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Confocal Imaging of Living Cells in Intact Embryos

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1. Introduction

How individual cells and tissues organize and coordinate to form the structure of a developing embryo is a major question in developmental biology. Equally intriguing is how cells in adult tissue respond during wound healing and tissue regeneration. In these morphogenetic processes there can be a wide variety of cell behaviors: individual cells may change shape or divide, migrate to different areas, or differentiate to form a pattern. Entire tissue layers can spread across a surface, fold up or cavitate to form a three-dimensional structure. For example, during formation of the neural tube in chick embryos, the neural epithelium folds up from a flat sheet of cells to form a tube with a hollow lumen. As the neural tube becomes shaped into various regions of the nervous system, the neural crest cells emerge from the dorsal midline of the neural tube and migrate to pattern the peripheral nervous system. A main theme in these examples is that the cell and tissue events are dynamic, on a time scale from minutes to days, covering spatial regions ranging in size from microns to millimeters.

It remains largely unknown how individual cells "know" where to go, when to move, and when to differentiate, or how tissue shape changes are coordinated. Some hints of the underlying processes have been obtained with light microscopy; however, this has been primarily limited to imaging thinly sectioned tissue or dissociated cells in culture. In these systems, it is difficult to know whether cells removed from their natural environment behave the same as they would in the intact embryo. The more relevant scenario is to image the living intact embryo, allowing the full interplay of cell movements, tissue interactions, forces, and signaling events. In those cases where the whole embryo cannot be studied, thick slice cultures offer a means to follow cells in a more natural setting. Both the intact embryo and thick slice cultures offer the chal-

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lenge of imaging through thick, highly scattering tissue while maintaining viability.

Because of its ability to image labeled structures deep within a thick specimen, the confocal microscope permits the imaging of cells and tissue within their own environment. Many different workers have used the optical sectioning ability of the confocal microscope to explore a wide variety of biological phenomena in living tissue ranging from the calcium dynamics in a living marine invertebrate embryo to the growth of axons and dendrites in cultured tissue slices of mammalian nervous tissue ***(1,2)***. Because confocal microscopy does not require fixing and clearing of the specimen it is ideal for recording cell and tissue dynamics in time-lapse. Just as a better understanding of construction would result from repeated observation of a rising building, rather than from merely examining the finished product, time-lapse data establish a spatial and temporal reference frame needed to link invaluable gene expression data with cell and tissue dynamics.

Performing confocal microscopy of living cells does not come without its share of difficulties, and special challenges arise in imaging explanted tissues or entire embryos. Issues such as maintaining the health of the tissue need to be balanced with short and clean optical paths. Many embryos, depending on their size, can be maintained intact and imaged in their own natural environment, such as zebrafish or sea urchin embryos. It is formidable to view and sustain other embryos that are less visually accessible, such as those vertebrates growing in an opaque egg or inside a host animal, such as a chick, mouse, or rat. In these cases, a special chamber may have to be constructed, and staining techniques may have to be developed to deliver a fluorescent dye to a specified area, perhaps deep within an embryo. Finally, although the confocal microscope can optically section through thick tissue, there is a trade-off between how deep into the tissue a particular objective can be used, its numerical aperture (NA), and the clarity of the image, making the choice of the objective lens and orientation of the embryo important. Here, we elaborate on the important aspects that should be considered for the imaging of live cells in intact embryos. We illustrate this with some example techniques we have used in our own research on imaging cell and tissue dynamics in early avian embryos.

2. Materials

2.1. Embryo Preparation

1. Howard Ringer's solution
2. Albumen waste container
3. Scotch tape (3M no. 810)
4. 18- and 25-gage needles; 1- and 3-mL syringes
5. Set of dissecting tools

6. India ink (Pelikan Fount no. 211143)
7. Filter paper (Whatman no. 1001-150; 150 mm diameter)
8. Pipetmen (P20, P200, P1000)
9. Sterile Pasteur pipet
10. Sterile plastic transfer pipet
11. 70% and 100% Ethanol
12. Sucrose (Fisher no. S5-500)
13. Fast Green FCF (Fisher no. 42053)
14. Culture medium
    a. Neurobasal medium (Gibco no. 21103-015)
    b. B-27 Supplement (Gibco no. 17504)
    c. L-glutamine (Sigma no. 3126)

2.2. Dyes

1. DiIC18(3) (Molecular Probes no. D-282)
2. DiI-CM (Molecular Probes no. C-7000; fixable)

2.3. Culture Chamber Components

1. Six-well plate (Falcon no. 3046)
2. Culture Insert (Millipore; Millicell-CM, PICM 030 50)
3. Circular glass coverslips (Fisher no. 48380 080; 25-mm diameter)
4. Fibronectin (Sigma no. F-2006)
5. Silicone grease (Dow Corning no. 79810-99)
6. Soldering tool

2.4. Microscopes and Thermal Insulation Components

1. Bio-Rad MRC-500 upright confocal microscope
2. Bio-Rad MRC-600 inverted confocal microscope
3. Optical Magnetic Disk Recorder (Panasonic no. TQ-3038F)
4. Rewritable Optical Disk (3M no. 15175; 590MB)
5. Incubator heaters (Lyon no. 115-20)
6. Cardboard (~4 mm thick)
7. Thermal insulation (Reflectix; 5/16 inch thick)
8. Velcro (McMaster-Carr no. 9489K65; 3/4 inch stick-on)

3. Methods

3.1. Whole Embryo Chick Explant Technique

We have developed a method of explanting the entire chick embryo including some of the surrounding blastoderm, which appears successful with embryos as early as five somites (**Fig. 1**). By keeping the embryo intact and spreading out the surrounding blastoderm onto a membrane surface, whole embryo explants develop at a growth rate similar to that of intact embryos incubated near the microscope stage, and maintain tissue integrity for approxi-

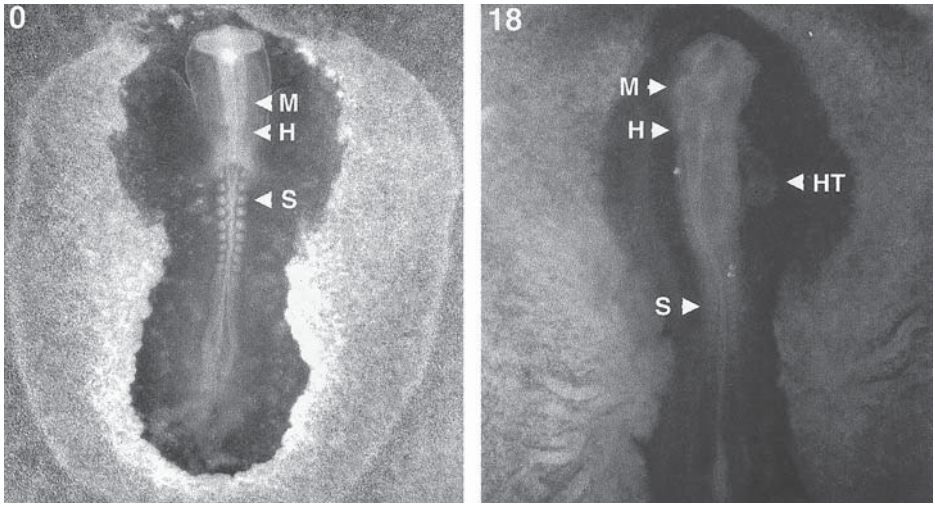

Fig. 1. Whole embryo chick explant culture. A typical whole embryo chick explant was positioned dorsal side down onto the culture insert membrane: (0 h) the embryo shown is a nine somite stage embryo; (18 h) after a time-lapse imaging session, the culture insert was removed from the six-well plate and flipped over to view the dorsal side of the embryo explant. The embryo explant has developed to 21 somites. Some of the anatomical features are labeled, including the midbrain (M), hindbrain (H), heart (HT), and somites (S). Scale bar = 1 mm.

mately 1–2 days in culture. The embryo explant can be placed either dorsal side up or down on the membrane. We describe this culture technique in detail.

1. About 1 h prior to removing the embryo from the egg, prepare the culture insert membrane. Coat the membrane surface with 200 µL of a fibronectin solution (20 µg/mL of fibronectin in Ringer's solution). The fibronectin solution helps to keep the embryo in place and makes the membrane surface transparent. The membrane is 200 µm thick and has a pore size of 0.4 µm. Place the culture insert in a Petri dish and cover to keep the fibronectin solution from evaporating. Rest the Petri dish slightly tilted so that the excess fibronectin solution runs to one side.
2. To remove the embryo from the egg, cut out an oval paper ring from filter paper. Make the inside diameter of the ring large enough to cover the entire embryo and the outside diameter to cover most of the surrounding blastoderm. Place the oval paper ring over the embryo and cut around the outside of the ring. With a pair of forceps, remove the oval paper ring from the egg, with the embryo attached, and place into a Ringer's solution. Separate the oval paper ring from the embryo with the forceps. Clear excess yolk platelets and India ink from the embryo by gently blowing Ringer's solution across the embryo with a P200 pipetman (set between 100 and 150).

3. Before placing the embryo on the insert membrane, pipet any excess fibronectin solution from the membrane with a pipetman. Use a plastic transfer pipet to transfer the whole embryo onto the culture membrane.
4. Gently spread out the surrounding blastoderm and the embryo onto the membrane with forceps. Remove excess solution with a pipetman placed at the rostral or caudal ends of the explanted embryo so that as the solution drains, the embryo maintains a straight line posture along the rostrocaudal axis. This naturally spreads out the explanted tissue without flattening the embryo and mimics the tension of the blastoderm that is normally stretched over the yolk sac of the intact embryo.
5. The culture medium that best supports the overall integrity and health of the chick explants is a defined medium consisting of Neurobasal Medium (Gibco), 98 mL: B27 supplement (Gibco), 2 mL; and 0.5 m*M* L-glutamine ***(3)***.

3.2. Culture Chambers for Chick Embryo Explants

One method we have used for chick embryo explants is based on a closed culture chamber design originally developed for light microscope imaging of trunk neural tube explants in chick ***(3)***. We have modified this chamber design for imaging chick morphogenesis with both inverted and upright confocal microscopes (*see* **Notes 1–3**). The closed chamber is made from a plastic six-well plate (Falcon) that together with the culture insert (Millipore) and embryo make up the culture system (**Fig. 2A**). We discuss our modifications in relation to the design criteria outlined previously.

1. To increase the resolution of the explant tissue during confocal imaging, the plastic bottom of one of the wells of the six-well plate is replaced with a glass coverslip (*see* **Note 4**). To make a neatly cut hole, press a soldering tool against a 5-cent coin placed on the outside wall of the plastic well. After the hole is made, use a fine sandpaper to smooth the area and wipe any debris away with a paper towel dipped in 70% ethanol. On the inside of the well with the hole, spread silicone grease around the circumference of the hole and attach a circular glass coverslip over the hole.
2. To prepare the six-well plate to support the embryo, the well with the glass coverslip is filled with 1.5 mL of tissue culture medium and the other wells are filled to two thirds with sterile water. Three support legs underneath the culture insert raise the membrane 1 mm off the culture dish bottom, allowing culture medium to be soaked up through 0.4 μm pores in the membrane. Sterile water in the other wells provides a hydrated atmosphere when the dish is sealed.
3. After the culture insert is placed in the well plate, a parafilm strip (5 cm × 40 cm) is wrapped around the sides of the dish. When sealed, the well plate provides a well oxygenated and hydrated environment for the embryo, which can be sustained for 1–2 days (*see* **Note 5**).
4. To maintain the temperature of the microscope stage at 37°C, the stage area is enclosed in a thermal insulation box (*see* **Note 6**). The walls of the box are made of cardboard pieces, cut to fit the individual microscope. Each piece of cardboard

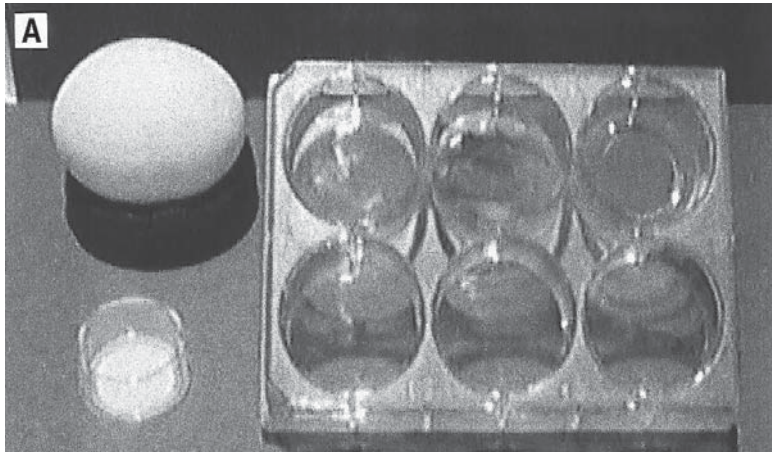

Fig. 2A

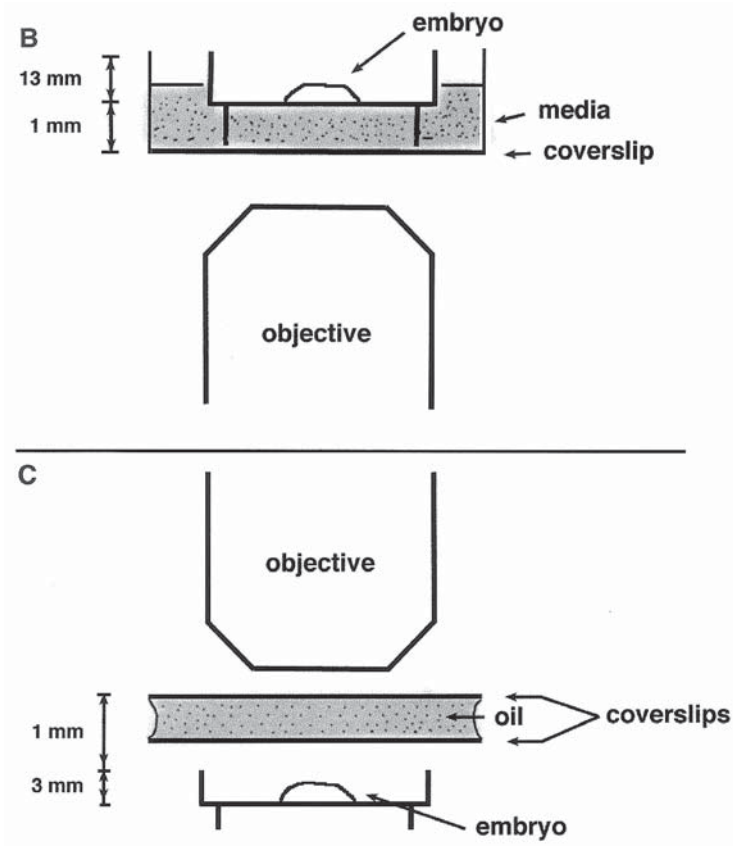

Fig. 2B,C

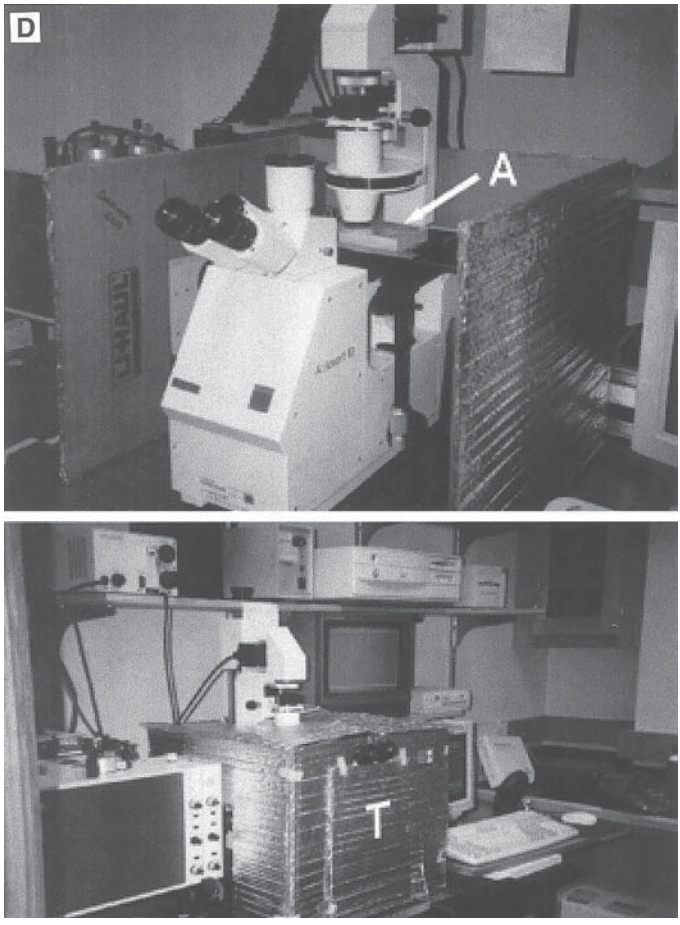

Fig. 2. Culture chamber components and microscopy setups. **(A)** Millipore Culture Insert and Falcon 6-well plate setup. **(B)** Schematic of microscopy setup for inverted microscope. **(C)** Schematic of microscopy setup for upright microscope. **(D)** Confocal inverted microscope with the six-well plate (A) on the microscope stage and thermal insulation box (T).

is covered with thermal wrapping (Reflectix Insulation) and Velcro strips are placed in strategic positions for easy assembly/disassembly. The insulation box is built large enough to enclose one or two incubator heaters (Lyon). The heaters are turned on approx 1–2 hours prior to imaging to allow a gradual warming of the stage and objectives. The insulation box has holes cut in the front, covered by flaps, to provide viewing through the eyepieces and adjusting the focus or position of the embryo explant.

5. With an inverted microscope, the light path to the embryo passes first through the glass coverslip, then through the tissue culture medium and membrane, and on to the embryo (**Fig. 2B**). Thus, the working distance between the embryo explant and an objective is slightly more than 1 mm (*see* **Note 7**). This means that we are limited to using low-magnification objectives (less than or equal to 10X) or long working distance high magnification objectives. Because we are interested in observing long duration events and preserving the morphology of the embryo, we sacrifice some working distance in exchange for growing the tissue on the membrane of a culture insert (*see* **Note 8**).

3.3. Adaptation of Culture Chamber for Upright Confocal Microscopy

To image from above we utilize the same closed chamber design but raise the culture insert so that the embryo is close to the roof of the well plate, minimizing the working distance. We describe the modifications to the basic chamber design below.

1. Complete **step 1** of **Subheading 3.2** in the same manner. The first change to the culture chamber design is to melt a hole in the top cover over one of the wells of the six-well plate and attach a glass coverslip over the hole on the inside. This will increase the image resolution when using the upright microscope.
2. Within this well, fit a plastic Petri dish, with a diameter slightly less than the inner diameter of the well, snuggly into the well. Make sure that the rim of the Petri dish is flush with the top of the well.
3. Shave off the top two thirds of the culture insert, leaving only the membrane legs and about 2 mm of plastic wall above the membrane. Use a fine sandpaper to smooth off the top of the wall and gently blow off any debris with canned air. Fill the Petri dish with 1 mL of culture medium and the other wells with sterile water. We use only 1 mL of culture medium as the walls of the modified culture insert are only 2 mm high.
4. The membrane can now be prepared and the embryo positioned on the membrane following **Subheading 3.1., steps 1–5**. The culture insert can then be placed into the well plate.
5. One of the basic problems with using an upright microscope, when viewing a live specimen requiring a warmed environment, is the formation of condensation on a surface over the embryo in the imaging pathway. In our design, condensation is likely to form on the glass coverslip attached to the cover of the six-well plate. To minimize condensation on the glass coverslip, we use a glass coverslip sandwich with a layer of heated oil in between. Secure the corners of a square 24 mm coverslip on top of the culture insert with silicone grease. Place a drop of warmed microscope immersion oil on top of this coverslip and then place the lid of the well plate gently on top. This should spread the drop of oil on the glass coverslip but not over the sides of the culture insert. This keeps the glass warm and prevents condensation from forming on the surface.
6. The working distance of approximately 2 mm is nearly the same for the inverted microscope chamber; however, the advantage is that in imaging from above, the

imaging pathway is only through glass and a culture membrane (**Fig. 2C**).

We have used the techniques described in **Subheadings 3.1–3.3** to image cell and tissue dynamics in time-lapse confocal microscopy during early chick nervous system development. We describe some of our imaging results (*see* **Notes 9–11**) and then present a summary, including some of the lessons we have learned (*see* **Note 12**).

4. Notes

1. *Tissue culture and chamber design considerations.* The more important aspects of designing a culture chamber are to maintain the health and integrity of an embryo or tissue, while allowing the best possible resolution from the confocal microscope. Details such as providing a surface for holding an embryo in place while supplying oxygen, water, and tissue culture medium in a temperature-controlled environment can be particularly challenging to incorporate simultaneously. Different sizes and shapes of embryos and the requirements of maintaining living cells within the framework of the confocal microscope have led to the design of many different culture techniques for live imaging. Chambers ranging from simple, sealed Petri dishes to sophisticated, manufactured setups that allow the simultaneous measurement of physiological parameters have been constructed (for a summary of different chamber designs, *see* **ref. *2***). Often a chamber may have to sustain tissue over long periods of time to capture a complete biological event. Thus, it is worth the time it takes to design a chamber that is flexible and simple enough to set up and use to perform many experiments, without extensive labor or cost. We describe some aspects of culture chamber design below.
2. *Surface for growing embryo or tissue.* There are three important criteria for a culture chamber. First, it must provide mechanical support of the embryo or tissue so that it can grow and remain in an orientation for imaging. Second, it must permit a supply of nutrients to the tissue. Third, the chamber must offer a clear light path to the specimen. A culture membrane, a collagen gel, or an agarose bed (supporting the embryo within a well or groove cut into the agarose) meet all three criteria. Make sure the culture membrane has pores large enough to allow nutrients to pass and is transparent when moistened and thin enough to image through. Agarose can be mixed up with a nutrient medium.
3. *Holding the embryo or tissue in place.* Tissue should be kept from drifting in the X–Y-plane or moving out of focus in the Z-direction, yet permitted the freedom to undergo the natural movements and experience the forces to which it might normally be subjected. For example, an embryo or tissue may drift due to thermal expansions, become unsettled by the flow of tissue culture media, or move because of natural growth movements. To minimize drift, a membrane surface or agarose bed may be precoated with a substance to which cells can attach. This coating may be a fibronectin solution or a plasma clot, or as simple as double-stick tape on a glass surface. An embryo or tissue can also be held in place with extra materials, such as filter paper strips or a blanket of overlying tissue.

Embedding tissue in agarose can hold the tissue in place, and may be used if the goal is to inhibit cell movement or tissue interaction.

4. *Keep a clean light path to the embryo.* The light path between the embryo and the objective lens should be as optically friendly as possible, with minimal light path scattering or distortion. Any medium in which you embed an embryo or use for nutrient supply should be optically clear. Indicator dyes can increase background fluorescence. Bubbles, yolk platelets, or any unnatural debris near the embryo should be removed. It is best if any plastic in the optical path is replaced with glass.
5. *Keep the tissue oxygenated and hydrated.* A reservoir of water and nutrients can be provided for in a closed chamber or can be perfused through the chamber during the imaging session. Choices concerning recycling or perfusing might be based on aspects such as the length of time necessary to maintain the tissue and whether the tissue requires a steady, new supply of nutrients for viability. These needs must be integrated with maintaining the position of the embryo and avoiding excessive condensation on imaging surfaces, which can be a major problem when the chamber is warmed.
6. *Temperature control.* Many embryos need to be maintained at a constant temperature to develop normally. Designs vary from a custom built, thermally insulated box surrounding both the microscope and a stand-alone heater to commercially available stage heaters with microsensors hooked up to an automated temperature control system. It is important that the design minimize thermal fluctuations and vibrations that could cause drift or decrease image resolution, especially at higher magnification.
7. *Objective lens magnification and working distance.* It is important to build the chamber so that it can accommodate a variety of microscope objectives. The maximum distance between the objective front surface and the specimen is called the working distance. A chamber design should try to maximize tissue viability and minimize working distance. Many open chamber designs allow high-magnification water objective lenses to be lowered into the culture medium directly above the specimen. Closed chambers, however, usually have a restriction such as a plastic lid that can limit working distance. If visualizing small details of cell behavior is desired, it is important to consider that high magnification objectives have much smaller working distances. A 10X (NA 0.25–0.30) objective lens can have a working distance of 2–3 mm, whereas a 40X Neofluar NA 0.75 can have a working distance of less than 1 mm. Long working distance (LWD) objective lenses can be used in sacrifice of image quality. The Nikon 40X LWD objective has a working distance of about 1 cm, allowing more flexibility with the chamber design, but the 0.5 NA reduces image quality. Zooming in with a low-power objective will magnify detail, and offers a different compromise, but there is a limit to the image quality.
8. *Other types of culture chambers.* In the case of aquatic embryos, such as sea urchin or zebrafish, methods for holding the embryo in place underwater have been developed. Sea urchin eggs have been attached to a glass coverslip by coating the coverslip with a thin layer of protamine sulfate, which rests above a hole cut in a plastic Petri dish ***(4)***. Zebrafish embryos have been placed in a triangular

well cut into an agarose bed, with a thin agarose blanket gently draped over the egg to hold it in place ***(5)***.

9. *Confocal imaging of chick embryo explants.* In the chick, there are two distinct segmentation processes which pattern the embryo during days 2–3 of development. In the rostral part of the embryo, the neural tube is shaped into seven repeated segments, called rhombomeres. The rhombomeres are thought to provide a spatial groundplan by which cells sense their position and differentiate into specific neurons. The rhombomeres are also the location for the emergence of a sub-population of migratory cells, known as the cranial neural crest cells, which emigrate from the neural tube to pattern the peripheral nervous system. It is largely unknown what cellular mechanisms shape the rhombomeres and how neural crest cells "know" when to leave the neural tube and where to go. Although molecular gene expression data are rapidly becoming known, there is a tremendous need to provide a spatial and temporal reference frame that could link the molecular and cellular data. We describe below examples from typical confocal imaging sessions that use the whole embryo chick explant technique and the culture chambers we described in **Subheadings 3.2** and **3.3** for both inverted and upright confocal microscopes.
10. *Inverted microscope.* Using the culture chamber design described in **Subheading 3.2**, we have imaged the shaping of the neural tube into the rhombomere segments. The inverted confocal microscope is outfitted with a thermal insulation box which surrounds an incubator heater and microscope stage area (**Fig. 2D**). By shining a light through the embryo, which is oriented dorsal-side down on the membrane, the transmitted light image reveals the outline of the neural tube walls. **Figure 3** shows the neural tube of a typical embryo explant before and after 10 h of time-lapse imaging. Notice how the neural tube walls have appeared to expand and constrict in places to shape the tube into segments.

    The shaping of the rhombomere segments takes just over 15 h, after which the neural tube begins to return to a less obviously segmented structure. Bright field images start to become hazy after 10–15 h in culture for reasons we do not yet understand. With this time constraint, we can watch the entire rhombomere segmentation in two separate time-lapse sequences. We typically record digital images every 5 min to optical disk (Pinnacle); NIH Image 1.60 ***(6)*** is used to play back the images in movie form. With a 10X Neofluar NA 0.30 and the aperture fully open, the Z-resolution is approx 30–40 µm.

    We have also imaged the movement of dye-labeled neural crest cells, using fluorescence confocal microscopy. An example of neural crest cell migration in a typical chick embryo explant is shown in **Fig. 4**. By injecting a fluorescent dye into the lumen of the neural tube, premigratory neural crest cells are labeled. Notice in **Fig. 4** that we can follow the trajectory of individual neural crest cells as they emerge from the neural tube and migrate into surrounding unlabeled tissue. We use a 10X Neofluar NA 0.30 objective lens and zoom in by a factor between 1 and 2 to observe individual or small groups of cells. The haziness we encountered after 10–15 h of time-lapsing does not seem to affect the confocal

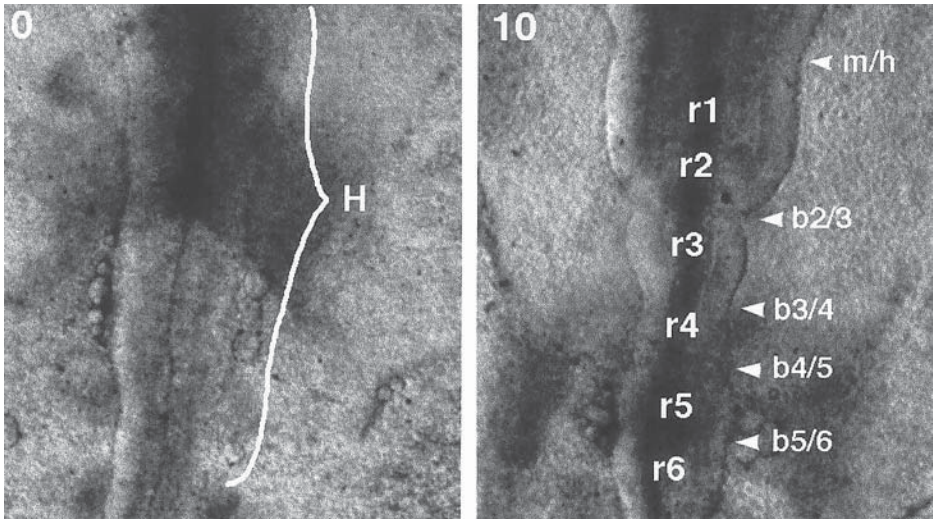

Fig. 3. Bright field images of hindbrain segmentation: (0 hrs) the hindbrain (H) of the neural tube appears as fairly unsegmented; (10 h) the neural tube shape changes give rise to the segmented structure of the hindbrain. The rhombomeres, r1–r6, are labeled as well as the boundary regions between them. For example, the boundary between r2 and r3 is labeled as b2/3, and the boundary between the midbrain and hindbrain is m/h. Figures 3 through 5 are examples from time-lapse confocal imaging sessions of typical whole embryo chick explants. Each image represents one confocal section taken with an inverted microscope.

fluorescence imaging, where time-lapse imaging sessions have lasted up to 18 h. The second segmentation process in the early developing chick embryo produces repeated clumps of cells or somites that form in pairs adjacent to the neural tube. The somite pattern is laid down in a regular sequence moving caudally down the anteroposterior axis of the embryo. Somites give rise to muscle, vertebrae, and skin. With the same imaging methods as described previously, we are able to watch the outline of a somite take shape from the onset of its budding into an individual clump and formation of the characteristic circular cross-sectional shape. **Figure 5** shows an example of somite formation in a typical embryo explant. Notice in the figure that three new somite pairs form in 4.5 h, which is the same rate as an ovo. In a 10-h time-lapse sequence we can observe approximately seven new somites and record digital images every 5 min to optical disk. Using a 10X Neofluar NA 0.30 provides a field of view large enough to capture the most newly formed somites and a portion of the unsegmented region.

11. *Upright microscope.* We have found that culturing a chick embryo dorsal side up on the membrane allows the embryo to better mimic its normal rotation. In the cranial region, this rotation turns the embryo so that the dorsal neural tube lays

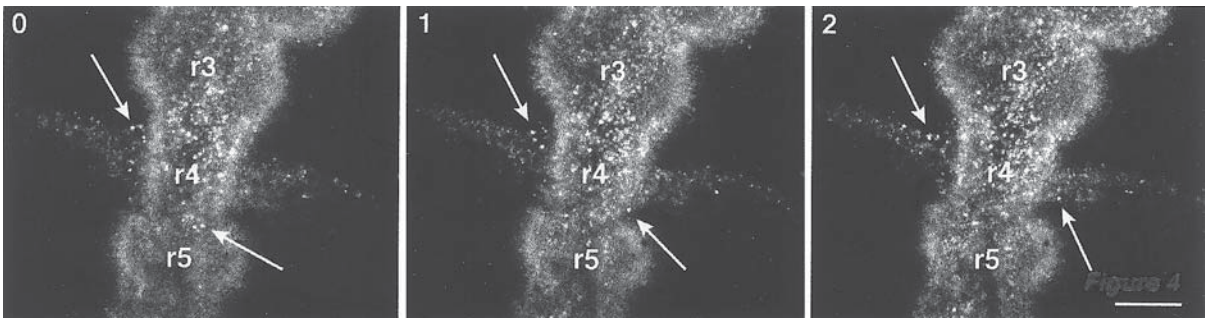

Fig. 4. Fluoresecent DiI injected into the neural tube labels cells within the neural tube and neural crest cells which are shown emigrating into the surrounding unlabeled tissue. The arrows follow individual neural crest cells leaving the neural tube from the region of rhombomeres r3, r4, and r5: (0 h) the **top arrow** identifies a neural crest cell after it has left the neural tube to join the population adjacent to r4, while the **bottom arrow** identifies a cell near the boundary between r4 and r5; (1 h) the **bottom arrow** shows that the neural crest cell has moved into r4, while the **top arrow** shows that the other neural crest cell has moved further away from the neural tube; (2 h) the **bottom arrow** shows that the neural crest cell is emigrating away from the neural tube having joined the stream exiting adjacent to r4. Scale bar = 100 μm.

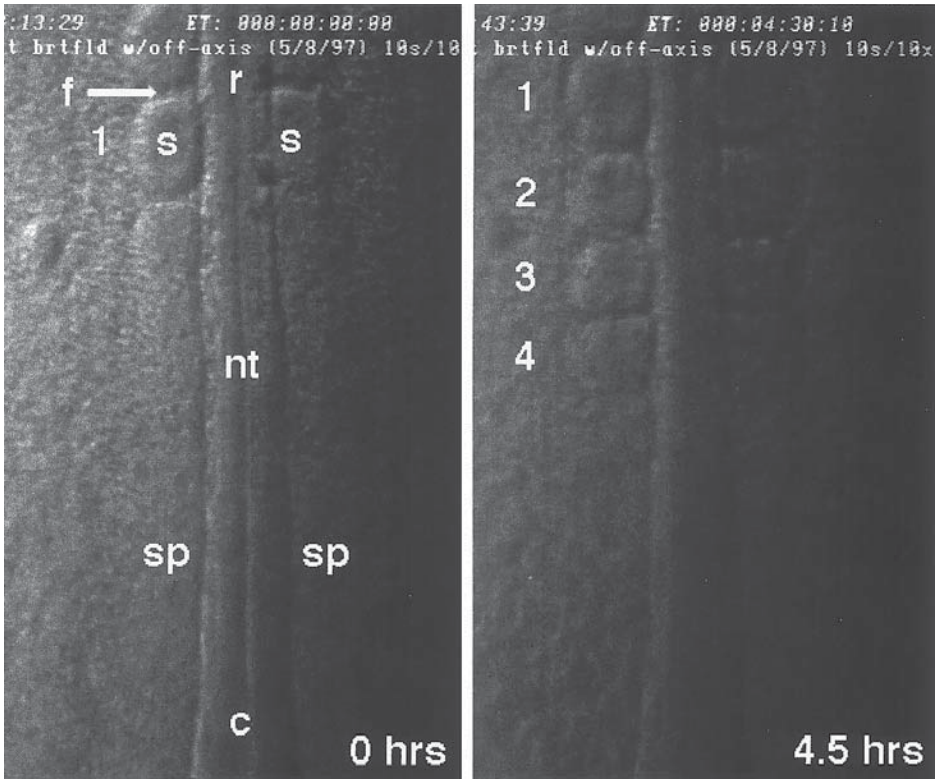

Fig. 5. Bright field images of somite formation: (0 h) the most newly formed somite pair is labeled by 1; (4.5 h) the fourth somite pair has formed. The rostral (r) portion of the embyro is at the top of the image, showing the intersomitic furrow (f), the unsegmented region called the segmental plate (sp). The bottom of the image is the caudal (c) portion of the embryo explant.

on its side. Although the neural crest cells move fairly planar as they exit the dorsal neural tube, their full migration pathway is from the dorsal side of the embryo all the way around to the ventral side. The rotation naturally exposes one side of the embryo, which allows us to follow more of the migration pathway of cranial neural crest cells as they reach the branchial arches. By using the optical sectioning capability of the confocal microscope, we have taken as many as 10 Z-sections of 10 μm each at each time point to capture a majority of neural crest cells and time-lapse sessions have lasted as long as 24 h. In **Fig. 6**, an example of a typical embryo explant shows the patterning of the cranial neural crest cells as the embryo explant begins rotating to the left. By 4 h into the time-lapse, a large number of neural crest cells have left the neural tube from the midbrain and rhombomere 1, r1, region. At 8 h, the natural rostral progression of the growing

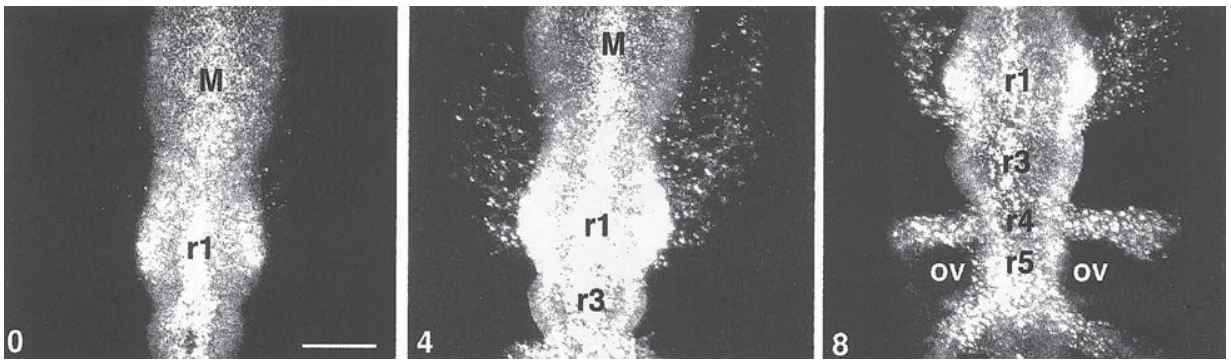

Fig. 6. DiI-labeled neural crest cells and cells within the neural tube of a typical embryo explant dorsal side up on a culture membrane. Each image represents 10 images, at 10 μm Z-increments projected onto one image, taken from a time-lapse confocal imaging session on an upright microscope: (0 h) the fluorescently labeled neural tube in the hindbrain region before many of the neural crest cells have left the neural tube; (4 h) neural crest cells are migrating into the surrounding unlabeled tissue from the midbrain and rhombomere 1 regions; (8 h) neural crest cell migration continues from the midbrain and rhombomere 1 regions. The natural growth of the embryo explant exposes the caudal rhombomeres and the other migrating neural crest streams adjacent to r4 and from r5 and r6. The midbrain (M), rhombomeres r1–r6, and the otic vesicle (ov) are labeled. Scale bar = 100 μm.

embryo explant exposes the pattern of neural crest cells adjacent to r4 and caudal to the otic vesicle.

We recorded fluorescence images to an optical magnetic disk recorder (OMDR; Panasonic) at 2-min intervals for time-lapse sessions that lasted as long as 24 h. Using an OMDR allows the rapid playback of frames and has the advantage of determining whether any adjustments to either the microscope or specimen need to be made during the time-lapse. We also recorded digital images every 2 min to optical disk (Pinnacle); NIH Image 1.60 ***(6)*** is used to play back the images in movie form.

12. *Summary.* We have outlined the important considerations for imaging living cells in intact embryos. This included addressing aspects of how to maintain the health and morphology of the tissue in combination with confocal microscopy.

As an example of the coordination between live embryos and imaging we discussed several examples from our work on imaging cell and tissue dynamics in the developing chick nervous system. Specifically, we discussed how chick embryos growing on a culture membrane maintain normal development until the period of natural rotation of the embryo, lasting from 1 to 2 days. Precoating the culture membrane with a fibronectin solution aids cell and tissue attachment to the surface and the pores of the membrane. Glass coverslips were used in place of plastic wherever appropriate and the culture membrane was transparent when moistened so that the imaging pathway was as clear as possible. The additional wells of the culture chamber held excess sterile water while tissue culture media was placed underneath the culture membrane to provide a moist and nutrient-rich environment. Although the closed culture chamber design limited our choices of objective lenses to low magnification or high magnification with long working distances, we were able to visualize the global and local cell movements we were interested in. In addition, the ease and simplicity of the culture chamber preparation decreased our experiment time. The six wells of the plate often provided a spare well to culture an additional embryo explant, which allowed us to choose between two embryo explants to image, once we visualized the explants on the microscope. Data from the nonimaged embryo explant would then be used for growth comparison to intact embryos, allowing two experiments to be performed during one time-lapse imaging session.

We utilized off-axis illumination to view the chick neural tube and somites in brightfield. This significantly improved the quality of an image by providing a 3D aspect to the outline of the tissue. For fluorescence imaging, we tried as often as possible to use the low-power setting on the laser which can help to prevent photobleaching of the specimen and prolong imaging sessions. We typically started each confocal imaging session using the highest neutral density filter and adjusted the gain and contrast so that the image displayed the widest possible spectrum on the 0–255 black–white scale. If the image appeared faint we would increase the amount of laser light to the specimen by decreasing the attenuation of the laser beam. We frequently used a 10X Neofluar NA 0.30 to visualize cell movements and found better resolution by increasing the zoom rather than switching to a 20X

LWD objective. We set the aperture to be fully open; although this translated into less confocal effect, it increased the sensitivity of the microscope and allowed for a maximum optical section thickness so that we could capture a majority of cell trajectories for longer periods of time.

There are several fluorescent labeling techniques ranging from iontophoretic labeling of small numbers of cells to bulk soaking of tissue, as well as different mixtures for effective delivery to various parts of the cell. We fluorescent labeled groups of neural crest cells by pressure injecting a large amount of dye into the neural tube and allowing the dye to be absorbed by cells. We found that buffering our DiI in warm sucrose [10 µL of DiI solution (0.5 mg of DiIC18(3) in 1 mL of 100% ethanol) in 90 µL of 0.3 *M* sucrose] worked best for labeling the neural crest cells and cells in the neural tube. We added a small amount (< 1 mg in 100 µL of dye solution) of Fast Green to the dye solution to make it easier to see the dye we were injecting into the neural tube.

During an imaging session, there are several ways to determine whether the tissue culture and chamber design are working effectively. First, one way to check the health of the embryo is to compare the embryo or cell movements at various spatial and temporal points to intact embryos. We used the formation of somites as a guide to the growth of the embryo explants. From time-lapse imaging data, embryo explants formed one new somite pair every 1.5 h, which is the same as in the intact embryo. In the formation of rhombomeres, we noted that the rhombomere boundaries formed in the same spatial order in the embryo explants as in intact embryos. The second way to check the health of the embryo is to watch for unexpected signs. The sudden appearance of uncharacteristic cell behavior or movements may be due to the onset of cell death, loss of health of the tissue, or tissue drift. Expansion or compression of cells or tissue may be due to thermal fluctuations. Tissue may start to go out of focus or the images become opaque as a result of tissue movement, condensation on a surface in the imaging pathway, or some other factor. We encountered an opacity of the image after approx 15 h during chick embryo explant imaging, which may be due to yolk platelets dissolving into the culture media.

In summary, advanced planning to address the challenges of imaging living tissue can save hours of laborious trial and error. The simpler the system, the easier it may be to troubleshoot. In the end, finding a robust culture chamber and confocal microscope setup leads to the flexibility to do many experiments and allows perturbations to the animal system with the benefit of watching cell and tissue dynamics in a living embryo.

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