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Visualizing Cell Contacts and Cell Polarity in *Caenorhabditis elegans* Embryos

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ABSTRACT

The *Caenorhabditis elegans* embryo is particularly amenable to microscopy and embryological studies because of its short developmental time, transparent shell, and nonpigmented cells. Within the embryo, contacts between cells often establish the polarization of neighboring cells. Experiments on cell contacts past the initial stages of cleavage can currently be performed on the embryo *in vivo*. The following techniques describe the process of making an agar mount for microscopic visualization, capturing 4D data on the microscope, identifying and highlighting cell contacts between blastomeres, and laser killing of blastomeres to inhibit cell contacts.

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INTRODUCTION

Understanding how cells in the embryo establish polarity and cell fate is important for fully understanding the process of development. *Caenorhabditis elegans* is an excellent model system for studying polarity in the embryo because of its invariant lineage and relatively few numbers of cells in the embryo (Sulston et al. 1983).

The proteins involved in polarizing the one-cell *C. elegans* embryo have been extensively studied and are well understood (reviewed in Gonczy and Rose 2005). Many studies have also examined the establishment of polarity of blastomeres later during embryogenesis. Several studies have shown that cell contacts between the P2 and the EMS blastomere permit and direct the polarity of the EMS spindle and establish the fate of the future daughter cells from EMS, the E and MS blastomeres (Walston and Hardin 2006). Through elegant blastomere isolation and recombination experiments, Goldstein showed the roles of both Src and Wnt signaling pathways in establishing polarity within the EMS blastomere (Goldstein et al. 2006). The four-cell *C. elegans* embryo is particularly amenable to both *in vivo* and *in vitro* experimentation. However, beyond the four-cell stage, *in vitro* embryological

experiments, such as those conducted by Goldstein, are increasingly difficult as individual cell identity is difficult or impossible to ascertain. Understanding how these cell contacts can lead to polarity and cell-fate differences is possible through *in vivo* experiments, such as examining cell–cell contacts and limiting those contacts through either genetic or mechanical manipulation. An example of this technique is the establishment of polarization of the ABar blastomere through contact with the C blastomere (Walston et al. 2004). To study the effects of this contact, several steps were required. Initially, analysis of images was conducted to determine the contact between the cells (Movie 15-1). The effects of this contact were abrogated through *pal-1* RNA interference, which alters the fate of the C blastomere (Movie 15-2). Second, the ABp blastomere was subjected to laser killing, creating steric hindrance between C and ABar (Movie 15-3). Similar techniques can be used to study contacts between blastomeres throughout development.

Here, we describe the techniques used to conduct these experiments including preparing embryos for visualization on an agar mount, collection of 4D data, analysis of 4D data, and ablation techniques for laser killing of individual blastomeres within the embryo. These techniques focus primarily on the use of differential interference contrast (DIC) microscopy because of its broad availability, common use for *C. elegans* imaging, and wide applicability to microscopic analysis of embryos of other organisms.

However, many of these techniques are also applicable to fluorescent imaging. The broad range of green fluorescent protein (GFP) markers and other labels that can be visualized in living embryos expands the possibilities of analysis of cell contacts and cell polarization.

Protocol A

An Agar Mount for Observation of *C. elegans* Embryos

The agar mount is an easy way to prepare *C. elegans* embryos for microscopy. The mount slightly embeds the embryo in agar to hold it in place. The mount also slightly compresses the embryo to provide consistent orientation of the embryo such that every embryo will be positioned with either its right side or its left side facing the objective. Other techniques can result in random orientations that complicate analysis and make identification of individual blastomeres more challenging.

IMAGING SETUP

For assembling the mount, a standard stereoscope is required for all steps with *C. elegans*. To identify early embryos (one to four cells), a total zoom of 80X or greater is recommended. We use Leica S8 APO microscopes with 10X eyepieces and Leica MZ12.5 microscopes with 16X eyepieces and 1.0X objective lenses.

MATERIALS

See the end of the chapter for recipes for reagents marked with <R>.

Reagents

Agar (5%)
C. elegans hermaphrodite (gravid)
M9 buffer <R>
Valap <R>

Equipment

See Imaging Setup
Calibrated glass pipettes, 50- μ L
Coverslips, 18 x 18-mm (#1)
Eyelash brush (eyelash glued to end of round toothpick)
Mouth pipette 15-in aspirator tube assembly
Microscope slides, 25 x 75 x 1-mm
Platinum wire pick
One inch of 30-gauge platinum or 90% platinum, 10% iridium wire is inserted into a 6-in pasteur pipette and is heated in a flame until the glass melts around the wire. Flat end hobby pliers or a small tack hammer are used to flatten the end of the pick.
Single-depression microslide, 3-mm
Syringes (1 cc) with 27 gauge x 1/2-in needles

EXPERIMENTAL METHOD

The total time needed for the experienced is 10–15 min and for the novice is 30 min.

1. Use a platinum wire pick to move approximately five gravid *C. elegans* hermaphrodites from a culture dish to a single-depression microslide mostly filled with M9 buffer.

The number of hermaphrodites needed will depend on the number of embryos required at the appropriate stage.

2. By holding a syringe with a needle in each hand, place the needles on either side of a hermaphrodite, and draw the flat sides of the tips of the needles across each other to cut the worm in half transversely (Fig. 1A,B). The embryos will be released from the halves of the hermaphrodite. Use an eyelash brush to carefully prod the halves to expel any remaining embryos.

It is important to cut as close to the vulva as possible to release newly fertilized embryos in the uterus. This step can also be conducted by cutting the worm in half with a #15 curved blade scalpel.

3. Sort embryos using the eyelash brush, and brush together into a group of approximately 10 embryos (Fig. 1C).

Embryos will tend to stick slightly to each other when grouped. If you desire a certain stage of embryogenesis, it is at this point that the embryo stage should be assessed and should be sorted appropriately. Two-cell stage embryos are the easiest developmental stage to collect.

4. Using laboratory label tape, tape two microscope slides parallel and one slide width apart on the laboratory bench. Place a third slide between the two taped slides (Fig. 2A). Using a 6-in pasteur pipette, place three to four drops of molten 5% agar onto the middle slide. Immediately lay the fourth slide perpendicular to the other three slides over the agar, and press it over the taped slides to flatten the agar before it cools (Fig. 2B).

5. Once the agar has set up, use a razor blade to trim excess agar from the edges of the slides.

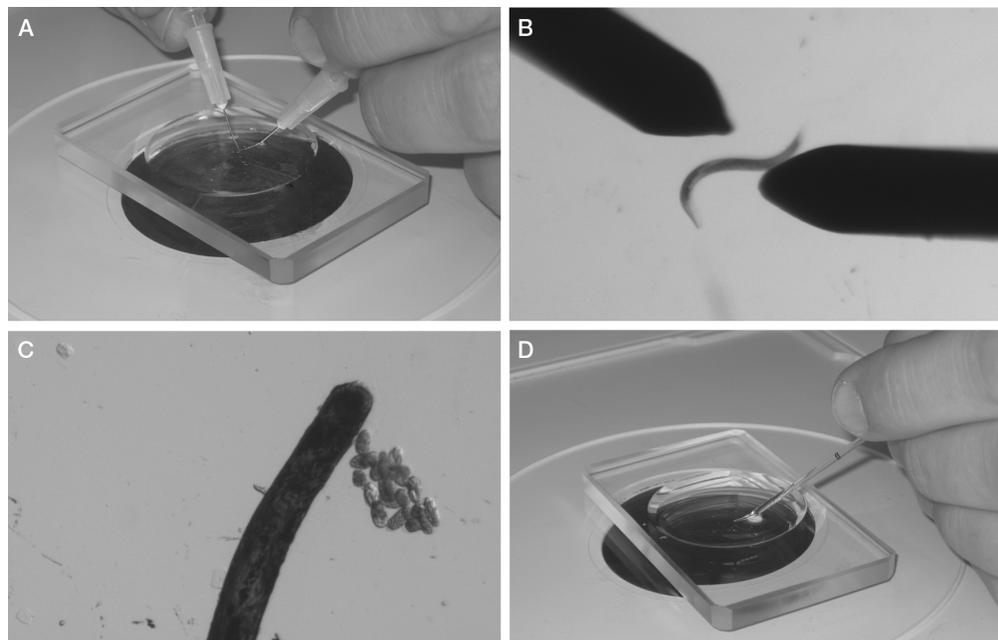


FIGURE 1. Isolation of *C. elegans* embryos and preparation for mounting on a slide. (A,B) Gravid hermaphrodites are cut in half with $27 \times \frac{1}{2}$ -in needles. (C) At a higher magnification, embryos are sorted and are grouped using an eyelash. (D) Embryos and M9 buffer are transferred using a mouth pipette.

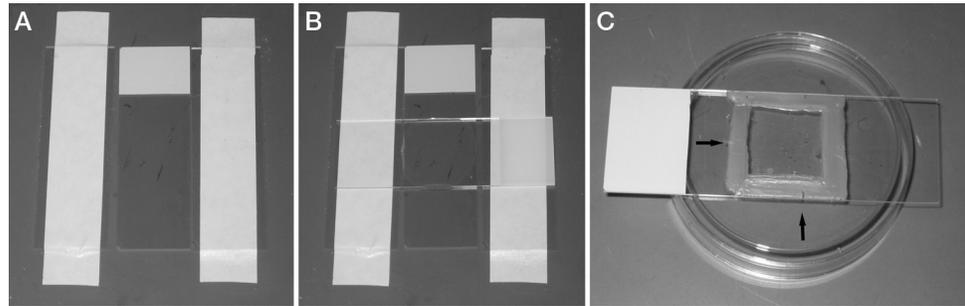


FIGURE 2. Making an agar pad. (A) Three slides are placed on the bench, and the outer two are taped down to the bench. (B) A drop of molten 5% agar is placed onto the middle slide. A fourth slide is then placed perpendicular to the three original slides. The top slide is compressed over the taped slides. (C) The finished slide is sealed with Valap. Using a toothpick to make hash marks in the Valap (arrows) aids in finding the grouping of embryos on the compound scope.

Carefully slide apart the untaped slides so the agar pad is left in the center of one slide.

See *Troubleshooting*.

6. Heat a glass 50- μ L pipette in a flame. Once the glass is soft and fluid, remove it from the flame, and quickly pull apart the ends. Break the two ends apart to create a pipette with a tapered end with a diameter of ~ 40 μ m. Place the pipette in a mouth pipette aspirator.
7. Using the mouth pipette, transfer the grouping of embryos (from Step 3) and ~ 20 μ L of M9 to the corner of the agar pad on the microscope slide (Fig. 1D).
8. Brush the embryos out of the M9 into the center of the slide using the eyelash. Position the embryos in a single layer side by side.
See *Troubleshooting*.
9. Set the edge of a coverslip at the side of the agar pad opposite the M9, and slowly drop it so that the coverslip lands on the embryos before it contacts the M9. Use a Kimwipe to wick excess buffer from the edges of the coverslip, and wick air bubbles from under the coverslip.
See *Troubleshooting*.
10. Trim excess agar from the edges of the coverslip using a razor blade. Seal the edges of the coverslip with melted Valap using a paintbrush (Fig. 2C).

TROUBLESHOOTING

Problem (Step 5): The agar pad dries to the slide before it can be used.

Solution: Make the pad immediately before use. Stereomicroscopes with light sources mounted under the stage have the potential to heat the stage after long use, which can quickly dry agar pads. Using a stereomicroscope with an external bulb or a cool temperature bulb will reduce this problem.

Problem (Step 8): Embryos fail to develop.

Solution: One-cell embryos are especially vulnerable to mechanical stress and are challenging to mount without killing. If studying a later stage of development, the likelihood of embryos surviving is markedly increased if two-cell or later stage embryos are used to make the mount. Groupings larger than 15–20 embryos often display increased lethality caused by oxygen starvation. By keeping groupings of embryos around 10 embryos, oxygen starvation should not be a problem.

Problem (Step 9): When coverslip is placed on slide, all the embryos wash to the edge of the coverslip.

Solution: Too much M9 buffer is used, and the M9 buffer is hitting the embryos before the coverslip can land on them and hold them in the agar.

Problem (Step 9): The slide has air bubbles under the coverslip.

Solution: Use more M9 buffer. This will allow M9 buffer to completely wash under the coverslip. However, too much M9 buffer will cause embryos to wash away (see previous problem).

DISCUSSION

Mounting *C. elegans* embryos on agar mounts provides a stable long-term environment for microscopic analysis of development. The slight compression from the coverslip will result in embryos reproducibly positioned with either the left or the right side facing toward the objective lens. During later stages of embryogenesis, embryos turn such that left-side views become dorsal views and right-side views become ventral views. Embryos on agar mounts will survive and will hatch from the eggshell on the mount. Embryos prepared with an agar mount are amenable to both light microscopy (with DIC optics) and confocal microscopy.

Alternative methods for mounting embryos, such as poly-L-lysine-coated slides with grease feet (Mohler and Isaacson 2010) or polymer beads (see Chapter 2), are often used to avoid compression to the embryo. However, these techniques typically result in random embryo orientation, which can complicate analysis of development. Additionally, the slight compression of the coverslip seems to have little to no effect on development; and, in most cases, avoidance of such compression is unnecessary.

In conclusion, preparing *C. elegans* embryos on an agar mount is a simple technique that can be easily mastered and is regularly performed by undergraduates in the investigators' laboratories. It provides a consistent embryonic orientation and environment that is suitable for long-term microscopy of *C. elegans* embryos.

Protocol B

Acquisition of 4D DIC Microscopic Data to Determine Cell Contacts in Embryos

Acquisition of stacks of images throughout the thickness of the embryo over time is a crucial method for identifying the positions and contacts between cells. Such 4D microscopy is a routine tool in laboratories that study early *C. elegans* development.

This protocol describes the use of a custom script within μ Manager's Beanshell scripting language. The script is helpful for reducing the number of shutter open/close events during 4D acquisition. Alternatively, the standard μ Manager package has been used successfully by one of us (Hardin) to acquire 4D footage, but this involves 20-fold more shutter open/close events than when the script is used. The script is available at <http://worms.zoology.wisc.edu/4d/4d.html>.

IMAGING SETUP

1. Microscopy/camera hardware: This protocol assumes a basic high-numerical-aperture (high-NA) microscope equipped with oil-immersion objectives and, optionally, an oilable, high-NA condenser from any of the major microscope manufacturers. We typically acquire 4D movies using a 60 \times –63 \times , 1.4–1.45-NA PlanApo objective. Older Newvicon video cameras, coupled to the video port on the microscope, are adequate for many applications, especially if they are equipped with a zoomable video lens attachment such as those sold by Nikon. In this case, an AG-5 digitizing board (Scion Corporation) or a similar video frame grabber can be used to digitize the video signal. Alternatively, modern cameras are almost exclusively charge-coupled device (CCD) cameras and have much higher spatial resolution than older video formats. We have successfully used cameras from Scion Corporation and QImaging. The mounting hardware for such devices differs depending on the microscope being used.
2. Cooled environment: We have found that *C. elegans* embryos can be imaged for long periods of time if the ambient temperature is reduced to $\sim 20^{\circ}\text{C}$.
3. z-axis controller/shutter/serial port: A variety of z-axis controllers are available from commercial sources (e.g., Prior Scientific, Ludl Electronics Products, Applied Spectral Imaging [ASI]). In addition, a shutter to block the transmitted light path between time points is strongly encouraged to minimize exposure of embryos to light and to heat.
4. Software: One of us (Walston) has used commercial software (IPLab) to acquire 4D footage. If an inexpensive alternative is desired, free software has been written by one of us (Hardin) as an alternative. Several options are available.
 - a. Legacy acquisition plug-ins for ImageJ: These plug-ins are available free of charge at the following URL: <http://worms.zoology.wisc.edu/research/4d/4d.html>. Full documentation of the plug-ins and detailed instructions for installation of ImageJ and QuickTime for Java are available at the same URL.
 - b. μ Manager: The public domain program μ Manager supports a variety of CCD cameras, z motors, and shutters. The μ Manager program can be obtained at <http://www.micro-manager.org/>.

MATERIALS

Reagents

C. elegans embryos, embedded as described in Protocol A
Immersion oil (Type DF oil is recommended)

Equipment

See Imaging Setup.

EXPERIMENTAL METHOD

The total time needed for the experienced is 5 min and for the novice is 15 min.

1. Turn on the z-axis and shutter control boxes and the CCD camera. Turn on the light switch on the microscope. Find a group of embryos using the 10X objective, before oiling the coverslip.
2. If a high-NA condenser is present, place a drop of oil on the condenser (for upright microscopes) or on the bottom of the slide (inverted microscopes). Carefully position the condenser so that it contacts the oil and spreads it uniformly between the condenser and the microscope slide.
3. Focus the condenser. The simplest method for achieving good condenser focus is to stop down the condenser using the iris diaphragm, closing it almost completely. Then the height of the condenser can be adjusted at high magnification until the octagonal outline of the diaphragm is in focus. When performed, open the condenser.
4. Once embryos have been located at 10X and the condenser has been focused, swing the 10X objective out of the way, and add a drop of immersion oil to the coverslip (upright microscope) or to the 60X objective lens (inverted microscope). We find that Type DF oil works well.
5. Carefully slide the 60X or the 100X objective into place (it should just clear the sealant on the slide, as long as it is not too thick). Make sure the correct condenser setting is selected to match the lens.
6. Refocus on the embryos, and refocus the condenser.
7. Open μ Manager. Use the “Live” button in the main μ Manager Studio window to display an image from the camera. If the “Autoshutter” option is not checked, click the “Open” button to open the shutter. Otherwise, it should open when the “Live” button is clicked. Optimize the positioning of embryos in the field using the stage controls on the microscope and/or by rotating the CCD camera gently by hand (if the mount supports this). Optimize the Nomarski optics through a combination of the following.
 - Center the condenser by closing it and by moving the octagon to the center of the field of view. Reopen the diaphragm to encompass the entire field of view.
 - Adjust the light level. High-quality Nomarski optics requires a substantial amount of light. Optimal settings must be empirically determined.
 - Adjust the exposure time, the gain, and other settings on the CCD camera within.
Use μ Manager a final time if needed.

See *Troubleshooting*.

8. Invoke the μ Manager 4D acquisition script. This protocol presupposes that a “favorite” has been created previously using the script window in μ Manager. This window is invoked using the “Tools → Script Panel” menu command in μ Manager. When the script window appears, select “Acquire_4D.bsh” from the list of favorites. Make sure that the cursor is blinking within the code of this script. Then click “Run”. Enter the desired parameters for time interval, number of time points, number of focal planes, and distance between focal planes. Enter the root

name for the images that will be collected. (Note: because most operating systems limit the total length of a file's name to 32 characters, the root name should be kept short.) If a shutter is being used, make sure that the "Use shutter" option is selected.

9. Click "OK." The parameters that have been entered will be displayed. If these are acceptable, click "OK." When prompted for a location to which to save images, make a new directory that will contain the images from the 4D sequence. Within the newly created directory, we recommend making two additional directories: (a) one called "working" and (b) one called "terminal." The latter is useful for acquiring a final z-stack of the terminal embryos. Typical settings for a long overnight movie are the following.

Number of time points: usually 200–300 for an overnight movie

Time interval (sec): usually 120–180

Number of shutters: 1

Number of focal planes: 20

Distance between focal planes: 1 μm

Root name: "working," or a short name of choice

Information for movie: Enter any pertinent information.

See *Troubleshooting*.

10. Once a directory is specified, the computer should start acquiring images. Status updates will be displayed in the ImageJ main window. To abort, click the "Stop" button in the "Script Panel" window.
11. When the movie is finished, we recommend collecting a terminal image. To do so, keep the field of view the same. Collect a second movie, specifying "1" as the number of time points. Save this movie in the "terminal" folder created previously.
12. To view the movie, there are several options available.
 - i. Raw 4D data sets: These can be viewed in one of several ways, including (a) importing the sequences as a "Virtual 5D Stack" within ImageJ, using the "Virtual 5D Stack" plug-in, available on the ImageJ web site, or (b) using the "Browse4D" plug-in available at <http://worms.zoology.wisc.edu/4d/4d.html>.
 - ii. Compressed movies: Movies can also be compressed to save disk space and can be viewed using QuickTime and the "QT4D Writer" and "QT4D Player" plug-ins available at the same URL. If this approach is being used, we typically save the movie using the same root name as the raw files with the word "movie" appended in the same directory created for the experiment. Although many compression algorithms are available, we typically use "Photo/JPEG," "Motion JPEGA," or "Motion JPEGB" compression, "grayscale," and "Medium" quality. This approach can compress movies 30-fold. To play compressed movies, use the QT4D Player plug-in. Select the desired movie. A graphical interface with clickable buttons or the arrow keys on the numeric keyboard can be used to navigate through movies. Once the movie has been successfully compressed and its quality verified, for routine purposes, it is now fine to delete the original files.

TROUBLESHOOTING

Problem (Step 7): No light appears to be reaching the camera.

Solution: Make sure the slider that diverts light from the microscope to the camera port is in the proper position and that the power supply to the camera is on. If the shutter has an external toggle switch, make sure that it is in the correct position. If the exposure time is set to too low a value, increase the exposure time using the controls in the Main μ Manager window.

Problem (Step 9): The plane of focus drifts systematically over time.

Solution: This often occurs in the first few minutes after making an agar mount. For this reason, it is advisable to check the focus several times during the first 15–20 min of acquisition. To reset the focus, open the shutter, and use the coarse focus on the microscope to refocus on the top focal plane.

Problem (Step 9): Temperature variation in the room results in inconsistent time course of development or variable phenotypes.

Solution: For best results, filming should take place in a room held at constant temperature, ~20°C. Make sure the air conditioner is on and that the door remains closed.

Problem (Step 9): After several hundred time points, μ Manager reports an error from which it cannot recover.

Solution: Some users have reported errors with μ Manager when using USB-to-serial port adapters. This is a known issue with μ Manager. Using a peripheral component interconnect (PCI)-based serial port card appears to alleviate this problem. Alternatively, acquire several shorter movies. We have successfully used this script under μ Manager for acquiring up to 150 time points with 25 focal planes/time point.

DISCUSSION

This procedure will result in the production of 4D data sets in the form of a series of consecutively named TIFF (tagged image file format) files that can be read by many different programs, including ImageJ, especially when supplemented with appropriate plug-ins. The reduced costs of such a system make this basic system feasible for teaching laboratories and research laboratories constrained by limited funds. Although we have described the use of such a setup for imaging *C. elegans* embryos, this apparatus is well suited for acquiring images of any transparent specimen.

Protocol C

Analysis of 4D DIC Microscopic Data to Determine Cell Contacts in Embryos

Identification of cell contacts is important for understanding how cells within the embryo can be polarized by their neighbors. This protocol will describe a technique for identifying cell contacts and for following those contacts through development. This protocol involves manual segmentation of the membrane of each blastomere from 4D DIC data sets (a wild-type example is shown in Movie 15-1; for comparison, a *pal-1*(RNAi) embryo is shown in Movie 15-2, and a laser-irradiated embryo is shown in Movie 15-3). Although this technique was performed with *C. elegans* embryos, it will work with any 4D DIC data set. The recent development of a pleckstrin homology (PH)-domain tagged::GFP expressed in *C. elegans* embryos simplifies this analysis (Audhya et al. 2005); however, many organisms lack appropriate GFP transgenes. The use of the GFP transgene advances other fluorescent techniques for labeling the membrane of embryonic cells that required using a laser to carefully permeabilize the eggshell to allow entrance of the dye into the embryo. The technique described below requires only easily obtainable 4D DIC data sets (see Protocol B).

IMAGING SETUP

Previously obtained 4D data sets are required. This technique will specifically address the use of DIC images but can also be extended to confocal images of transgenic embryos.

MATERIALS

Equipment

See Imaging Setup

Computer with image analysis software program and drawing or photograph editing program

A variety of programs can be used for this technique. The image analysis program must have the ability to handle and to easily navigate 4D data sets. The investigators regularly use ImageJ and occasionally use BD Biosciences IPLab. For the drawing or photograph editing program, the investigators have used commercially available Adobe Photoshop and Adobe Photoshop Elements, and freely available versions of ImageJ and GIMP.

Tracing tool

A mouse can be used for this; however, the investigators have had significantly more success using a pen and tablet (such as a Wacom tablet) or the trackpad on a laptop computer.

EXPERIMENTAL METHOD

The total time needed is 15–20 min per time point.

1. Open the 4D data set in the image analysis program. Navigate through time points to the time of interest, and identify the cells of interest. Scroll through the focal planes identifying the focal plane with the closest contact or the clearest contact between the cells.

See *Troubleshooting*.

To analyze cell contacts, start the method at least two to three time points before contact or suspected contact.

2. Export all images from that focal plane either to individual frames or to a stack of images.
3. Open the exported images in one of the photograph editing programs. Starting with the first image, select the freehand drawing tool in the software, and use the trackpad or the mouse to trace around the membrane of one of the cells in the embryo.

The first image analyzed may not be the first time point. In several cases, the investigators found it easier to trace the best contact time point first and to work in either direction in time after that.

4. Selecting a different color, repeat for the other cell being studied for contact. Maintain the color scheme throughout the analysis.
5. Repeat for each time point in the analysis.

See *Troubleshooting*.

TROUBLESHOOTING

Problem (Step 1): Difficulty determining where the cell membranes are for each cell.

Solution: It is highly recommended to have both the single frame and the 4D data set open at the same time. Membranes that are hard to pick out in the individual frame often are much more obvious in that frame when it can be visualized within the context of the 4D data sets. Often examining neighboring images (either adjacent time steps or adjacent focal steps) can clarify the membrane boundaries in the individual frame. Using a fluorescent membrane marker, such as a PH domain::GFP transgene, highlights membrane boundaries simplifying analysis of cell contacts.

Problem (Step 5): This process takes a lot of time, is there a quicker way to do this?

Solution: Several *C. elegans* laboratories, including the investigators' laboratories, are working on automated or semiautomated segmentation techniques for identifying cell boundaries in images. Until the quality of these techniques can be tested and can be shown to be accurate, hand segmentation of the images is the most accurate way, although it is time consuming. As mentioned above, membrane-based GFP markers also simplify this process and reduce the time for image analysis.

DISCUSSION

This procedure will result in the identification of cell membranes and potential contacts between different blastomeres in the embryo. Although described here for use with *C. elegans* embryos, it could be conducted with any 4D DIC data set. This technique requires particular attention to detail to correctly identify the boundaries of each cell. However, if both a static image and the 4D data set are compared simultaneously, identification of cell boundaries can be completed accurately. Images that use GFPs that label cell boundaries or cell membranes make identification of the boundaries straightforward and require tracing of the boundaries only to highlight and to identify particular cells.

Protocol D

Laser Killing of Blastomeres in *C. elegans*

Blastomere isolation and recombination experiments have led to a wealth of understanding of the events in the four-cell *C. elegans* embryo. However, identifying individual blastomeres after isolation at stages past the four-cell stage is limited. In addition, removal of blastomeres from their native surroundings can interfere with many cell contacts besides the contacts of interest. An alternative approach to studying cell interactions within the *C. elegans* embryo is to use laser ablation of individual cells. Laser ablation can be used to kill one of two cells in contact with each other to understand what happens when a cell no longer signals to its neighbor. Additionally, killing a cell that is between two cells that will eventually contact each other can result in the corpse of the cell forming a steric barrier between the cells preventing the contact. The following protocol describes laser ablation of embryos mounted on an agar mount.

IMAGING SETUP

The laser ablation of *C. elegans* blastomeres requires DIC optics with an ~60x–100x high-NA objective lens. The microscope also requires a tunable dye laser attachment for the lasing and a camera to observe progress of laser killing. A common setup for ablation of *C. elegans* consists of a Micropoint tunable dye laser (Photonic Instruments, Arlington, IL), which includes a 337-nm nitrogen pumping laser and a dye cell filled with Coumarin 440 dye (5 mM in methanol). The intensity of the laser spot can be attenuated using a density gradient filter that slides to generate a spot of the appropriate diameter and intensity as judged by cracking of a coverslip (see below).

MATERIALS

Reagents

C. elegans embryos mounted on an agar mount slide (see Protocol A)

Equipment

See Imaging Setup

EXPERIMENTAL METHOD

The total time needed is 15–30 min.

1. Select embryos on an agar mount.
2. Place embryos on the microscope, and locate them on the slide.
3. Focus the image on the top focal plane of the embryos.
4. Move the focus slightly above the embryos (essentially focusing on the coverslip of the slide), and move the microscope stage to remove embryos from the field of view.
5. Activate a single pulse of the laser. If focused properly, the laser should crack or poke a small hole in the coverslip when pulsed.

See *Troubleshooting*.

6. Note on the monitor where the pulse cracked the coverslip.
If using a monitor with a glass screen, marking the spot on the screen with a marker directly on the screen is convenient.
7. Move the microscope stage back to the embryos, and focus on the nucleus of the blastomere to be targeted.
The target nuclei should be in interphase of the cell cycle and should not be undergoing division.
8. Lase the nucleus of the target cell with ~10 pulses/sec until charcoal buildup can begin to be seen within the nuclei, ~10–15 sec.
See *Troubleshooting*.
9. Record embryonic development with 4D microscopy.

TROUBLESHOOTING

Problem (Step 5): No hole or crack appears in the coverslip when targeting the laser.

Solution: If no hole appears in the field of view, either the laser is not aimed properly down the objective within the field of view, or the focus is incorrect. The microscope must be focused on the coverslip. If the laser is properly positioned, slightly adjust the focus with the fine-focus control until the coverslip is in focus.

Problem (Step 8): The embryo explodes when laser ablation is attempted.

Solution: If focused too close to the coverslip while targeting the nuclei within a particular embryo, the embryo will rupture as the laser cracks the coverslip. To prevent this, target focal planes of those nuclei deeper within the embryo.

Problem (Step 8): The cell fails to die and continues through development.

Solution: If the target nucleus is not lasered sufficiently, it can recover, and the cell will resume mitotic divisions while the embryo progresses through development. Additionally, some cells progress through one final cell division before halting any future divisions. Depending on the particular nature and goals of the experiment, this may or may not be acceptable, and the cell should not be considered killed until cell divisions are halted. Many laser-killed blastomeres will display Brownian motion in which particles in the cytoplasm undergo rapid shaking movements following ablation. This should not be confused with cell division.

Problem: The entire embryo dies rather than an individual blastomere being killed.

Solution: Contrary to the previous problem of not lasering the nucleus long enough, if the entire embryo dies, it is usually caused by excessive lasering triggering embryonic arrest. Practice and experience will provide the best experience with gauging the appropriate amount of lasering to trigger cell death without killing the entire embryo.

DISCUSSION

In cases in which blastomere isolation is not feasible, an alternative is laser killing of particular blastomeres to understand the effects of those blastomeres on development or to create a barrier with the killed cell that prevents other cells from contacting each other. This technique does not require dissecting the embryo, and identification of individual blastomeres at the time of the killing and subsequent to the killing simply requires tracing the lineage of the remaining blastomeres. It can be conducted on embryos throughout development to study cells at a variety of stages of development. This technique can be conducted before 4D imaging or in the middle of collection of a 4D data set without removing the embryo from the microscope if it is set up with both the laser and the 4D imaging equipment.

RECIPES

CAUTION: See Appendix 7 for proper handling of materials marked with <I>. Recipes for reagents marked with <R> are included in this list.

M9 Buffer

Reagent	Amount
KH ₂ PO ₄	3 g
Na ₂ HPO ₄ <I>	6 g
NaCl	5 g
MgSO ₄ <I>, 1 M	1 mL
H ₂ O	to 1 L

Valap

Weigh out a mixture of Vaseline, lanolin, and paraffin (1:1:1[w/w/w]), and place the component in a glass or ceramic vessel. Melt the mixture on a hot plate over medium to low heat until completely liquid. The wax mixture should spread smoothly and should dry quickly on a glass slide. If it hardens too quickly, then add more Vaseline and lanolin. If it does not harden fast enough, then add more paraffin. Valap is solid at room temperature; just before use, warm it at low setting on a hot plate.

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MOVIE LEGENDS

Movies are freely available online at www.cshprotocols.org/imaging.

MOVIE 15.1. Contact between the C blastomere and the ABar blastomere directs the spindle orientation of ABar division in a wild-type *Caenorhabditis elegans* embryo. (*Left*) Contact between the C blastomere (membrane near contact outlined in blue) and the ABar blastomere (membrane near contact outlined in green). Focal depth (18

μm) of greatest contact is shown. (*Right*) Shortly after contact between the C and the ABar blastomeres, the mitotic spindle of the ABar blastomere (right line) is shifted toward the contact resulting in a cell division that is perpendicular to orientation of division of the neighboring ABpr blastomere (left line). Focal depth is $7.5 \mu\text{m}$.

MOVIE 15.2. Disruption of the fate of the C blastomere using *pal-1(RNAi)* knockdown results in delayed and minimal contact between the C and the ABar blastomeres before division of ABar causing the ABar spindle to be misaligned. (*Left*) Contact is minimal and is delayed between the C blastomere (membrane near contact outlined in blue) and the ABar blastomere (membrane near contact outlined in green). Focal depth ($14 \mu\text{m}$) of greatest contact is shown. (*Right*) In lieu of a polarizing signal from the C blastomere, the ABar blastomere (right line) adopts the default spindle orientation and divides parallel with the ABpr blastomere (left line). Focal depth is $7 \mu\text{m}$.

MOVIE 15.3. Laser killing of ABp creates a barrier between contact with the C and the ABar blastomeres in *Caenorhabditis elegans* embryos. The ABp blastomere (labeled with a red x) was laser killed at the four-cell stage of embryogenesis, just before the start of the movie. The corpse of ABp prevents contact between the C blastomere (membrane near closest contact outlined in blue) and the ABar blastomere (membrane near closest contact outlined in green). Focal depth ($10.5 \mu\text{m}$) of closest proximity between the two blastomeres is shown. As a result, the ABar blastomere aligns its spindle in the default orientation (line) and divides parallel with the other AB granddaughter cells (not shown).