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Cell Lineage Analysis

Videomicroscopy Techniques

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1. Introduction

Complete or partial embryonic cell lineages are available for several animal model systems. In the case of the nematode *Caenorhabditis elegans*, the entire embryonic cell lineage has been determined and is largely invariant ***(1)***. This makes lineage analysis a potentially useful tool for assessing mutant phenotypes in *C. elegans*. Indeed, lineage analysis of some mutants has shown that one cell can be transformed into a different cell resulting in duplication or absence of certain tissues ***(2,3)*** (**Fig. 1**).

The wild-type lineage was originally determined by direct observation under a light microscope. This was a very slow process since only one or two of the >500 total embryonic cells could be followed per embryo. Determining the lineage of mutants by direct observation would be even slower and more difficult than in wild-type for at least two reasons:

1. Many of the mutants that display lineage defects will be recessive zygotic lethal mutations. These mutations must be maintained as heterozygotes, which results in only one of four embryos displaying a lineage defect.
2. Mutant phenotypes are not always 100% penetrant, which means every lineage would have to be followed multiple times.

The advent of four-dimensional (4-D) microscopy ***(4)*** makes cell lineage analysis of mutants much more practical. This technique allows development of embryos to be recorded in three dimensions (3-D) over time. Multiple cells can then be followed in a single embryo, making lineage analysis much simpler and faster. This technique can be used for a variety of other experiments, including analysis of morphogenetic movements ***(5)***, analysis of cytoplasmic flow in early embryos ***(4)***, cell ablation studies ***(5)***, and analysis of migration of individual cells ***(6,7)***. Here we will describe how we prepare mounts, record embryos, and analyze recordings to determine the cell lineage of mutant *C. elegans* embryos. Although some of the techniques described here are specific to *C. elegans*, the apparatus and software can be adapted easily to a variety of experimental uses.

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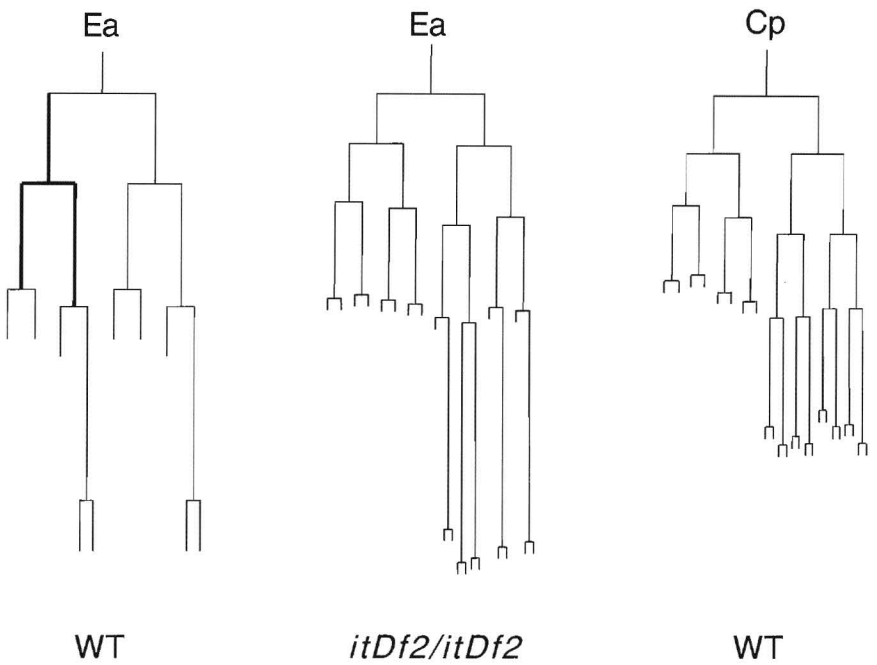

Fig. 1. Examples of lineage trees from wild-type and *itDf2* embryos. This figure is modified from **ref. 2**. The E lineage in *itDf2* homozygous embryos is different than the E lineage in wild-type embryos, but similar to the wild-type C lineage *(2)*. Bold lines indicate the part of the wild-type lineage that was followed in **Fig. 3**. The E lineage produces intestine in wild-type animals and the C lineage normally produces epidermal and muscle cells *(1)*. *itDf2* homozygotes produce muscle and epidermis from the E lineage instead of intestine *(2)*. This is consistent with the observed lineage transformation shown here.

2. Materials

2.1. Embryo Preparation

1. 3-mm single-depression microslide. (VWR Scientific, West Chester, PA).
2. M9 solution: 3 g KH_2PO_4; 6 g Na_2HPO_4; 5 g NaCl; 1 mL of 1 *M* $MgSO_4$; 1 L H_2O
3. Scalpel (Feather Disposable, #15, curved blade).
4. Mouth pipet. A 20-µL micropipet (Fisher Scientific, Pittsburgh, PA) is drawn out over a flame and broken in half; only one half is used. A small piece is broken off of the tip to provide an opening that is >40 µm in diameter. The capillary tube is inserted into one of the plastic adapters supplied with the capillary tubes. This assembly is inserted into a piece of rubber tubing. A second plastic adapter is inserted into the other end of the tubing and is used as a mouthpiece to apply suction.
5. 5% agar (Difco Bacto-Agar, Difco, Detroit, MI).
6. Microscope slides (Fisher*brand* Colorfrost [Fisher Scientific], precleaned, 25 × 75 × 1 mm).
7. Eyelash brush. This consists of an eyelash glued to the end of a toothpick. (glue: Devcon Duco® Cement, Danvers, MA).
8. 18 × 18-mm cover slips (Corning No. 1½, Corning Glassworks, Corning, NY).
9. Platinum wire pick. A one inch long piece of platinum wire (Fisher, 32 g) is inserted into the end of a short Pasteur pipet and held over a flame until the glass melts around the wire holding it in place. A pliers with a flat face is used to press the tip of the wire flat.

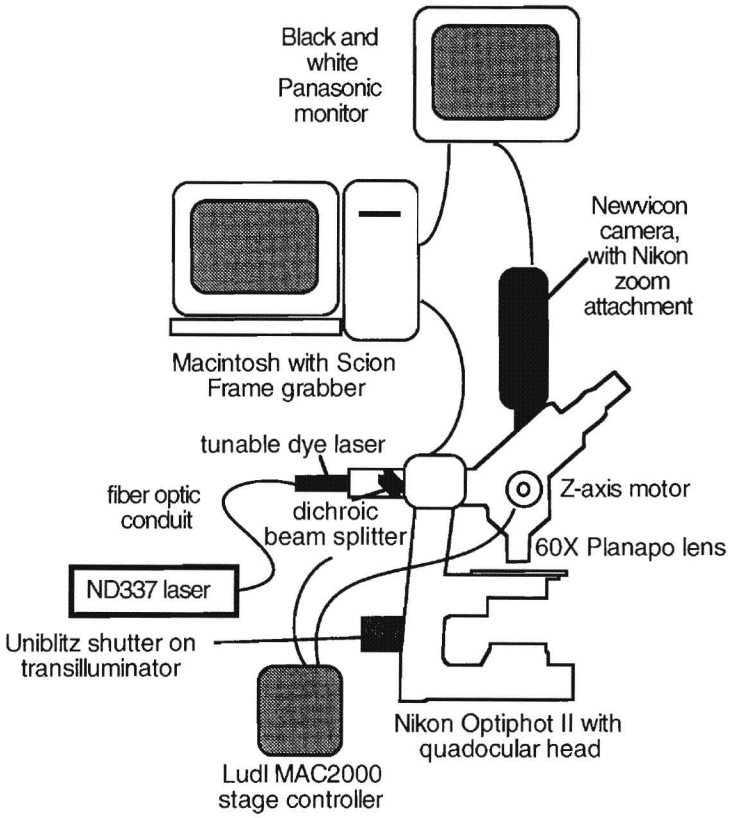

Fig. 2. Schematic diagram of integrated 4-D microscopy setup. The video camera with zoom attachment sends signals to a TV monitor, which is used to set up optics. The video signal is then sent to the computer and optics on the computer screen are adjusted to match the monitor. Parameters are then specified for the modified version of NIH Image, which is used to control the Ludl box and the shutter. Also shown is a fiber-optic delivered laser (Photonic Instruments, Chicago, IL), which allows ablations to be performed followed by immediate imaging.

2.2. Recording and Lineage Analysis

1. Nikon Optiphot-II microscope equipped with differential interference contrast optics, a 60X oil immersion 1.4 NA planapochromat lens (Nikon Corp., Tokyo, Japan), a Ludl Z-axis stage controller operated by a Ludl Mac2000 control box (Ludl Electronic Products Ltd., Hawthorne, NY), and a Uniblitz electronic shutter on the transilluminator port (Vincent Associates, Rochester, NY) (**Fig. 2**).
2. Shutter and Z-axis motor are controlled by serial cable connections to a Macintosh computer and a Scion LG3 8-bit frame grabber (Scion Corp., Frederick, MD) (**Fig. 2**).
3. Device control and image acquisition are accomplished using NIH Image. NIH Image is a public domain image analysis program written by Wayne Rasband available via anonymous ftp from www.zippy.nimh.nih.gov. A modified version of NIH Image is used for QuickTime movie construction and playback, and is available on request.
4. Newvicon video camera (Dage-MTI [Michigan City, IA], 13.5 W, 120 V) with a Nikon zoom attachment (**Fig. 2**).
5. Panasonic black-and-white TV monitor (**Fig. 2**).

3. Methods

3.1. Embryo Preparation

1. Five to 10 young adult *C. elegans* hermaphrodites are removed from plates with a platinum wire pick and deposited into a depression slide containing approx 0.5 mL of M9 buffer.
2. Animals are cut in half transversely with a scalpel midway along the A-P axis of the animal to release embryos into the solution. This is done under a dissecting microscope (*see* **Note 1**).
3. A strip of colored laboratory label tape is placed on the bottom of two slides. These slides are placed on a hard flat surface with an untaped third slide between and parallel to them. Two or three drops of melted 5% agar are put on the center of the third (middle) slide, and a fourth slide is placed over the agar perpendicular to the third slide. The top slide is pressed down firmly to produce a thin agar pad between slides 3 and 4. The slides are carefully pulled apart such that the agar pad is left on the center of one slide.
4. Under the dissecting microscope, 8–10 2-cell embryos are collected from the M9 solution with a mouth pipetter into a small volume (<20 µL) and deposited onto a corner of the agar pad.
5. Embryos are brushed away from the main puddle of liquid toward the center of the pad using an eyelash. Typically two embryos are placed side by side; this allows recording of two embryos at a time. The liquid surrounding the embryos is allowed to dry, which embeds the embryos in the agar.
6. Once the liquid has dried a drop of M9 solution is put on top of the embryos and a cover slip is gently placed over the embryos. It is important to avoid getting air bubbles near the embryos; air bubbles make it impossible to obtain good optics later in the procedure.
7. Excess agar is trimmed away from the edges of the cover slip with a razor blade; care must be taken not to move the cover slip. Melted Vaseline is applied to the edges of the cover slip with a paint brush. This provides a seal that keeps the agar and embryos from drying out (*see* **Note 2**).

3.2. Recording and Lineage Analysis

1. The slide is placed on the stage of the microscope with a drop of immersion oil between the slide and the condenser lens. A 10X objective is used to locate embryos. Embryos are centered in the field of view, a drop of immersion oil is put on the cover slip and the 60X objective is swung into place (*see* **Note 3**).
2. Optics are optimized by focusing the condenser lens and rotating the Nomarski prism until the desired level of contrast is obtained. The microscope lamp is never set above 6 (50% of maximum) to avoid heat damage to the embryos.
3. Light is then sent to the video camera and embryos are observed on a TV monitor. The camera is rotated so that the anterior/posterior (A/P) axis of the embryos is vertical. If only a single embryo is used the A/P axis should be horizontal on the monitor. The zoom is then adjusted until the embryos fill the screen without any edges being cut off.
4. Contrast and gain knobs on both the camera and the monitor are adjusted to obtain the best image. We prefer a high-contrast image to make the nuclei stand out but not to the extent that they look distorted.
5. To set up a recording, NIH Image is opened, the image on the monitor is captured and displayed on the computer screen, and contrast and gain are adjusted on the computer until the image resembles what is observed on the TV monitor.

6. The 4-D recording macro is then invoked, using the appropriate menu option in NIH Image. Parameters for the recording are specified including number of focal planes, distance between focal planes (micrometer), time interval between z-series (seconds), number of timepoints and root file name. The software uses all of this information and issues the appropriate serial commands to control the focus motor (telling it how often and what distance to move), and the shutter (so that light is not hitting the embryos between z-series acquisitions). The focus is adjusted so that the top surface of the embryo is in focus, the shutter is closed, and the recording is started. The motor automatically resets to the top focal plane after each scan. For a lineage quality, recording scans are typically taken starting with a four-cell embryo every 45 s, 23–25 focal planes and 1.0 μm apart, for approx 6 h.
7. Images are stored directly on the hard drive, which requires approx 1.5 GB of hard drive space. There are options for reducing the amount of space required for lineage recordings (*see* **Note 4**). Files are later compressed and transferred either to recordable CDs or magneto-optical disks.
8. Images can be played back as movies within a specially modified version of NIH Image. A single focal plane can be followed forward or backward in time. At each timepoint, all of the different focal planes of a recording can be scanned. This allows us to follow a specific cell throughout the recording, even though it may move through different focal planes.
9. To analyze the lineage, a cell of interest is selected at the beginning of the recording and followed through time until it divides (**Fig. 3**). The division is carefully followed to make sure that both daughters can be clearly identified. The time, focal plane, and location at which the cell divided are documented so that it can be returned to later without having to retrace the entire lineage. This is accomplished by placing transparency film over the computer monitor and tracing the positions of the nuclei immediately after the division.

   One of the daughter cells is then chosen and monitored until it divides. One daughter cell from each successive division is followed until the end of the recording is reached or the cell is lost. Because the positions of cells are charted after each division, it is possible to go back to earlier timepoints in the recording to pick up a sister cell and follow its lineage. This can be done until all of the cells of interest have been lineaged (*see* **Note 5**).
10. Lineage trees are then drawn either manually or using available commercial software that can be used to trace and plot lineage trees semiautomatically *(7)*. Lineages are then compared to the wild-type lineage to assess any differences (**Fig. 1**) (*see* **Notes 6** and **7**).

4. Notes

1. Cutting worms with a scalpel in liquid can be difficult because the worms are constantly moving. Pinning them against the glass with the blade helps. Also, lifting the scalpel blade in and out of the liquid causes the animals to move. Once the blade is submerged it should be kept under water until cutting is finished. Finally, serotonin may be added to the M9 solution; this drug causes the worms to lay eggs quickly so young embryos can be obtained without cutting worms *(9)*.
2. The steps from making the agar pad to sealing the slide with Vaseline must be done as quickly as possible to prevent the pad from drying out. Ideally, the entire process from collecting two cell embryos to starting a recording should be done in 30 min or less. This ensures that embryos will be four or eight cells when the recording is started, which makes identification of cells easier when lineaging.
3. Any source of vibration will cause blurring of images. If vibration is a problem an isolation table can be purchased to stabilize the microscope, or the microscope can be placed on an apparatus as simple as a large, heavy board on top of inflatable rubber donuts to eliminate vibration.

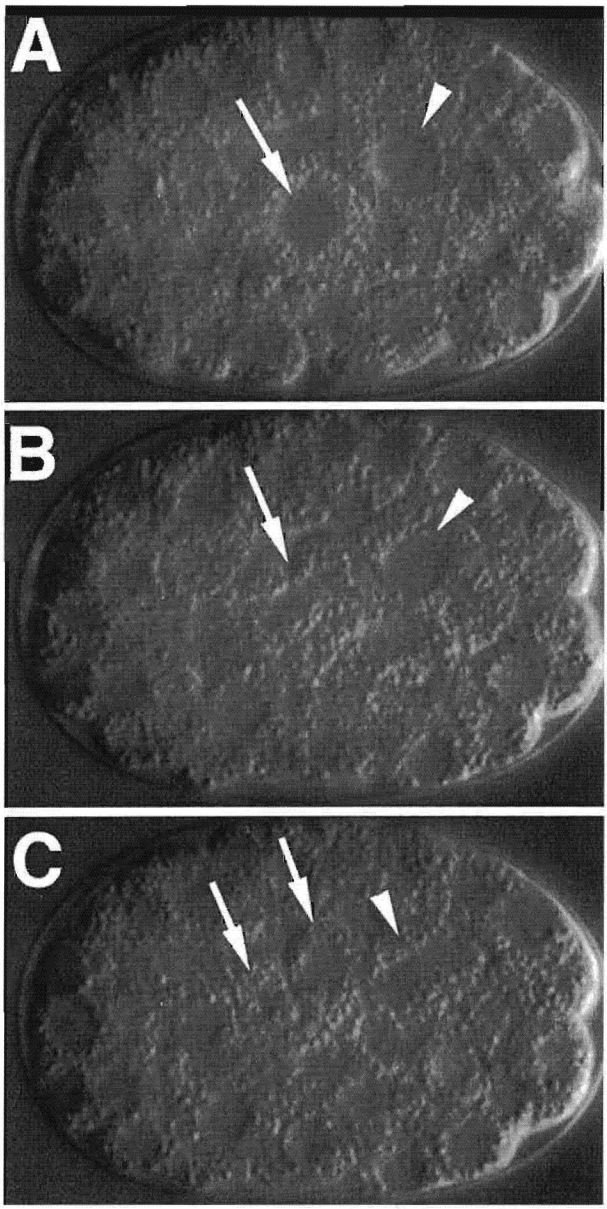

Fig. 3. Images of a wild-type embryo recorded for lineage analysis with the integrated 4-D microscopy system. Anterior is to the left and dorsal is to the top. Images display how the lineage of the cell Eal can be followed. Standard nomenclature was used to name cells *(1)*. **(A)** Arrow indicates nucleus of Eal and arrowhead indicates nucleus of Epl as determined by lineage analysis, starting with a 28-cell embryo in which the precursor cells Ea and Ep can easily be identified. **(B)** The same embryo approx 5 min later; Eal is dividing (arrow indicates furrow between forming cells) and Epl has not yet started to divide (arrowhead indicates nucleus). **(C)** Same embryo approx 2 min later; arrows indicate the progeny of Eal, the anterior daughter Eala (left) and the posterior daughter Ealp (right). Only the nucleus of Ealp can be distinguished in the focal plane shown. Epl is in the process of dividing (arrowhead). At this point, to continue lineaging, either Eala or Ealp would be followed through the recording until it divides. The other cell would be ignored until later but could easily be identified if time, location on the screen, and focal plane of the division are all documented.

4. Several things can be done to reduce the amount of hard drive space that a lineage recording occupies. Early in development, cells are bigger and divide more slowly, so it is possible to record using only 15 focal planes instead of 25 and scans that are 1–1.5 min apart instead of 45 s. With the described system this can only be accomplished by setting up two separate recordings. The second recording has to be set up quickly so that cells do not divide or move during setup. There are other NIH Image macros and standalone software for this recording system ***(10)*** that allow change of parameters during a single recording. Available standalone software compresses the images before storing them to the hard drive, which significantly reduces the amount of space occupied ***(11)*** (*see* Chapter 26). Finally, because early embryonic development is well described, it is possible to wait until the embryos have 28 cells to start recording. Cell position is essentially invariant until this time, and diagrams exist showing the position of each cell up to this stage (J. Priess, personal communication). This saves about 1 h of recording time. The diagrams are available on request.
5. During a recording, embryos turn either ventral side up or dorsal side up. Embryos that are left side up at the 4- or 28-cell stage usually become dorsal views; embryos that are right side up tend to become ventral views. It is often difficult to lineage deep into the embryo, especially in older embryos. If comprehensive lineaging of several cells is necessary, it is helpful to do separate recordings of both dorsal- and ventral-view embryos. This ensures that every cell is in the top half of the embryo in one recording or the other.
6. For many other types of experiments in *C. elegans*, including following cytoplasmic streaming in early embryos, cell migrations, and following development of embryos in which cells have been killed with a laser or embryos that have been laser-permeabilized and treated with drugs; 4-D microscopy can be used. Many of the procedures described in this chapter can be modified for different experiments. For example, laser-permeabilized embryos would explode on the agar mounts described here and have to be mounted on poly-lysine coated cover slips and placed over a slide with grease feet ***(5)*** or a depression slide ***(12)*** to prevent compression. This type of experiment also requires that a laser be attached to the microscope. For an example of the use of our integrated 4-D workstation for this sort of experiment, *see* **ref. *5***. A schematic diagram of this system is shown in **Fig. 1**.

   To look at early events in single-cell *C. elegans* embryos, M9 solution cannot be used, because embryos do not have completely developed egg shells. More complex solutions that better mimic the internal conditions of the embryo must be used for these experiments ***(13)***.

   Our laboratory has also used 4-D microscopy to analyze gastrulation events in normal and laser-ablated sea urchin embryos. Conditions for mounting embryos are significantly different than those described here ***(14)*** (*see* Chapters 2 and 3).
7. A variety of systems exist for doing 4-D recordings ***(4,5,7,9,15–17)***. The original analog system was developed by John White ***(4)***. Advantage of the analog system are that recorded images can be played back at high speed and image quality is good. The main disadvantage of this system is that recordings cannot be deleted and storage discs become a major expense. Software has been developed for use with the original analog system that allows computer-generated lineage trees and 3-D reconstructions of nuclear positions to be produced ***(7)***.

   More recently, several digital systems have been developed, including the one described here. These have the advantage of using erasable media. Playback of recordings is slower (though improving) and image quality may be slightly lower than analog systems using existing technology. However, new software and better computers are starting to overcome these problems (*see* **ref. *10*** for discussion).

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