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Culture and Manipulation of Embryonic Cells

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Abstract

The direct manipulation of embryonic cells is an important tool for addressing key questions in cell and developmental biology. *C. elegans* is relatively unique among genetic model systems in being amenable to manipulation of embryonic cells. Embryonic cell manipulation has allowed the identification of cell interactions by direct means, and it has been an important technique for dissecting mechanisms by which cell fates are specified, cell divisions are oriented, and morphogenesis is accomplished. Here, we present detailed methods for isolating, manipulating and culturing embryonic cells of *C. elegans*.

I. INTRODUCTION: USES AND LIMITATIONS OF THE SYSTEM

The direct manipulation of embryonic cells, long considered a primitive tool of experimental embryology, has re-emerged as an important tool that can be used together with modern methods in model organisms to address long-standing questions in cell and developmental biology (Fraser and Harland, 2000; Weaver and Hogan, 2001). In studying embryos of many species, methods of fragmenting and culturing embryonic tissues or cells have long been useful for addressing questions of blastomere autonomy in early and later embryogenesis, for exposure to drugs or other agents that perturb specific processes, and for direct labeling of DNA or RNA. Nematode embryos are surrounded by an outer chitinous eggshell and an inner vitelline envelope (Chitwood and Chitwood, 1974). For C. elegans workers, the small size of the embryo and the impermeability of the eggshell and vitelline envelope has made such experiments difficult. A method of permeabilization and blastomere isolation, a culture system that will support further cellular development and differentiation, and assay methods for assessing the degree of development and its relative normality after experimental manipulation are minimal requirements for a satisfactory C. elegans embryonic culture system. Methods of isolating early blastomeres have included crushing of the eggshell and extrusion (Laufer et al, 1980; Schierenberg, 1987), laser ablation of neighboring blastomeres within an intact eggshell (Sulston et al, 1983; Priess and Thomson, 1987), laser puncturing of the eggshell producing extrusion (Schierenberg, 1987), and digestion of the eggshell followed by shearing or manual stripping of the vitelline envelope (Cowan and McIntosh, 1985; Edgar and McGhee, 1988, respectively). This last method is described in detail below.

Permeabilization of complete embryos can be achieved by removing the eggshell and vitelline envelope. In addition, one-cell embryos within the shell can be permeablized to certain drugs such as cytochalasin D by gentle pressure on an overlying coverslip (Hill and Strome, 1990), although older embryos are resistant. RNAi of a gene that does not affect early development but results in permeable embryos has also been used (Goehring et al, 2011).

Normal development of *C. elegans* embryos follows an invariant cell lineage, with characteristic asymmetric divisions, division times, and cleavage planes (Sulston et al, 1983). The first four cleavages generate a set of founder cells for specific tissues (Figure 1). Cell proliferation continues to about 500 cells (approximately 5 hr post-fertilization at 25°C), followed by a period of rapid morphogenesis and elongation without much further cell division from five to seven hours after fertilization (Figure 2). Hatching occurs at 12 hours.

The media and simple culture chambers described in this chapter will support cell division of intact embryos without a vitelline envelope from the one or two-cell stage to approximately the normal cell number of 550 cells as assayed by nuclear counts, although at a division rate that ranges from normal to about 50% slower than normal (Figure 3, 4H). Even without the enclosure of the vitelline envelope, early blastomeres divide along the characteristic axes, with the normal relative timing of divisions, and with the appropriate asymmetric cleavages for their lineage-specific patterns (Figure 3). The nuclear movements in the early P and EMS blastomeres, in which the P₁ and P₂ nuclei move toward the AB and EMS contact boundaries respectively, also appear normal (not shown).

At the 16 to 28 cell stage, an embryo devitellinized at two cells appears as an elongate "neck" of P₁ derivatives ending in a small ball of 4 C cells, reflecting the longitudinal division axes in the P lineage. The 16 AB descendants, attached at the MS end, form a larger ball as a result of their orthogonal cleavage pattern (See Figure 3E-M). Cell movements are observed at the 28-cell stage when gastrulation would normally occur, bringing the posterior-most cells into contact with MS blastomeres (Lee and Goldstein, 2003). At this stage, the embryo rounds up, in a process similar in appearance to compaction in the mouse blastula. However, further morphogenesis does not occur, probably because the lack of vitelline envelope enclosure precludes the usual cell contacts (Schierenberg and Junkersdorf, 1993). Furthermore, if the removal of the vitelline envelope is performed before 8 to 12 cells, correct specification of early blastomeres can be impaired by loss of specific normal cell contacts (Schnabel, 1991, 1994). Although the cuticle does not form around the embryo, cuticular blebs can occasionally be seen. Twitching is sometimes observed, indicating muscle differentiation. Histochemical or antibody staining and observation of gut granules or GFP markers (Goldstein, 1995a, for example) reveals patches of contiguous cells expressing differentiation markers for gut (Figure 4A,E), hypodermis (Figure 4B,F), or muscle (Figure 4C,D,G), representing the approximate number of cells appropriate for differentiated tissues. By the criteria of increasing antibody staining and cell integrity, the embryos will live about 48 hours before cells begin to lyse.

In culture, variations in embryo viability may be due to the media quality, the physiological state of the mothers, and unavoidable variability in the permeabilization procedure. A fraction of devitellinized embryos inevitably die early due to rough handling. However, in a good preparation over 90% of intact devitellinized embryos will continue division and differentiation, and the yield from a single preparation can be as high as 100–200 embryos. In less optimal preparations, cell division will often stop one to two divisions early, at approximately 300 cells, but differentiation generally still proceeds. Separation of blastomeres or drug treatments can reduce survival. Embryos devitellinized during early cleavages may show aberrant cleavage patterns in experiments in which they are compressed in the permeabilization pipette. Cells manipulated too soon after division -- within 2-3 minutes after cytokinesis -- may rejoin and subsequently undergo a tetrapolar cleavage, which erases P lineage differentiation. In spite of such caveats, this method allows one to produce a large number of permeabilized staged embryos at the dissecting microscope relatively quickly, making it possible to obtain significant numbers of embryos to determine statistical significance in experiments in which occasional variations are inevitable. See Edgar and McGhee (1988), Goldstein (1992, 1993), and Edgar et al (1994) for early experimental uses of embryonic culture to study cell fate specification, and Goldstein (1995b), Herman (2002), Christensen et al. (2002), Nance et al. (2005) and Shaham (2005) for more recent uses in studying additional topics including cell polarization, morphogenesis, neuronal physiology, and in microarray profiling of transcripts in cells, and Zhang et al (2011) for isolation and culture of larval cells.

The protocols presented here are ones that either of us have used successfully. In cases where we have used multiple methods independently to achieve the same aim, each method is presented. In some cases, it might be possible and even trivial to adapt these protocols for new uses, for example by combining parts of different protocols that are presented below. Although some such alterations might seem obvious, we present only those methods that we have already tested.

II. EMBRYO DEVITELLINIZATION AND BLASTOMERE ISOLATION

In the procedure described here, embryos are stripped of the eggshell and vitelline envelopes by treating the eggshell with chitinase and mechanically stripping off the vitelline envelope. Batches of up to about 200 embryos can be prepared at the dissecting microscope, although if staged embryos are selected, the yield becomes lower; e.g. a good preparation yields 20 to 40 2-cell embryos. Blastomeres may be isolated manually following this denuding. The procedure takes from 15 to 40 minutes, depending on the number of worms cut. Some key materials are shown in Figure 5.

A. Materials

Gravid well-fed hermaphrodites—Worms (raised at 16°C) in their second day of laying give the maximum yield of early embryos. Such cultures are best maintained by transferring agar chunks daily from starved L1 plates and retransferring no more than 200 worms to a fresh plate at L3 or L4. Using young adults that have just started laying can increase the proportion of early embryos in the first few cleavage rounds.

Equipment—Dissecting microscope, lightly siliconized or untreated depression slide (2 or 3 well types with a shallow depression are convenient; Fisher 12-565B); mouth pipette apparatus (Sigma A5177) and drawn-out capillaries of proper size (Fig. 5); #15 scalpel and handle or two 22-gauge syringe needles; damp box (clear plastic box with damp paper and supports for slides); pipetters; culture slides or chambers (see next section); eyelash mounted on toothpick or fine pulled glass needle.

Solutions (see Section VII for formulations)—Egg salts and egg salts with tetramisole (Sigma T1512; optional); freshly made 1:9 solution of NaOCl with 4–5% free chlorine (Fisher SS 290-1 or Aldrich 23,930-5) in egg salts; EGM (Edgar's growth medium); optional silicon oil (Sigma M6884, or Dow Corning) or Voltalef 3S oil (uses of oil can be deleted if care is taken to avoid excess evaporation during procedure by use of a damp box between steps); chitinase-chymotrypsin solution.

B. Procedure

- Transfer 20 to 80 gravid worms to a 50–100 µl drop of egg salts in one well of the depression slide. Add 50 µl of egg salts with optional tetramisole (approximately 100 µg/ml), then cut worms roughly in half with the scalpel or two syringe needles used like scissors, working at the dissecting microscope. Many eggs will be released by cutting, and additional eggs can be recovered by breaking uncut uteri with the pipette tip or a glass needle (see Notes 1 and 2 below).
- 2. Collect embryos of desired stages in a mouth pipette with small diameter (~50–100μm; see Note 2) capillary and transfer them to a 100 μl drop of NaOCl solution in the other depression well. Incubate for 2.5–3 minutes. Treating with NaOCl solution for longer than 4 minutes can result in aberrant development.
- 3. Meanwhile, set up a 30 μl drop of EGM, a 30 μl drop of egg salts, and a 15 μl drop of chitinase/chymotrypsin on depression slides; optionally, coat each drop with 3 μl silicon oil. Transfer the embryos quickly through the EGM and egg salt rinses and into the chitinase/chymotrypsin drop. Eggshell digestion will normally take 4 to 8 minutes and is evident when the embryos round up slightly; 3-cell embryos will have a cloverleaf shape (see Note 3 below). Grouping embryos of specific stages can be done during this digestion if staging is important.
- 4. Move embryos gently through a 30 μl rinse drop of EGM and into a 30 μl drop of EGM under silicon oil. Remove the vitelline envelope by drawing individual embryos in and out of a narrow-bore pipette (~25μm, slightly smaller than the embryo diameter) which shears the tough envelope and strips it off (see Note 4 below). The following criteria indicate both a good preparation and a good batch of culture medium: a) embryos show very little lysis, initially or after overnight culture; b) embryos continue to divide on schedule; c) intact embryos after overnight incubation have more than 300 cells, and ideally more than 500; d) gut granules are visible under polarized light in more than 90% of these embryos (if gut granules are missing, this can usually be attributed to an early tetrapolar cleavage as noted above).

5. If isolated blastomeres are needed, separate cells of two-, three-, or four-cell embryos with an extremely fine eyelash or a fine pulled glass needle. Alternatively, cells can be loosened from each other and separated by pulling embryos several times in and out of a glass needle that has a ~30–40μl opening, just wider than the diameter along the short axis of an embryo (see Note 5 below).

6. Transfer to fresh EGM in culture chambers (see Section III).

C. Notes

- 1. Tetramisole (Sigma T1512, a 10 mg/ml stock solution in H₂0 diluted to approximately 100 μg/ml in egg salts) addition is optional; this drug paralyses the worms and makes cutting easier. Cut immediately: if too contracted, the worms won't release many eggs. Use a fresh scalpel blade each day, as they corrode quickly. Excess tetramisole will result in a precipitate forming in the NaOCl solution.
 - For maximum yield, more eggs can be released by treating cut worms for approximately 1 minute with an equal volume of NaOCl solution added directly to the cutting drop. As soon as eggs are released, add the same volume of EGM as NaOCl used to prevent further egg damage. This treatment will kill pronuclear stages and damage one-cell embryos. The 3 minute NaOCl treatment is still necessary before treating with chitinase for a uniform digestion. For eggs earlier than 2-cell, in which the shell is still somewhat permeable, add an equal volume of EGM as soon as the worms are cut. After this, many can survive the 3 minute NaOCl and chitinase treatment, and some will still be at the one-cell stage after chitinase treatment and removal of the vitelline envelope if you work fast.
- 2. Satisfactory transfer pipettes are made from SMI Micropipettor 5–30 µl capillaries (Fisher 21-380-9C) or World Precision Instruments 4-inch capillaries (1B100F-4), by double-pulling over a very small flame. This is done by first heating and pulling gently to make a thin center section, next cooling briefly, and then reheating more gently while keeping the capillary under tension for the final pull. The ideal pull produces a pipette with a distinct shank and a narrow gradually tapering section of about 3/4 to 1 inch. A small gas burner can be made by mounting a large syringe needle in a cork, with a screw clamp on the tubing to adjust gas flow. Alternatively, pipettes of consistent size can be made by pulling on an automated puller. Before use, break the capillary to the desired tip, about 100–150 μm (3 or 4 times egg diameter) by pinching between your thumbnail and fingertip or using a razor blade under a dissecting microscope. A microforge microscope can help in making consistent pipettes, although it is not necessary. Keep the pipette diameter small to minimize liquid transfer. If eggs stick to the inside of the pipette, many can be recovered by flushing with the NaOCl solution or the EGM as you go along. Expect to change pipettes frequently, and have a good supply pulled before a working session.

3. If the chitinase digestion doesn't work within 8 minutes, it will probably not work at all. The commonest problems are the hypochlorite or the physiological state of the worms (first day laying seems to give tougher eggs). Hypochlorite should be an unexpired batch, and it usually becomes poor a month or so before the expiration date. Keep the stock refrigerated in a dark vented bottle, filled to near the top. Mix a 3 ml tube just before starting, and keep it on ice; it will work for about 3 hours and should then be replaced.

After enzyme treatment and especially after permeabilization, the embryos are quite sticky and will clump; this can be minimized by pipetting one or only a few at a time. Small clumps can be broken up by expelling from the pipette with a little force a few times.

4. Permeabilization pipettes are pulled by hand in the same manner as the transfer pipettes, using Kwik-fil injection capillaries (1B100F-4 from World Precision Instruments). The inner thread may help cut the vitelline envelope. The ideal pull gives a long gradual taper in the thin section so that it can be cut to the diameter desired. Pipettes are cut with a fresh scalpel blade on Parafilm under the dissecting scope. An adaptor for the mouth pipette can be made with small-bore Tygon tubing threaded onto a syringe needle attached to a mouth tube. Fill the pipette tip with EGM back to the wider bore and test on the first batch of chitinased embryos; recut the pipette tip if it is too small. The ideal size will vary according to the age of the embryo you are trying to get; the very early stages are more fragile and need a slightly larger bore; embryos larger than eight cells compress with less damage and will often come out of the larger pipettes with the vitelline envelope intact. Such nonpermeabilized embryos will continue to develop to the hatching stage.

Use a syringe on the mouth pipette for filling and cleaning permeabilization pipettes; the actual permeabilization is done by mouth air pressure. Pipettes can sit in air for several hours without drying out, as the bore is so small, but as they dry they will eventually clog. Store pipettes (a good one is worth guarding, and will last several weeks) by flushing the tip several times with distilled water and suspending the pipette tip in a tube of sterile distilled water or 0.1 M HCl, using a tape "flag" on the pipette so that it doesn't sink in completely.

If the pipette is right, it takes only a few minutes to permeabilize a batch of embryos by individually sucking them into the pipette and gently expelling them. They will emerge more or less compressed depending on the inner diameter of the pipette, but round up again within a few minutes. If there is a lot of lysis with permeabilization, either the digestion was incomplete or the pipette bore was too small. As the cell membranes are quite labile for 4 to 5 minutes after cytokinesis begins, embryos devitellinized during and just after cleavage may lyse or blastomeres may fuse, later undergoing an abnormal tetrapolar cleavage. If critical, check during an experiment and eliminate such embryos.

5. A very fine eyelash (a traditional electron microscopy tool) mounted on a toothpick with glue is the most satisfactory tool for rapid manual blastomere

separation; a glass needle also works, but breaks easily. Eyelashes can be cleaned with alcohol and a tissue. Blastomeres will lyse if separated just after a division; 5 to 10 minutes after cleavage is the best time for successful manipulations, unless you need earlier separations. AB/P₁ separations are easiest. 4-cell embryos can also be separated. Alternatively, to get P₂ and EMS blastomeres, P₁ can be separated after the first division and EMS/P₂ after the second. P lineage blastomeres can be recognized by relative size and their linear arrangement. With low yield, MS, E, P₃ and C can be separated from the 4 descendants of an initial isolated P₁ blastomere. Cell identifications made on the basis of cell size can be confirmed by examining for distinct cell cycle periods.

III. CULTURE METHODS

For overnight culture, or observation over several hours, several types of culture chambers have proved useful. The simplest is Teflon-coated immunology slides with multiple wells (Cel-Line HTC Super-cured, available in many configurations from Cel-Line Associates, Newfield, NJ), with 30 to 100 μ l of EGM pipetted onto a well and covered with 3 μ l of silicon oil. Embryos can be observed by dissecting scope or at $10\times$ or $16\times$ on the compound microscope without a coverslip and subsequently recovered or manipulated further. Alternatively, a coverslip can be added to an oil-covered drop of 10 to 20 μ l EGM, so that the drop does not spread beyond the boundaries of the well. However, such embryos are almost impossible to retrieve.

A second way of making a coverslipped chamber is to use a gelatin-subbed slide with spacer strips to raise the coverslip: 1/2" 3M Drafting Transfer Tape, available from art supply stores, makes a good spacer. Embryos are pipetted into 5 to $10~\mu l$ EGM on the subbed slide and covered with 3 μl silicon oil. The coverslip is applied gently, then pressure-sealed to the tape before the oil reaches the tape. The open ends are sealed with oil or thin plastic wrap. Again, it's hard to retrieve these embryos. This mount works well at 40X but is too thick for 100X.

A third way of making a coverslipped chamber is to begin by scraping the four corners of an untreated coverslip on a hand-softened ball of ordinary children's plasticine clay, to create clay feet that will support the coverslip. About $10-15\mu l$ of EGM is put in the center of the coverslip on the same side as the clay feet, and cells or embryos are added to the center of the EGM drop. A slide is then placed on the coverslip and pushed down until the preparation is the desired thickness. Thickness can be controlled carefully if the volume of fluid needed to fill the space under the coverslip for a desired thickness is calculated, and then the slide is pushed down until the fluid fills the space under the coverslip, if care is taken to not alter the volume significantly by adding too much medium when transferring embryos and evaporation is prevented (for example, a $20\times20\text{mm}$ coverslip with $12\mu l$ of EGM would be precisely $30\mu m$ high after flattening until the fluid filled the space under the coverslip, since $20\text{mm}\times20\text{mm}\times0.030\text{mm}=12\text{mm}^3$ or $12\mu l$, as $1\mu l=1\text{mm}^3$). These coverslips can be sealed but kept gas-permeable for microscopy by painting liquid vaseline petroleum jelly (kept at 65°C) across each of the four sides of the coverslip. Such thin preparations are suitable for a 100x objective on an upright or inverted microscope. On an upright microscope with a short

working distance objective lens such as a typical 100x lens, if the cells move as you focus, this means the objective is pushing on the coverslip, and the most likely cause is that your preparation was not made not sufficiently thin.

For viewing at 100X using an inverted microscope, where gravity positions the cells against the coverslip, another satisfactory culture chamber consists of an aluminum or plastic slide $25\text{mm} \times 75\text{m} \times 5\text{mm}$, drilled with a 16mm diameter central hole. This hole is covered on the bottom with a 20 mm square coverslip (acid-washed and baked) sealed with vacuum grease. $30 \, \mu \text{l}$ of EGM is pipetted on, embryos are added, and the slide is quickly sealed to prevent evaporation, using a $16 \, \text{mm}^2$ coverslip with several $2 \, \mu \text{l}$ drops of silicon oil pipetted around it to seal. If slides are moved carefully, embryos or fragments will stay fairly well put and can be identified by their position, although they should be well separated initially.

All unsealed preparations should be kept in a damp chamber when not being observed. Damp paper towels in a plastic box, with risers such as plastic pipettes to keep the slides from getting wet, work well. Incubate at 16^C rather than 20°C for optimum viability.

IV. DRUG TREATMENTS

After removal of the vitelline envelope, embryos can be exposed easily to various drugs by adding the drug to the medium. Cytochalasin D, aphidicolin, nocodazole, α -amanitin, and blockers of nucleic acid or protein synthesis have been used in various protocols (Edgar and McGhee 1988; Edgar et al 1994, Edgar, unpublished results). Dosage should be titrated by the physiological effects in the medium used. Particularly in the case of α -amanitin, high doses are needed to produce saturation effects in a rich medium, since much of the drug seems to be absorbed to media components. When removing embryos from drug exposure, pipette them through several rinses.

Embryos can also be permeabilized to drugs without chitinase treatment and removal of the vitelline envelope. For this method, treat embryos for 1 minute in 1:9 NaOCl solution on the depression slide. At 1 minute add an equal volume of EGM to block the hypochlorite. Using a gelatin-subbed slide with two 3M tape spacer strips, put the embryos into a 3 to 5 ul drop of EGM between the tape strips, add a coverslip and seal it to the tape by pressure with a dissecting needle. Press down gently on the coverslip with the needle while adding 30 µl EGM at the top edge. This should stick the embryos to the slide. To permeabilize the embryos, press harder on the coverslip until the embryos flatten slightly and rebound when released, repeating 2 or 3 times. Nonpermeabilized embryos will develop to hatching; permeabilized embryos usually fail to hatch. Permeabilization can be monitored by adding 0.0002% Nile Blue A (make stock in 10% EtOH) to the medium; this vital dye is taken up only by permeabilized embryos and collects in the gut cells. Once the embryos are fixed to the slide and permeabilized, fresh medium with or without drugs can be flushed through the open ends of the chamber by adding liquid at one side and pulling through with a tissue at the other. When changing the media, use several 30 µl flushes. Fixation and staining can be done in the same manner.

One advantage to this preparation is the embryos remain stuck (with luck) to the gelatin, so that individual embryos can be followed through a procedure. The caveats are that permeabilization may be uneven from embryo to embryo; remnants of the shell and vitelline envelope may prevent rapid and equal access of the drug to all cells; and in some embryos the vitelline envelope appears to reseal, as occasionally a hatching L1 with a blue gut is observed if Nile blue is present.

V. RADIOACTIVE LABELING

Both UMP and UTP labelled with ³H or ³²P are readily taken up by embryos after removal of the vitelline envelope, and can be visualized by autoradiography (Edgar et al 1994); one supposition as to how the triphosphate gets in is that cell membranes may be slightly leaky.

Radioactive labelling requires extreme care, since working with small numbers of embryos must be done under the dissecting scope and it is virtually impossible to avoid all mouth pipetting. Likewise, wearing gloves makes the manipulation very difficult and probably increases the chance of contamination. It can be done most safely using the Cel-Line slides and moving embryos in small batches from drop to drop. A plastic shield should be fitted around the dissecting microscope for labelling with ³²P. Appropriate safety precautions should be taken. The mouth pipette should be fitted with a cotton plug or a very long tube, and care should be taken never to overfill the glass tip.

VI. FIXATION, CYTOCHEMISTRY AND ELECTRON MICROSCOPY

A. Fixation methods

The method of choice for fixation of devitellinized embryos is paraformaldehyde with glutaraldehyde. This method gives the best morphology for further Nomarski observations, and allows one to keep track of a small number of the embryos. If ethanol fixation is required, as for certain antibodies, it should follow a brief (1 minute) paraformaldehyde fixation to attach the embryos to a slide. Embryos can be slide-mounted either at the time of fixation, or, as is often desirable after culture in which individual embryos have been followed, fixed in place in the culture chamber.

1. Fixation in place—Very gently pipette 1 to 2 volumes of fix solution (See Section VII; 3% paraformaldehyde is preferable with this method because of dilution) around the edges of the EGM drop containing the embryos, so as not to disturb them. Alternatively, pipette embryos into 50 µl drops of 2.25% fix on a Teflon slide, separating them as desired for identification. Covering with oil is not necessary for the short fixation. Fix at room temperature or on an aluminum block on ice for 3 to 5 minutes. For antibodies, room temperature for 5 minutes generally works well. After fixation, carefully remove approximately half the liquid and replace with phosphate buffer; do this at least 3 times. This should be done with the mouth pipette if you don't want to shift the embryos; if all embryos in a drop are equivalent, you can use a Pipetman. Keep in a damp box until further processing. A damp box can be made by putting a wet paper towel in the bottom of a plastic box, putting a plastic support (for example, the insert from a tip box) in to hold the slide

above the wet paper towel, and putting the cover on the plastic box, so that humidity from the wet paper towel can fill the box.

2. Fixation with attachment to a slide—These preparations are easier to handle than loose embryos, although sometimes antibody staining is more successful if the embryos are not flattened.

On a gelatin-subbed slide, identify the area to be used by scribing a circle on the back with a diamond-point pencil, and pipette 3 to 5 μ l fix solution (2.25% paraformaldehyde) on the subbed side. Pipette the embryos into the fix, let them settle, remove liquid if the volume is large, then cover the drop with a small cover slip about 5 mm² (cut with a diamond point from a larger coverslip.) As the liquid spreads to the point where the embryos flatten slightly, press lightly on the coverslip with a dissecting needle if the embryos are not contacting the slide (sometimes crystals or lumps need to be flattened). If there is too much liquid, pull off some with a tissue; if too little, work fast! Quickly add 30 μ l additional fix to float up the coverslip. Fix for five minutes at room temperature, (fixation time can be modified for specific methods). After floating the coverslip, it can be pressed down again lightly with a needle to stick floating embryos to the slide, to transfer those stuck to the coverslip back to the gelatin, or to flatten for procedures such as nuclear counts. After fixing, transfer the slide to a Coplin jar containing phosphate buffer or PBS (depending on the procedure), making sure the coverslip slides off on immersion. Slides can be collected and held at this point, as long as a day or two in the refrigerator.

- **3. Ethanol or methanol fixation**—Use the procedure above, and after attaching the embryos to the slide with a small coverslip and pressure, fix the slide in a Coplin jar of ethanol or methanol at 4°C for 10 minutes. Further treat by air-drying or by rehydration through an alcohol-buffer series: 2 minutes each 95%, 90%, 75%, 50%, 30%, PBS or phosphate buffer.
- **4. Fixation and then attachment to a slide**—Polylysine can be used to stick embryos to a slide, but embryos will stick to a polylysine coated slide poorly until after fixative is washed out. This method uses depression slides with agarose-coated wells so that embryos do not have a chance to stick to a glass slide until after the fixative is well washed out. The conditions presented here are optimal for extracting cytosolic molecules and preserving cytoskeletal structure.

Depression slides for fixatives are agarose-coated by pipetting hot 0.8% agarose in CSB buffer (10mM MES pH6.1, 125mM Kcl, 3mM MgCl, 2mM EGTA, 10% sucrose) into each well briefly and letting the excess drip out while the agarose is still fluid. Depression slides for washes are prepared similarly with 0.8% agarose in PBS. These coated slides should be prepared fresh and kept in a damp box to prevent dessication of the agarose surface. Embryos should be moved to 4% paraformaldehydeand 0.1 mg/ml L-alpha-lyso-phosphatidyl choline (L-alpha-lysolecithin) in CSB buffer for 2 minutes to fix and permeabilize. Transfer the embryos to another agarose coated well with 0.2% gluteraldehyde in CSB for 10 minutes. Wash out the gluteraldehyde with 4 changes of PBS in the wells coated with agarose prepared in PBS. Embryos are then moved to a drop

of PBS on a polylysine-coated slide, to which they should stick. Polylysine coated slides are prepared by placing 1–2 μ l of 0.1% (w/v) poly-L-lysine in dH20 on a slide, putting a coverslip on top, and then heating this at at least 100°C until the polylysine solution dries. The coverslip (which will not stick tightly to the slide when the solution has dried) is removed then. Polylysine coated slides can be kept at room temperature for weeks until needed. Multitest slides with 15 flat wells each surrounded by a hydrophobic coating (MP Biomedicals 6041505) can be coated and used.

B. Immunostaining

Devitellinized and cultured embryos that are healthy, or even drug-treated, can be successfully stained for many markers of differentiated tissue types using fairly standard methods. The methods below were originally adapted from Susan Strome (Strome and Wood 1983). Various dilutions of primary and secondary antibodies, detergents, blocking agents, incubation times and incubation temperatures should be tested to optimize staining with the particular antibodies being used.

Embryos on standard glass slides are treated in a standard manner by wiping around the previously marked specimen area and incubating in 30–50 μ l drops in a damp box; washes can be done in Coplin jars. Embryos in drops can be handled by removing and adding liquid or by transferring into fresh drops using the mouth pipette.

- 1. In a Coplin jar, permeabilize fixed embryos to antibodies by treating 5 minutes in 0.5% Triton X-100 in PBS.
- 2. Rinse in PBS.
- **3.** On the slide, block with normal goat serum (or serum corresponding to the secondary antibody) diluted with an equal volume of 0.1% Triton-PBS (TPBS) for 1 hour at room temperature or 30 min at 37°C.
- **4.** Replace with primary antibody diluted appropriately in TPBS and incubate as appropriate for the particular primary antibody.
- **5.** Wash 3 times 15 minutes in PBS or TPBS in a Coplin jar at room temperature.
- **6.** Repeat blocking step if background has been high.
- 7. Incubate on the slide with secondary antibody diluted in TPBS, normally 45 minutes to 2 hours at room temperature. Secondary antibodies from Jackson Laboratories work well.
- **8.** Wash at least 3 times 10 minutes in PBS.
- **9.** Proceed with development of secondary antibody if using peroxidase or phosphatase systems.
- 10. Stain for DNA with 0.5 to 1 μ g/ml 4',3-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 5 minutes, if desired, and then wash in PBS again.

11. At this point, loose embryos can be observed under an inverted microscope or coverslipped, or mounted to slides with a 1 minute paraformaldehyde fix as described above. If refixed, rinse in PBS before sealing.

12. Mount in Gelutol or other glycerol mounting media.

C. Cytochemical staining

- **1.** β -galactosidase—Embryos are handled on slide mounts or in drops, exactly as for antibody staining, with paraformaldehyde fixation giving the best morphology. Following fixation and a rinse in PBS of 5 minutes or longer, incubate with β -galactosidase stain solution (see Section VII) at room temperature in a damp box. The time of staining will vary with different markers. Follow with DAPI staining as above, and mount.
- **2. Gut esterase**—Fix only 3 minutes, on ice; wash 5 min in 125 mM phosphate buffer (4 °C), and stain in a drop of pararosaniline stain solution (see Section VII) on ice for no more than 2 hours. Rinse, DAPI stain 5 minutes, and mount.
- **3. Combinations**—Most of these staining methods can be combined successfully, although their order is important. Gut esterase is very labile, and if stained for, this should be the first procedure. If esterase staining is used in combination with X-gal blue staining (the American flag effect) the esterase staining should be stopped by a PBS rinse before it gets very red, so as not to obscure the β -galactosidase precipitate. β -galactosidase stain is then added and incubated for the appropriate length of time. Unfortunately, esterase and antibody staining cannot be combined, as the esterase staining solution seems to destroy antigenicity. Antibody staining should follow the β -galactosidase staining if a visible-light system is used. A peroxidase-conjugated secondary antibody works well. Alternatively, if a polyclonal primary antibody is used as a marker, β -galactasidase can be stained using a mouse monoclonal anti- β -galactosidase (Promega Z3781), diluted 1:300 and incubated 3–4 hours at room temperature, 37°C for 1 hr, or 4 °C overnight, followed by a fluorescent secondary antibody. Two primary antibodies can be incubated together if conditions are the same; otherwise do the longer incubation first. The two secondaries can be incubated together.

VII. SOLUTIONS AND CULTURE MEDIA

The following formulations have proved satisfactory with the procedures detailed in preceding sections. EGM (Edgar's growth medium) has been developed and modified over several years: a simplified version suitable for short-term culture is included as well as the current optimal version.

Egg Salts

118 mM NaCl; 48 mM KCl; glass distilled $\rm H_2O$. Autoclave. This salt mixture is preferable to M9 for cutting worms or for NaOCl dilutions, as it is more physiologically isotonic than M9 for embryos.

Egg Salts with tetramisole

100 µg/ml tetramisole, from 5 mg/ml stock solution.

Egg Buffer

118 mM NaCl; 48 mM KCl; 2 mM CaCl₂; 2 mM MgCl₂; 0.025 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.4); glass distilled H₂O. Filter-sterilize or autoclave before adding sterile Hepes. Permeabilized embryos will be relatively happy for an hour in this buffer.

Chitinase-chymotrypsin Solution

Chitinase, 10 units (the best currently available is Streptomyces griseus chitinase, Sigma C6137); α -chymotrypsin (Sigma Type II, C4129) 10 mg; egg buffer 1 ml; penicillinstreptomycin (Sigma P3539) 10 μ l. Mix on ice, let sit several hours or overnight, centrifuge if cloudy, and filter-sterilize. Store refrigerated. This works better after a day or so, and can be frozen in 100 μ l aliquots for convenience. Avoid repeated freeze-thaw cycles.

Edgar's Growth Medium (EGM), 10 ml

In the tradition of naming media used for embryology experiments after the scientists who have developed the media, one of us (B.G.) re-named the Embryonic Growth Medium developed by Lois Edgar (Edgar, 1995) as "Edgar's Growth Medium". A medium derived from EGM by Chris Shelton, Shelton's Growth Medium (Shelton and Bowerman, 1996) is discussed below.

PVP (polyvinylpyrrolidine)	50 mg
L-tyrosine	0.5 mg
Stock salts	840 μ1
.25M Hepes pH 7.4	1000 µl
Inulin (5 mg/ml in H ₂ O)	1000 µl
Ammo acid stock	1250 µl
Tissue-culture grade H ₂ O	1320 µl
Fetal bovine serum (FBS)	4000 µl
LPSR-1 (Sigma L9263)	1000 µl
(optional: reduce FBS to 3 ml if LPSR-1 is used)	
Penicillin-streptomycin (Sigma P3539)	$100~\mu l$
Galactose stock	$100~\mu l$
Base mix	$100~\mu l$
L-glutamine (14 mg/ml in H ₂ O; mix fresh)	$100~\mu l$
BME vitamins (Sigma B6891)	50 µl
$0.5M\mathrm{Na_2}\mathrm{HPO_4}$	$40~\mu l$
Pyruvic acid (14 mg/ml in H ₂ O; mix fresh)	$20~\mu l$
1 MMgSO ₄	$20~\mu l$
1 MCaCl ₂ (optional)	$20~\mu l$
Lactate syrup (Sigma L4263)	10 μl
Trace minerals	10 μl
Chicken egg yolk dilution or cholesterol 3-sulfate stock	50 µl

or chemically defined lipid mix, Gibco BRL or Sigma L-5146 $\,$ $\,$ 100 μl

Use tissue-culture grade chemicals (e.g. Sigma's tissue culture grade). Weigh PVP and tyrosine into a tube and add other components in the order listed with egg yolk last. Let sit on ice 1–3 hours, centrifuge at 5000 rpm for 5–10 minutes, and filter-sterilize. Store refrigerated; this medium is good for a month or longer.

Because this recipe is a tissue culture medium "made from scratch", it is complicated. However, stock solutions can be made up once every several years and frozen or refrigerated as designated below. A batch of medium takes less than an hour to put together.

Stock Solutions for EGM

- 1. Stock Salts (100 ml): 70 m 1*M* NaCl; 30 ml 1*M* KCl. Use tissue-culture grade H₂O and autoclave. Refrigerate.
- **2.** Base Mix (100 ml): tissue-culture H₂O, 100 ml; adenine, 100 mg; ATP, 10 mg; guanine, 3 mg; hypoxanthine, 3 mg; thymine, 3 mg; xanthine, 3 mg; uridine, 3 mg; ribose, 5 mg; deoxyribose, 5 mg. Autoclave and refrigerate. Good indefinitely.
- 3. Amino acids (100 ml): tissue-culture H₂O, 100 ml; DL-α-alanine, 90 mg; L-arginine, 100 mg; L-asparagine, 50 mg; L-cysteine (free base), 24 mg; glycine, 40 mg; L-histidine (free base), 50 mg; DL-isoleucine, 50 mg; L-leucine, 25 mg; L-lysine, 15 mg; DL-methionine, 30 mg; DL-phenylalanine, 50 mg; L-serine, 40 mg; DL-threonine, 120 mg; L-tryptophan, 4 mg; DL-valine, 40 mg. Filter-sterilize, aliquot at 1250 μl in Eppendorf tubes for convenience, and store at -20°C.
- 4. Trace mineral stock (1000×): tissue-culture grade H_2O , 100 ml; $ZnSO_4.7H_2O$ (5×10⁻³M), 144 mg; $FeSO_4.7H_2O$ (1×10⁻³M), 28 mg; $MnSO_4.H_2O$ (5×10⁻³M), 85 mg; Na_2SeO_3 (1×10⁻⁴M), 1.7mg. Filter-sterilize and store refrigerated. Replace when precipitate is seen.
- 5. PVP: polyvinylpyrrolidine MW 40,000 (Sigma P0930, embryo-tested). Store at room temperature or -20°C. The Sigma P0930 can be dissolved at 50 mg/ml in tissue culture H₂O and used to replace 1 ml of the H₂O in the recipe; filter, sterilize, and store this stock solution at 4°C.
- 6. Inulin: 5 mg/ml (Sigma I3754) in tissue-culture grade H_2O . Inulin (not insulin), like the PVP, adds osmolality. Autoclave to dissolve. Refrigerate or keep at room temperature. This solution tends to precipitate at 4°C, or to grow things at room temperature.
- **7.** Galactose: 100 mg/ml in tissue-culture grade H₂O. Autoclave, aliquot to 1 ml, and freeze; refrigerate after thawing.
- **8.** Fetal bovine serum (FBS): Heat treat at 56°C for 30 minutes. The quality of serum is critical, and if not good, embryos tend to bleb at early cleavages and lyse. You may need to try several batches; however, you need so little that a

- tissue culture lab may be willing to let you try their sera. Freeze in 3 or 4 ml aliquots at -20° C.
- **9.** LPSR-1 is a low-protein serum replacement with extra growth factors. Its use is optional but may improve the medium somewhat, depending on your batch of serum.
- **10.** Water: Quality of H₂O is very important, so either buy certified tissue-culture H₂O or get some from a tissue-culture lab.
- 11. Egg yolk: Separate yolk of an egg, roll on paper towel to remove all white, and mix an aliquot 1:1 with sterile H₂O, as otherwise it is too thick to pipette. While this sounds arcane, it is a source of cholesterol and carrier lipoproteins, and apparently important for hypodermal cell development, as hypodermal cells detach in low cholesterol media. Cholesterol 3-sulfate (Sigma C9523) or a defined cell culture lipid mix (Gibco BRL or Sigma L-5146) seems to work nearly as well; for cholesterol, make a stock at 25 mg/ml in 30% ethanol. This will not dissolve completely but can be used as a slurry. Store at 4°C.
- 12. Additional Notes: For convenience, freeze serum, amino acids, and LPSR-1 in aliquots for one batch of medium. Small aliquots of vitamins, galactose, and base mix can be refrigerated several months once thawed. A few components, as noted, must be mixed fresh.

This is a trial-and-error medium based on earlier *C. elegans* media (Laufer et al, 1980), mouse embryonic culture media, and Leibovitz's L-15 media, which is an insect tissue culture medium. The osmolality without PVP, inulin, and serum is 291 mM, the same as the standard tissue culture medium M199, but embryos seem to do much better with these additives, so that one suspects that normal osmolality within the vitelline envelope is very high. The L-15 medium works reasonably, although not as well as EGM, in a mix of 600 μ l L-15 (Gibco 320–1415 liquid, 430–1300 dry), 100 μ l inulin mix, 100 μ l 0.25 M Hepes (pH 7.4), 200 μ l FBS per ml. Add 10 μ l base mix and 5 μ l egg yolk or cholesterol for better results. Also see Shelton's Growth Medium, below.

A quick trial of medium can be made by cracking eggs under a cover slip in medium and observing early cleavages in partial embryos or isolated blastomeres. There should be continuing division without much delay, and very little membrane blebbing at division. Blebbing and early lysis is probably a serum problem, but may be due to high divalent cations. The added Ca²⁺ and Mg²⁺ can be reduced or omitted to control blebbing at division. If blastomeres are observed to shrink or swell, overall osmolarity may be adjusted by 5 to 20 mM with stock salts. Refrigerated medium sometmes looks contaminated because cholesterol precipitates out over several days; if the embryos aren't dying, the medium can be refiltered and used.

Minimal EGM

This simplified version is suitable for short-term culture of permeabilized embryos, e.g. in preparation for fixation or for a few hours. It does not support development as satisfactorily for overnight culture as EGM. For 10 ml: PVP, 50 mg; inulin (5 mg/ml), 1 ml; stock salts,

840 μ l; 0.25M Hepes (pH 7.4), 1 ml; FBS, 4 ml; penicillin-streptomycin, 100 μ l; galactose (100 mg/ml), 100 μ l; lactate syrup, 10 μ l; glutamine (14 mg/ml), 100 μ l; pyruvic acid (14 mg/ml), 20 μ l; H₂O, 2830 μ l.

Shelton's Growth Medium (SGM)

This version is simpler to prepare than EGM, and it supports development fairly well (Shelton and Bowerman, 1996), although late differentiation markers that develop in EGM do not appear in SGM. Inulin, 1 ml of 5 mg/ml stock; PVP powder, 50 mg; BME vitamins (Gibco BRL) 100 μ l; chemically defined lipid concentrate (Gibco BRL) 100 μ l; 100× concentrated Pen-Strep (Gibco BRL) 100 μ l; Drosophila Schneider's Medium (Gibco BRL) 9 ml. Before use, heat-treated fetal calf serum (Gibco BRL) is added, and the tonicity of the medium is adjusted by varying the percentage of FCS added. 35% FCS (volume/volume) is typically used.

2.25% Paraformaldehyde Fix (20 ml)

Add 2 drops 1M NaOH to 10 ml $\rm H_2O$, heat to boiling, and let cool to about 70°C. Meanwhile, weigh 450 mg paraformaldehyde, add to the cooled $\rm H_2O$, and swirl to dissolve. Add 10 ml 250 mM phosphate buffer, pH 7.2–7.4. Filter through Whatman paper and add 80 $\rm \mu l$ 25% glutaraldehyde (or equivalent) to 0.1% final glutaraldehyde concentration. This will keep about a week in the refrigerator.

Gelatin-subbed slides

Hydrate 2% gelatin (Sigma G1890 seems best) in H_2O for a few minutes, then melt at 65°C. Put 15 μ l on one end of a slide with a Pipetman, and draw a second slide lengthwise as if making a blood smear, to give an even coating. Air dry, preferably several weeks for best sticking. This formulation will not kill embryos.

β-galactosidase stain (1 ml)

250 mM phosphate buffer, (pH 7.2–7.4), 500 μ l; H₂O, 378 μ l; 1M MgCl₂, 10 μ l; 1% sodium dodecyl sulfate, 4 μ l (for subsequent antibody staining, substitute Triton X-100 at final concentration of 0.1%); Fe stock (100 mM potassium ferrocyanate, 100 mM potassium ferricyanate, in H₂O; freeze aliquots, store current stock at 4°C), 50 μ l; 2% X-gal in dimethylsulfoxide (store at –20°C), 12 μ l. Mix fresh on day of use.

Esterase Stain (1 ml)

4% NaNO₂ (stock good one week at 4°C), 50 μl; pararosaniline [stock: stir 400 mg pararosaniline HCl (Sigma P1528) with 8 ml H₂O overnight, add 2 ml concentrated HCl, and stir several hours; centrifuge and filter; best after aging at several weeks at 4°C], 50 μl; 2.8% Na₂HPO₄, 1ml; 0.2MNaOH, 20 μl; α-naphthyl acetate (20mg/ml in acetone), 2 μl. Mix NaNO₂ and pararosaniline to diazotize; then add other components in order given. Keep on ice; good for about 1 hr.

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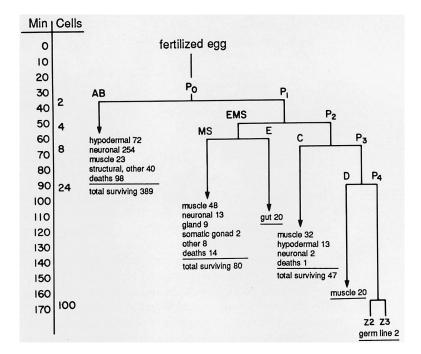


Figure 1. Early lineage of *C. elegans* embryos, indicating differentiated tissues derived from each of the eight founder cells.

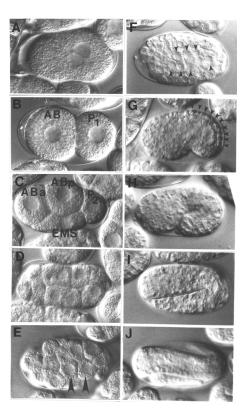


Figure 2. Embryonic development of *C. elegans* within the eggshell. (A) One-cell stage; (B) two-cell stage; (C) four-cell stage; (D) 16-cell stage; (E) onset of gastrulation at 28 cells, (arrowheads indicate the two E cells); (F) mid-proliferation stage (arrowheads indicate E cells in gut primordium); (G) late comma stage (arrowheads indicate hypodermal cells at dorsal midline); (H) 1.5-fold stage, with elongation beginning; (I) 2-fold stage; (J) pretzel or 3-fold stage. Anterior left, dorsal top in all photos. Photos by Francine Storfer-Glazer.

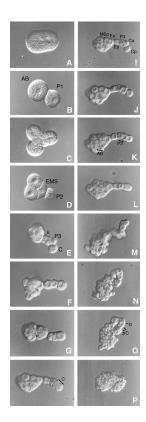


Figure 3.

Early divisions in devitellinized cultured embryos. Embryo in (G)-(P) was cultured in the presence of 200 µg/ml of α -amanitin; embryos cultured with or without α -amanitin behave indistinguishably up to about 100 cells, and then cease division. Founder cells and their progeny are labeled in some of the panels; double-ended arrows indicate cleavage axes. (A) First cleavage, t=0 (approximately 40 minutes after fertilization); (B) 2-cell stage, 10 minutes; (C) second cleavage, AB cell dividing, 20 minutes; (D) 4-cell stage, 25 minutes; (E) 8-cell stage, 57 minutes; (F) 12-cell stage, with eight AB cells, four P lineage cells, 73 minutes; (G) 12-cell stage, 81 minutes; (H) C division, 93 minutes; (I) 15-cell stage, 97 minutes; (J-L) 16- to 24-cell stages with P_3 and the 8 ABs dividing, 105, 110 and 120 minutes, respectively; (M) 187 minutes, (N) 200 minutes. (O) 230 minutes, note gastrulation-like movements beginning (in M) at 46 cells (32 AB, 4C; D, P4, 4E, 4 MS cells), in which P-lineage cells move to contact MS and Eaa; (P) same embryo after 16 hours showing α -amanitin arrest at approximately 100 cells. See Figure 4D for an embryo cultured overnight without drug treatment.

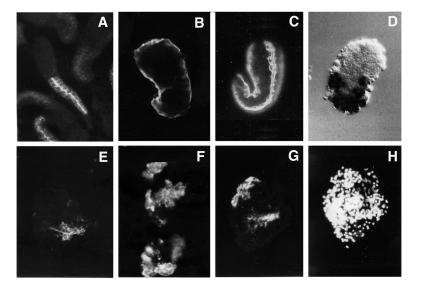


Figure 4. Antibody staining for differentiated tissue markers in normally developing embryos (A, B, C), and embryos devitellized at 2–4 cells and cultured overnight (E, F, and G). (A, E) monoclonal antibody J126 (S. Strome), specific to gut: (B, F) Monoclonal antibody J130 (S. Strome) to an early hypodermal protein; (C, G) Monoclonal antibody 5.6.1.1 (David Miller) to myosin heavy chain A. (D) cultured embryo viewed with Nomarski, stained for β -galactosidase expression from Ce MyoD-lacZ fusion construct specific for body muscle precursors (from M. Krause); (H) cultured embryo DAPI stained for nuclei.

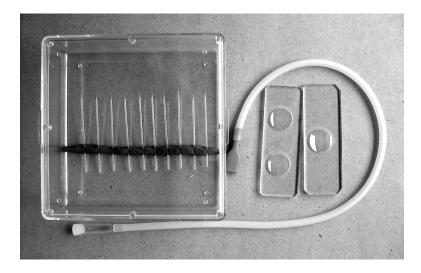


Figure 5.Some of the materials used: a mouth pipette, two depression slides, and a box of pulled glass needles.