

Incubate dechorionated eggs with 1 μM Syto12 in SW for 20 min. Wash once with SW (34).

3.3.5. Analysis of Surface Movements

Place eggs or embryos in a coated Petri dish containing finely ground Nile Blue particles in SW. Particles of chalk or charcoal or a 1 μM suspension of fluospheres (Molecular Probes) can also be used (54, 55). Depending on the density of particles you want to attach on the surface, either let eggs fall on particles for a low density or gather eggs and particles to the centre of the dish using a gentle swirling motion if you want many surface particles attached. Wash several times with SW and select embryos with the desired number and position of attached particles for observation.

3.3.6. Endoplasmic Reticulum

Prepare a saturated solution of DiI-C16(3) in Wesson cooking oil (**Section 2.3**). Micro-inject a small oil droplet into the egg or embryo. Allow the dye to diffuse in the tubes and sheets of the continuous ER network for 15–30 min prior to observation (33, 56) (**Fig. 14.1i**, **j**).

3.3.7. Calcium

Calcium-sensitive dyes (34, 57) (*see* **Note 13**) that are cell permeable do not function in *Phallusia* eggs, so the free acid forms must be injected. It is best to use dextran-coupled dyes because they remain cytoplasmic unlike non-coupled dyes which enter large organelles such as yolk vesicles. Inject an amount of Calcium-Green dextran or Fura-2 dextran equivalent to approximately 0.1–0.2% egg volume to give final concentration in the egg of 10–20 μM (**Fig. 14.1k**). Wait 30 min for diffusion of the dye before imaging. Calcium signals traverse the eggs within 10–20 s and images must be acquired every few seconds in order to observe wave-front propagation.

3.3.8. Mounting Live Embryos for Imaging

See chambers for live imaging in **Sections 2.1** and **3.1**.

3.4. Blastomere Isolations and Ablations

Thanks to the stereotyped cell division pattern of ascidian embryos, it is possible to isolate or ablate a blastomere with the certainty of its identity (**Fig. 14.1r**). We find it possible to isolate individual blastomeres up to the 32 cell stage for embryos of *Ciona* and *Phallusia*.

3.4.1. Blastomere Isolations by Cutting

Isolation is carried out on a 1.5% agarose-coated Petri dish. Place an embryo at the centre of dish and identify the blastomere of interest. Place a glass knife between the blastomere of interest and a neighbouring blastomere and then press the needle to separate them. By repeating the procedure on isolated portions of embryos, a single blastomere can be isolated. One can also perform blastomere isolation on embryos mounted in a wedge as described above for **Section 3.2**, using the same glass capillary needles and hydraulic micro-manipulator as for micro-injection.

3.4.2. Embryo Dissociation Using Calcium-Free Sea Water

Rear embryos in SW as explained in **Section 3.1**. At the desired stage, pipette a few embryos into a large volume of calcium-free sea water (EMC). Transfer them a second time into fresh EMC to ensure elimination of calcium. Using a fine-coated glass capillary (**Sections 2.1**, steps 9 and 10), pipette the embryos roughly up and down until blastomeres separate. Return isolated blastomeres to SW for further divisions and observation. Embryos chosen for dissociation should be early in the division cycle (prior to nuclear envelope breakdown), since towards mitosis the lateral connections between blastomeres become tightly sealed up.

3.4.3. Blastomere Ablations

Ablation is carried out on a 1.5% agarose-coated dish in a hole made with a tungsten needle. The hole should be only slightly bigger than the ascidian embryo. Place an embryo in the hole made in the agarose. Using a stream of SW from micro-pipette, rotate the embryo, so that the blastomere of interest faces you. Ablation of a blastomere is achieved by injecting water into the blastomere of interest until it bursts. With our stereo-microscope setup, it is possible to ablate individual blastomeres from embryos up to the 64 cell stage.

3.5. Fixing and Immuno-labelling Eggs and Embryos

Fixation and permeabilization procedures followed by fluorescent labelling using antibodies can be carried out on large populations of ascidian eggs and embryos or a small number of injected eggs and embryos in order to analyse the distribution of macromolecular structures. Usually, the localization of the cytoskeleton, organelles, proteins and mRNAs can be best imaged using a confocal microscope (33, 58, 59). Use of different primary

antibodies made in rabbit, mouse and rat allows one to distinguish several different proteins or macromolecular structures in the same sample.

3.5.1. Fixation

Primary antibodies are initially tested on embryos fixed in two different ways, some antibodies will work for immuno-labelling with one of these fixatives but not the other (*see* **Note 14**).

1. Methanol fixation: collect dechorionated eggs, embryos or tadpoles in a small volume of SW (less than 100 μL) and plunge them into cold (–20°C) methanol fixative stored in screw cap tubes. Store the tube at –20°C until use.
2. Formaldehyde fixation: add a small volume (less than 100 μL) of dechorionated eggs, embryos or tadpoles into formaldehyde fixative stored in screw cap tubes. Fix for 2 h at RT or ON at 4°C with shaking (20 rpm). Place the tubes upright in order to allow fixed eggs and embryos to settle to the bottom. Wash three times in PBS and store 1–3 days at 4°C. For longer storage, one can replace the PBS with ethanol by a graded series (25, 50, 75, 100%) of consecutive washes and then place at –20°C (*see* **Note 15**).

3.5.2. Immuno-labelling

Labelling of fixed samples is typically performed in volumes of 50–100 μL in 0.5 mL tubes or in multiwell plates (Falcon, flexible plate U-bottom). For all incubations >30 min, the multiwell plate should be placed in a "humid chamber" made by lining an appropriately sized plastic box or Petri dish with wet paper towels. As examples we provide standard methods for labelling two cell structures (microtubules and mitochondria) and for determining the localization of a protein of interest (aPKC: polarity protein atypical protein kinase C) in methanol-fixed samples (**Fig. 14.1o–q**).

1. Sample rehydration, permeabilization and blocking: transfer the desired amount of fixed eggs and/or embryos to a multiwell plate. Re-hydrate samples in 1:1 ethanol/PBS-Tw solution, followed by three washes in PBS-Tw. The samples are then blocked by washing three times for 10 min in PBS-Tw-BSA with shaking (20 rpm).
2. Primary antibody labelling: dilute primary antibodies which label mitochondria (NN18, raised in mouse) and tubulin (YL1/2, raised in rat) in PBS-BSA and add to the fixed samples to give a final volume of 50–100 μL per well. Incubate ON (*see* **Note 16**) at RT with shaking (20 rpm). Resuspend the samples a few times during the incubation. Wash five times in PBS-Tw.
3. Secondary antibody labelling: dilute secondary antibodies in PBS-BSA and add to samples. Use an antibody raised against rat immunoglobulin (pre-absorbed to minimize mouse cross reaction, **Section 2.5**) coupled with rhodamine

and an antibody raised against mouse immunoglobulin (pre-absorbed to minimize rat cross reaction, see material) coupled with Cy5 (**Fig. 14.1o**). Incubate 4 h at RT with shaking (20 rpm), resuspending gently from time to time.

3.5.3. Amplification with Biotin/Streptavidin or TSA Method

To detect some non-abundant proteins, it may be necessary to amplify the signal of the secondary antibody. Amplification procedures also allow one to use less primary antibody, thus reducing background and conserving precious antibodies.

1. Amplification using biotin/streptavidin: secondary antibodies coupled to biotin bind multiple fluorescent streptavidins and therefore increase the signal compared to fluorophore-coupled secondary antibodies (but *see* **Note 17** for biotin/streptavidin limitations). As an example, to detect aPKC first incubate with a primary antibody raised in rabbit against a conserved aPKC peptide sequence (1/100 dilution). After washing as described above, incubate samples for 4 h with a biotin-conjugated anti-rabbit antibody. Then dilute the fluorescein-labelled streptavidin in PBS-BSA (**Fig. 14.1p**), add to samples and incubate for 2 h at RT with shaking (20 rpm) and occasional resuspension.
2. Amplification using tyramide system amplification (TSA): secondary antibodies coupled to peroxidase (HRP) react with a detectable substrate to give enzymatic amplification (*see* **Note 5**). In the TSA method, a labelled tyramide derivative covalently couples to amino groups of adjacent proteins when activated by peroxidase. This allows strongly amplified and high-resolution labelling, without the signal diffusion that can occur with more commonly used methods based on precipitation of coloured HRP substrates (*see* **Note 5** for TSA limitations). Incubate samples labelled with anti-aPKC antibody (1/500 dilution) with a HRP-coupled secondary antibody (1/100 dilution) for 4 h at RT. Dilute 1 μL of 30% H_2O_2 in 200 μL of amplification buffer (provided in the kit). Next, add 1 μL of the intermediate H_2O_2 dilution (in order to have a H_2O_2 final concentration of 0.0015%) and 1 μL of Alexa488-tyramide (reconstituted following the manufacturer's recommendations) to 100 μL of amplification buffer. Mix and apply immediately to the samples. Reaction time must be determined for each antibody but 20 min is a good starting point. We have observed TSA reaction times ranging from 5 min to 2 h (*see* **Note 5**) (**Fig. 14.1q**).

3.5.4. Labelling Chromosomes

Wash immuno-labelled samples in PBS-Tw. Incubate with Hoechst 33342 diluted in PBS for 15 min at RT with shaking (20 rpm) (**Fig. 14.1o**). DNA labelling can also be achieved by mounting embryos directly in Vectashield mounting medium with DAPI (Vector Laboratories).

3.5.5. Actin Microfilament Labelling with Phalloidin in Formaldehyde-Fixed Samples

Formaldehyde fixation allows one to label actin using fluorescent phalloidin (*see* **Note 6**) (**Fig. 14.1n**) in addition to immunolabelling of proteins.

1. Transfer the desired amount of fixed embryos to a multiwell plate.
2. Wash with PBS-Tw, then permeabilize with PBS-Tr (*see* **Note 6**) for 30 min with shaking (20 rpm).
3. Block with three times 10 min washes in PBS-Tw-BSA.
4. If desired, first immuno-label the protein of choice as described above.
5. Label actin with phalloidin just before staining DNA and mounting: dilute phalloidin coupled with rhodamine in PBS-BSA and add to egg or embryo samples. Place the multiwell plate in a humid chamber and incubate 2 h at RT with shaking (20 rpm), resuspending from time to time. Wash once with PBS-Tw.
6. Immediately label DNA with Hoechst as above (step 4) and mount as described below (**Section 3.5.6**). Phalloidin-labelled samples should be imaged soon after labelling as the signal decreases rapidly with time.

3.5.6. Mounting and Imaging

Wash samples three times with PBS-Tw followed by three washes with PBS. Put 20 μL of Citifluor mounting medium on a glass slide. Deposit approximately 20 μL of labelled eggs or embryos in the Citifluor drop. To create a spacer, add tiny feet to a cover-slip (22 mm × 22 mm, #1) by swiping each of the corners in modelling clay. Place the cover-slip on the drop of Citifluor containing the sample and press gently on the four clay feet with forceps in order to fix the cover-slip on the slide and immobilize eggs or embryos (*see* **Note 18**). Seal with nail polish. Observe labelled samples in a fluorescence or confocal microscope with appropriate laser settings to discriminate between fluorophores.

3.6. Isolated Cortices: Preparation, Labelling and Imaging

A special advantage of the ascidian model is that isolated cortical fragments can be prepared from eggs and early embryos (2–16 cell stages) (**Fig. 14.1s**). Because isolated cortex preparations are extremely thin (0.5–5 μm), they are ideal for fluorescent and confocal microscopy and provide very high-resolution images for determining the association of macromolecules with the major components of the cortex (plasma membrane, ER and microfilaments) (60). Furthermore, isolated cortices constitute an "open-cell preparation" which does not require permeabilization via exposure to detergents or organic solvents that have deleterious effects on cell structures (such as ER).

3.6.1. Preparing and Fixing Cortices

Isolated cortical fragments are prepared as described in our previous publications (33, 53, 61) and in the BioMarCell web site

(*see* **Note 1**) with some modifications. The procedure is best done by watching all steps under a stereo-microscope. Ascidian eggs and embryos are deposited with a mouth pipette on a glass cover-slip into a large drop of calcium-free sea water (EMC). Wait 20–30 s for eggs or embryos to settle and attach onto the cover-slip. Replace EMC by isotonic Buffer X using a gentle stream from a Pasteur pipette coming from the side of the cover-slip (do this carefully in order to avoid breaking eggs and embryos). Wash again with Buffer X. Then shear eggs and/or embryos with a vigorous stream of Buffer X using a Pasteur pipette. Wash the lawn of isolated cortical fragments quickly with Buffer X. Label the ER with DiI if necessary (see below) and then fix with CIM fixative for 30 min at RT in a humid chamber using gentle shaking (5 rpm). Wash one time with CIM solution and three times in PBS (*see* **Note 19** for microtubule preservation and immuno-labelling of unfixed cortices). ER can be labelled with DiO after fixation (see below).

3.6.2. Endoplasmic Reticulum Labelling

There are three ways to visualize the contiguous ER network on isolated cortices (**Fig. 14.1s–u**).

1. Labelling of ER with CM-DiI-C16(3) ("fixable DiI") before fixation: remove Buffer X from just-isolated cortices (see above). Dilute 1.7 μL of the CM-DiI stock solution in 0.5 mL of Buffer X and emulsify by passing the liquid through a fine gauge needle. Apply to living isolated cortices and incubate for 1 min. Wash one time with Buffer X and fix the cortices with CIM fixative.
2. Labelling of ER with DiO-C6(3) after fixation: prepare isolated cortices as described above and fix them with CIM fixative. Wash one time with CIM solution, followed by three washes in PBS. Expose fixed isolated cortices to DiO-C6(3) diluted in PBS (0.2 μg/mL) for 10 s. Wash three times with PBS. Observe the cortices and ER networks immediately. If isolated cortices are to be immuno-labelled (see below), DiO labelling should be performed after immuno-labelling just before mounting.
3. Labelling of ER with DiO-C6(3) without fixation: remove Buffer X from just-isolated cortices. Expose living isolated cortices to DiO diluted to 0.2 μg/mL in Buffer X for 10 s. Wash one time with Buffer X and observe immediately.

3.6.3. Mitochondria Labelling

Some cortices (those made at 2 cell stage particularly) retain plaques of mitochondria-rich myoplasm. It is possible to image these mitochondria by isolating cortices from embryos which were previously labelled with DiO-C2(3) as in **Section 3.3** above.

3.6.4. Actin Labelling Using Phalloidin

The best actin labelling is obtained when performed on freshly isolated cortices before fixation. Live cortices are exposed for 2 min to Buffer X containing fluorescent phalloidin (prepared as described in **Section 2.5**) followed by one wash with Buffer X and fixation with CIM fixative. Then wash one time with CIM solution, three times in PBS and observe labelling with a fluorescence or confocal microscope.

3.6.5. Immuno-labelling of Cortices

Cortices can be labelled for microtubules, ribosomes (on rough ER), mitochondria and other constituents using antibodies (33, 59, 60). As an example, we provide methods for labelling aPKC and microtubules on fixed isolated cortices (**Fig. 14.1v**).

1. Prepare and fix isolated cortices as described above (step 1). For labelling of microtubules, we recommend adding EGTA and taxol at 10 mM and 1 μg/mL, respectively, in Buffer X during the process of shearing eggs and embryos and during subsequent washes (*see* **Note 19**). Cover-slips are positioned sample-side-up on a parafilm layer in Petri dish made into a humid chamber.
2. Block with three washes of PBS-BSA for 10 min with gentle shaking (5 rpm).
3. Dilute aPKC and YL1/2 antibodies in PBS-BSA and add to samples a volume of 30–50 μL per cover-slip is sufficient. Incubate antibody-covered cover-slips in the humid chamber for 1 h at RT with gentle shaking (5 rpm).
4. Wash three times in PBS.
5. aPKC protein can be visualized using either fluorescent secondary antibodies or biotin/streptavidin amplification system. In the case of biotin/streptavidin labelling, dilute biotin-coupled secondary antibody in PBS-BSA and add to sample. Use a Cy5 conjugated secondary antibody for visualizing microtubules. Incubate with secondary antibodies for 1 h at RT with gentle shaking (5 rpm).
6. Wash three times in PBS.
7. Dilute fluorescein-conjugated streptavidin in PBS-BSA, add to sample and incubate 1 h at RT with gentle shaking (5 rpm).
8. Wash three times in PBS.

3.6.6. Mounting and Imaging

Put a 10 μL drop of Citifluor in the centre of a microscope slide. Remove excess solution from the labelled isolated cortices on the cover-slip, but do not allow it to dry out. Carefully position the cover-slip unto the Citifluor drop and remove excess liquid with absorbent paper. Seal with nail polish. Because isolated cortex preparations are extremely thin (0.5–5 μm) they can be imaged

at high resolution with a regular fluorescent microscope. Confocal microscopy allows very high resolution co-localization of ER, ribosomes, microfilaments, microtubules and/or any mRNAs and proteins of choice.

4. Notes

1. Useful links:
   - Tunicate Portal: http://www.tunicate-portal.org/index.htm
   - Ascidian News: http://depts.washington.edu/ascidian
   - ANISEED, Ascidians Network for In Situ Expression and Embryological Data: (62): http://aniseed-ibdm.univ-mrs.fr/
   - GHOST, *Ciona intestinalis* genomic and cDNA resources (63): http://ghost.zool.kyoto-u.ac.jp/
   - FABA, Four-dimensional Ascidians Body Atlas (64, 65): http://chordate.bpni.bio.keio.ac.jp/faba2/2.0/top.html
   - DBTGR, DataBase of Tunicate Gene Regulation (64): http://dbtgr.hgc.jp/
   - CIPRO: an integrated protein database of the ascidian Ciona intestinalis. http://cipro.ibio.jp/2.5/
   - CITRES Ciona Intestinalis Transgenic line RESources http://marinebio.nbrp.jp/ciona/index.jsp
   - JGI *Ciona* genome browser: http://genome.jgi-psf.org/Cioin2/Cioin2.home.html
   - BioMarCell, Ascidians Film Archive (32): http://biodev.obs-vlfr.fr/recherche/biomarcell/
   - BioDev Research Unit, Villefranche-sur-Mer marine station (McDougall/Yasuo/Sardet labs): http://biodev.obs-vlfr.fr/recherche_en.htm
   - Ascidian Stock Center at UC Santa Barbara: http://www.ascidiancenter.ucsb.edu
   - Dutch ascidians (about species worlwide): http://www.ascidians.com
   - Friday Harbor Centre for Cellular Dynamics methods: http://raven.zoology.washington.edu/celldynamics/downloads/index.html
2. mRNA detection using in situ hybridization is not described in the present chapter. Protocols can be found in previous publications (58, 66–68) and in our chapter

"Localization and anchorage of maternal mRNAs to cortical structures of ascidian eggs and embryos using high resolution in situ hybridization" in a separate volume of *Methods in Molecular Biology* (44).

3. Commercial antibodies and streptavidin are generally supplied with sodium azide as preservative (or thimerosal in case of HRP-coupled secondary antibodies and streptavidins), allowing them to be conserved for several months at 4°C. If preservatives are not included by the manufacturer, add sodium azide or thimer at 0.02% to the antibodies before aliquoting and freezing.
4. Although the monoclonal NN18 antibody was originally produced against vertebrate neuro-filaments, in ascidian embryos this antibody recognizes a mitochondrial ATP synthase subunit (personal communication of T. Nishikata).
5. Many TSA kits corresponding to various fluorophores and haptens exist. Although we generally use the Alexa488-TSA kit from Molecular Probes, we sometimes use Cy3-TSA and Cy5-TSA kits from PerkinElmer since they are cheaper and provide intense red and far red fluorescent signals. Biotin-TSA reaction followed by fluorescent streptavidin labelling does not give a much better result than direct fluorophore-TSA based reactions. Quenching of endogenous peroxidases is not necessary for ascidian eggs and early embryos when TSA method is used. TSA amplification increases with time and is not linear with respect to quantity of targeted protein in the cell; therefore it is important to limit reaction times if the purpose is meaningful comparisons of protein amount. We sometimes observe high background staining with the TSA system and it may be necessary to further dilute the primary antibodies for optimization of signal-to-noise ratio. When the confocal microscope is set to high sensitivity we have noticed the presence of nuclear background labelling using the TSA reaction.
6. Use of fluorescently coupled phalloidins is a convenient way to label actin microfilaments; however, note that phalloidin labelling should not be performed on fixed samples which have been dehydrated with alcohol (instead, a treatment with Triton aids in permeabilization). For samples which have been fixed in methanol or stored in ethanol, it is possible to use an anti-actin antibody (Calbiochem CP01) (59) to label microfilaments.
7. For horizontal injections, one can also use a "ledge" chamber as in Jaffe and Terasaki, 2004 (46). A small piece of

coated cover-slip is pressed onto double stick tape (which acts as the spacer) to give an overhanging side, so that the eggs line up under or next to this ledge.

8. Injection quantification for fluorescent proteins produced after mRNA injection can be performed as in Levasseur and McDougall, 2000 (69). A calibration curve is made by measuring the fluorescence from egg-sized droplets containing known concentrations of GFP protein. The droplets are formed by extruding different GFP solutions from a micro-injection needle under silicone onto cover-slips treated with dimethyldichlorosilicane (2% in 1,1,1-trichloroethane) to remove the surface tension, so that the egg-sized bubbles remain spherical. Accurate production of egg-sized droplets is important since the fluorescence intensity is proportional to total volume as well as to concentration. Using the same imaging parameters (filters, exposure time, binning, etc.) measure the fluorescence of the injected egg and compare this value to the calibration curve to determine the concentration of the fluorescent protein in vivo.
9. In vivo labelling techniques work best for transparent eggs and embryos of *Phallusia* (33, 55), whereas for *Ciona* eggs and embryos the opacity of yolk vesicles and intrinsic autofluorescence hinder the detection of fluorescent labelling unless it is near the surface. Fixed *Ciona* samples are suitable for fluorescent detection of proteins or mRNAs; however, signal intensity and resolution are greater in transparent eggs and embryos of *Phallusia* (58).
10. Live embryos can only be labelled for short periods (30 min) with Hoechst because it inhibits DNA replication and results in DNA bridges.
11. FM 4-64 tends to concentrate at the centrosomes if embryos are crowded on the slide and dying.
12. Concanavalin A (non-succinylated) and wheat germ agglutinin (WGA) also label membranes of fertilized eggs and embryos, but they should not be used on unfertilized eggs because they induce capping and cause eggs to be activated (70).
13. A ratiometric method should be used when one wishes to quantify calcium signals or to control for artefacts due to sample thickness, cytoplasmic domains, or dye concentration. The eggs can be injected either with the ratiometric dye Fura-2 dextran or with a mixture of Calcium-Green dextran and the calcium-insensitive dye Texas-Red dextran. Acquire fluorescent images simultaneously with appropriate excitation and emission filters.

14. Methanol treatment causes instantaneous fixation by dehydration and a good permeabilization of eggs and embryos. Microtubule structures are well preserved by methanol fixation. A high concentration of EGTA (50 mM) is added to the methanol as tubulin is one of the major targets of the potent calcium-dependant protease. Formaldehyde treatment causes progressive fixation by making intra- and inter-molecular bonds. It is well suited for maintaining the integrity of the cell cortex and in particular microfilament labelling using phalloidin. Fixations for electron microscopy of whole eggs and embryos and cortices prepared from them are not detailed here but can be found in our previous publications (33, 61).
15. The ethanol dehydration step after formaldehyde fixation further permeabilizes the samples and improves signal for many antibodies and for labelling of structures deep within the embryo (but is not compatible with phalloidin labelling, *see* **Note 6**). As for methanol-fixed embryos, ethanol-dehydrated samples should be re-hydrated into PBS before immuno-labelling.
16. Incubation with antibodies can be shortened to 2 h at RT but labelling structures situated deep within the embryo is better with ON incubation at RT or 4°C.
17. Although biotin/streptavidin gives good amplification of signals, note that streptavidin attaches to mitochondria yielding background labelling. Therefore this method is not appropriate for detection of cytoplasmic proteins.
18. It is important that the embryos be slightly compressed and snug between slide and cover-slip if an oil immersion objective is to be used. This is not necessary for an air objective which does not touch the cover-slip.
19. Calcium ions are implicated in microtubule depolymerization and we observed that adding EGTA to Buffer X helps to preserve microtubules on isolated cortices. Moreover addition of taxol, a drug which stabilizes microtubules, gives better results than EGTA alone. Also note that it is also possible to immuno-label unfixed live cortices (an open-cell preparation) by diluting primary and secondary antibodies in Buffer X instead of PBS and using brief incubation times (5 min separated by quick washes with Buffer X). Observe live-labelled cortices within 30 min of making cortices, since as time passes the ER network will start vesiculating.

Acknowledgements

We thank personnel of the Developmental Biology unit (BioDev, Villefranche-sur-Mer Marine Station, CNRS/UPMC UMR7009, France) for helpful discussions and previous members of the Villefranche lab, Fabrice Roegiers and François Prodon. We thank M. Khamla for help with graphics. The laboratories of C. Sardet, A. McDougall and H. Yasuo are supported by ANR (Agence Nationale de la Recherche), CNRS-ACI (Centre National de la Recherche Scientifique—Action Concertée Incitative), UPMC (Université Pierre et Marie Curie, Paris 06), ARC (Association pour la Recherche sur le Cancer) and AFM (Association Française contre les Myopathies).

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