

Video Article

Time-lapse Microscopy of Early Embryogenesis in *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans has often been used as a model system in studies of early developmental processes. The transparency of the embryos, the genetic resources, and the relative ease of transformation are qualities that make *C. elegans* an excellent model for early embryogenesis. Laser-based confocal microscopy and fluorescently labeled tags allow researchers to follow specific cellular structures and proteins in the developing embryo. For example, one can follow specific organelles, such as lysosomes or mitochondria, using fluorescently labeled dyes. These dyes can be delivered to the early embryo by means of microinjection into the adult gonad. Also, the localization of specific proteins can be followed using fluorescent protein tags. Examples are presented here demonstrating the use of a fluorescent lysosomal dye as well as fluorescently tagged histone and ubiquitin proteins. The labeled histone is used to visualize the DNA and thus identify the stage of the cell cycle. GFP-tagged ubiquitin reveals the dynamics of ubiquitinated vesicles in the early embryo. Observations of labeled lysosomes and GFP::ubiquitin can be used to determine if there is colocalization between ubiquitinated vesicles and lysosomes. A technique for the microinjection of the lysosomal dye is presented. Techniques for generating transgenic strains are presented elsewhere (1, 2). For imaging, embryos are cut out of adult hermaphrodite nematodes and mounted onto 2% agarose pads followed by time-lapse microscopy on a standard laser scanning confocal microscope or a spinning disk confocal microscope. This methodology provides for the high resolution visualization of early embryogenesis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2852/>

Protocol

1. Nematode cultures

1. Obtain the appropriate *C. elegans* strain from the *Caenorhabditis* Genetics Stock Center (CGC) or from a colleague.
2. Grow nematodes on NGM agar plates seeded with an OP50 bacterial lawn (3). For analysis of GFP strains growth at 25°C is recommended.
3. The day before your microscopy experiment, pick at least 40 L4 larvae onto seeded plates and place the plates at 25°C overnight. These worms will be young adults for the experiment.

2. Injections

If it is desirable to view structures such as lysosomes or mitochondria, adults can be injected with fluorescent dye prior to visualization.

1. Prepare dried agarose injection pads. (this can be done one to many days beforehand)
 1. Prepare molten 2% agarose in water. With a Pasteur pipette put 2 drops onto a 22X54 mm coverglass. Place another coverglass cross-wise on top of the drop. In order to achieve the proper thickness, press on the top coverglass until the diameter of the pad is around 1.5 cm. Let sit for 5-10 minutes.
 2. Remove top coverglass. Dehydrate the agarose pad by placing it in an 80°C oven for 1 hour or allow to sit on benchtop overnight.
2. Prepare Mitotracker or Lysotracker solution.
 1. Dilute fluorescent agent in Egg Buffer. We typically use a 1:10 dilution of Mitotracker or Lysotracker.
 2. The injection needle is made from a 1.2 OD glass capillary. Pull the injection needle using a needle puller.
 3. Use a pulled Pasteur pipet to back fill the needle with the diluted dye.
 4. Place needle in a light-free, humidified chamber until use.
3. Pre-Injection: Setting up the microscope and needle.
 1. Mount injection needle onto injection apparatus. Our apparatus is a Narishige direct drive micromanipulator mounted onto the stage of a Nikon TE200 inverted microscope equipped with DIC imaging capability.

2. Connect the needle to a 1.2 mm ID tube that is connected to the pressure regulator. Either compressed air or nitrogen can be used as an external pressure source. Set the regulator to 20-25 psi.
 3. Place 2 drops of heavy mineral oil (Parafin oil) onto the injection pad.
 4. Place the injection pad onto the microscope and lower the needle into the oil. Check to make sure fluid flows freely from the needle when pressure is applied. Apply injection pressure and observe whether fluid flows out of the needle. If not, you will need to gently break the end of the needle. The needle can be broken by gently driving the tip into a small bit of broken coverglass placed on the injection pad. After the needle is flowing, move on to the next step.
4. Injection.
1. Approximately 1 hour prior to viewing embryos, transfer young adult worms into the oil drop on the injection pad. Perform this transfer using the dissecting microscope. Use a platinum wire worm pick with a pointed tip for transferring worms.
 2. Arrange 3 – 10 worms so that they are lying directly on the pad. For injection, it is easiest if the worms are lined up parallel to one another and a little less than one worm length apart. Once the worms are on the injection pad it is important to work quickly and complete the injection process before the worms perish from desiccation.
 3. Place the coverglass with worms onto the microscope stage of the injection microscope. Focus into the central part (rachis) of the distal gonad tube. Then use the micromanipulator to move the tip of the needle into the same focal plane. Move the stage horizontally so that the worm is punctured by the needle. Once the tip of the needle is inside the rachis, apply pressure and allow the gonad to fill with the dye mixture. Inject both gonad arms of all worms on the injection pad.
 4. After injection, use a pulled Pasteur pipet to deliver about 0.5 ml of Egg Buffer to the worms. This will keep them from dying of dehydration and allow them to recover from the injection procedure.
 5. Allow the worms to recover for 1-2 hours at room temperature in a light-free, humidified chamber.

3. Agarose pad for viewing embryos

1. Prepare molten 2% agarose in Egg Buffer.
2. With a Pasteur pipet, put 3 drops of molten agarose onto a clean microscope slide.
3. Place slide between two other slides that have one layer of labeling tape covering them. These serve as spacers that will ensure that your agarose pad is the correct thickness.
4. Place a second clean microscope slide perpendicularly down onto the agarose drops. Press down until top slide rests on tape from guide slides.
5. Just before you are ready to mount embryos for observation, remove the top slide.

4. Mounting embryos for viewing

1. If worms have not been injected, follow the next two steps. Otherwise skip them.
 1. Choose young adult worms from the plate that was prepared the day before. You should be able to see embryos inside the young adults.
 2. Pick 5-20 worms onto an unseeded NGM plate. Allow the worms to crawl around on the unseeded plate for a few minutes so that the worms will lose most of the bacteria that is stuck to them.
2. Obtain young embryos from adult worms
 1. Place a 5-20 μ l drop of Egg Buffer in the middle of a 18 mm² glass coverglass (a coverglass thickness of 1.5 is preferable).
 2. Move worms from the unseeded plate or injection pad into the drop of Egg Buffer.
 3. Cut open worms with a 26 gauge hypodermic needle. Cut open the worm near the vulva. You should see embryos spill out of the worm.
3. Mount onto microscope slide
 1. Invert the microscope slide that was prepared with the agarose pad and lower it onto the coverglass with embryos.
 2. If extended time lapse is required, use petroleum jelly to seal the edges of the coverglass to prevent desiccation.

5. Microscopy

1. Finding the appropriate embryos.
 1. Place slide onto microscope stage. Scan with 10X lens to find embryos.
 2. To find early stage embryos, search for embryos that are in the process of completing maternal meiosis. Meiotic embryos can be distinguished from later embryos because they have not undergone shortening. (After embryos have completed meiosis, you can see a significant gap between the cell membrane and the egg shell at the anterior end.)
 3. Once you have identified an appropriately staged embryo, move to a higher magnification lens for recording of embryogenesis.
2. Imaging
 1. In this example, we are imaging embryos that express the mCherry::H2B and GFP::Ub tagged proteins. We are using the Zeiss LSM700 conventional laser scanning microscope.
 2. Images are collected using the 63X 1.4NA lens. The 488 and 555 lasers are used with minimal laser power and maximum gain. A Z stack is collected every 10-15 seconds. Depending on the extent of photobleaching, 30-60 time points are collected. Z stack images are collapsed into a maximum projection image for the final time-lapse video.

6. Representative Results:

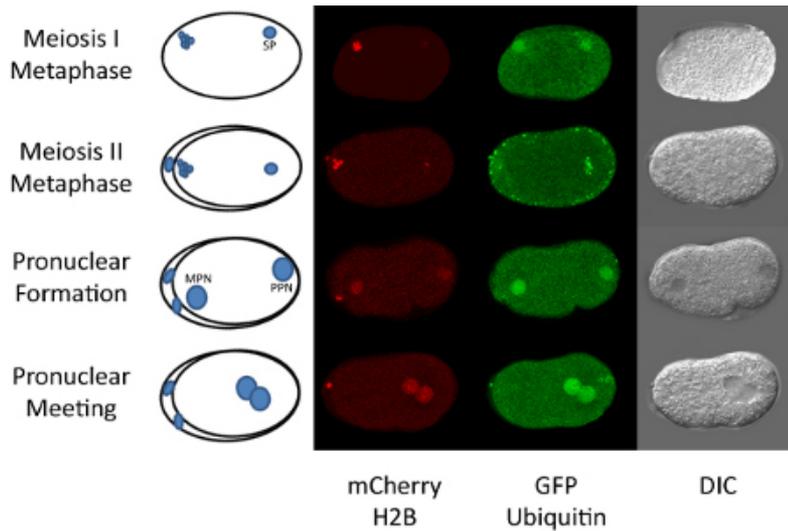


Figure 1. Fertilization to First Mitosis in *C. elegans*.

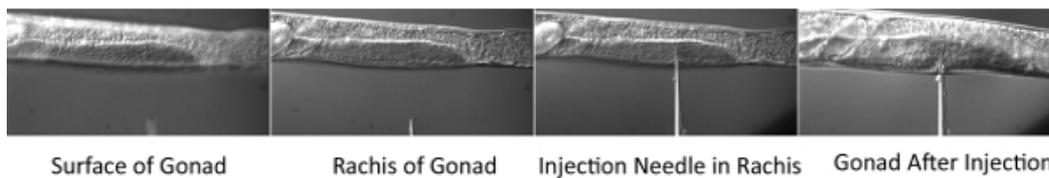


Figure 2. Microinjection procedure.

Supplemental Video 1. Time-lapse video of GFP::ubiquitin plus Lysotracker Blue in a meiotic embryo. [Click here to view the video.](#)

Discussion

After fusion with the sperm, the zygote undergoes a series of dynamic events. These events transform the oocyte from a relatively static cell into the rapidly developing embryo. In many organisms, cytoplasmic rearrangements are critical for establishing embryonic polarity and for egg activation. The *C. elegans* embryo provides an excellent opportunity for observing these early events of egg activation and embryogenesis. The embryo is transparent and early developmental events occur relatively rapidly after fertilization. *C. elegans* researchers have generated many useful transgenic strains that express fluorescently labeled proteins involved in early development. Using these strains along with RNAi or mutants provides a powerful system for dissecting the molecular pathways that underlie development of a multicellular organism (5, 6, 7, 8). This video article presents a practical approach to using these tools and techniques to record events during early embryogenesis in *C. elegans*.

The mature *C. elegans* oocyte is arrested in prophase of meiosis I and is fertilized in the spermatheca of the adult hermaphrodite. After fertilization, the oocyte proceeds through the remainder of meiosis I and II. These stages can be observed using fluorescently labeled histone H2B (5). During completion of maternal meiosis, the sperm DNA remains condensed (see Figure 1). After the completion of meiosis, the maternal and paternal DNA becomes decondensed and the two pronuclei form. The maternal pronucleus migrates towards the paternal pronucleus and they join together near the center of the embryo. Mitosis quickly ensues after pronuclear meeting. All of these events occur in less than an hour. Thus, time-lapse microscopy of this sequence of events can be readily accomplished. Performing this technique requires certain facility in nematode culturing as well as basic microscopy skills in addition to access to a confocal microscope.

The example presented here utilizes a transgenic *C. elegans* strain that expresses two fluorescent proteins, GFP::ubiquitin and mCherry::H2B. A confocal laser scanning microscope is used to observe the dynamic localization of these two proteins. In addition, we show that injection of fluorescently labeled Lysotracker can be used to follow the fate of lysosomes in the embryo after fertilization. Injection of a labeled tracker can also be performed for visualization of mitochondria or local calcium concentrations (9). In theory, the injection protocol could be used to view any type of fluorescently labeled molecule in the embryo. This might include labeled small molecules, proteins, lipids, etc. In some cases, it may not be necessary to actually inject labeled trackers as the worms will readily uptake some dyes such as mitotracker (10). However, we have tried both soaking and injection of lysotracker and have found much better results with injection for visualization in embryos.

Technical Considerations:

The technical challenges presented with this procedure include the microinjection technique as well as the difficulty in imaging very early embryos. Regarding microinjections, the thickness of the agarose injection pad is critical for successful injections. If the pad is too thick, the worms will desiccate and die quickly. If the pad is too thin, the worms will not stick to the pad during the injection process.

The early embryos can be sensitive to environmental conditions such as temperature and buffer conditions. Wild type embryos should initiate decondensation of the sperm DNA and pronuclear migration within a minute or so of completing meiosis II (4). Within 20 minutes, the first cytokinesis should begin. If embryos are exposed to very high laser intensity or are overheated or crushed during mounting, this process may be disrupted. Such embryos will arrest development. If this occurs, the experiment should be repeated with decreased laser power or a thicker agarose pad.

C. elegans embryos do not secrete their egg shell until shortly after fertilization. During the short time between fertilization and the secretion of the egg shell, embryos are not viable outside of the mother (11). Some labs have avoided this challenge by imaging embryos *in utero* (5, 12). *In utero* imaging offers certain advantages such as the ability to capture events that occur during or just after fertilization. However, this technique requires the use of a microscopy system that allows for deep tissue imaging. Systems such as two-photon or multi-photon are especially suited for this (13, 14). When using a conventional laser-scanning or spinning disk confocal microscope, the best images are obtained from embryos that have been cut out of the adult worm as described here.

Imaging System Considerations:

The type of confocal imaging system used depends upon the users' needs. In general, laser scanning systems are recommended if one wishes to acquire images with the highest resolution possible. On the other hand, spinning disk systems are better suited for imaging highly dynamic processes as image acquisition is relatively rapid and photobleaching/phototoxicity is reduced.

The specific imaging parameters will vary depending upon the experimental design and the confocal imaging system employed. In multidimensional imaging, one needs to consider the number of channels, focal planes, and time-points being collected. Due to the limiting effects of photobleaching and phototoxicity, a finite number of images can be acquired in any given experiment. Thus, if you choose to increase the number of channels to be acquired, you will likely need to reduce the number of focal planes and/or time points and vice versa. The total number of images that can be acquired will be influenced by the intensity of the fluorophore being imaged as well as the sensitivity of the confocal microscope being used. More intense fluorophores and more sensitive instruments will require shorter exposure times and thus produce less photobleaching and phototoxicity.

A large number of imaging systems are available. We will give details on the systems that have been used in our own laboratories. For imaging worm embryos by spinning disk confocal microscopy, we use a Nikon TE2000U inverted microscope equipped with a 60X/1.4 N.A. Plan Apo objective. The microscope is connected to a Yokogawa CSU10 spinning disk unit, a Hamamatsu C9100-13 EM CCD camera, and a Spectral Applied Research LMM5 laser merge module containing four solid state lasers with output at 405, 491, 561 and 655 nm. Using this system we can capture one or two channels, six to twelve focal planes, and 25 to 50 timepoints (at 30 to 60 second intervals). A typical exposure time is 100 to 200 milliseconds.

For imaging embryos with laser scanning confocal microscopy, we use a Zeiss LSM700 equipped with 2 channels and four solid state lasers (405, 488, 555, and 635 nm). This system is attached to a Zeiss Axio Observer inverted microscope with a Plan-Apo 63X/1.4NA objective used for embryo imaging. The system uses the Zeiss ZEN software for image acquisition which also provides limited image processing and analysis. When imaging with two fluorochromes, we typically gather 1-5 focal planes (~0.5 μm apart) and collect a Z stack every 15 seconds. Collections are continued until significant photobleaching has occurred. To generate the final time-lapse video, Z stack images are consolidated using the maximum projection tool in the ZEN software. Videos are exported from the software as avi files.

Disclosures

No conflicts of interest declared.

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