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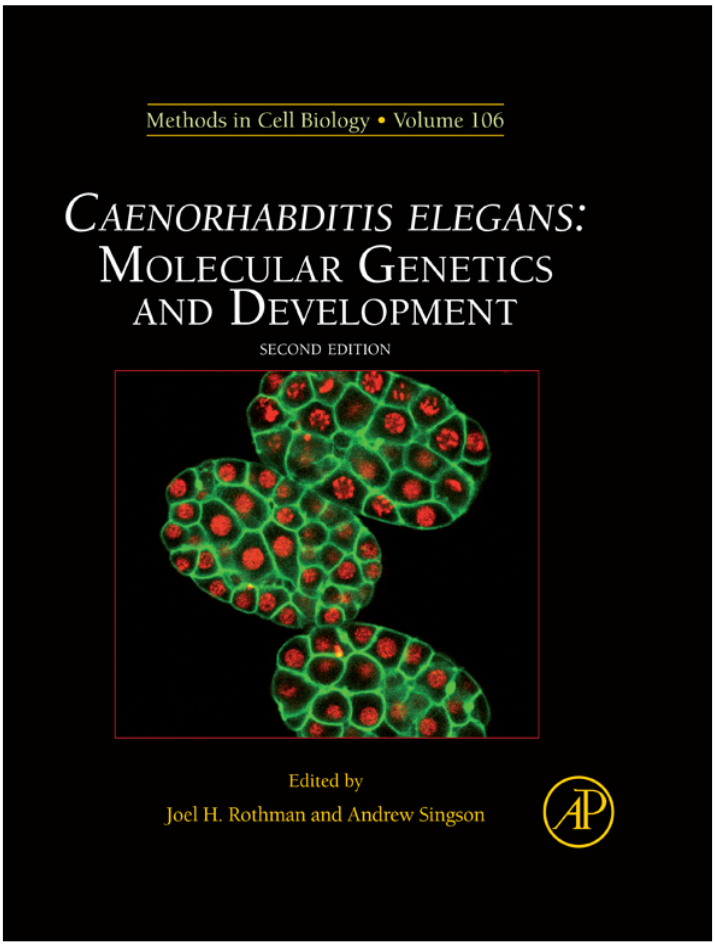

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CHAPTER 14

Imaging Embryonic Morphogenesis in *C. elegans*

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Abstract

The *Caenorhabditis elegans* embryo is well suited to morphogenetic analysis via modern microscopy, due to its short generation time, transparency, invariant lineage, and the ability to generate transgenic embryos expressing various fluorescent proteins. This chapter provides an overview of microscopy techniques for imaging embryonic morphogenesis, including making agar mounts, capturing four-dimensional (4D) data using Nomarski microscopy, imaging of actin in embryos, factors important for optimizing 4D fluorescence microscopy, and recent techniques that leverage fluorescence microscopy for intracellular imaging of cellular components during morphogenesis.

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I. Introduction

Caenorhabditis elegans has obvious advantages that make it well suited for analyzing morphogenesis of living embryos. Its organizational simplicity, transparency, and essentially invariant development enabled the determination of the complete embryonic cell lineage (Sulston *et al*., 1983). Such invariant development allows the assessment of mutant phenotypes at the level of single cells in *C. elegans.* The wild-type embryonic lineage was originally determined by direct observation using Nomarski microscopy. This was a very slow process, since only one or two of the >500 total embryonic cells could be followed per embryo. More recently, the use of histone::GFP and other technologies, along with automated analysis, has streamlined lineaging even further. These developments have been discussed elsewhere (Murray *et al*., 2008; see the chapter by Cowan and Chisholm, this volume). The analysis of postmitotic movements of cells in embryos has likewise benefitted from technological advances. The advent of four-dimensional (4D) microscopy made analysis of morphogenesis much more practical by using computer-controlled equipment to record development of embryos in three dimensions over time. Simultaneous software advances made analysis of 4D movies practical (Thomas *et al*., 1996).

The fundamental concepts of microscopy that apply to any context in *C. elegans* are covered elsewhere in this volume (see the chapter by Maddox and Maddox, this volume). In this chapter, we focus on the uses of modern microscopy specifically for imaging later morphogenesis in *C. elegans* embryos, after many embryonic cells have undergone their terminal divisions. We describe preparation of standard agar mounts and other approaches for immobilizing embryos and treating embryos prior to performing routine 4D microscopy, discuss simple methods for capturing 4D movies, and discuss various probes for imaging fluorescently tagged cells or structures in living embryos. We also describe one way of performing correlative fluorescence and transmission electron microscopy (F-TEM) during embryonic morphogenesis.

Since the most dramatic movements occur among hypodermal cells during this period of embryonic development, much of this chapter focuses on techniques that are particularly useful for analysis of hypodermal cells. Because the terms "hypodermal" and "epidermal" are used interchangeably, this chapter will use the latter for better consistency with standard usage in other organisms (see Chisholm and Hardin, 2005 for discussion).

II. Methods

A. Mounting Embryos for Imaging Morphogenesis

1. Agar Mount (Modified from Heid and Hardin, 2000)

The agar mount is a simple way to prepare *C. elegans* embryos for microscopy. The basic technique is presented elsewhere in this volume (see the chapter by

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Maddox and Maddox). Here we provide additional details regarding this key technique as it relates to analyzing morphogenesis. Agar mounts have several key advantages for analyzing morphogenesis. First, the mount slightly compresses the embryo, holding it in place. Second, such compression produces a consistent orientation convenient for imaging many aspects of embryonic morphogenesis. As the processes of morphogenesis proceed, either the dorsal or ventral surface of the embryo will be against the coverslip. After ventral enclosure is complete, the embryo then turns on its side, such that every embryo will be positioned with either its right or left side facing the coverslip. For many morphogenetic events, especially those involving the embryonic epidermis, such mounts are very useful (see Chisholm and Hardin, 2005 for details of the basic movements associated with epidermal morphogenesis). For some events, other orientations of the embryo may be preferable, and for these purposes, other mounting techniques may be used (see below).

Imaging setup

For assembling the mount, a standard stereomicroscope is required. To identify early embryos (1–4 cell), a total zoom of 80× or greater is recommended. We have typically used either a Wild MZ5 microscope with 20× oculars or Leica MZ12.5 microscope with 16× oculars.

Materials

i. **Reagents:**

Agar (5% w/v)
M9 buffer:

3 g KH_2PO_4
6 g Na_2HPO_4
5 g NaCl
1 mL 1 M $MgSO_4$
1 L H_2O

Valap:

Equal parts by volume of vaseline, lanolin, and paraffin.
Heat thoroughly until melted and mix.

ii. **Equipment:**

Calibrated glass pipettes (50 μL)
Coverslips (18 × 18 mm, No. 1)
Eyelash brush (eyelash glued to end of round toothpick)
Mouth pipette 15″ aspirator tube assembly
Microscope slides (25 × 75 × 1 mm)

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Platinum wire pick: 2.5 cm of 30 gauge platinum or 90% platinum/10% iridium wire inserted into a 6″ Pasteur pipette and heated in a flame until the glass melts around the wire. Flat-end hobby pliers or a small tack hammer can be used to flatten the end of the pick.
Single-depression microscope slide (3 mm)
Syringes (1 cc) with 27 gauge × $^{1}/_{2}$″ needles

Method

i. Use platinum wire pick to move approximately five gravid *C. elegans* hermaphrodites from culture dish to single-depression microscope slide containing M9 buffer. The number of hermaphrodites needed will depend on several factors, including the number of embryos of an appropriate age desired and the gravidity of the worms being used.
ii. Holding one syringe and needle in each hand, place one on either side of a hermaphrodite and draw flat sides of tips of needles across each other to cut the worm in half transversely. The embryos will be released from the halves of the hermaphrodite. Use eyelash brush to carefully prod 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 (Fig. 1).
iii. Sort embryos using eyelash brush and brush together into group of approximately ten embryos. Embryos will tend to stick slightly to each other when grouped. If one desires a certain stage of embryogenesis, it is at this point that embryo stage should be assessed and sorted appropriately. Two-cell stage embryos are the easiest developmental stage to collect.
iv. Using colored 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. Using a 6″ Pasteur pipette, place three to four drops of molten 5% agar onto the middle slide. Immediately lay fourth slide perpendicular to other three slides over agar and press over taped slides to flatten agar before it cools.
v. Once agar has set up, use a razor blade to trim excess agar from edges of the slides. Solidifed agar in the slide "sandwich" can be left assembled until embryos are ready to be added to the pad. Carefully slide the untaped slides apart so agar pad is left in center of one slide.
vi. Heat glass 50 μL pipette in flame. Once glass is soft and fluid, remove from flame and quickly pull apart ends. Break two ends apart to create a pipette with a tapered end with a diameter of approximately 40 μm. Place pipette in mouth pipette aspirator.
vii. When ready, carefully slide the untaped slides apart so agar pad is left in the center of one slide. Using mouth pipette, transfer the grouping of embryos (from step iii) and approximately 20 μL of M9 to the corner of agar pad on the microscope slide.

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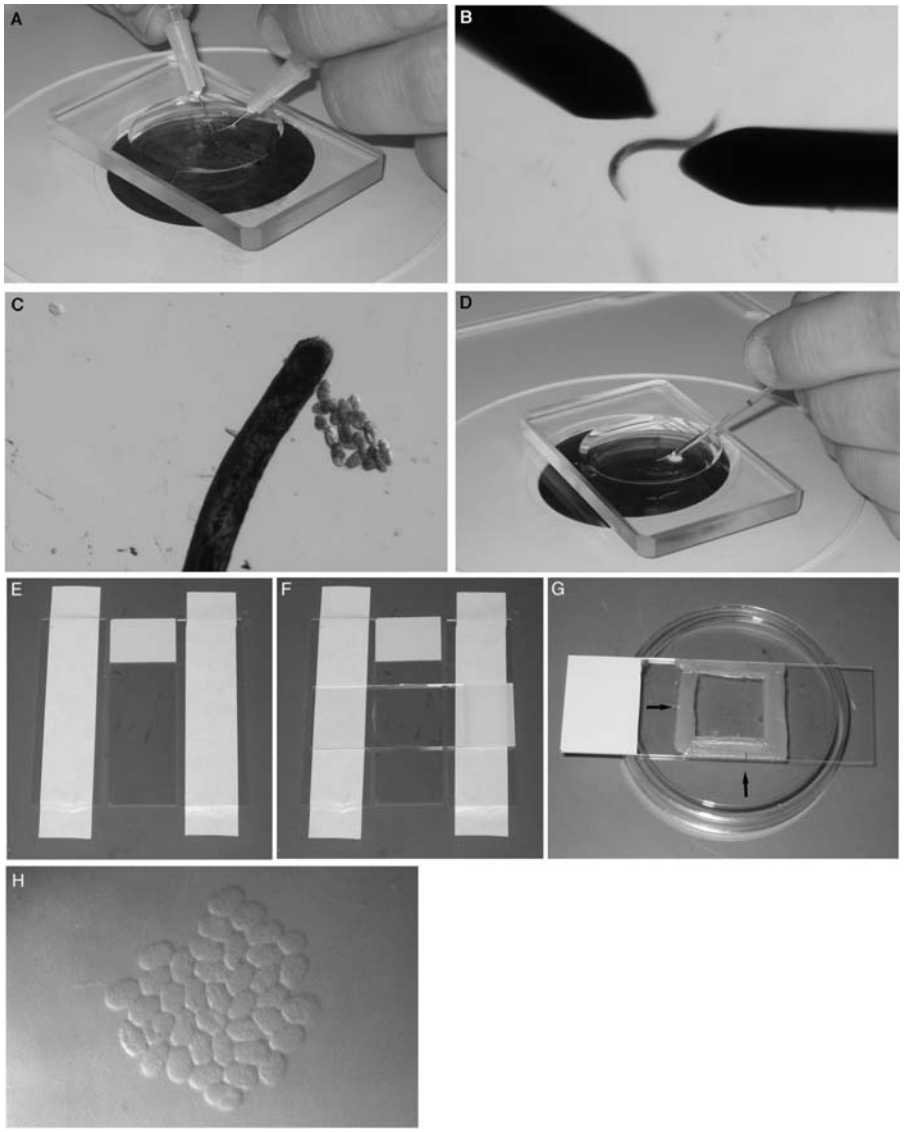

Fig. 1 Making a standard agar mount. (A, B) Gravid hermaphrodites are cut in half with 27 × $^{1}/_{2}''$ needles. (C) At a higher magnification, embryos are sorted and grouped using an eyelash. (D) Embryos and M9 buffer are transferred using a mouth pipette. (E) Three slides are placed on the bench and the outer two are taped down to the bench. (F) 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. (G) 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 microscope. (H) A low- magnification view of embryos within a completed mount. Embryos are grouped tightly together (image courtesy of T. Loveless).

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viii. Brush embryos out of M9 into the center of slide using eyelash. Position embryos in a single layer side-by-side. We find that especially in the case of embryos in which only a percentage show a phenotype of interest (e.g., homozygous mutant progeny from heterozygous mutant mothers, weak RNAi, etc.), that a large contiguous grouping of embryos is useful (Fig. 1H).
ix. Set the edge of a coverslip at the side of the agar pad opposite the M9 and slowly drop so that the coverslip lands on the embryos prior to the M9. Use a Kimwipe to wick excess buffer from edges of coverslip and wick air bubbles from under coverslip.
x. Trim excess agar from edges of coverslip using a razor blade. Seal edges of the coverslip with melted VALAP using a paintbrush.

Troubleshooting

i. Problem: 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 due to oxygen starvation. By keeping groupings of embryos to less than 20 embryos, oxygen starvation should not be a problem.
ii. Problem: The agar pad dries on 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.
iii. Problem: 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.
iv. Problem: The slide has air bubbles under the coverslip.
Solution: Use more M9 buffer. This will allow M9 buffer to completely wash under 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 right side facing toward the objective lens. During later stages of embryogenesis embryos turn such that left-hand views become dorsal views and right-side views become ventral views. Embryos on agar mounts will survive and hatch from the eggshell on

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the mount. Embryos prepared with an agar mount are amenable to both light microscopy (with differential interference contrast optics) or confocal microscopy. Preparing *C. elegans* embryos on an agar mount is a simple technique that can easily be mastered and is regularly done by undergraduates. It provides a consistent embryonic orientation and environment that is suitable for long-term microscopy of *C. elegans* embryos.

2. Other Mounting Methods

For many morphogenetic events, agar mounts are convenient because they produce uniform orientation of developing embryos. However, there may be times when more randomized orientations are desired. Examples include imaging of the anterior of the embryo during head enclosure, or events during gastrulation when an *en face* view is desired. For these cases, other mounts are more useful. We discuss two types here. Because protocols for producing these mounts are published elsewhere, we only briefly mention them here.

a. **Simple poly-L-lysine mount** (see Mohler and Isaacson, 2010 for further details)
The simplest approach is to mouth pipette embryos in random orientations onto a poly-L-lysine coated coverslip supported by grease feet above a microscope slide.

   i. Spread a small volume of a 1 mg/mL stock of poly-L-lysine onto coverslips. Allow the coverslips to air-dry for >1 h. Poly-L-lysine is typically applied from a premixed stock solution in distilled water (Sigma). Frozen stocks can be aliquoted and stored at $-20\ ^{\circ}C$ indefinitely. Avoid refreezing.
   ii. Cut gravid hermaphrodites at the vulva in M9. Mouth pipette embryos in a small volume (approx. 3 μL) of water onto precoated coverslips.
   iii. Pipette a ring of silicon oil around the drop, and four dots of silicon vacuum grease (Dow Corning) to the corners of the coverslip. It is typically convenient to insert vacuum grease into a 10 mL plastic syringe without needle, from which it can then be extruded. The grease "feet" provide a spacer that allows a microscope slide to be affixed to the coverslip.
   iv. Inverted a slide over the coverslip to form the mount. Press gently to allow fluid to contact the slide.

b. **Liquid mount with bead spacer** (see Murray *et al.*, 2006 for further details)
As an alternative to the simple poly-L-lysine mount method, polymer beads are added to the medium to serve as spacers between the coverslip and the slide to prevent the embryo from being excessively compressed. If the bead diameter is <25 μm (e.g., 20 μm), then this will result in slight compression of the embryos and results similar to the standard Agar mount. If larger diameter beads are used (e.g., 30 μm), then poly-L-lysine should be used as above.

   i. Prepare a 1:30 dilution of 20 μm polymer beads (Polysciences, Inc., Warrington, PA; catalog #18329-5) mixed with M9 in a microfuge tube.

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ii. The bead mixture should then be resuspended with a pipette tip prior to use and added to the coverslip as above. Embryos can then be pipetted into the drop of bead slurry.
iii. No grease feet are needed using this method. Instead, seal the edges as described above for the standard agar mount, using Valap.

B. 4D Nomarski Imaging of Morphogenesis

1. Introduction

By its very nature, developmental biology requires thinking in four dimensions. Not only do embryos change dramatically over time, as the seemingly featureless single-celled zygote is transformed into an embryo with recognizable body axes and organ systems, but this remarkable transformation occurs in three spatial dimensions. The coordinated changes that occur within the developing embryo include carefully orchestrated signaling events, changes in gene expression, and morphogenetic movements, that is, regulated cell divisions and cell movements that sculpt the basic body plan as a recognizable organism emerges.

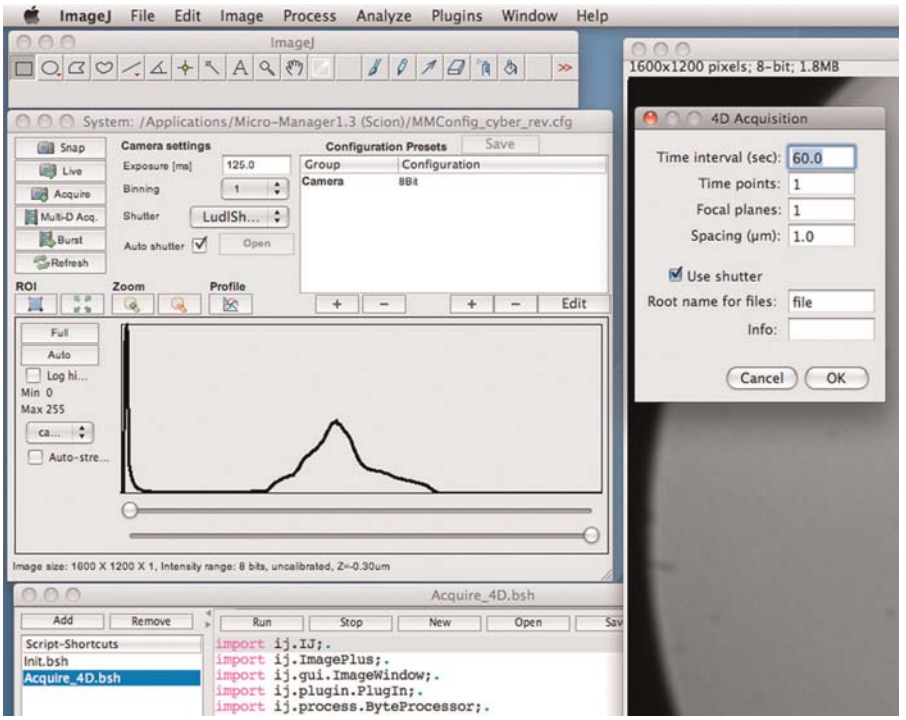

Fig. 2 4D acquisition script running within MicroManager. A screen shot of the Micro-Manager Beanshell script for 4D acquisition running under Mac OS X 10.5. The script is available at http://worms.zoology.wisc.edu/4d/4d.html. (For color version of this figure, the reader is referred to the web version of this book.)

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2. Acquisition of 4D Nomarski Images During Morphogenesis

Introduction

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 "four-dimensional" (4D) microscopy is a routine tool in laboratories that study early *C. elegans* development.

Imaging Setup

i. *Microscopy/camera hardware*: This protocol assumes a basic high numerical aperture (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 60–63×, 1.4–1.45 N.A. 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 Corporation. In this case, an AG-5 digitizing board (Scion Corporation) or similar video frame grabber can be used to digitize the video signal. Alternatively, modern cameras are almost exclusively CCD cameras, and have much higher spatial resolution than older video formats. We have successfully used cameras from Scion Corporation and QImaging Corporation. The mounting hardware for such devices differs depending on the microscope being used.

ii. *Cooled environment*: We have found that *C. elegans* embryos can be imaged for long periods of time if the ambient temperature is reduce to approximately 20 °C.

iii. *Z-axis controller/shutter/serial port*: A variety of Z-axis controllers are available from commercial sources (e.g., Prior, Ludl, 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 heat. A number of shutters are available. We have used shutters from Ludl and Vincent Associates.

iv. *Software*: Many commercial software packages can be used to acquire 4D footage. If an inexpensive alternative is desired, I have written free software as an alternative. Several options are available:

   (a) Legacy acquisition plugins for ImageJ: These plugins are available free of charge at the following URL: http://worms.zoology.wisc.edu/research/4d/4d.html. Full documentation of the plugins and detailed instructions for installation of ImageJ and Quicktime for Java are available at the same URL. Image J can be obtained at http://rsbweb.nih.gov/ij/

   (b) Micro-Manager: The public domain program Micro-Manager, which is built on top of ImageJ, supports a variety of CCD cameras, Z motors, and shutters, and, with some effort, novices can extend its functionality using the Beanshell scripting language or through the Java plugin architecture supported by ImageJ. Micro-Manager has a major advantage over other free

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alternatives, in that it is being continuously updated to support new hardware. The Micro-Manager program can be obtained at the following URL: http://www.micro-manager.org/. The remainder of this section will describe the basic use a Beanshell script written within the Micro-Manager environment as an example of how to acquire 4D data.

Methods

This protocol describes the use of a custom script within Micro-Manager's Beanshell scripting language. The script is helpful to reduce the number of shutter open/close events during 4D acquisition. Alternatively, we have used the standard Micro-Manager package successfully to acquire 4D footage, but this cannot be customized, and does not permit custom file naming conventions and directory structure. The script is available at http://worms.zoology.wisc.edu/4d/4d.html

i. *Setting up a 4D acquisition sequence*

a. 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 10× objective, prior to oiling the coverslip.
b. If a high NA condenser is present, place a drop of oil on the condenser (for upright microscopes) or 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 microscope slide.
c. 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 done, open the condenser.
d. Once embryos have been located at 10× and the condenser has been focused, swing the 10× objective out of the way and add a drop of immersion oil to the coverslip (upright microscope) or the 60× objective lens (inverted microscope). We find that Type DF oil works well.
e. Carefully slide the 60 or 100× 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.
f. Refocus on the embryos, and refocus the condenser.
g. Open Micro-Manager. Use the "Live" button in the main Micro-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:

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- Center the condenser by closing it and 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, gain, and other settings on the CCD camera within Micro-Manager a final time if needed.

h. Invoke the Micro-Manager 4D acquisition script (Fig. 2). This protocol presupposes that a "favorite" has been created previously using the script window in Micro-Manager. This window is invoked using the "Tools -> Script Panel" menu command in Micro-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, makes sure that the "Use shutter" option is selected.

i. 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 (s): 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.

j. 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.

k. When the movie is finished, we recommend collecting a terminal image stack. 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.

l. To view the movie, there are several options available:

   (i) Raw 4D datasets: These can be viewed in one of several ways, including (a) importing the sequences as a "Virtual 5D Stack" or Hyperstack within

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ImageJ, using the "Virtual 5D Stack" plugin or ImageJ itself, available on the ImageJ web site, or (b) using the "Browse4D" plugin available at http://worms.zoology.wisc.edu/4d/4d.html.

(ii) Compressed movies: Movies can also be compressed to save disk space and viewed using QuickTime and the "QT4D Writer" and "QT4D Player" plugins available at the same URL. The advantage of postacquisition compression is that the storage requirements are greatly reduced, and, if QuickTime is used, the entire dataset does not need to be imported into RAM or imported as a virtual stack. Unfortunately, Quicktime for Java has been deprecated by Apple Computer, Inc. If QuickTime compression 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," compression, "gray scale," and "Medium" quality. This approach can compress movies 30 fold. To play compressed movies, use the "QT4D Player" plugin. 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. The up and down arrow keys can be used to scroll up and down through the focal planes; the left and right arrow keys scroll backwards and forwards in time. Once the movie has been successfully compressed and its quality verified, for routine purposes it is now fine to delete the original files.

Troubleshooting

i. Problem: 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 Micro-Manager window.

ii. Problem: 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.

iii. Problem: 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, approximately 20 °C. Make sure the air conditioner is on and that the door remains closed.

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iv. Problem: After several hundred time points Micro-Manager reports an error from which it cannot recover.
Solution: Some users have reported errors under Micro-Manager when using USB-to-serial port adapters. This is known issue with version of Micro-Manager prior to 1.4. Using a PCI-based serial port card appears to alleviate this problem. Alternatively, acquire several shorter movies. We have successfully used this script under Micro-Manager for acquiring up to 150 time points with 25 focal planes/time point. We have had success acquiring long movies with Micro-Manager 1.46, Mac OS X 10.7, and a KeySpan USB-to-serial adapter.

Discussion

This procedure will result in the production of 4D datasets in the form of a series of consecutively named TIFF files that can be read by many different programs, including ImageJ, especially when supplemented with appropriate plugins. The reduced costs of such a system make this basic system feasible for teaching laboratories and research laboratories within limited funds. While we have described the use of such a setup for imaging *C. elegans* embryos, this apparatus is well suited to acquiring images of any transparent specimen.

3. Introducing Pharmacological Agents During 4D Acquisition (Adapted from Williams-Masson *et al.*, 1997)

Introduction

This procedure assumes a fairly standard laser ablation setup. Many *C. elegans* laboratories use a nitrogen laser to pump a tunable dye laser routed through the epifluorescence light path of the microscope. The dye cuvette typically contains Coumarin 440 dye, which can be obtained from Sigma or other suppliers, and is reconstituted in methanol at a concentration of 5 mM in methanol (see the protocol in Walston and Hardin, 2010 for further details). A common version of this setup is sold by Photonic Instruments, a division of Andor Corp., under the trade name Micropoint.

Methods

1. Creating the mount

   i. Cut gravid hermaphrodites at the vulva in water. Mouth pipette embryos in a small volume (approx. 3 μL) of water onto coverslips precoated with 1 mg/mL poly-L-lysine. Poly-L-lysine is typically applied from a premixed stock solution in distilled water (Sigma).
   ii. Allow embryos to settle for 30 s and then treat for 2 min with 100 μg of FITC-conjugated poly-L-lysine (Sigma). FITC-poly-L-lysine enhances the

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ability of a standard ablation laser tuned to 440 nm using Coumarin dye to perforate the eggshell.

iii. Rinse embryos three times with embryonic growth medium (EGM; for a detailed recipe, see Shelton and Bowerman, 1996) + 3 μg/mL Nile Blue A (Sigma) + 1–2 μg/mL cytochalasin D or nocodazole. The addition of Nile blue allows the assessment of perforation of the eggshell; permeabilized embryos will take up the dye into granules in gut cells.

iv. Cover with a 30 μL drop of EGM plus drug. Stock solutions of 2 mg/mL cytochalasin D or nocodazole (Sigma) in DMSO can be stored at 4 °C.

v. Pipette a ring of silicon oil around the drop, and four dots of silicon vacuum grease (Dow Corning) to the corners of the coverslip. It is typically convenient to insert vacuum grease into a 10 mL plastic syringe without needle, from which it can then be extruded. The grease "feet" provide a removable spacer that allows a microscope slide to be affixed to the coverslip.

vi. Invert a slide over the coverslip to form the mount. Press gently to allow fluid to contact the slide.

2. Perforating the eggshell

i. Determine the position of the ablation beam by moving to a region of the mount away from the embryos. Focus on the coverslip, and crack the coverslip using the laser. In the case of the standard manual Micropoint laser, a sliding neutral density grating can be used to attenuate the beam strength. Beam amplitude should be just sufficient to crack the coverslip. If more power is desired later, then the grating can be adjusted accordingly.

ii. Select an embryo of the desired developmental stage. Find a region of the embryo where there is a space between the cells and the eggshell. Embryos are typically oriented within the eggshell such that there is a larger space between the anterior end of the embryo and the eggshell than in other regions of the embryo, making this region convenient for laser irradiation.

iii. Position the embryo so that the eggshell of this region is over where the ablation laser will hit.

iv. Using a foot pedal or push button, pulse the laser. Usually only one hit is necessary, but sometimes more pulses are required.

v. It is often possible to tell that the eggshell is perforated because the embryo will move slightly toward the perforation site. However, we have found that sufficiently small holes will not induce this response, yet dye penetration will nevertheless be observed.

vi. If needed, additional perforations can be induced to increase the rate of penetration of the compound of interest. However, we have found that the number of viable embryos obtained in these cases goes down markedly.

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vii. These mounts can be imaged using 4D microscopy. We have also found it possible to remove the coverslip and process embryos for phalloidin staining (see above; for examples, see references).
viii. Embryos can be scored after 4 h for blue gut granules, which indicate that sufficient permeabilization was achieved for Nile Blue A penetration.

Discussion

Performing long-term 4D filming after perforation of the eggshell is difficult. Many embryos show abnormalities in subsequent development. Extensive negative controls (i.e., perforation of the eggshell in the presence of carrier, such as DMSO, alone) are therefore highly advisable. If sufficient precautions are taken, however, it is possible to perform pharmacological inhibition followed by 4D filming, as we have shown in several circumstances (Thomas-Virnig *et al.*, 2004; Williams-Masson *et al.*, 1997).

C. Fluorescence Imaging of Morphogenesis

1. Introduction: Imaging Modalities for 4D Fluorescence Imaging

Nomarski microscopy, while a daily workhorse for imaging morphogenesis and performing basic phenotyping, is limited. Refractile elements in the cytoplasm of embryonic cells, combined with the inherent curvature of the embryo, limits the resolution of the standard Nomarski microscope. In addition, the epidermis is exceedingly thin (less than 0.5 μm in some cases), making it difficult to resolve. Fluorescence imaging of specific structures in embryos, combined with confocal or multiphoton microscopy, overcomes these challenges. The chapter by Maddox and Maddox in this volume covers basic modalities of fluorescence microscopy. Here we discuss several useful strategies for visualizing cells and subcellular structures during morphogenesis, an alternative phalloidin staining procedure, and a simple strategy for analyzing fluorescence recovery after photobleaching (FRAP) data.

Given the wide array of genetically encoded fluorescent probes avaialble (see below), 4D datasets of fluorescent specimens acquired using confocal, multiphoton, or widefield deconvolution techniques have several advantages over 4D datasets acquired using transmitted light optics, such as Nomarski microscopy. First, such techniques permit much more refined optical sectioning of the specimen with little contribution by out-of-focus information. Secondly, it is much easier to understand the distribution of the fluorescent signal from a 3D reconstruction of a sample than a 3D-stack of DIC images (for an attempt at the latter, see Heid *et al.*, 2002). For thin specimens imaged within 5 μm of the coverslip such as *C. elegans* embryos, oil immersion optics and a high NA lens (NA = 1.4–1.45) are typically the best choice.

Although the use of fluorescent probes present several key advantages, it also presents several challenges for 4D imaging of morphogenesis. Because *C. elegans*

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embryos are not flat, multiple focal planes must be acquired at each successive time point, requiring repeated exposure of the region of interest (ROI) to excitation photons that compromise embryo viability. In addition, the photobleaching that accompanies repeated observation in 4D experiments complicates image quantification. If photobleaching is not too severe, it is sometimes possible to normalize the signal from bleached specimens using several algorithms (e.g., see the ImageJ plugin that is part of the McMaster Biophotonics suite of routines: http://www.macbiophotonics.ca/imagej/).

Several imaging modalities have been used frequently for analyzing morphogenesis. For the physical basis of each, readers should consult the chapter by Maddox and Maddox in this volume. Here we will focus on issues related to filming morphogenesis.

Laser scanning confocal microscopy (LSCM)

In some cases LSCM suffices. Depending on the density of fluorophore labeling and the nature of the tagged moiety, many experiments have been performed using rather unremarkable equipment, such as a Bio-Rad 1024 or similar older laser scanning confocal devices. Interested readers are urged to consult older reviews describing methods of confocal imaging in *C. elegans* (e.g., Mohler, 1999). LSCM is typically the mode of choice for performing single focal plane photobleaching experiments (see below). For long-term 4D acquisition in which many focal planes are acquired, LSCM quickly leads to arrest of embryos. The time required to induce arrest varies, depending on the ambient temperature and the probe being imaged. However, in general LSCM is not well suited to long-term viability of embryos filmed over many hours.

Multiphoton excitation laser scanning microscopy (MPLSM)

Multiphoton laser-scanning microscopy has several potential advantages over LSCM for live imaging of embryos. MPLSM excites fluorescence using a series of short, high-energy pulses of near-infrared photons from a mode-locked laser. MPLSM has a key advantage for live embryo imaging experiments in *C. elegan*s. In a two-photon microscope the probability of excitation varies as the inverse fourth power of the distance from the focal plane. Photons are thus only absorbed in a very small volume centered on the plane of focus, eliminating photobleaching and photodamage caused by excitation of fluorophores above and below the plane of focus. The resulting improvements in viability can be quite dramatic. In our laboratory, *C. elegans* embryos expressing a GFP-tagged junctional protein survive for 30–90 min when imaged using a Bio-Rad 1024 CLSM at low power (10%; J. Hardin, unpublished) but the same embryos can be imaged for many hours using MPLSM (Raich *et al.*, 1999). An example is shown in Fig. 3 (see Köppen *et al.*, 2001 for more details).

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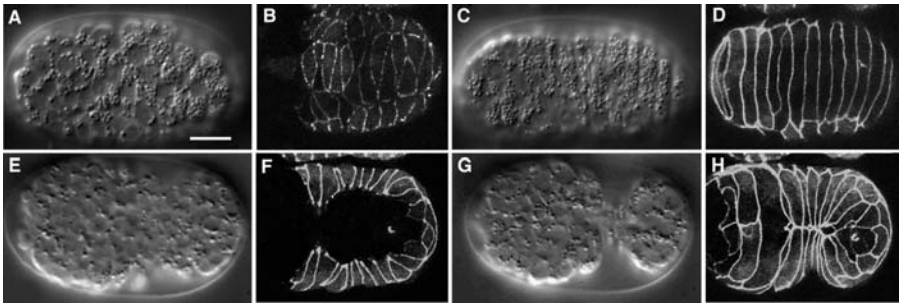

Fig. 3 Comparison of Nomarski 4D imaging (A, C, E, G) with the use of DLG-1::GFP as a junctional marker (B, D, F, H). (A, B) Early dorsal intercalation of epidermal cells. (C, D) Intercalation complete. (E, F) Early ventral enclosure. (G, H) Ventral enclosure complete. Bar = 5 μm.
(Adapted from Chisholm and Hardin, 2005).

Although MPLSM can be superior to CLSM for many applications, there are several issues that can make MPLSM less than optimal. First, the typical MPLSM device is expensive, placing it out of reach of most individual laboratories. In contrast, individual labs can often afford disk-scanning confocal microscopes, a factor that is particularly important for live embryo studies, which often monopolize microscope time. Second, for fluorophores that emit in the red portion of the visible spectrum, the wavelengths needed to generate a two-photon event are longer than those produced by the Ti:Sapphire lasers commonly used in commercial MPLSM devices. For such probes, Nd::YLF lasers, which emit at 1047 nm, are very effective (Mohler and White, 1998), but may not be readily available.

Disk-scanning confocal microscopy

For the developmental biologist, spinning disk systems based on Yokagawa scanheads are an inexpensive alternative that provides many of the benefits of more elaborate technologies, such as MPLSM. Because disk-scanning systems use an off-the-shelf focus motor, CCD, filter wheel, and shutter components, commercial imaging packages or freeware packages such as Micro-Manager can be used to drive data acquisition. In our laboratory, disk-scanning technology has largely replaced both CLSM and multiphoton microscopy for routine 4D data acquisition during morphogenesis (for examples, see Figs. 4A,B, 5, and 6B,C). The data generated by spinning disk can also be improved via postacquisition deconvolution, making its Z resolution comparable to LSCM or MPLSM.

Considerations for optimizing 4D data during morphogenesis

There are several thorough treatments of empirical considerations that lead to high-quality 4D fluorescence dataset (e.g., Hardin, 2006). Here, we will focus on

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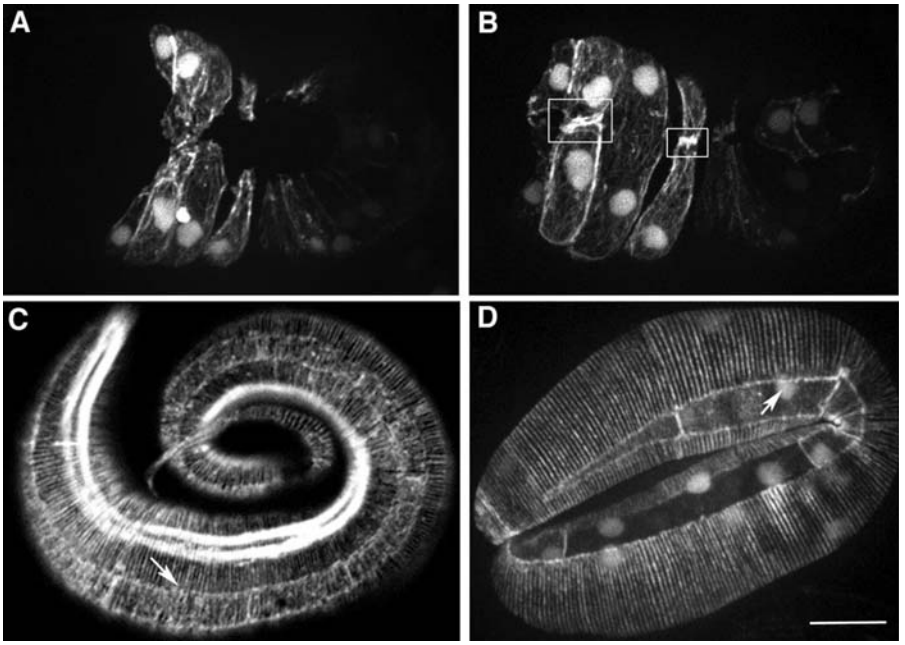

Fig. 4 Imaging of F-actin during morphogenesis. (A, B) F-actin dynamics during ventral enclosure imaged in a wild-type embryo expressing *vab-10::ABD::GFP*. Images were acquired using a Perkin-Elmer UltraView LCI system, with Hamamatsu Orca II-ER camera. The boxed regions in (B) show extensive actin accumulation between two pairs of ventral epidermal cells. Elapsed time between (A) and (B) is 1380s. (From Lockwood *et al.*, 2008). (C) Phalloidin staining of an elongated embryo. Circumferential filament bundles (CFBs) are clearly visible throughout the epidermis. The arrow points to junctional actin. (From Costa *et al.*, 1998). (D) A twofold stage embryo expressing VAB-10ABD::GFP. CFBs are prominent; arrow points to junctional actin. Image courtesy of R. Zaidel-Bar. Bars = 5 μm.

several key factors affecting 4D fluorescence acquisition during morphogenesis. First, as with 4D Nomarski imaging, ambient temperature must be controlled carefully. The temperature must be kept below 25 °C; for long films, a temperature closer to 20 °C is advisable. This is often not possible in shared user facilities, in which elevated temperatures suited to tissue culture work are the focus. Second, despite theoretical calculations of voxel sampling in Z stacks of fluorescent images, it is typically advisable to acquire very closely spaced optical sections if one is imaging events in the epidermis. We have found that focal planes spaced 0.5 μm apart or less are necessary, due to the extreme thinness of the epidermis. Finally, modern lenses with newer coatings make a significant difference. For very high-resolution filming of cytoskeletal elements or thin structures, we have found that very high NA lenses are helpful. In particular, we have found that lenses designed for total internal reflection microscopy (TIRF), but without the internal optics for TIRF itself, provide

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excellent results, such as recent vintage Nikon NA 1.45 oil immersion "TIRF" lenses.

Ultimately, the choice of a particular imaging modality is largely based on empirical issues, including the type of probe being imaged. We now turn to types of probes useful for analyzing morphogenesis.

2. Probes for Visualizing Morphogenesis

Introduction

The discovery and widespread use of variants of the green fluorescent protein (GFP) as a tag for visualizing gene expression and protein localization within living organisms has revolutionized live embryo imaging, including in *C. elegans*. Many of the probes we use routinely for analyzing morphogenetic events are genetically encoded. However, several other techniques have proven useful for studying morphogenesis in *C. elegans*.

Junctions

For studying morphogenetic movements in embryos, junction-localized FPs are extremely useful. Our laboratory used junction-localized FPs to study epithelial sheet movement in *C. elegans* early on, and these tools have become standard among *C. elegans* researchers. These include AJM-1 (Mohler *et al.*, 1998), DLG-1 (Köppen *et al.*, 2001), and HMP-1/α-catenin (Raich *et al.*, 1999). Others include JAC-1/p120ctn (Pettitt *et al.*, 2003) and HMR-1/cadherin (Achilleos *et al.*, 2010). The use of epithelial junctional markers is particularly useful for following cellular movements at single-cell resolution during events such as dorsal intercalation, ventral enclosure, and the early steps of elongation. The use of such markers provides much clearer views of morphogenetic movements than can often be achieved with Nomarski microscopy (Fig. 3). We have also found that it is possible to create transgenic lines expressing more than one FP-tagged junctional marker, such as *dlg-1::dsRed* and *hmp-1::gfp* (Zaidel-Bar *et al.*, 2010). In some cases, however, especially if two junctional proteins physically interact, coexpression can lead to artifactual aggregation of proteins (C. Lockwood and J. Hardin, unpublished).

Actin and tubulin

i. Actin

We have employed two basic methods to visualize the F-actin cytoskeleton in embryos during morphogenesis: (i) phalloidin staining of fixed specimens and (ii) imaging of the F-actin binding fragment of the spectraplakin, VAB-10, fused

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to GFP. Since phalloidin staining of F-actin containing structures is such an important technique for studying epidermal morphogenesis, we provide this alternative procedure to that described by Maddox and Maddox in Chapter XX. An example of an embryo stained with phalloidin to reveal actin-rich structures is shown in Fig. 4C.

(a) *Phalloidin staining for analyzing morphogenesis*
Method

1. Make solutions and coat ring slides with 25 μL poly-L-lysine.
2. Obtain eggs from adults by bleaching. Make sure to sweep embryos off the plate using a glass pipette.
    a. Hint: It is sometimes advantageous to bleach embryos for a slightly longer period of time to further weaken the eggshell. This can be done by allowing the embryos to remain in the bleach solution for another minute after the worm carcasses have all been dissolved.
3. Wash embryos at least 2× with ddH_2O. We find they stick to the slide better when using water, not M9.
4. Add embryos to ring slides that have been precoated with poly-L-lysine. Let sit 5–10 min to give embryos time to adhere.
    a. Hint: It helps the embryos to adhere when the slide is rinsed with ddH_2O before adding the embryos.
5. Remove the water from the ring slide. The bulk of the liquid can be poured off into a liquid waste container, if care is taken to avoid mixing embryos from one ring to another. Use a Kimwipe to gently remove the remaining liquid.
6. Add 45–60 μL of the fix/permeabilization solution to each ring.
    a. Incubate the slides for 20 min in a humid chamber at room temperature. If there are many embryos, it is advisable to incubate 2–5 min longer.
    b. A simple humid chamber can be made by placing wet paper towels in the bottom of a Tupperware container.
7. Wash slides 2 × 5 min with 1× PBS by adding 70 μL of PBS to each ring per wash. Remove liquid after each wash as above.
8. Add 60 μL phalloidin solution to each ring.
    a. Incubate 1–2 h in a humid chamber at room temperature in the dark (we usually incubate for 90 min). Alternatively, incubations can be carried out at 4 °C overnight in the dark, which can reduce background.
9. Wash slides 2 × 5 min with 1× PBS while rotating.
10. Dry off slide using a Kimwipe. Mount by inverting an 18 mm square coverslip with 8 μL SlowFade over each sample. Seal edges with nail polish.

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Reagents
Bleach Solutions

1. Bleach solution:
   a. 0.4 mL bleach
   b. 0.4 mL 10N KOH
   c. 3.2 mL water
2. Fix/permeabilization solution:
   a. 200 μL 10% PFA
   b. 10 μL 10% Triton X-100 (Sigma) or 5 μL 10 mg/mL lysolecithin in chloroform (Sigma). Lyoslecithin is much gentler than Triton, and will preserve fine structure, but also results in less overall extraction, leading to higher background signal in some cases. For preserving fine filaments in the epidermis, lyoslecithin is preferable.
   c. 48 μL 0.5 M PIPES (pH = 6.8)
   d. 25 μL 0.5 M HEPES (pH = 6.8)
   e. 1 μL 1 M $MgCl_2$
   f. 10 μL 0.5M EGTA
   g. 196 μL ddH_2O
3. Phalloidin solution:
   a. 6 μL 6.6 μM Alexa-488
   b. 114 μL 1 × PBS

Other solutions:

1. 10% PFA
   a. 1 g paraformaldehyde
   b. 30 μL 5N NaOH
   c. 10 mL 60 mM PIPES (1.2 mL 0.5M PIPES + 8.8 mL ddH_2O)

   Incubate ~30 min in 65 °C water bath until PFA is dissolved (note: do not exceed 65 °C!)
2. 10% Triton
   a. 1 mL Triton X-100
   b. 9 mL ddH_2O

   Place on a nutator platform to mix thoroughly
3. 0.5 M PIPES
4. 0.5 M HEPES
5. 1 M $MgCl_2$
   a. Dissolve 203.3 g of $MgCl_2 \cdot 6\ H_2O$ in 800 mL of dH_2O. Adjust the volume to 1 L with dH_2O. Dispense into aliquots and sterilize by autoclaving or filtering. Keep aliquots closed when they are not being used.

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6. 0.5M EGTA

   a. Add 190.175 g ethylene glycol bis(beta-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to 1 L of dH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by either autoclaving or filtering.

7. 1× PBS

   a. 137 mM NaCl
   b. 2.7 mM KCl
   c. 10 mM Na_2HPO_4
   d. 2 mM KH_2PO_4

   Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2 PO_4 in 800 mL dH_2O. Adjust the pH to 7.4 with HCl. Add dH_2O to 1 L. Autoclave to sterilize.

(b) *VAB-10ABD::GFP and other GFPs:* Phalloidin staining is indispensible for studying the F-actin cytoskeleton during morphogenesis. However, it is not suitable for capturing rapid, dynamic movements involving the actin cytoskeleton. GFP-tagged versions of monomeric subunits of actin, such as *act-2* (Willis *et al.*, 2006) can be useful for some purposes, but the GFP likely affects polymerization dynamics. An alternative approach for *in vivo* analysis in living embryo exploits F-actin-binding proteins. Two that have been used during early development are moesin::GFP (Velarde *et al.*, 2007) and Lifeact::GFP (Pohl and Bao, 2010). For the study of morphogenesis, the spectraplakin VAB-10, which contains both actin- and tubulin-binding motifs, was exploited by the Labouesse laboratory to produce a construct containing the actin-binding domain (ABD) of VAB-10 tagged with GFP or mCherry (Gally *et al.*, 2009). We have used this construct to image filopodial dynamics during ventral enclosure (Fig. 4A,B), as well as circumferential filaments network and junctional actin during elongation (Fig. 4C, D). Use of the VAB-10 constructs requires care; we have found that excessive imaging can lead to lethality in strains containing *vab-10ABD* transgenes. For short periods of time, however, these constructs are invaluable for imaging rapid actin dynamics. In addition, the original *vab-10ABD* strains are mosaically expressed. For some applications, this is actually an advantage, but for others it can be somewhat problematic.

ii Tubulin: Numerous antibodies can be used to visualize tubulin during morphogenesis, using standard freeze-cracking (Miller and Shakes, 1995). Alternatively, there are several GFP constructs that have been successfully used to visualize epidermal cells. A particularly useful set of such constructs have recently been published that use the *lbp-1* promoter to drive expression of either tubulin subunits or plus-end tracking proteins predominantly in epidermal cells. These include *Plbp-1::gfp::β-tubulin* and *Plbp-1::ebp-1/EB 1::gfp* (Fridolfsson and Starr, 2010). These

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should be especially useful for analyzing microtubule dynamics during morphogenesis.

iii Cell cortex/membranes: Several markers have been particularly useful for visualizing the cell cortex or membrane. These include a *ced-10/Rac::gfp* translational fusion (Liu *et al.*, 2007) and various PH-domain constructs fused to mCherry or GFP (e.g., Audhya *et al.*, 2005). An example of the use of *ced-10::gfp* is shown in Fig. 5. The interested reader is encouraged to consult the primary references for further details. An alternative technology for visualizing membranes involves the use of lipophilic dyes, such as the red dye FM4-64, which has been used to visualize cell fusion in the dorsal epidermis. Since the eggshell is impermeable to such dyes, they must be introduced via laser perforation of the eggshell as described above. In the case of FM4-64, its excitation wavelength requires the use of fairly specialized lasers if multiphoton excitation is used (Mohler *et al.*, 1998).

iv Cytosolic markers: To developmental biologists, fusing the coding region of EGFP to the regulatory DNA associated with a gene of interest (i.e., GFP "reporter constructs") is often used to assess the tissue-specific and temporal patterns of transcriptional activation of a gene. Such information provides valuable information about how the expression of a gene is regulated. However, such transcriptional reporters can also be invaluable for live embryo imaging for several reasons. First, such reporter constructs result in the expression of GPF in the cytosol; because GFP is fairly small, these reporters are capable of percolating into small volumes within the cytoplasm, including fine protrusions extended by cells as they migrate. Second, the highly specific pattern of expression of some genes allows either many or a very small number of cells to be visualized against a dark background,

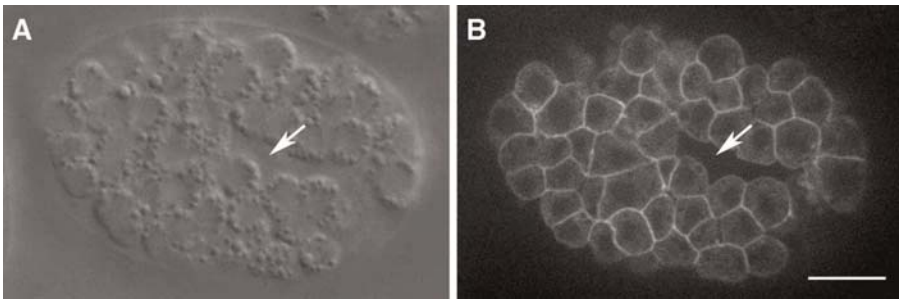

Fig. 5 CED-10/Rac::GFP allows imaging of cell cortices during morphogenesis. The same embryo expressing *ced-10::gfp* (Liu *et al.*, 2007) was imaged using fluorescence and Nomarski microscopy. The arrows indicate the closing ventral gastrulation cleft. Images were acquired using a Perkin-Elmer UltraView LCI system, with Hamamatsu Orca II-ER camera. Images courtesy of R. Zaidel-Bar. Bar = 5 μm.

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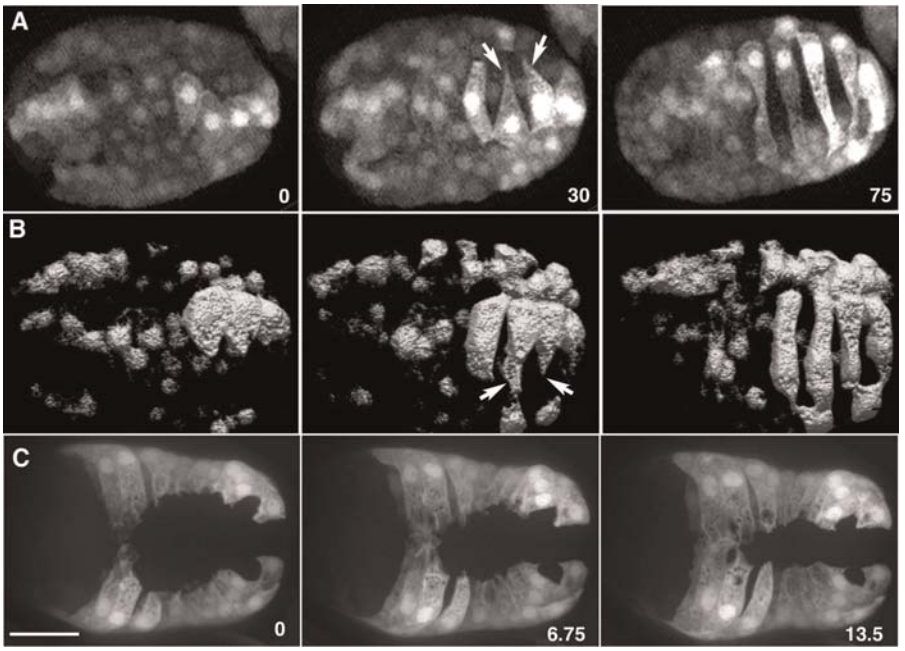

Fig. 6 Cytosolic markers allow imaging of cell motility and cell movement in embryos. (A, B) Frames from 4D movies of dorsal intercalation in embryos expressing *lbp-1p::gfp*, which is expressed in a subset of dorsal epidermal cells. (A) An embryo imaged using two-photon excitation microscopy. *z*-stacks were subsequently projected using a maximum intensity procedure. (From Heid *et al.*, 2001). (B) A similar embryo imaged using a Perkin-Elmer UltraView LCI system (with Yokagawa CSU10 scanhead; images courtesy of T. Walston). The dataset was subsequently subjected to surface rendering using Volocity software. Fine protrusions are visible in both cases. It is clear in (B) that the protrusions are wedge-shaped in the *z*-dimension. (C) Frames from a 4D movie of ventral enclosure in an embryo expressing a *Pdlg-1::gfp* reporter. Elapsed time in minutes is shown. *z*-stacks were acquired at 50 s intervals, 20 focal planes/stack; acquisition time/image, 300 ms. Fine details of protrusions are visible against a dark background using this particular transcriptional reporter. (Images courtesy of M. Sheffield.) Bar = 10 μm.

dramatically improving the effective contrast of the specimen being imaged. Third, imaging cytosolic GFP reporters typically does not cause as much photodamage as with GFP translational fusions. We have frequently used two different markers to image events in the epidermis: *Plbp-1::gfp* (Heid *et al.*, 2001) and *Pdlg-1::gfp* (Sheffield *et al.*, 2007). An example of the former is shown in Fig. 6A–B, and the latter in Fig. 6C. An extremely useful adjunct to the use of cytosolic markers is the use of a voxel rendering program, such as Volocity (Perkin-Elmer). Fig. 6B shows the results this procedure in the case of intercalation of dorsal epidermal cells. The result is a striking 3D view of cells as they intercalate.

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3. FRAP

Introduction

In addition to imaging specific structures within embryos during morphogenesis, fluorescence can also be used to study the dynamics of redistribution of molecules in embryos using FRAP. GFP can be used very successfully in studies involving FRAP (Lippincott-Schwartz *et al.*, 2003), which is easily done via repeated scanning of a selected ROI in the LSCM. FRAP has been used in several contexts in *C. elegans*, particularly in one-celled zygotes (e.g., Labbe *et al.*, 2004).

Methods

1. *Acquiring FRAP data*
   The acquisition of the raw data to be analyzed in a FRAP is highly dependent on the apparatus and acquisition software, so it will not be discussed here in detail. We have been able to successfully photobleach single junctional domains in embryos undergoing morphogenesis. In our case, these experiments have been performed using an Olympus Fluoview 1000 with SIM scanner for rapid photobleaching with simultaneous imaging (Fig. 7A,B). In general, the conditions that allow for successful imaging via laser scanning confocal microscopy pertain. We have found that single focal planes can be imaged in embryos in agar mounts quite successfully. We have also found several specific considerations to be important in performing FRAP during morphogenesis:

   a. During morphogenesis, embryos may move/twitch slightly. The bleach zone ROI must be chosen such that the bleach zone does not move out of the ROI. For FRAP of junctional GFPs, this can be accomplished by altering the size and/or aspect ratio of the ROI. Note that as a result, FRAP is practically limited to early phases of elongation; after this time, embryos twitch too much to make reliable measurements.
   b. Embryos in which FRAP is performed at temperatures exceeding 27 °C may show erratic results. This is likely due to overall sickness of embryos at elevated temperatures. If possible, filming should occur at $\leq$25 °C.

2. *Analyzing FRAP data: Simplified FRAP analysis using ImageJ*
   Once obtained, analysis of the recovery kinetics of a bleached region in a FRAP experiment proceeds along similar lines. There are many options for analyzing data from FRAP experiments. The technique we describe here is a free, semi-automated solution suitable for use by undergraduates, and relies on plugins we have adapted from original Java routines written by Tony Collins, McMaster University (Collins, 2007). This plugin is available for download from http://worms.zoology.wisc.edu/research/4d/4d.html. To use, install the plugin by copying to a convenient directory within the Plugins directory in ImageJ. For more information, see the ImageJ web site (http://rsbweb.nih.gov/ij/).

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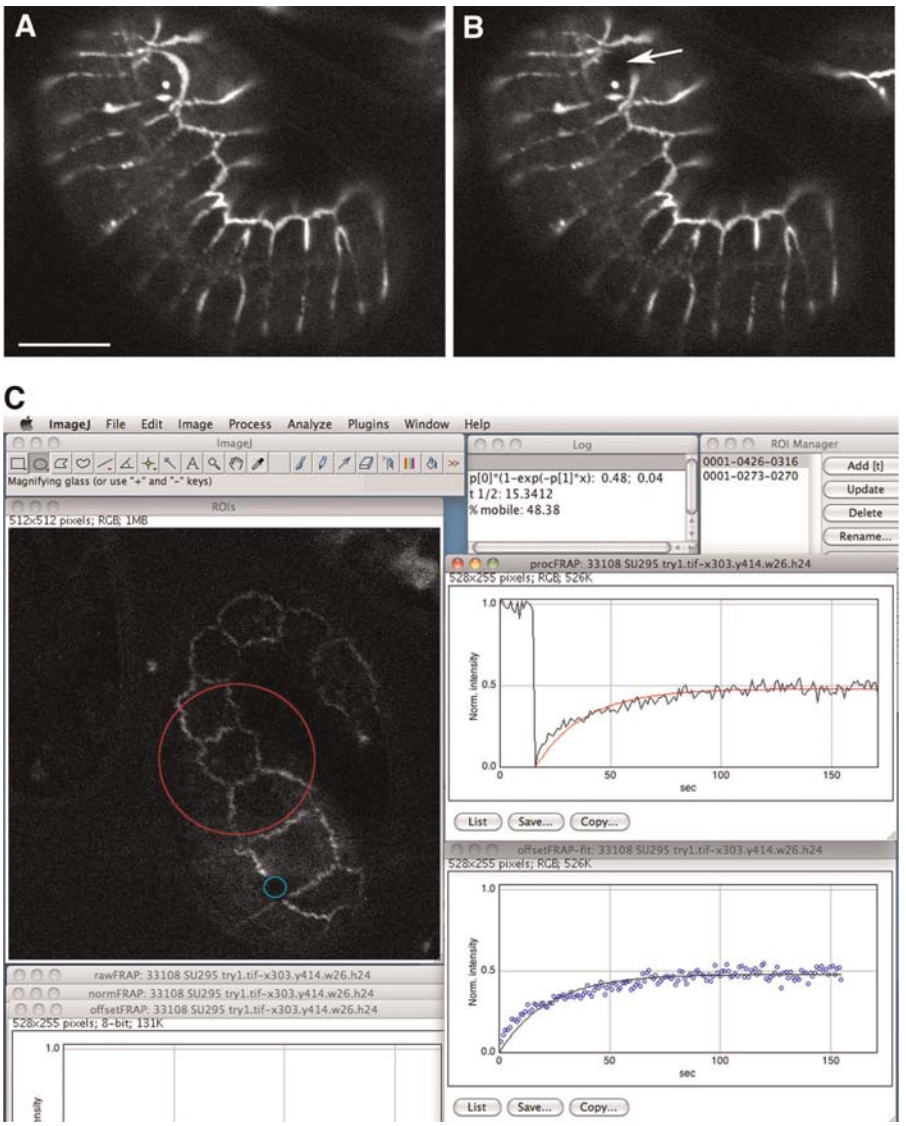

Fig. 7 Fluorescence recovery after photobleaching (FRAP) analysis of junctional protein mobility. (A) An embryo expressing *hmr-1::gfp* immediately prior to bleaching (image courtesy of J. Keegan). (B) The same embryo after bleaching. The arrow indicates the region that was bleached. Bar = 5 μm. (C) Screen capture of the output from the ImageJ-based FRAP profiler plugin. The example shown is analysis of mobility of *jac-1::gfp* (raw images courtesy of A. Lynch). (For color version of this figure, the reader is referred to the web version of this book.)

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3. *Importing data from the confocal microscope*

   a. *Invoke the LOCI Bio-Formats Importer plugin within ImageJ*: Many standard installations of ImageJ, or the package installer based variant of ImageJ, Fiji (http://pacific.mpi-cbg.de/wiki/index.php/Fiji) may have Bio-Formats Importer installed, or it can be obtained from http://www.loci.wisc.edu/software/bio-formats. The Bio-Formats plugin can read many data formats, including most raw image formats generated by standard confocal microscopes. We have tested this code using datasets from an Olympus Fluoview 1000 (.oif format files), but the basic approach should work for other types of datasets.
   b. *Import the dataset as an image stack into ImageJ*: After opening the Bio-Formats plugin, select a file within the directory containing the dataset, and import the data. Our setup uses a SIM scanner for photobleaching. The frame in which the bleach is applied often has high background. This frame can be deleted. The Bio-Formats plugin also often imports a few extraneous files into the stack that should be deleted before proceeding.

4. *Selecting ROIs for FRAP analysis*

   a. Open the ROI Manager in ImageJ: Once the stack has been opened and cleaned up, open the ROI Manager [ImageJ→Analyze→Tools→ROI Manager].
   b. Define bleach zone ROI: Navigate to the frame immediately after the bleach pulse was applied. Select the elliptical marquee tool in ImageJ from the main ImageJ window, and drag out a ROI that encompasses the bleach region. Once selected, add it to the ROI Manager list by typing [t].
   c. Define control ROI: A second ROI must be selected which serves as a control for the overall bleaching of the specimen during postbleach filming. This ROI can be a large region of the embryo. For FRAP of junctions, we typically select a large, but comparable, region containing the unbleached junctions. Once selected, add it to the ROI Manager list by typing [t].
   d. Select the bleach zone ROI in the ROI Manager list to reselect it.

5. *Output of the plugin*

   1. Run the FRAP Profiler plugin [ImageJ→Plugins→ (location where the plugin was installed)]. A dialog will appear with pull-down menus for selecting various options. These include:

      i. Single or double exponential curve fitting. Choose the latter if it appears, or there is physical reason to suspect, a two-step recovery process.
      ii. The real-world time interval between successive images in the movie.
      iii. The option to produce an image showing the bleach ROI and the control ROI overlaid on the first postbleach image.

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2. The FRAP Profiler plugin generates several windows (Fig. 7C):
   i. A log file with the parameters of the curve fit (half-life, immobile fraction, and constants). For a single exponential curve fit, the form of the equation with constants is $p[0]*(1–e\ (–p[1]*x)$.
   ii. If the option was selected, a window containing an image showing the two ROIs.
   iii. Graphs of (i) the raw FRAP data; (ii) normalized FRAP data; and (iii) curve fits of the normalized data, including curves with the first postbleach time point set as the origin (i.e., $t = 0$). By clicking the "List. . ." button, the data in the latter can be exported to a spreadsheet or curve-fitting program for further analysis.

D. Correlative Fluorescence and TEM During Morphogenesis (see Sims and Hardin, 2007 for more details)

1. Introduction

This final section describes high-pressure freezing (HPF) techniques for correlative light and electron microscopy on the same sample, starting with embryos mounted for 4D microscopy. Fluorescence information from a whole mount can be displayed as a color overlay on transmission electron microscopy (TEM) images to generate what we have termed fluorescence-integrated TEM (F-TEM) images. An alternative for postembedding correlative TEM is described elsewhere (Sims and Hardin, 2007). The method we describe here uses a thin two-part agarose pad to immobilize live *C. elegans* embryos for LSCM, HPF, and TEM. Pre-embedding F-TEM images display fluorescent information collected from a whole mount of live embryos onto all thin sections collected from that sample. For typical uses in our laboratory, this method relies on creating a strain with a rescuing array containing a GFP tagged protein of interest, often rescuing a lethal mutation (e.g., *ajm-1*; Köppen *et al.*, 2001). Embryos that have lost the rescuing transgene arrest. Alternatively, transgenes can be used as simple markers to identify the genotype of embryos. Because *C. elegans* embryos are difficult to fix with conventional chemical fixation, HPF is the method of choice. The agarose mount we describe for embryos is used for ultrastructural analysis, using freeze substitution with 1% osmium and 0.1% uranyl acetate. The agarose pad is made of a thin base of high-strength agarose. The thin base layer provides the strength and toughness to keep the mount intact. An agarose pad composed only of low-melting agarose would not hold the pad together well enough to allow transfer to a HPF specimen carrier. Additionally, it is necessary for the top agarose layer to be very thin to allow imaging in a confocal microscope. The method described here is specifically designed to be used at the conclusion of a 4D experiment. An alternative correlative procedure has been described elsewhere (Kolotuev *et al.*, 2010).

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2. Materials

General Materials

1. Glass slides, glass coverslip (22-mm square), and standard implements for preparing embryos for agar mounts (see the first section of this chapter).
2. High-pressure freezing specimen carriers.
3. l-hexadecene. l-hexadecene is used to coat the top specimen carrier to promote release of carriers after freezing and as filler in the bottom carrier as described by McDonald (1999)
4. High-pressure freezer (we used the BAL-TEC HPM 010).
5. Petri dishes to make a humid chamber (95 and 60-mm diam. dishes).
6. 20-mL scintillation vials for resin infiltration.
7. 3-Aminopropyltriethoxy-silane, 3-APTS (Sigma, A-3648).
8. Agarose (1% gel strength of 1000 g/cm^2 or greater for base; Invitrogen, cal. no. 15510-027). The strength of agarose can be tested empirically by making a thin pad of agarose over a slide. If the entire pad can be lifted off the slide without tearing, using a razor blade under one corner of the pad, it is strong enough to be used as a base for the correlative pad. We start with a 5% solution of agarose and dilute to between 4% and 5% with additional 0.1M HEPES as needed to form the base pad.
9. Low melting temperature agarose (Sigma, A-9539) to immobilize embryos.
10. 2 mL polypropylene vials with screw cap lids for freeze substitution.
11. Styrofoam box, dry ice, rotary shaker in 4 °C cold room. Freeze substitution using a Styrofoam box has been described by McDonald (1999). We fit the aluminum block into a tight-fitting piece of Styrofoam, which holds the block in place as the dry ice sublimes. We do not monitor the temperature during freeze substitution.
12. Disposable polyethylene pipettes (Fisher, cat no. 12-711-7). Any 3 mL or smaller disposable pipette with its own bulb should suffice. The use of disposable pipettes for dispensing fixatives avoids contaminating more expensive pipettes.
13. Microtiter plate shaker and smaller Styrofoam box in −20 °C freezer.
14. Single-edged razor blade. A single-edged razor blade is used to cut out a small piece of agarose containing the embryos to fit within the 2 mm specimen holder. The edge of a razor blade or a fine-tipped weighing spatula can also be used to transfer to the specimen carrier.
15. Sharpened tooth picks.
16. Rain-X (UnelkoCorp., Scottsdale, AZ) to coat slides for flat embedding. Two standard 1 × 3-inch microscope slides are coated with Rain-X or Teflon release agent using a cotton tipped applicator or Kimwipe. Coat each slide 3× and buff clear with a Kimwipe. This coating prevents the epoxy resin from gluing the slides together.
17. Clear acetate tape (Scotch brand or similar) for use as a spacer.

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18. Richardson's stain for thick sections. To prepare, make: A. 1% methylene blue in 1% borax (w/v in dH20) and B. 1% Azure II in dH20. Mix equal volumes of A and B and apply to sections with a syringe equipped with a syringe filter to remove precipitates.

Media

1. Use 0.1 HEPES (*N*-Hydroxyethylpiperazine-N′-2-ethanesulfonate) buffer to prepare agarose. A stock solution of 1*M* HEPES buffer is prepared by the addition of a solution of HEPES acid to a solution of HEPES base (sodium salt): 13.01 g of HEPES sodium salt is added to 50 mL of dH_2O.
2. The freeze substitution medium is 1% osmium tetroxide with 0.1% uranyl acetate in acetone (McDonald, 1999). Briefly, to prepare 25 mL of 1% osmium with 0.1% uranyl acetate, cool 24 mL of EM-grade acetone in a disposable 50 mL polypropylene tube on crushed dry ice. If pure OsO_4 crystals are not consolidated in the bottom of the vial, freeze the unopened vial of solid osmium in liquid nitrogen. Osmium crystals will fall to the bottom of the ampule. Add 1 to 2 mL of the cold acetone to the ampule, mix, and add back to the 50 mL tube of acetone on dry ice. Repeat until all of the osmium is dissolved in 25 mL of acetone. Add the UA in methanol (0.025 g UA in 1 mL of methanol) to the acetone, keep cold on dry ice. Add 1 mL of freeze substitution mix (1% osmium tetroxide with 0.1% uranyl acetate in acetone) to each 2 mL substitution vial and freeze in liquid nitrogen.

3. Methods

a. *An Agarose Mount for Live Embryos*

   i. A thin (high-strength) agarose pad is formed over a standard glass microscope slide. Agarose is dissolved in 0.1M HEPES (neutral pH) buffer to a final concentration of 4% to 5%. The thickness of the pad can be controlled by adding a single layer of cellulose tape over two slides on either side of the slide to be coated. Add 100 μL of melted agarose to the top of the center slide and compress the hot agarose to the thickness of a layer of Scotch tape (approximately 60 μm) with a fourth slide resting on the two adjacent tape-covered slides. Allow the agarose to solidify before sliding the top slide off. Place the slide with the agarose pad in a humid chamber. A humid chamber can be made by placing a 25-mm diameter Petri dish inside a 95-mm Petri dish and adding water to cover the bottom of the large dish.
   ii. *C. elegans* embryos are obtained by cutting open gravid hermaphrodites in a watch glass filled with distilled water as described for standard agar mounts. Only one group of approximately 10 embryos should be used. This grouping typically fills the field of view when using a 60–63× objective. Larger groups of embryos are impractical and difficult to navigate in the TEM.
   iii. Place 70 μL of 5% low melting temperature agarose dissolved in 0.1M HEPES along one edge of the agar pad. Quickly position a glass coverslip

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over the low-melt agarose to spread it out before it solidifies. Spacers can be used on either side of the slide to obtain the correct thickness. Use the edge of a slide resting on two spacer slides on either side of the slide containing embryos to apply pressure to the coverslip and compress the agarose to a uniform thickness. Ideal mount thickness is 100 μm, which is the thickness of the smallest HPF specimen carrier configuration. To obtain agarose of the right consistency, heat the low melting temperature agarose to boiling in a glass test tube. The agarose is most easily dispensed by cutting 5–10 cm off the end of a yellow pipette tip and preheating the tip by rotating in the hot agarose. Fill the tip with 70 μL of hot agarose and then dispense the agarose in a line along the edge side of the slide, over the high-strength agarose. The actual volume of agarose dispensed on the pad is less than 70 μL, as about half (or more) of the agarose stays in the tip. Use the 70 μL as a starting point and adjust as needed. This small volume cools rapidly, so the coverslip must be quickly placed over the low melting temperature agarose and gently pressed down to spread a thin layer of agarose around the embryos. A microscope slide turned on edge can be used to apply pressure to the coverslip on either side of the embryos but not directly over the embryos. The top layer of agarose is thinner than the high melting temperature agarose used to make the base. If the mount is too thick, embryos will be beyond the focal depth of the confocal microscope, making it impossible to acquire a fluorescent image. Note that covering embryos with hot low melting temperature agarose does not work with embryos that have been bleached, because they are too fragile.

iv. Seal the edges of the slide with Valap to prevent dehydration.

v. Acquire a focal series through the group of embryos at 1 μm intervals using the appropriate excitation wavelength (488 nm for GFP). A transmitted light image can be acquired simultaneously with each fluorescent image of GFP expression. In our experiments, embryos must attain a minimum age to be able to identify "mutant" from rescued embryos. The transmitted light image can be used to confirm the developmental stage of embryos.

b. HPF and Freeze Substitution

i. Configure HPF specimen carriers to provide a 100- or 200-μm deep well. To allow access to the agarose-immobilized embryos, scrape the Valap off the slide (which has already been imaged in a confocal microscope), and push the coverslip horizontally off the agarose pad using a single-edged razor blade. Cut out a small square of the agarose including the embedded embryos (maximum size is 2 mm diameter) and transfer this small pad to a bottom specimen carrier using a razor blade. The agarose pad can be pushed into a specimen carrier using a sharpened toothpick that has been coated with 1-hexadecene to keep the toothpick from adhering to the agarose pad. Loading tiny samples into specimen carriers becomes a battle with surface tension. Adhering carriers to the tops of Petri dishes with double stick tape holds carriers in place during the loading process. Double

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stick tape is available in two types: "permanent" and a less aggressive "removable" (Scotch 667 from 3M). The less aggressive tape holds carriers without having to fight to remove them for loading in the freezing holder, just prior to freezing. The specimen carrier can be filled with bacteria and 1-hexadecene.

ii. HPF is preformed in a BAL-TEC HPM 010. Readers unfamiliar with HPF and general issues related to successful specimen preparation should consult McDonald (1999), including filling of specimen carriers, removal of the specimen holder, and separation of specimen carriers.

iii. The use of a brass bottom and aluminum top (or vice versa) allows for quick identification of the bottom carrier, which is transferred, while under LN_2, to a polypropylene freeze substitution vial filled with frozen 1% osmium tetroxide and 0.1% uranyl acetate in acetone. The vial is capped and transferred to an aluminum block also cooled in liquid nitrogen.

iv. The empty holes in the aluminum block are filled with liquid nitrogen and the block is wrapped in aluminum foil and packed with crushed dry ice in a Styrofoam box taped to a rotary shaker. The box is shaken at 100 rpm for 3 to 4 days at −80 °C.

v. Shake vials on a microtiter plate shaker at 100 rpm for 2 to 3 days at −20 °C.

vi. Warm to 4 °C overnight, transfer to room temperature, and rinse with three to four changes of dry acetone. Agarose blocks can usually be identified by the presence of the embryos.

c. *Epoxy Infiltration and Polymerization for Agarose-Embedded Embryos*

i. Transfer freeze substituted specimens into 20-mL scintillation vials (wash and oven dry) containing 30% Epon in acetone (EM grade) rotating on a rotary mixer for 4 h to overnight at room temperature.

ii. 50% and 75% Epon in acetone for 2 h each at room temperature.

iii. Three changes of 100% Epon for 1 h each at 50–60 °C.

iv. Transfer the resin-infiltrated agarose pad to a Rain-X or Teflon coated slide with some fresh resin. Place two thicknesses of Parafilm on both ends of the slide as a spacer and place a second coated (Rain-X) slide over the Epon-infiltrated agarose.

v. Polymerize on a flat surface in a 60 °C oven for 24 to 48 h.

vi. Remove the resin from between the slides, rough up one side by scraping a razor blade across the surface, and mount/glue on a blank Epon block for sectioning.

vii. One side of the now polymerized agarose pad containing embryos is roughed up to remove residual Rain-X or Teflon release agent and to increase the surface area for adhesion to an Epoxy blank. The small sample piece can be attached to the blank using glue or additional Epoxy resin followed by polymerization in a 60 °C oven. This orients the embryos parallel with the cutting plane to obtain a similar orientation in the TEM as the view already obtained by LSCM.

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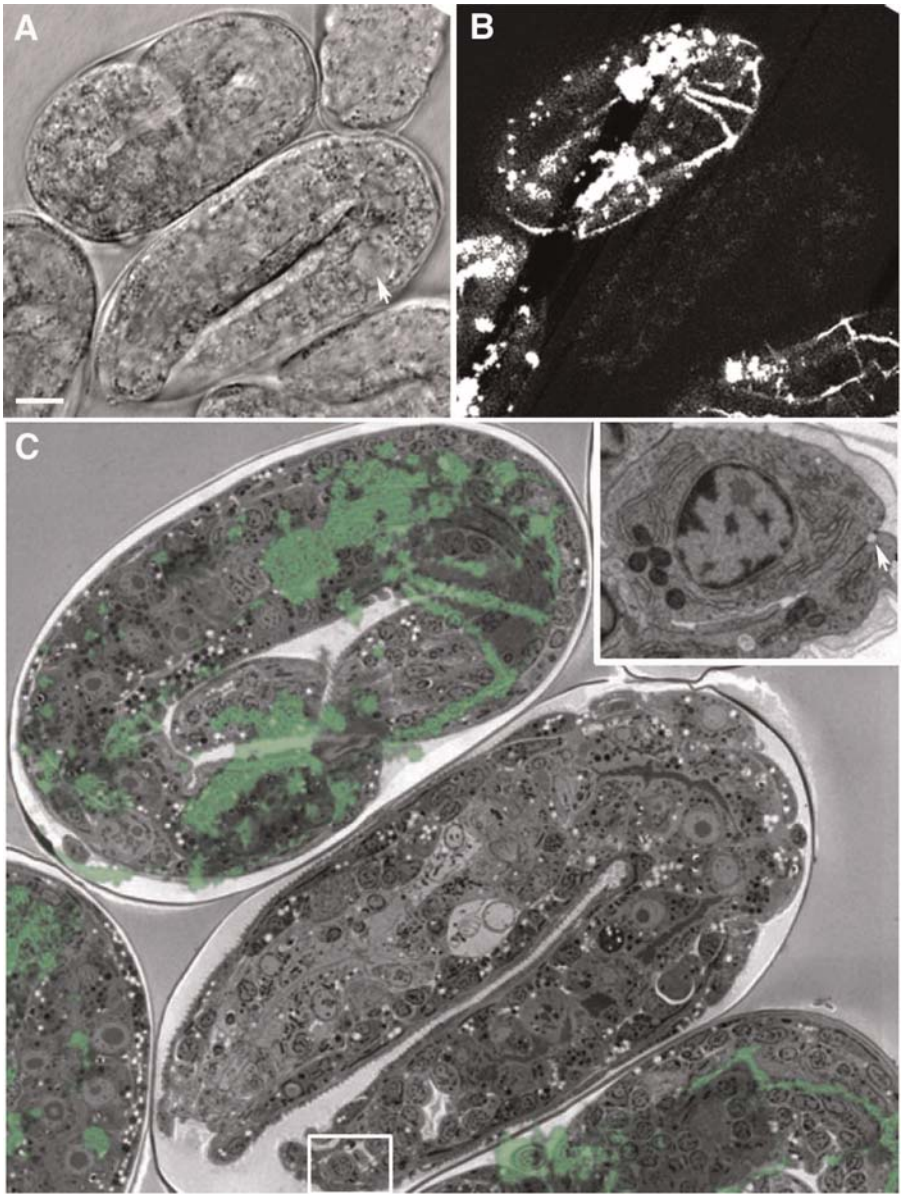

Fig. 8 (A) A transmitted light image of live *ajm-1(ok160);ajm-1::gfp* embryos embedded in agar. An embryo lacking *ajm-1* function has developed a characteristic vacuole (arrow). (B) A maximum intensity projection of AJM-1::GFP expression in the same embryos. Transmitted light and fluorescent images were obtained simultaneously on a Bio-Rad 1024 confocal using 488 nm excitation. Embryos that have lost the transgene can be easily distinguished from those that express it. (C) A TEM image of the same embryos after HPF and embedding in Epon. The fluorescence signal can be superimposed on the low-magnification transmission electron micrograph. The inset shows the small area within the box. The arrow points to epithelial cell membrane separations associated with the loss of *ajm*-1 function. Bar = 5 μm. (For color version of this figure, the reader is referred to the web version of this book.)

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viii. Cut 0.5-μm sections and place on 3-APTS coated coverslips. To coat 12-mm round coverslip with 3-APTS: clean 12-mm round coverslips in detergent solution. Rinse 10× in dH2O. Dehydrate using three changes in absolute ethanol. Coat slides in 2% solution of 3-APTS in dry acetone for several minutes. Rinse twice in dH2O. Spread out coverslips on clean filter paper and dry at room temperature. Stain thick sections with Richardson's stain. Rinse coverslips gently after staining with distilled water. Cut thin and/or semithin sections after embryos are detected in thick sections by light microscopy.

d. *TEM*
Collect thin sections on formvar-coated slot grids to provide an unobstructed view of an entire section. Stain thin and ultrathin sections for 10–20 min in 1% aqueous uranyl acetate, followed by 3 min in Reynolds' lead before viewing in a TEM. Thinner sections may require longer staining times. Large montage images can be collected manually and "stitched together" in Photoshop (Adobe) or collected and montaged automatically with analySISTM or similar software. When comparing different embryos within the same mount, it is helpful to make a map using a transmitted light image of the embedded group of embryos. Number the "mutant" and rescued embryos to keep track of higher magnification images. Because a TEM image may be a mirror image of the transmitted light image, flip the image in Photoshop with the same numbering scheme to be prepared for either orientation.

e. *Overlaying Correlative Microscopic Images*
A single transmitted light image is typically chosen from the multiple focal planes acquired based on features of interest that were in focus. The fluorescent image is a brightest point *z* projection (ImageJ) of all fluorescent images acquired. Because embryos are alive at the time of image capture, and hence may be moving, the fluorescent image(s) may be blurred. The detection of GFP confirms the presence of the rescuing DNA and definitively identifies the genotype of embryos. Confocal fluorescent and transmitted light images are manipulated with ImageJ and Adobe Photoshop. LSCM images are prepared using ImageJ. Some of the light (LSCM) and EM images collected may be mirror images of each other; they can be corrected in Photoshop. LSCM and TEM images are aligned starting with the TEM image, which usually contains more pixels. Fig. 8 demonstrates the results of this correlative method.

III. Summary

C. elegans embryos are a powerful model system for imaging detailed cell movements, intercellular dynamics, and the overall shape changes that occur during morphogenesis. With the widespread use of genetically encoded fluorescent markers and the ability to perform correlative electron microscopy on transgenic embryos, the virtues that originally led to the selection of *C. elegans* as a model organism have been expanded. In the future, other extensions of live imaging, such as *in vivo*

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fluorescence resonance energy transfer (FRET) and super resolution microscopy, will be added to the routine repertoire of developmental biologists using *C. elegans* to study morphogenesis.

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