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**A simple method to identify ascidian brain lineage cells at neural plate stages following
in situ hybridisation**

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Running head: In situ hybridisation in ascidian brain precursors

Abstract

The technique of in situ hybridisation can be used to visualise the spatial and temporal pattern of gene expression during development. Ascidians are invertebrate chordates that develop into a tadpole larvae with a fixed cell cleavage pattern. The knowledge of the cell lineage allows the earliest steps of cell fate specification to be followed at a single cell resolution. This protocol describes preparation of *Ciona intestinalis* embryos, classical in situ hybridisation protocol coupled with nuclear staining and a guide to identify gene expression in specific precursors of the developing brain at neural plate stages of development.

Keywords:

Ciona, ascidian, brain, sensory vesicle, in situ hybridisation, neural plate, development

1 Introduction

Ascidians are marine invertebrate chordates, within the subphylum (or phylum) tunicata (1). At the larval stage of development, ascidians have a chordate body plan in miniature, in the form of a tadpole (Fig. 1A). Running centrally along the tail is a notochord, flanked by muscles and overlain by a dorsal hollow neural tube. The neural tube forms by neurulation, the rolling up of a neural plate (2). One particular advantage of ascidian development is that it proceeds with an invariant cleavage pattern and fixed cell lineage, which is well described, particularly for neural lineages (3, 4). Many molecular tools and computational tools are available for ascidians (5–9) and the gene regulatory networks for a number of tissues are well deciphered (10–12). Several species of solitary ascidians (e.g. *Ciona* sp., *Halocynthia roretzi*, *Phallusia mammilata*) are commonly used for developmental biology studies. Their cell lineages and tadpole body plans appear remarkably similar despite large evolutionary distances separating them (13), allowing knowledge gained from one species to be tentatively applied to others. However, the molecular mechanisms patterning the extremely well-conserved body plans are not always necessarily conserved (14, 15).

The central nervous system (CNS) of ascidian larvae consists of, along the anterior posterior axis, a sensory vesicle, a narrow neck region, a trunk ganglion, and a tail nerve cord (Fig. 1A). The CNS of a *Ciona intestinalis* larvae, a commonly studied ascidian species, provides the second example of an animal, and the only chordate, in which the wiring between neurons, or connectome, has been described (16). The *Ciona* CNS is made up of a total of 330 cells, of which 177 are neurons (16, 17). 143 of these neurons reside in the sensory vesicle, or larval brain. A striking feature of ascidian brains is that they are highly asymmetric (16). Most obvious of these asymmetries are the sense organs. While the developmental origin of the two larval brain pigmented cells is dorsal, residing initially in the lateral borders of the neural plate, their final position is highly asymmetric. On the right hand

side of the brain is the ocellus, consisting of one cup shaped pigmented cell, around 30 photoreceptors and 3 lens cells. Anchored in the ventral sensory vesicle is the second pigmented cell, the single celled otolith, thought to sense gravity and responsible for the upward swimming behaviour of newly hatched larvae (**18**). On the left wall of the sensory vesicle is another sense organ, made up of the coronet cells, which may constitute a pressure receptor (**18, 19**). These brain asymmetries are seen only later in development, during larval stages, and rely on cell-signalling events and a clockwise rotation of the anterior neural tube (**4, 20, 21**).

At the 8-cell stage of ascidian development, four recognisable ‘founder lineages’ are born, the A-, B-, a- and b- lineages, with A- and B- situated in the vegetal pole and a- and b- lineages in the animal pole (Fig. 1A). The CNS develops from the A-, a- and b- lineages. At the mid-gastrula stage the neural plate has 6-rows of cells aligned in a grid-like array (Fig. 1C). The bilateral columns of cells are named column 1 for the medial-most column, to column 4 for the lateral-most column. Along the posterior to anterior axis, rows of cells are named rows I-VI. Only the first four rows of cells will give rise to the central nervous system with rows V and VI giving rise to a specialised region of epidermis (reviewed in (**10**)). Rows I and II are A-lineage derived and will generate the posterior-most part of the sensory vesicle, and the ventral and lateral rows of the CNS posterior to the sensory vesicle. Row III and IV are a-lineage origin and will generate the anterior sensory vesicle including the pigmented cells and the oral syphon primodium (mouth), which is continuous with the neural tube (**22**). The b-line cells border the neural plate and this lineage will generate the dorsal most row of the neural tube from the posterior sensory vesicle to the tail nerve cord. This chapter focuses on identifying cells within the a- and A- neural lineages during the neural plate stages.

Whole mount in situ hybridisation (ISH) is an invaluable method to detect gene expression during development. Due to the well-described cell lineages, the transcriptional

states of identified brain precursors can be tracked precisely during early development. This is particularly easy at neural plate stages when nuclear staining can be used to identify cells. Here I describe our laboratory's whole mount ISH protocol on neural plate stage embryos and a guide on how to identify cells within the neural plate. The ISH protocol is based on one developed by Wada et al (23), with several modifications added over the years. Recognising individual cells within the neural plate is made possible by following the published pattern of neural plate divisions (24, 25) as well as the time lapse imaging of transgene expression, available online (26, 27). While this protocol is a guide for neural plate stages, it is also possible to identify individual cells at later stages of development by coupling nuclear staining with ISH (28, 29). Coupling fluorescent ISH with immunodetection of transgene expression driven by lineage specific gene markers is also a good technique used to identify cells (30, 31).

I predict that that classical ISH will continue to prove invaluable, to understand how different cell types are generated during development, for the continued deciphering of gene regulatory networks and for confirming and analysing the newly generated single cell RNA-seq data. One major future challenge will be to link the early cell lineages, gene expression patterns and gene regulatory networks precisely to the individual neurones making up the CNS connectome. Lineage analysis continues to make progress in this area (20).

2 Materials

All solutions are made with Millipore filtered deionised water.

2-1 Embryo preparation

1. Room at 18°C.
2. Dissection scissors.
3. Artificial sea water (ASW): 420mM NaCl (24.55g/l), 9mM KCl (0.67g/l), 10mM CaCl₂ (1.47g/l of CaCl₂·2H₂O); 24.5mM MgCl₂ (4.98g/l of MgCl₂·6H₂O), 25.5mM MgSO₄ (6.29g/l of MgSO₄·7H₂O), 2.15mM NaHCO₃ (0.18g/l), 0.05g/l Kanamycin, 10mM Hepes (diluted from frozen aliquoted stocks of 1M Hepes pH8.0), 0.1mM EDTA (diluted from a 0.5M stock solution pH8.0).
4. 6cm or 10cm petri dishes (depending on number of embryos to be collected) coated in 1% agarose in artificial sea water. 1% agarose is heated in ASW using a microwave, allowed to cool to around 50°C and poured in to petri dishes to a thickness of 1-2mm.
5. Dechoriation solution: 0.05% pronase E (Sigma P5147), 1% sodium thioglycolate (Sigma T0632) in ASW. This is stored at 4°C for up to a week. Take care to bring aliquot to room temperature prior to dechoriation.
6. Corex glass centrifuge tubes, 15ml.
7. Pasteur pipettes pre-soaked in tap water for at least 48 hours to prevent embryos adhering to the glass surface (pipettes improve with re-use)
8. Squeezy wash bottle (500ml) filled with ASW.
9. 1N NaOH solution.
10. 1M Tris pH9.5 solution.
11. 15ml Falcon tubes.
12. 3ml plastic transfer pipette.

13. 1.5ml microcentrifuge tubes.
14. Stereo microscope (we use Leica S8AP0).

2-2 Embryo fixation and storage

1. Low adhesion 1.5ml micro-centrifuge tubes (e.g. Eppendorf DNA LoBind Tube.)
2. Home made glass micropipette: glass tube outer diameter 5mm, inner diameter 3mm, pulled under a flame to make tapered end and fitted with rubber tube that is stapled at one end (Fig. 2A). Micropipettes are pre-soaked in tap water for at least 48 hours to prevent embryos adhering to the glass surface (pipettes improve with re-use).
3. Artificial seawater with 0.5% BSA. *See Note 1.*
4. Fix solution: 4% paraformaldehyde, 0.5M NaCl, 0.1M MOPS pH7.5. *See Note 2.*
5. Ethanol (100%).

2-3 In situ hybridisation

1. Ethanol
2. PBST: 137mM NaCl, 8.1mM Na₂HPO₄, 2.68mM KCl, 1.47mM KH₂PO₄, 0.1% Tween20.
See Note 3, 4.
3. Proteinase K 2µg/ml in PBST. *See Note 5.*
4. Postfix solution: 4% paraformaldehyde in PBS. *See Note 2.*

5. Hybridisation solution: 50% formamide, 6x SSC, 5x Denhardt's solution, 1mg/ml torula yeast RNA, 0.1% tween, 2.5 % dextran sulphate. *See Note 3, 6, 7.*
6. Anti-sense RNA probe. *See Note 8.*
7. Wash solution 1: 50% formamide, 5xSSC, 0.2% SDS. Preheated to 50-55°C. *See Note 9.*
8. Wash solution 2: 50% formamide, 2x SSC, 0.2% SDS. Preheated to 50-55°C. *See Note 9.*
9. Wash solution 3: 2x SSC, 0.1% tween 20. Preheated to 50-55°C.
10. Wash solution 4: 0.2x SSC, 0.1% tween 20. Preheated to 50-55°C.
11. RNase solution (Optional): 20µg/ml RNase A in 2xSSC. *See Note 10.*
12. Blocking buffer: 0.1M Tris pH7.5 (diluted from a 1M stock solution), 0.15M NaCl (diluted from a 5M stock solution), 0.5% Roche blocking reagent. *See Note 11.*
13. Anti-Digoxigenin-AP fab fragments (Roche, Sigma).
14. TMNT solution: 100mM NaCl, 50mM MgCl₂, 100mM Tris, pH8.0 (all diluted from 1M stock solutions), 0.1% Tween.
15. NBT/BCIP solutions (e.g. Promega S3771).
16. Workshop modified manual centrifuge (Hettich Zentrifugen 1011; Fig. 2B).
17. Glass Pasteur pipettes, pre-soaked in tap water.

2-4 Mounting, photography and staging

1. Glass slides.
2. Cover slips 22x22mm.

3. Depression slide.
4. Vaseline.
5. Vaseline dispenser (5ml syringe fitted with 200 μ l pipette tip; Fig. 2C).
6. 80% Glycerol.
7. Stereo microscope (we use Leica S8AP0).
8. Epifluorescent microscope with a DAPI filter and camera (we use Olympus Bx51 fitted with a Leica DFC310FX camera).
9. Vectashield-DAPI (Vector laboratories) or Hoechst 33342 (10 μ g/ μ l stock).

3 Methods

3-1 Embryo preparation

Entire procedure is conducted in an 18°C room.

1. Eggs are manually dissected from adult *Ciona intestinalis* using dissection scissors and transferred to a 6cm petri dish (not agarose coated as chorionated embryos do not stick to the plastic).
2. Sperm is removed by cutting a small slit in the sperm duct and removing sperm with a 20 μ l micropipette. Take care not to suck any other debris with the sperm. Clean sperm will keep for a few days at 4°C in a microcentrifuge tube. Take care when handling sperm so it does not come into contact with the eggs.
3. Transfer the eggs to the Corex glass centrifuge tube with a little sea water.

4. To 10mls of dechoriation solution in a 15ml Falcon tube, add 11 drops of 1N NaOH using a 3ml plastic transfer pipette. Mix. *See Note 12.*

5. Add approximately 1ml of dechoriation solution to the eggs in ASW and mix gently using a glass pasteur pipette.

6. Allow eggs to settle in the centrifuge tube.

7. Remove as much sea water as possible and add 3-4ml of dechoriation solution. Pipette up and down very gently. Continuously take a few eggs and observe under the stereomicroscope to monitor dechoriation (Fig. 3). Firstly, the follicle cells fall off, leaving the egg surrounded by chorion (Fig. 3B). From this moment, it is important to very carefully monitor the dechoriation. When most eggs have lost their chorion (Fig. 3C), remove as much solution from the eggs as possible and gently fill the tube to 10ml with ASW from a squeeze wash bottle. Dechoriation should not take more than 5 minutes. Wait for eggs to fall to the bottom of the tube. Turn or tilt the tube from time to time to prevent eggs sticking to the sides of the tube. Wash and repeat three times. It is important to remove eggs from dechoriation solution as quickly as possible for good quality eggs.

8. Place eggs in an agarose-coated petri dish.

9. For fertilisations, add 500µl of ASW to a 1.5ml microcentrifuge tube, 25µl of 1M Tris pH9.5 and a drop of sperm. Mix. Add some of the sperm solution to the eggs. Under a stereomicroscope it should be possible to see the sperm vigorously swimming and the eggs move or spin around as they are hit by the sperm. Wait 10 minutes and transfer eggs to a clean agarose-coated petri dish. Eggs should be sufficiently dispersed so that they do not touch one another. Embryos that touch will fuse. To facilitate later staging in the stage series, it is better to proceed with batches of embryos that develop synchronously.

3-2 Embryo fixation and storage

1. It will take approximately 5 hours at 18°C to reach gastrula stage (3-row neural plate) and 6 hours to reach 6-row neural plate stage. From just before 5 hours, observe stage under stereomicroscope, batches of embryos should be fixed in low adhesion micro-centrifuge tubes every 15 minutes until around 9 hours of development to cover all neural plate stages. Pick up embryos with home made glass micropipette (Fig. 2A). Place embryos in a 1.5ml microcentrifuge tube that has been pre-coated in ASW-BSA, wait till embryos sink to the bottom, remove as much ASW as possible and then add fix solution. *See Note 1.*
2. Fix embryos overnight at 4°C. *See Note 13.*
3. Wash embryos in 0.5-1ml 25% ethanol (in water) twice, 50% ethanol twice, 75% ethanol twice and 100% ethanol twice. Embryos are stored at -20°C in 100% ethanol. These stocks can be stored for many years. I have achieved good ISH results on embryos stored in this way for more than 10 years.
4. To process stored embryos for ISH, a few embryos are taken with a pipettelman from the stock tube and place in a fresh tube with 500µl of ethanol.
5. It is best to separate the stages of embryos into individual tubes so the temporal sequence of cell divisions can easily be tracked, facilitating cell identification. Even with synchronised embryos, there will still be some differences in the exact stage of the embryos in a given time point.

3-3 In situ hybridisation

Unless otherwise stated, all washes are carried out at room temperature using 500µl of wash solution. In between each wash, the embryos are spun to the bottom of the microcentrifuge tube using a workshop modified hand centrifuge (Fig. 2B). Solution is carefully removed with a pasteur pipette. Holding tubes over black coloured material makes it easier to see embryos.

Day 1.

1. Rehydrate embryos in 75% ethanol (in PBST), 50% ethanol/PBST and then 25% ethanol/PBST, followed by 3 rinses in PBST. Each wash should be for 5 minutes.
2. Incubate embryos in 100µl of 2µg/ml of proteinase K in PBST for 25 minutes at room temperature followed by three PBST washes *see* **Note 5**.
3. Post-fix embryos in 100µl of 4% paraformaldehyde in PBS for 1 hour.
4. Wash 2x 5 minutes in PBST.
5. Incubate embryos in 100µl hybridisation solution for 10 minutes. Gently tap the tubes to ensure that the hybridisation solution mixes well with the small amount of PBST (and embryos) that remain in the tube. It should be possible to see the two solutions ‘swirl’ as they mix.
6. Replace hybridisation solution with 100µl fresh hybridisation solution and incubate at 50-55°C for 1 hour.
7. Heat probe solution to >95°C for 5 minutes. Cool rapidly.
8. Change solution to 100µl hybridisation solution containing 50ng of RNA probe (*See* **Note 8**). Incubate at 50-55°C for at least one night and up to three nights. Longer incubations give better results but are not necessary for many probes used.

Day 2.

8. Carefully remove probe solution and keep it at -20°C. Probes can be re-used many times and results become cleaner with repeated use.
9. Wash embryos in Wash solution 1 for 20 minutes at 50-55°C. Repeat.
10. Wash embryos in Wash solution 2 for 20 minutes at 50-55°C. Repeat. *See Note 10.*
11. Wash embryos in Wash solution 3 for 20 minutes at 50-55°C. Repeat.
12. Wash embryos in Wash solution 4 for 20 minutes at 50-55°C. Repeat.
13. Wash in PBST for 5 minutes.
14. Incubate in 100µl blocking buffer for 30 minutes.
15. Replace with fresh 100µl blocking buffer containing 1/2000 anti-digoxigenin-AP antibody and incubate overnight at 4°C. *See Note 14.*

Day 3.

16. Wash 3x 5 minutes in PBST.
17. Wash 3x 1 hour in PBST.
18. Wash in 100µl TMNT solution, 2x 5 minutes.
19. Replace solution with 100µl TMNT solution containing 6.6µl NBT and 3.5µl BCIP per ml of TMNT.
20. Incubate in the dark until the desired staining intensity has been reached. *See Note 15.*
21. Post fix embryos in 100µl of 4% paraformaldehyde in PBS for 1 hour at room temperature or overnight at 4°C. If embryos are to be processed for nuclear staining, embryos should be

stored in fix until further processing. Parafilm is placed around the top of the microcentrifuge tube and embryos can be stored for up to a few weeks at 4°C. *See Note 16.*

3-4 Mounting and photography

1. Fixed embryos are rinsed in PBST (3 washes) to remove fixative.
2. Remove as much PBST as possible and replace with a drop of Vectashield-DAPI (from the supplier's pipette). You can also incubate embryos overnight in Vectasheild-DAPI.
3. Transfer embryos in Vectashield-DAPI into a depression slide containing a small pool of Vectashield-DAPI. When not under stereomicroscope protect from light with an aluminium foil covered petri-dish lid.
4. Take one embryo and place in a tiny drop of Vectashield-DAPI onto a glass slide.
5. Take a coverslip and, using a Vaseline dispenser (Fig. 2C), place one dot of Vaseline on each corner of the coverslip.
6. Place the coverslip over the embryo so that the embryo is in the centre (Fig. 2D).
7. Under a stereomicroscope carefully press down until the coverslip touches the Vectashield liquid (without squashing the embryo). Gently adjust the position of the coverslip until the embryo is in the desired orientation for photography. Pushing on the side of the coverslip with a 200µl micropipette cone is my preferred method.
8. Photograph the embryo first under bright field, then UV fluorescence for nuclear stain. The embryo should be fixed in position for this process. It is important to obtain a good brightfield image first as recent lots of Vectashield-DAPI occasionally cause the ISH stained embryos to

darken after UV exposure (e.g. Fig. 4). While this effect only takes place from time to time, photographing the bright field first ensures the sample is not lost. *See Note 17 and 18.*

9. Alternatively, nuclei can be stained with Hoechst. After fixation and washing in PBST, embryos are placed in 100µl PBST with 1/1000 dilution of the Hoechst stock solution for 15 minutes in the dark. Embryos are then washed 2x 10 minutes in PBST, then washed overnight at 4°C in PBST. After the overnight wash, embryos are placed in 100µl 80% glycerol for 30 minutes and mounted as described above in 80% glycerol. *See Note 17 and 18.*

10. To make ISH plus DAPI images, an overlay is made in Photoshop (Fig. 5). Open both images in Adobe Photoshop or similar program.

11. Change the DAPI image to grey-scale.

12. Adjust contrast and light for both images.

13. Copy and paste grey-scale DAPI image on top of the bright field image.

14. Using the 'calques' window, adjust the transparency of the grey-scale image (to around 50%) to see at the same time both the nuclei and the ISH stain.

15. Fuse visible calques to make one image.

16. Adjust brightness, contrast, rotate and crop image to desired size.

3-6 Staging.

Comparing a new ISH plus DAPI stained embryo series to the documented pattern of cell divisions should enable the precise identification of which cells express a given gene. The pattern of cell divisions is illustrated in Figs 6-9 and described below. In Figures 6-9 I have

provided, as a guide, DAPI stained images showing the progression of neural lineage divisions from approximately stage 11 (early gastrula) to stage 15 (mid neurula) of development (32). In Figures 6 and 8, at the top, are DAPI ISH stained embryos, beneath is a drawing highlighting the nuclei in the neural plate and surrounding cells, with the corresponding rows of neural plate cells labelled, the brain precursors outlined in red and the embryo outlined in blue. At the bottom is a schematic drawing of the neural plate with the cell divisions shown and brain precursors outlined in red. Figure 6 covers stages 11-13 ('3-row' to '6-row' neural plate stages). Figure 7 shows the same embryos with ISH signals, detailing expression patterns in the neural plate cells on the schematic drawing (bottom) by grey filled boxes. Similarly Figure 8 covers stages 13-15 ('6-row' to '12-row' neural plate stages) with Figure 9 illustrating the same embryos with ISH signals.

At the early gastrula stage, the neural cells are arranged in three rows, row I/II (precursors to rows I and II), row III/IV and row V/VI (Fig. 1B, Fig. 6A). Cells within each row of the neural plate divide in a stereotypical fashion (Fig. 6) (24). Firstly, cells in row I/II divide (with the medial columns of cells starting to divide before the lateral columns of cells) (Fig. 6B). Then cells in row V/VI and finally cells in row III/IV divide (Fig. 6C, D). This gives rise to the 6-row neural plate with the 6 rows (I-VI) of cells aligned along the posterior-anterior axis, respectively (Fig. 1C; Fig. 6E).

Between the 6-row and 12-row neural plate stages, row II cells start to divide first followed by row I (Fig. 8A-C). Next, rows III, V and VI divide approximately at the same time, but variably, depending on the embryo (Fig. 8 D-G). Row VI is often hard to see as it is situated close to the anterior tip of the embryo (grey boxes in Fig. 8 F, H, I). The variability in the exact temporal sequence of divisions of rows III, V and VI is made clear in Figure 8. In Fig. 8D-G, rows I and II have divided. In Fig. 8D, row VI cells are dividing before rows III and V. The embryo in Fig. 8E on the other hand shows division of row V before rows III and

VI. In Fig. 8F, cells in rows III and V are dividing (row VI is not visible). In Fig. 8G, cells in rows V and VI are dividing before row III. Nonetheless, it is still relatively easy to identify each cell nuclei. The divided row I nuclei are small and bright and the medial four columns of cells are curved along the dorsal marginal lip (e.g. Fig. 8D,E). Divided row II cell nuclei are large and diffuse also providing good landmarks for orientation. Row IV is the last row of cells to divide (Fig. 8H) rapidly followed by another division of row II cells (Fig. 8I). At the mid neurula stage (Fig. 8I) the brain precursors are in '12-row' stage. At this stage, row III cells serve as good landmarks: the nuclei of column 3 sister cell pairs are separated, while the nuclei of the anterior column 1 sister cells are more compacted and closer together than those of the posterior sister cells (Fig. 8J, row III in red). At later stages, the exact identification of lateral row I and II cells becomes difficult with this technique due to their intercalation (grey boxes). While using the terms 3-row, 6-row and 12-row stages are useful for illustration purposes, it should be noted that the cells are dividing continuously and there is no 'pause' at these stages.

4 Notes

Notes:

- 1.** BSA is added to ASW to help prevent the embryos from sticking to the sides of the microcentrifuge tubes prior to fixation. Low adhesion 1.5ml tubes should be pre-rinsed in 1ml of 0.5% BSA in ASW. Leave some BSA-ASW (100-500µl) in the tube to add the embryos to. Add embryos near the bottom of the tube with the home-made micropipette, they will sink rapidly. Remove as much liquid as possible prior to fixation.
- 2.** Paraformaldehyde is prepared as a 40% solution with heating at 65°C and stirring to dissolve with addition of NaOH (to pH7.5) and frozen in small aliquotes. Alternatively, it can

be bought commercially. NaCl is prepared as a 5M stock solution and MOPS buffer is as a 1M pH7.5 stock solution.

3. Tween20 is viscous, so a 20% solution is prepared, which is much easier to pipette accurately.

4. PBS is made as a 20X solution (160g/l NaCl, 28.84g/l Na₂HPO₄·2H₂O, 4g/l KCl, 4g/l KH₂PO₄) and diluted as required. We do not adjust the pH.

5. Proteinase K treatment makes embryos stick to each other. Therefore we opted for a light proteinase K treatment at room temperature instead of 37°C. If embryos appear 'sticky' after ISH, try reducing the proteinase K concentration or incubation time.

6. We buy commercially prepared 20x SSC solution. We prepare 100x Denhardt's solution (2% Ficoll 400, 2% polyvinylpyrrolidone, 2% BSA), which is stored frozen in aliquots. Torula yeast RNA is prepared as a 100mg/ml stock and stored frozen according to the published protocol (33).

7. We add dextran sulfate to our hybridisation solution following (34). After testing several probes ('easy' and 'difficult') we found that adding dextran sulfate improved some probes tested but had little effect on others. Since it did not appear to have any detrimental effects, we systematically add it to our hybridisation solution.

8. Antisense RNA probe synthesis is carried out using standard procedures (e.g. (35)). We use ProbeQuant G-50 micro-columns to purify probes, according to the manufacturers instructions. While we use 50ng probe in 100µl of hybridisation solution as standard, this is sometimes modified during trouble-shooting. If a probe gives rise to too much 'background' then it is diluted further (i.e. 25µg, 10µg, 5µg per 100µl hybridisation solution) and tested to determine the best probe concentration. Frequently we found that dilution of the probe

concentration resulted in dramatic improvement for 'difficult probes'. However, this results in longer colour reaction times.

9. We reduced the amount of SDS in the original protocol for the washes from 1% to 0.2%.

This decreased the time taken for the colour reaction without significantly increasing background for several probes tested.

10. Optionally RNase treatment can be carried out between wash solutions 2 and 3. However, we found that for standard ISH for several genes tested, RNaseA treatment reduced foreground without significantly decreasing background. However, when we use ISH to detect *lacZ* driven by upstream regulatory sequences, I found that RNase treatment was essential. If background is high, it is worth testing if RNase treatment improves the result. RNase treatment is carried out after wash solution 2 washes. Embryos are rinsed in wash solution 3 at room temperature three times. Then 100µl RNase A solution is added (20µg/ml RNase A in 2XSSC) for 20 minutes at 37°C. Embryos are then rinsed 3x5 minutes in room temperature in wash solution 3 before re-joining the basic protocol with the 2x20 minutes wash solution 3 washes at 50-55°C.

11. Roche blocking reagent is made as a 10% stock solution in maleic acid buffer (0.1M Maleic acid pH 7.5; 0.15M NaCl) with heating and vortexing. Solution is stored in frozen aliquots.

12. Whole mount ISH is performed on embryos developed from dechorionated eggs. However, it is known that the chorion is essential for the later left-right asymmetry of the ascidian larval brain (36). This technical caveat should certainly be considered when analysing embryos developed from dechorionated eggs, particularly if one is interested in left-right patterning/asymmetry. This issue can be avoided by dechorionating embryos just prior to fixation (21, 36).

13. Embryos can alternatively be fixed for 90 minutes at room temperature.

14. For the standard protocol the embryos are incubated in the antibody solution overnight at 4°C. This incubation can be extended to two nights or even three and good results can be obtained. It is also possible to incubate for 1.5 to 2 hours at room temperature but I would not recommend this for 'difficult' probes.

15. During NBT-BCIP staining, embryos are checked approximately every 30 minutes. Incubations can take as little as 30 minutes or up to 3-6 days. When long incubations are required, embryos are rinsed and stored in PBST at 4°C overnight and then steps 18-20 followed the next morning. After a few days, if there is no background staining, embryos can be left overnight in the colour reaction solution. However, the solution should be changed regularly (minimum morning and evening). For 'difficult probes' if background levels start to rise, it is best to immediately wash embryos in PBST overnight at 4°C and then resume staining. Once high background levels are reached, it is much more difficult to remove the background, but washing overnight (or several nights) in PBST at 4°C or room temperature with agitation can sometimes rescue an experiment.

16. If nuclear staining is not required, embryos can be washed with PBST to remove fix and stored in 80% glycerol. Embryos stored in this way remain stable and can be re-photographed even after several months of storage.

17. The choice between Vectasheild-DAPI and Hoechst is difficult. The quality of the nuclear staining is similar. The advantage of Vectasheild is that ~~embryos can be mounted immediately without the overnight wash~~ and it is easier to handle than glycerol. The disadvantage is that, sometimes, for unknown reasons, the embryos go dark after UV exposure. This problem will affect all embryos in a batch or none and only happens from time to time. Each manipulator should test which of these techniques is best suited to him/her. Adjusting the amount of

glycerol between 50 and 80% is also possible as decreased transparency (with 50% glycerol) is sometimes better for probes with staining in internal tissues.

18. The 'going black' issue was also noted by another ascidian researcher (Hiroki Nishida and Shih Yu, Osaka University, Japan; *personal communication*). Their current method of DAPI staining and mounting embryos to avoid this problem is as follows. Embryos are first stained with DAPI (Final concentration 0.1-0.2 µg/ml PBS) for 20 minutes and then washed with PBS once for 10 minutes. Embryos are mounted in 80% glycerol (in PBS) 0.5% n-Propyl gallate. Embryos treated by this method usually show a strong background just after mounting in 80% glycerol, but the background decreases in one day after mounting.

5. References

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Figure Captions

Fig. 1 The ascidian central nervous system. A) A tadpole larvae (top) with the CNS outlined with a black dotted line. The anterior otolith and posterior ocellus pigmented cells are clearly visible. The very narrow tail nerve cord (posterior), only four cells in cross-section, is indicated by a simple dotted line. Below the photograph, the CNS outline is labelled to show the main subdivisions of the CNS. The sensory vesicle or brain is drawn below, with the contribution from the a-, b- and A-lineages indicated by dotted red lines. An 8-cell stage embryo is drawn on the right, showing the four founder lineages, of which three contribute to the brain. B-D) 3-row, 6-row and 12-row neural plate stages. An embryo drawing for each stage is shown with the a- and A-line neural plate cells that contribute to the brain coloured in red. Below are schematics of the neural plate, on the left showing the column and row names and on the right the left hand side brain precursors of the neural plate is drawn showing cell names (37). The right hand side of the bilaterally symmetrical neural plate has identical cell names, except the cell name is underlined.

Fig. 2. Equipment. A) Hand made micropipette for transferring live embryos. B) Workshop modified manual centrifuge. Two of the four 15ml tube holders are removed. The remaining two are replaced with racks for holding microcentrifuge tubes. 40 tubes can be spun at the same time with this system. C) Vaseline dispenser. D) Slide ready for photography. The

embryo is in a tiny drop of Vectashield in the centre of the coverslip. The coverslip is held by four dots of Vaseline and pushed down until it touches the Vectashield drop. Gentle sideways movements of the coverslip are used to adjust the position of the embryo in the drop.

Fig. 3. Dechoriation progression. A) Firstly, eggs are covered in follicle cells. B) Follicle cells fall off but eggs remain surrounded by chorion. C) Eggs are coming away from the chorion and should now be washed quickly. D) Dechorionated eggs.

Fig. 4. Vectashield-DAPI problem. Embryo stained with *DllB* probe prior to (A) and after (B) UV exposure.

Fig. 5. Adobe Photoshop DAPI-ISH overlay. A) Screen shot of *Gsx* ISH in Photoshop. B) Screenshot of DAPI image after conversion to greyscale. C) Overlay of DAPI shot (50% transparency) and ISH image. D) Contrast, light adjusted, rotated and cropped image of *Gsx* ISH with DAPI stain.

Fig. 6. Stages 11-13 DAPI images (3-row stage to 6-row stage). Top: DAPI-stained images. Embryo is outlined in blue, brain precursors are outlined in red. Middle: drawings of neural plate and surrounding nuclei of the corresponding embryo (top). The embryo is outlined in blue, the brain precursors are outlined in red and rows of cells are indicated. Dividing cell nuclei are connected by black bars. Bottom: schematic drawings of neural plates representing the embryo above. A) 3-row neural plate. B) The medial 3 pairs of cells (columns 1-3) of row

I/II are dividing. C) Row V/VI is dividing, one cell in row III/IV is dividing. D) Row III/IV is dividing. E) 6-row neural plate stage.

Fig. 7. Stages 11-13 ISH images. Same embryos as Fig.6, but showing ISH-DAPI merged images. On the schematic drawings (bottom), the cells expressing each gene are coloured in grey.

Fig. 8. Stages 13-15 DAPI images (6-row to 12 row stage). Top: DAPI-stained images.

Embryo is outlined in blue, brain precursors are outlined in red. Middle: drawings of neural plate and surrounding nuclei of the corresponding embryo (top). The embryo is outlined in blue, the brain precursors are outlined in red and rows of cells are indicated. Nuclei of cells which have divided from the 6-row neural plate stage are connected by black bars. Bottom: schematic drawings of neural plates representing the embryo above. Cells shown in light grey could not be clearly identified on the DAPI image. A) Row II cells start to divide. B) Row II cells are dividing. C) Row II cells have divided, row I cells are dividing. D-J) Row I and II cells are divided. D-G) Embryos are at a similar stage, but show cell divisions in different orders between rows III, V and VI. D) Cells in row VI are dividing. E) Cells in row V are dividing. F) Cells in rows III and V are dividing (row VI not visible). G) Cells in rows V and VI are dividing. H) Cells in rows III and V have divided and cells in row IV are dividing. I) Cells in all rows have divided, row II cells have divided again. J) Top: close up of embryo and nuclei drawing in (I) showing characteristic organisation of row III cells. Bottom two panels: the characteristic organisation of row III cells is even clearer in this embryo, in which there is no ISH stain. Brain precursors are outlined in red and row III cell nuclei are coloured in red in the nuclei drawing.

Fig. 9. Stages 11-13 ISH images. Same embryos as Fig.6, but showing ISH-DAPI merged images. On the schematic drawings (bottom), the cells expressing each gene are coloured in

grey. In J the close up of (I) is shown with the stained cells indicated in purple on the nuclei drawing.

Table Captions

Table 1.

Table 1 lists the common gene name used and the unique gene identity number for *Ciona robusta* for annotation.

Table 1. Unique identifiers for *Ciona robusta* genes presented

Gene name	<i>Ciona robusta</i> Unique gene identity
<i>Fgf9/16/20</i>	Cirobu.g00004295
<i>Snail</i>	Cirobu.g00005955
<i>ZnF266</i>	Cirobu.g00001049
<i>Delta-like</i>	Cirobu.g00012743
<i>Msx</i>	Cirobu.g00005203
<i>Foxb</i>	Cirobu.g00006425
<i>EfnA.b (ephrin Ab)</i>	Cirobu.g00005364
<i>Foxc</i>	Cirobu.g00012813

<i>Gsx</i>	Cirobu.g00005160
<i>DllB</i>	Cirobu.g00008660

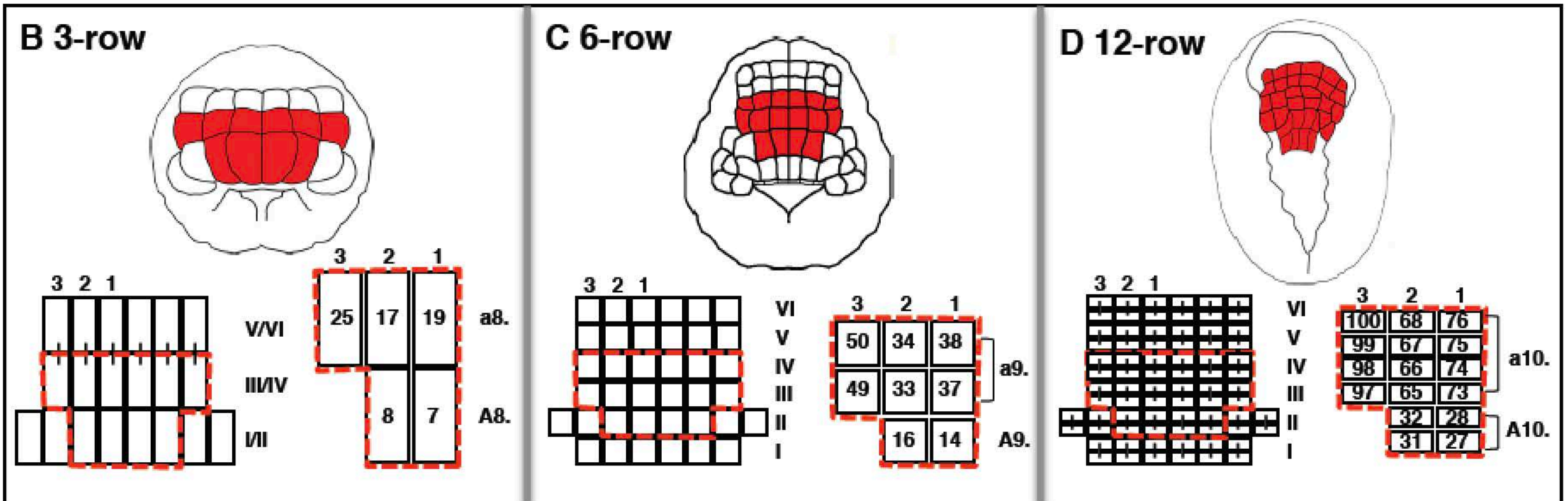
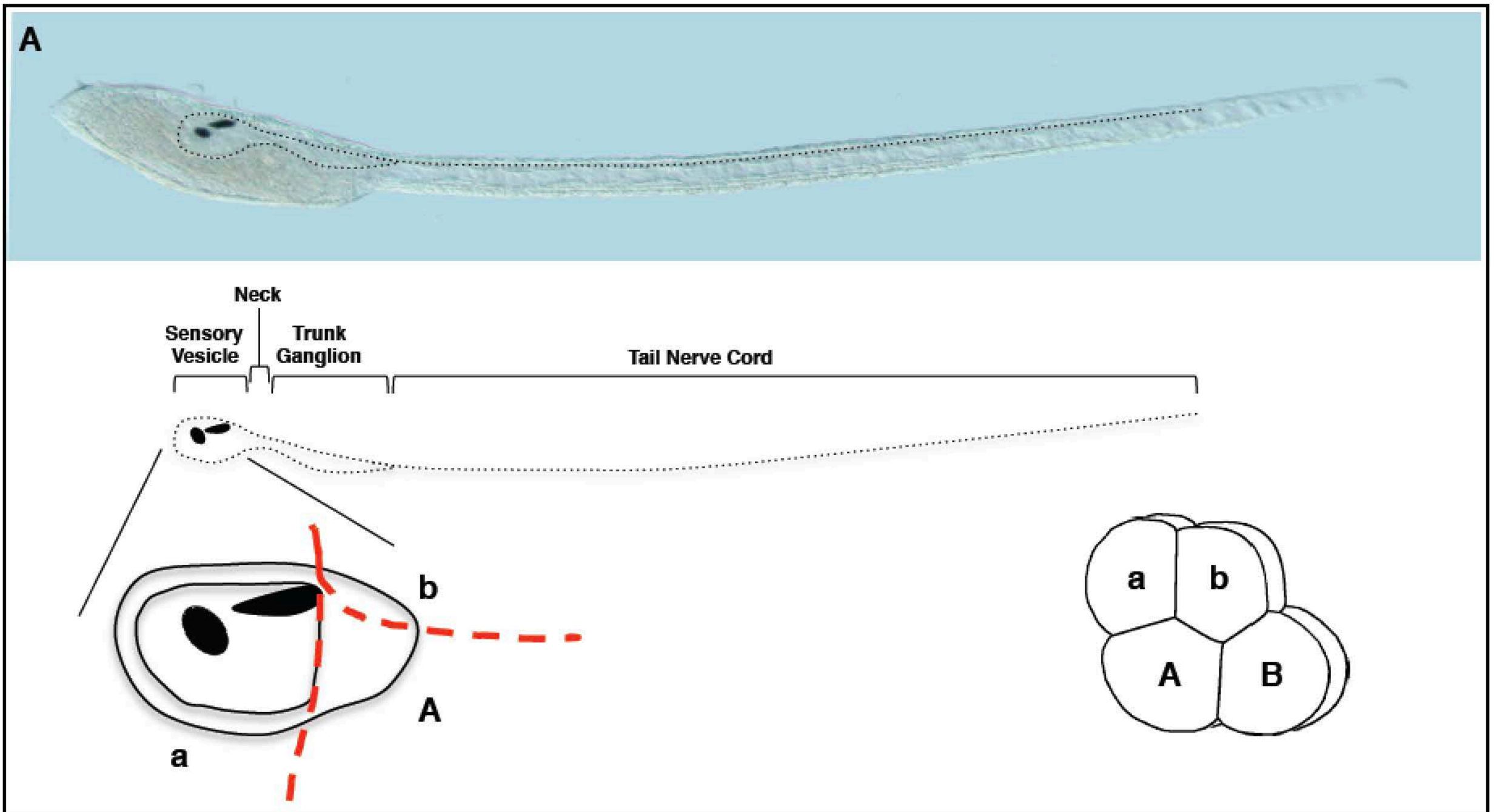


Figure 1

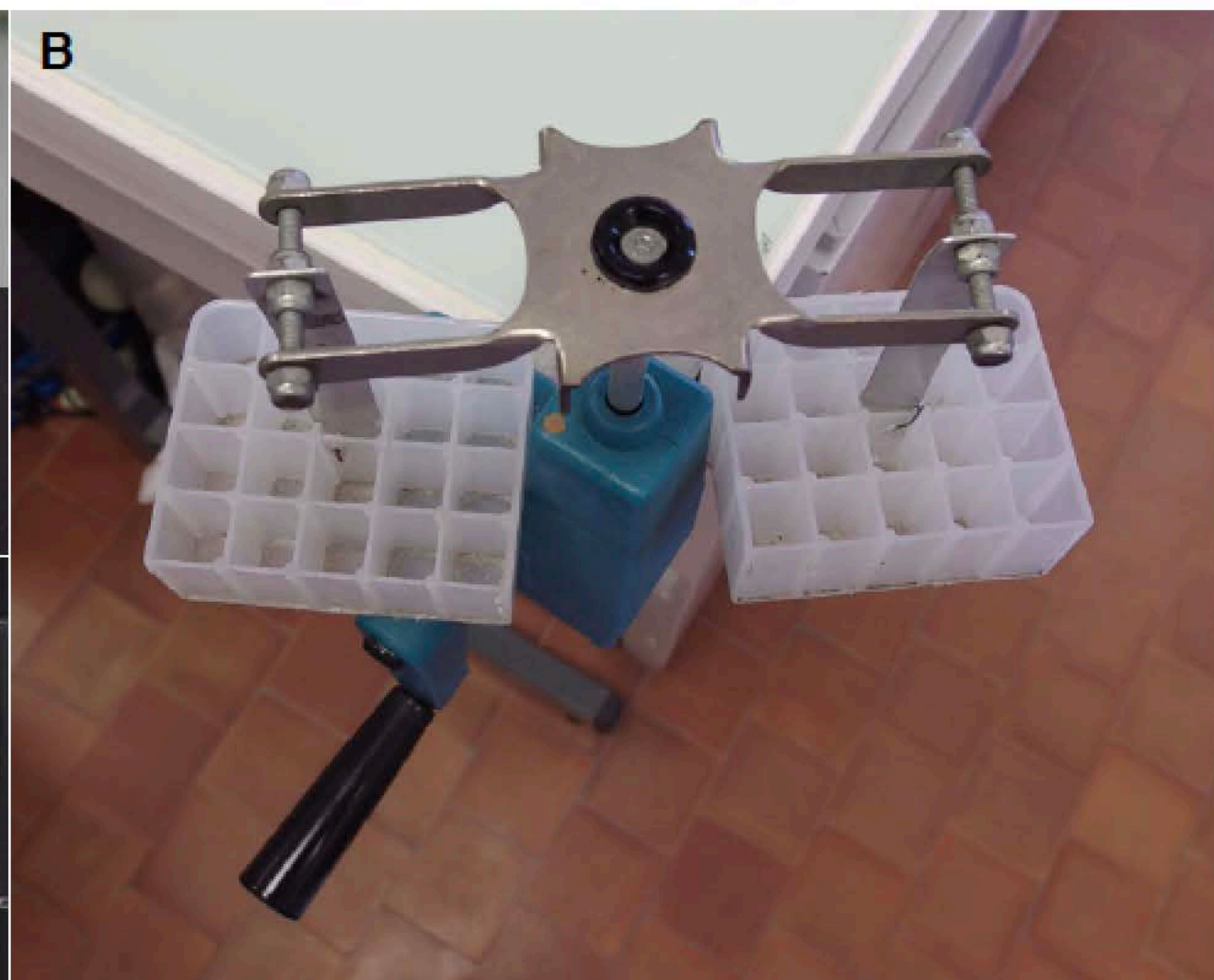
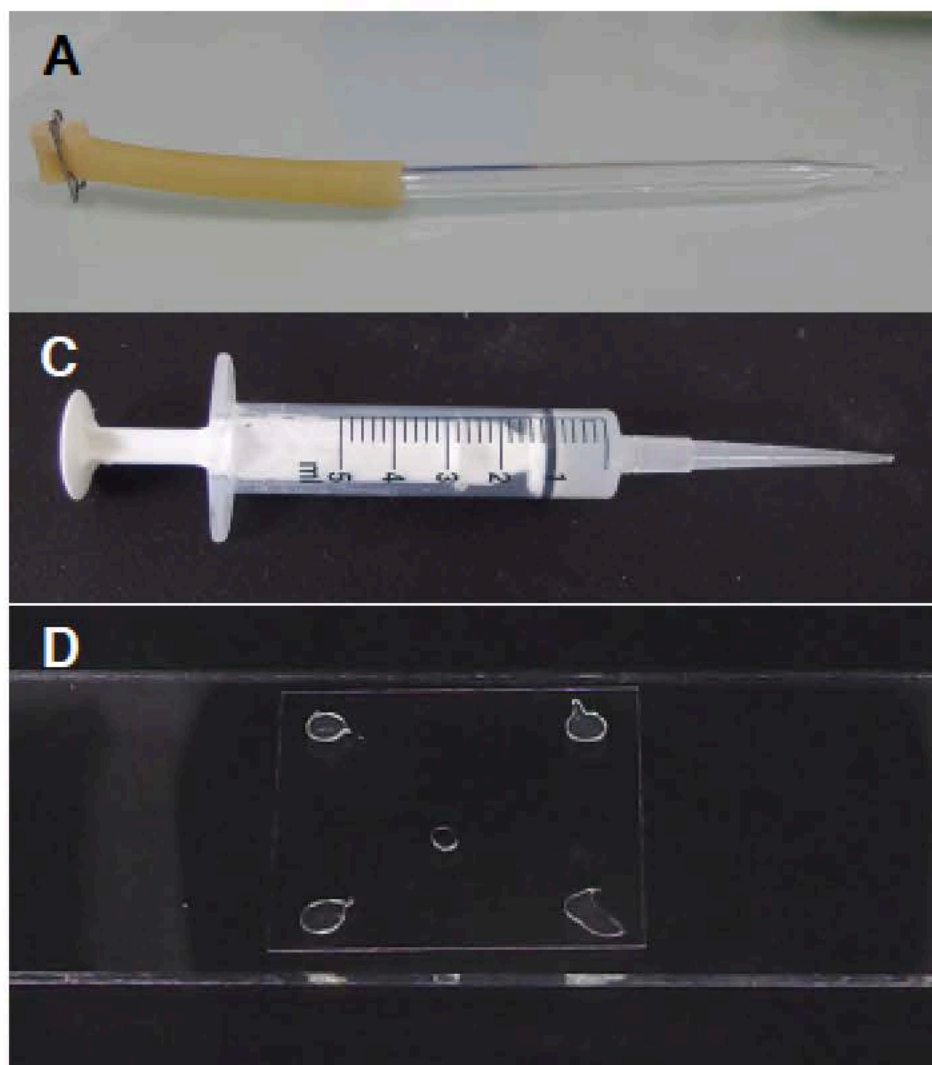


Figure 2

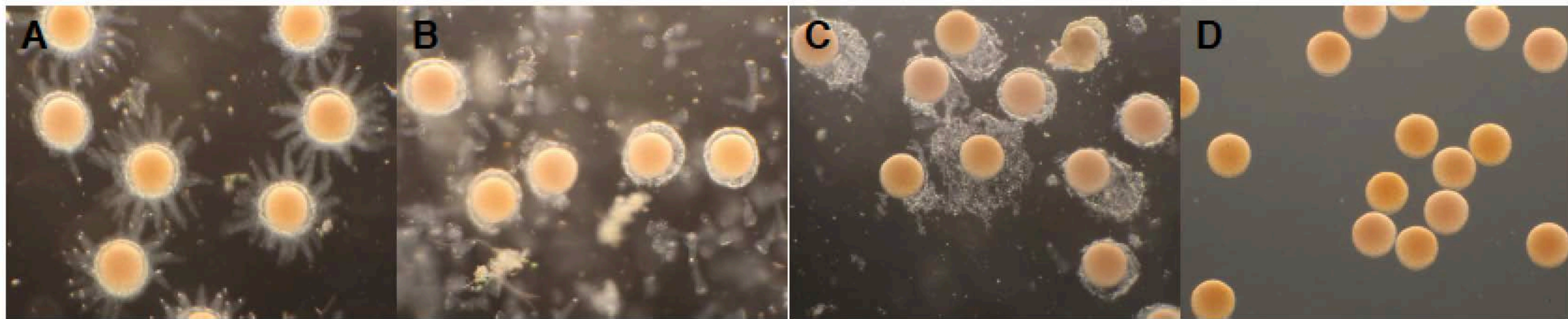


Figure 3

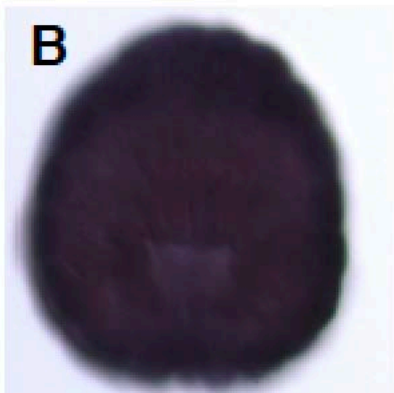
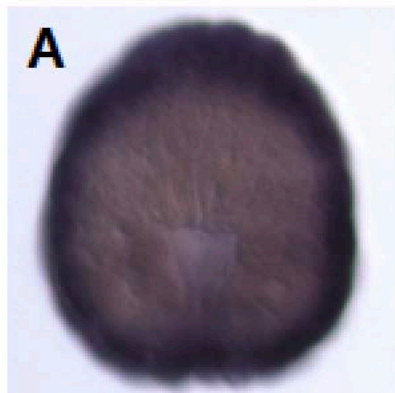


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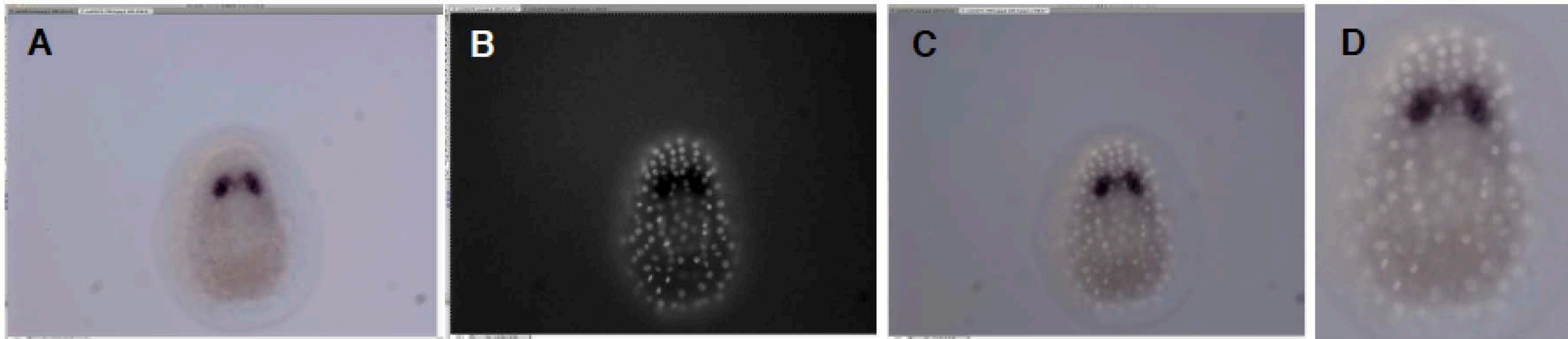


Figure 5

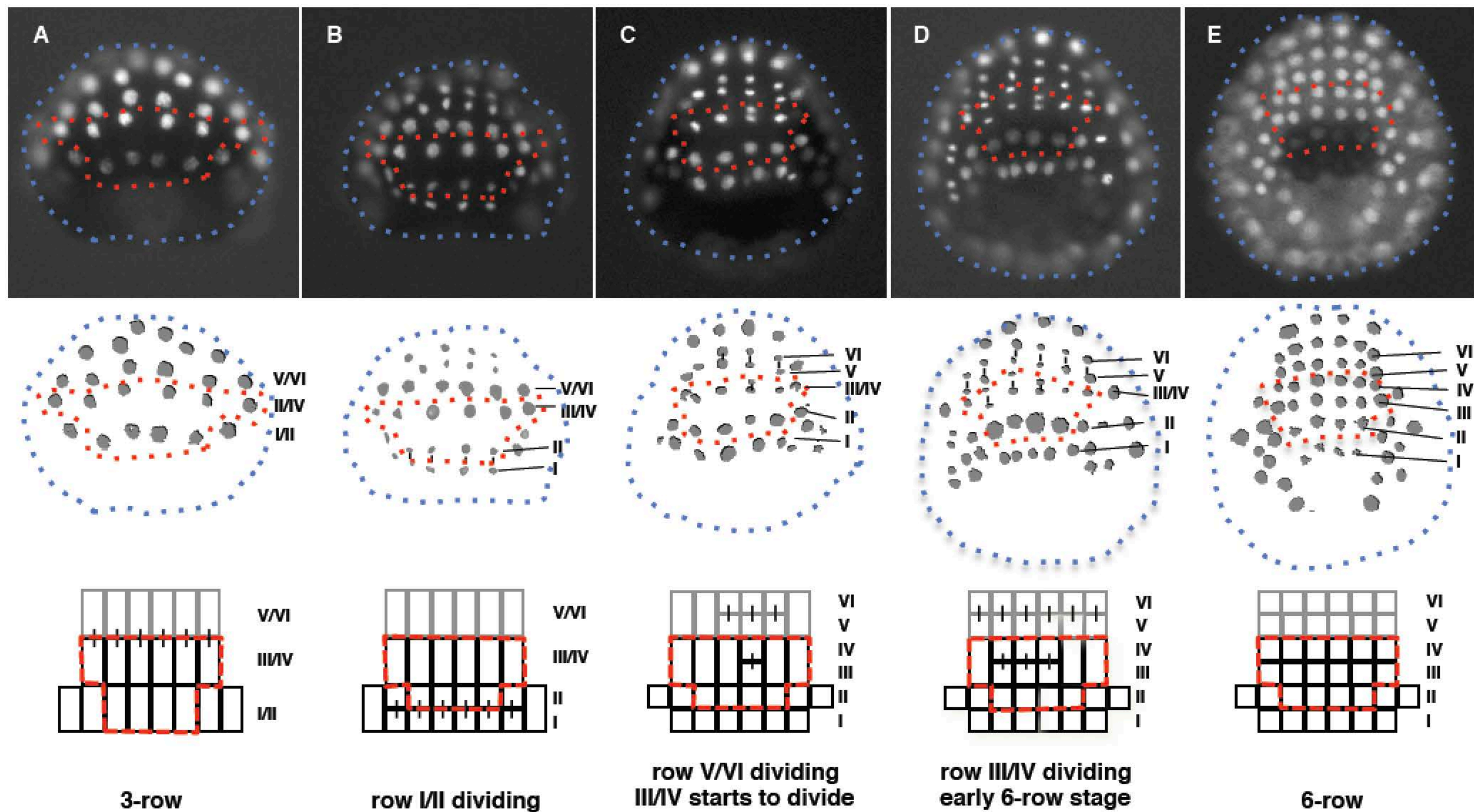
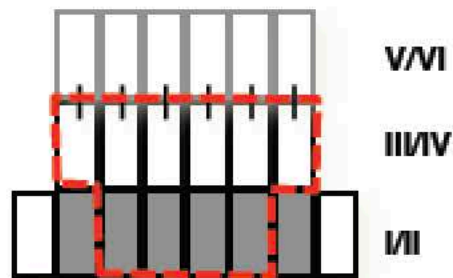
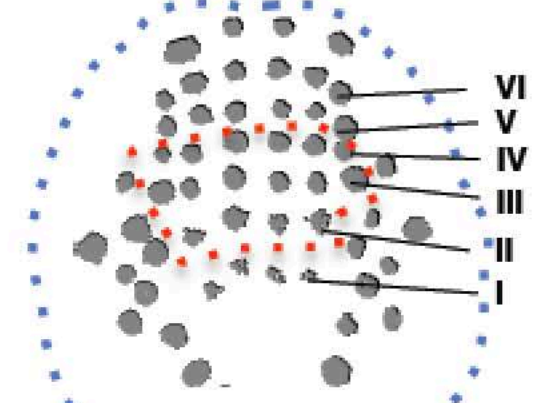
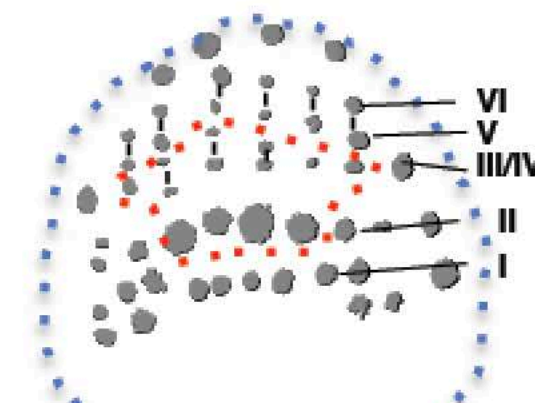
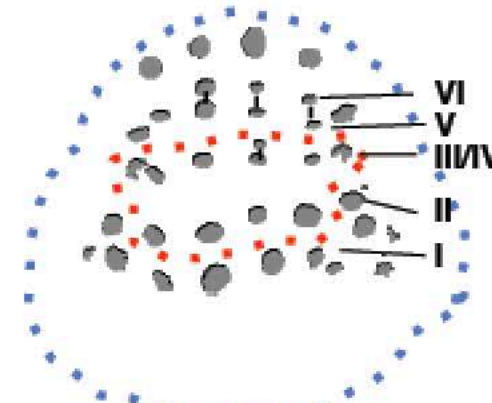
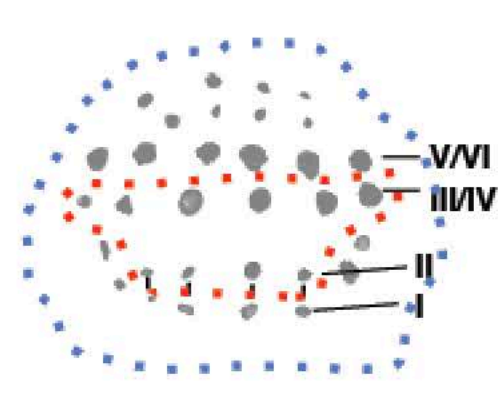
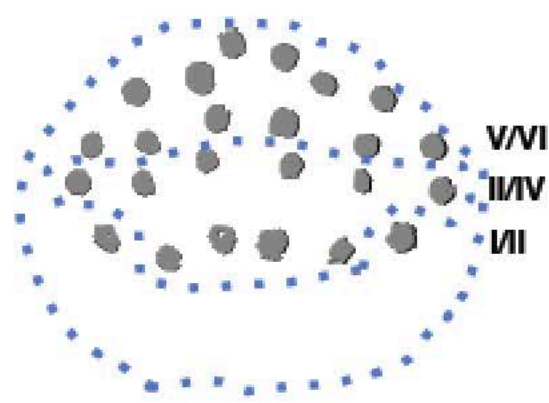
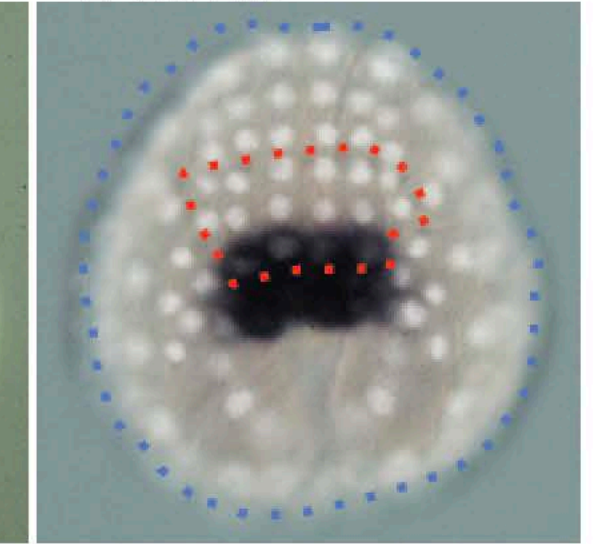
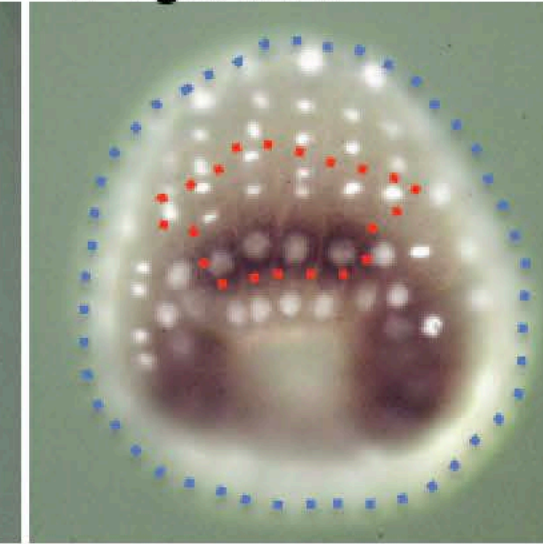
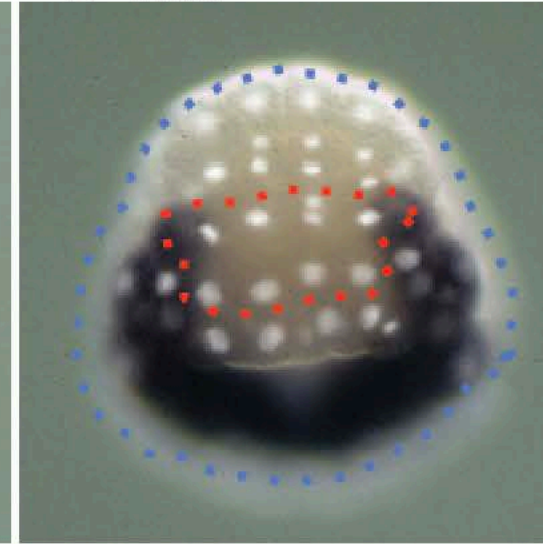
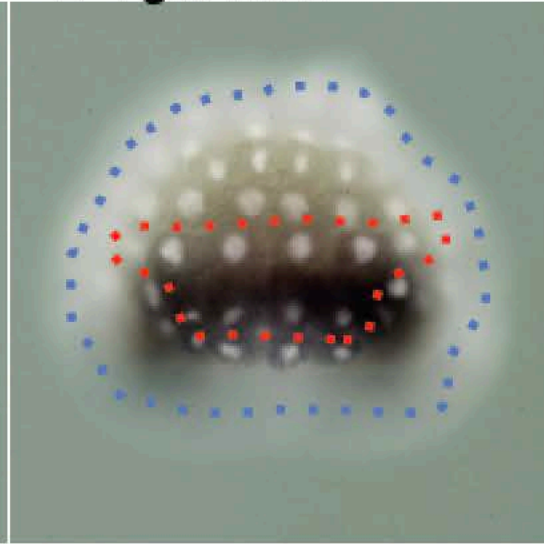
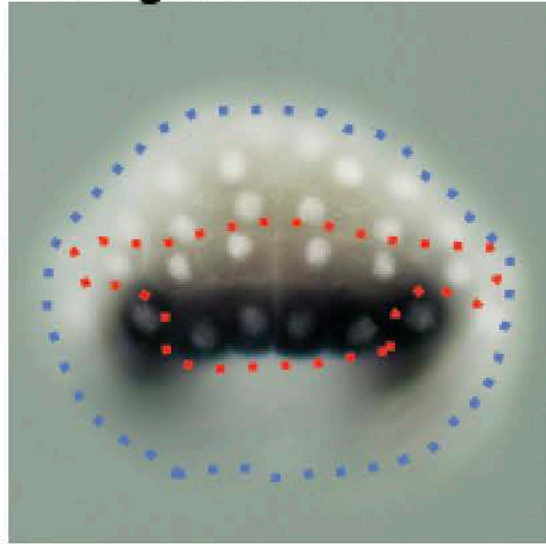
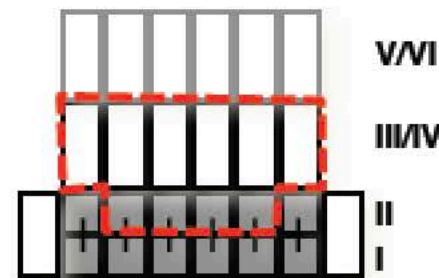
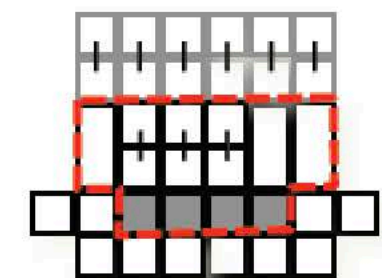
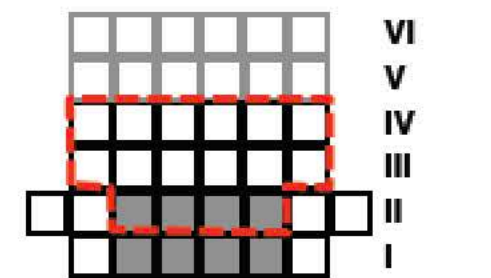


Figure 6

A- *Fgf9/16/20***B- *Fgf9/16/20*****C- *Snail*****D- *Fgf9/16/20*****E- *ZnF266*****3-row****row VII dividing****row V/VI dividing
III/IV starts to divide****row III/IV dividing
early 6-row stage****6-row****Figure 7**

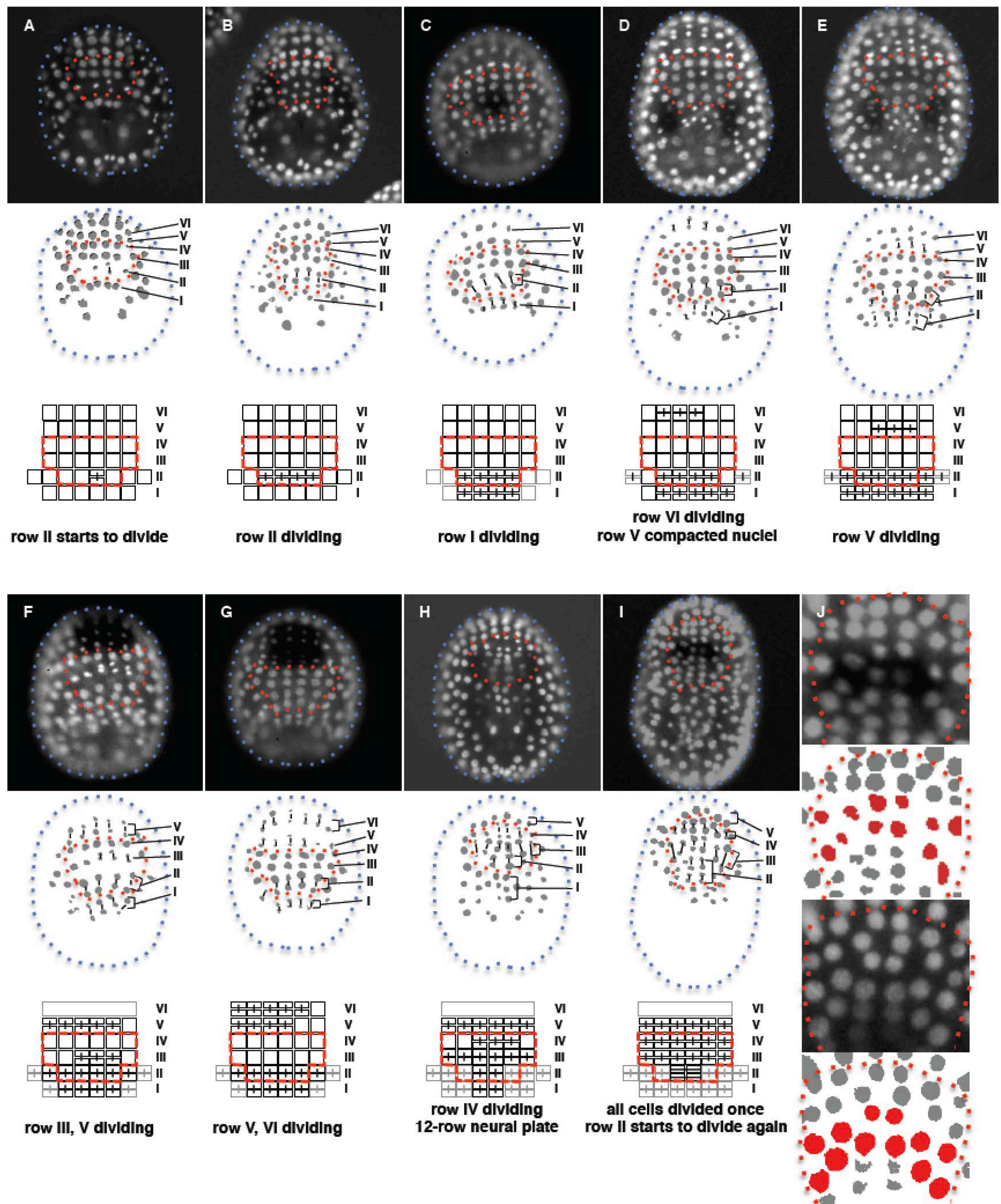
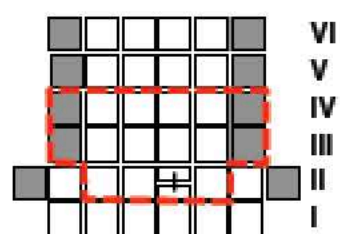
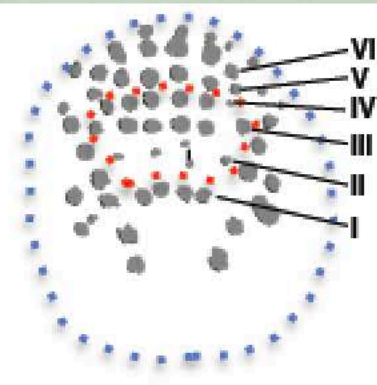
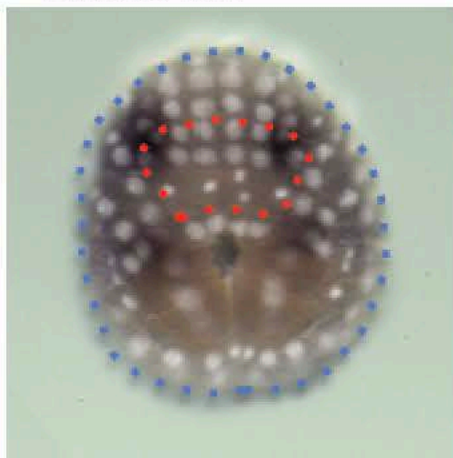
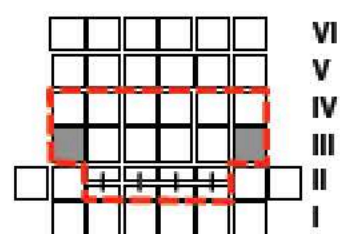
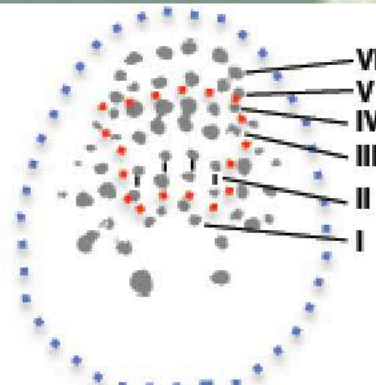
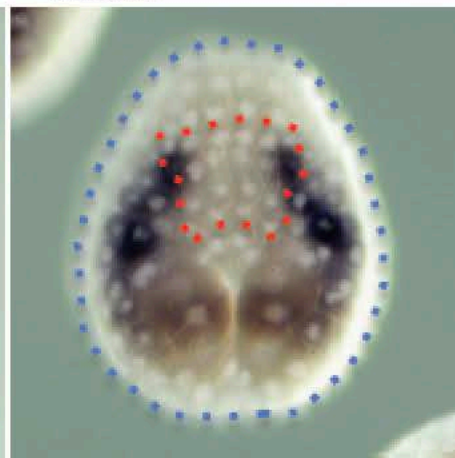


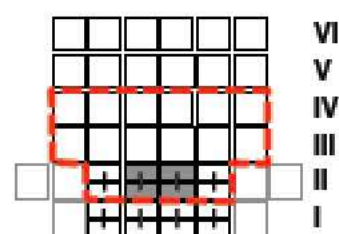
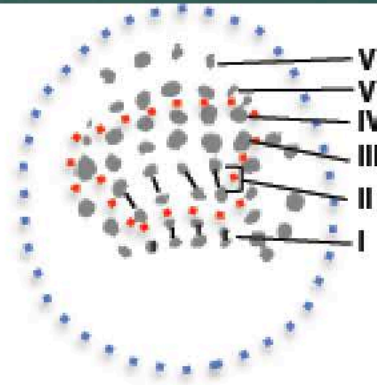
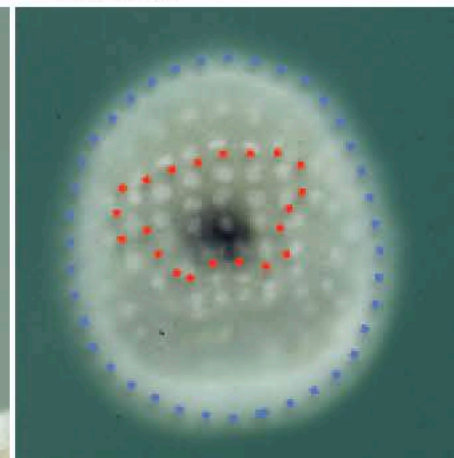
Figure 8

A *Delta-like*

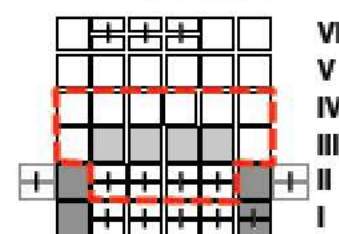
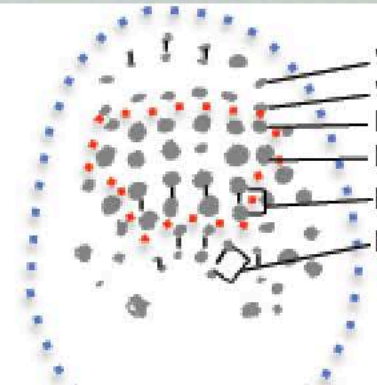
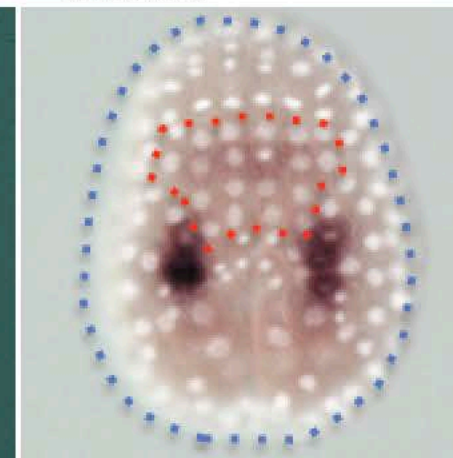
row II starts to divide

B *Msx*

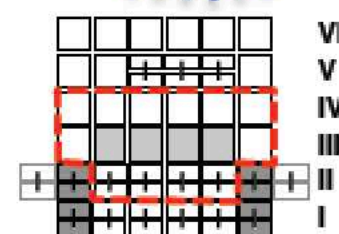
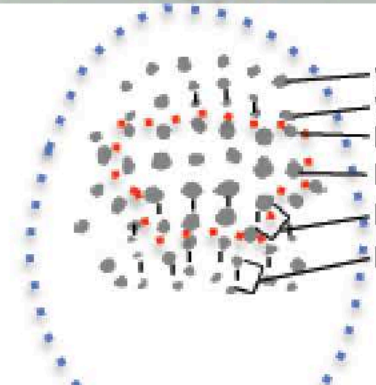
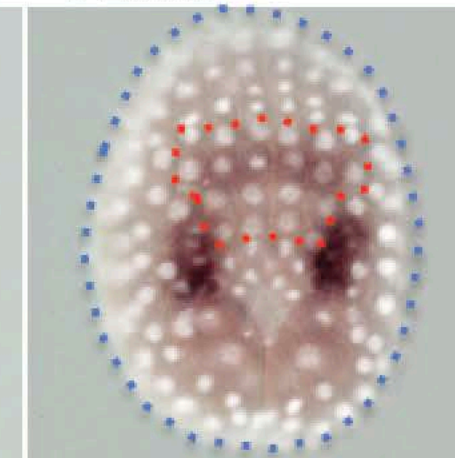
row II dividing

C *Foxb*

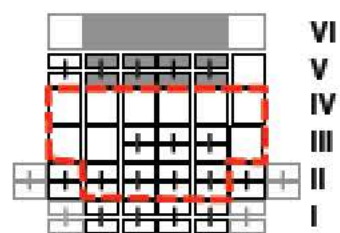
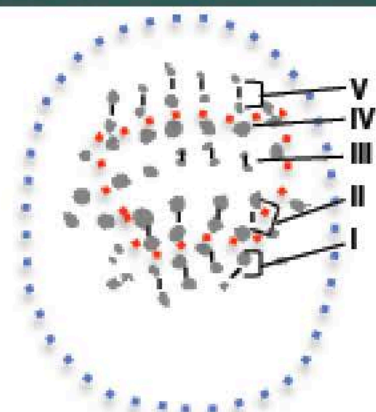
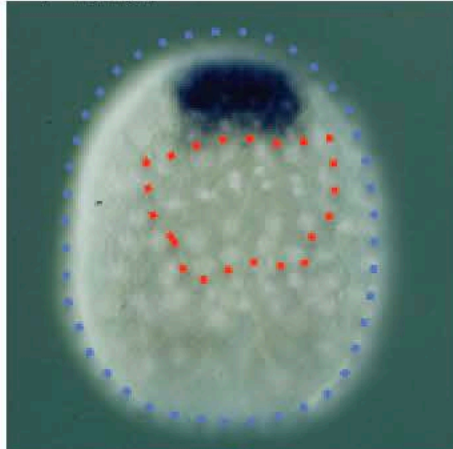
row I dividing

D *Efna.b*

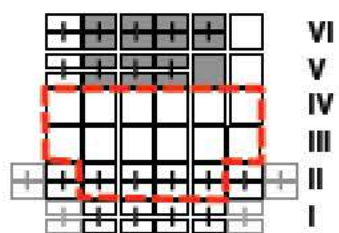
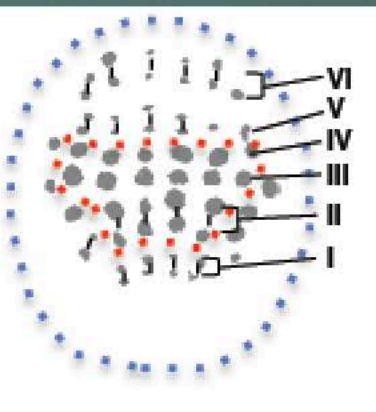
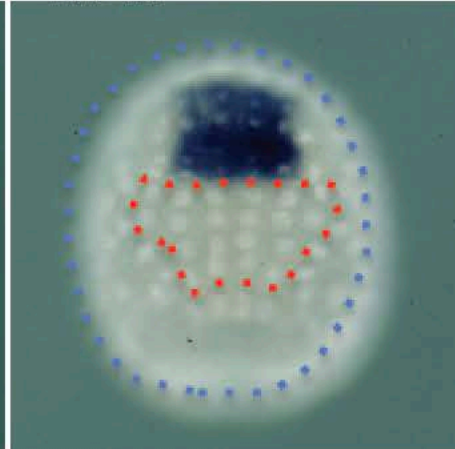
row VI dividing
row V compacted nuclei

E *Efna.b*

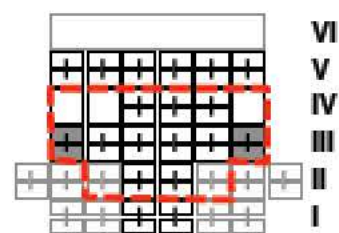
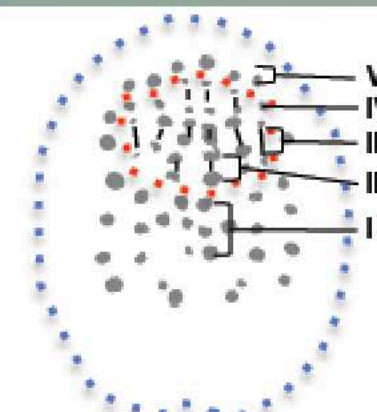
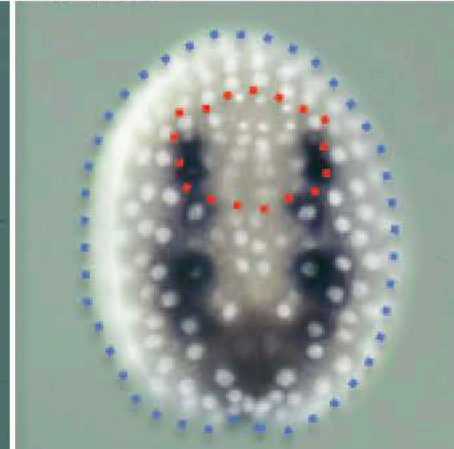
row V dividing

F *Foxc*

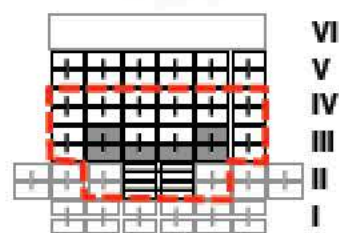
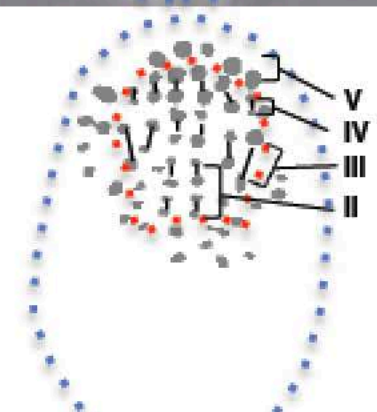
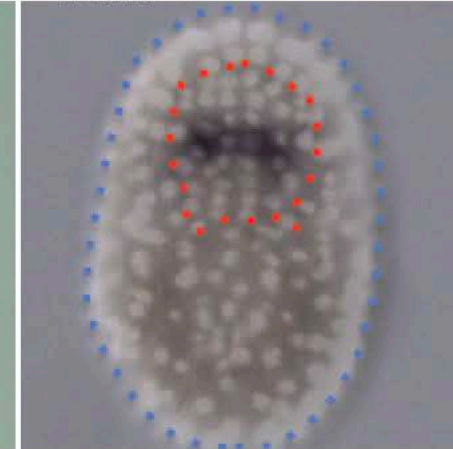
row III, V dividing

G *Foxc*

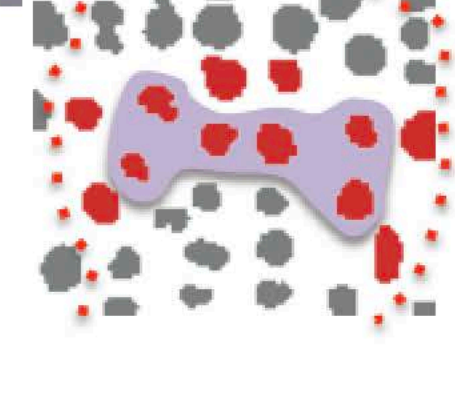
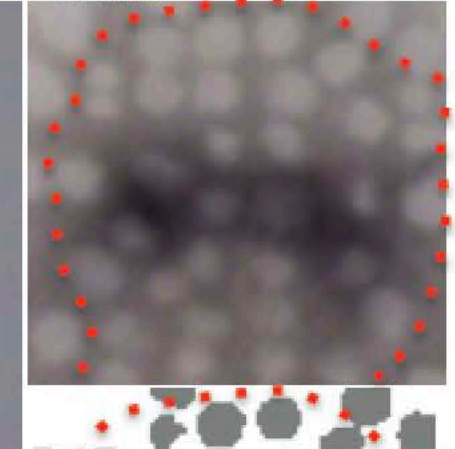
row V, VI dividing

H *Msx*

row IV dividing
12-row neural plate

I *Gsx*

all cells divided once
row II starts to divide again

J *Gsx***Figure 9**