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Cold Spring Harb Protoc 2010; doi: 10.1101/pdb.prot5541

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Protocol

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

Timothy Walston and Jeff Hardin

INTRODUCTION

The *Caenorhabditis elegans* embryo is particularly amenable to microscopy and embryological studies because of its short developmental time, transparent shell, and nonpigmented cells. 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. Differential interference contrast (DIC) microscopy is the focus here because of its broad availability, common use for *C. elegans* imaging, and wide applicability to microscopic analysis of embryos of other organisms. This protocol describes the use of a custom script within μ Manager's Beanshell scripting language. The script is helpful for reducing the number of shutter open/close events during 4D acquisition.

RELATED INFORMATION

The standard μ Manager package (without the custom script) has been used successfully to acquire 4D footage, but this involves 20-fold more shutter open/close events than when the script is used. The script is available at <http://worms.zoology.wisc.edu/research/4d/4d.html>. A method for preparing live embryos for microscopy is described in **An Agar Mount for Observation of *Caenorhabditis elegans* Embryos** (Walston and Hardin 2010a). Related protocols include **Analysis of 4D DIC Microscopic Data to Determine Cell Contacts in *Caenorhabditis elegans* Embryos** (Walston and Hardin 2010b) and an ablation technique for killing individual cells within the *C. elegans* embryo, described in **Laser Killing of Blastomeres in *Caenorhabditis elegans*** (Walston and Hardin 2010c).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

- C. elegans* embryos, embedded as described in **An Agar Mount for Observation of *Caenorhabditis elegans* Embryos** (Walston and Hardin 2010a)
- Immersion oil (Type DF)

Equipment

- Cooled environment (~20°C)
- Microscope, equipped with camera and computer

This protocol assumes a basic high-numerical-aperture (high-NA) microscope equipped with oil-immersion objectives and, optionally, an oilable, high-NA condenser from any of the major microscope manufacturers.

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011 (in press).
Cite as: *Cold Spring Harb Protoc*; 2010; doi:10.1101/pdb.prot5541

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We typically acquire 4D movies using a 60X-63X, 1.4-1.45 NA PlanApo objective. Older Newvicon video cameras, coupled to the video port on the microscope, are adequate for many applications, especially if they are equipped with a zoomable video lens attachment such as those sold by Nikon. In this case, an AG-5 digitizing board (Scion Corporation) or a similar video frame grabber can be used to digitize the video signal. Alternatively, modern cameras are almost exclusively charge-coupled device (CCD) cameras and have much higher spatial resolution than older video formats. We have successfully used cameras from Scion Corporation and QImaging. The mounting hardware for such devices differs depending on the microscope being used.

Software, for 4D footage acquisition and image processing

This article describes the use of μ Manager (www.micro-manager.org/), a public domain program that supports a variety of CCD cameras, z motors, and shutters. The Legacy acquisition plugins for ImageJ (<http://worms.zoology.wisc.edu/research/4d/4d.html>), a public domain program that supports a variety of CCD cameras, z motors, and shutters. The Legacy acquisition plugins for ImageJ (<http://worms.zoology.wisc.edu/research/4d/4d.html>) are also freely available. Full documentation of the plugins and detailed instructions for installation of ImageJ and QuickTime for Java can be found at the same URL. The "Browse4D," "QT4D Writer," and "QT4D Player" plugins for viewing movies are also available at the same URL. Software for acquiring 4D footage is also available commercially (IPLab, BD Biosciences).

z-axis controller/shutter/serial port (e.g., Prior Scientific, Ludl Electronics Products, Applied Spectral Imaging [ASI])

A shutter to block the transmitted light path between time points is strongly encouraged to minimize exposure of embryos to light and to heat.

METHOD

Image Acquisition

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

1. Turn on the z-axis and shutter control boxes and the CCD camera. Turn on the light switch on the microscope.
2. Find a group of embedded *C. elegans* embryos using the 10X objective, before oiling the coverslip.
3. If a high-NA condenser is present, place a drop of immersion oil on the condenser (upright microscope) or on the bottom of the slide (inverted microscope). Carefully position the condenser so that it contacts the oil and spreads it uniformly between the condenser and the microscope slide.
4. Focus the condenser.
 - i. Stop down the condenser using the iris diaphragm, closing it almost completely.
 - ii. Adjust the height of the condenser at high magnification until the octagonal outline of the diaphragm is in focus.
 - iii. Open the condenser.
5. Once embryos have been located at 10X and the condenser has been focused, swing the 10X objective out of the way and add a drop of immersion oil to the coverslip (upright microscope) or to the 60X objective lens (inverted microscope).
6. Carefully slide the 60X or the 100X objective into place (it should just clear the sealant on the slide, as long as it is not too thick). Make sure the correct condenser setting is selected to match the lens.
7. Refocus on the embryos, and refocus the condenser.
8. Open μ Manager.
 - i. Use the "Live" button in the main μ Manager Studio window to display an image from the camera.
 - ii. 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.

9. 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).
10. Optimize the Nomarski optics through a combination of the following:
 - i. Center the condenser by closing it and by moving the octagon to the center of the field of view. Reopen the diaphragm to encompass the entire field of view.
 - ii. Adjust the light level. High-quality Nomarski optics requires a substantial amount of light. Optimal settings must be empirically determined.
 - iii. Adjust the exposure time, the gain, and other settings on the CCD camera within. Use μ Manager a final time if needed.
See Troubleshooting.
11. Invoke the μ Manager 4D acquisition script.
This protocol presupposes that a "favorite" has been created previously using the script window in μ Manager.
 - i. Invoke the script window by using the "Tools \rightarrow Script Panel" menu command in μ Manager.
 - ii. When the script window appears, select "Acquire_4D.bsh" from the list of favorites.
 - iii. Make sure that the cursor is blinking within the code of this script, and then click "Run."
 - iv. Enter the desired parameters for time interval, number of time points, number of focal planes, and distance between focal planes.
 - v. Enter the root name for the images that will be collected.
Because most operating systems limit the total length of a file's name to 32 characters, the root name should be kept short.
 - vi. If a shutter is being used, make sure that the "Use shutter" option is selected.
 - vii. Click "OK."
 - viii. The parameters that have been entered will be displayed. If these are acceptable, click "OK."
12. When prompted for a location to which to save images, make a new directory that will contain the images from the 4D sequence.
13. Within the newly created directory, make two additional directories: one called "working" and one called "terminal."
The latter is useful for acquiring a final z-stack of the terminal embryos (see Step 15).
14. Assign the following typical settings for a long overnight movie:
 - i. Number of time points: usually 200-300 for an overnight movie.
 - ii. Time interval (sec): usually 120-180.
 - iii. Number of shutters: 1.
 - iv. Number of focal planes: 20.
 - v. Distance between focal planes: 1 μ m.
 - vi. Root name: "working," or a short name of choice.
 - vii. Information for movie: enter any pertinent information.
*After 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.
See Troubleshooting.*
15. When acquisition of the movie is finished, collect a terminal image.
 - i. Keep the field of view the same.
 - ii. Collect a second movie, specifying "1" as the number of time points.
 - iii. Save this movie in the "terminal" folder created in Step 13.

Viewing the Movie

The movie can be viewed as raw 4D data sets (Step 16) or as a compressed movie (Step 17). Compressing the movies saves disk space.

16. View raw 4D data sets in one of several ways, including the following two options:
 - i. Import the sequences as a “Virtual 5D Stack” within ImageJ, using the Image5D plugin (available on the ImageJ Web site at <http://rsb.info.nih.gov/ij/>).
 - ii. Use the “Browse4D” plugin (available at <http://worms.zoology.wisc.edu/research/4d/4d.html>).
17. Compress the movies before viewing by using QuickTime and the “QT4D Writer” and “QT4D Player” plugins (available at <http://worms.zoology.wisc.edu/research/4d/4d.html>).
 - i. 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.
 - ii. Use “Photo/JPEG,” “Motion JPEGA,” or “Motion JPEGB” compression, “grayscale,” and “Medium” quality.

Many compression algorithms are available, although we typically use the above options. This approach can compress movies 30-fold.
 - iii. View the compressed movie using the “QT4D Player” plugin.
 - iv. Select the desired movie, and use the graphical interface with clickable buttons or the arrow keys on the numeric keyboard to navigate through the movie.
 - v. After the movie has been successfully compressed and its quality verified, for routine purposes, it is now fine to delete the original files.

TROUBLESHOOTING

Problem: No light appears to be reaching the camera.

[Step 10]

Solution: Consider the following:

1. 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.
2. If the shutter has an external toggle switch, make sure that it is in the correct position.
3. If the exposure time is set to too low a value, increase the exposure time using the controls in the Main μ Manager window.

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

[Step 14]

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

Problem: The plane of focus drifts systematically over time.

[Step 14]

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

Problem: 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, $\sim 20^{\circ}\text{C}$. *C. elegans* embryos can be imaged for long periods of time at a reduced ambient temperature. Make sure that the air conditioner is on and that the door remains closed.

DISCUSSION

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

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