Video Article

Imaging *C. elegans* Embryos using an Epifluorescent Microscope and Open Source Software

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Abstract

Cellular processes, such as chromosome assembly, segregation and cytokinesis,are inherently dynamic. Time-lapse imaging of living cells, using fluorescent-labeled reporter proteins or differential interference contrast (DIC) microscopy, allows for the examination of the temporal progression of these dynamic events which is otherwise inferred from analysis of fixed samples[1,2]. Moreover, the study of the developmental regulations of cellular processes necessitates conducting time-lapse experiments on an intact organism during development. The *Caenorhabiditis elegans* embryo is light-transparent and has a rapid, invariant developmental program with a known cell lineage[3], thus providing an ideal experiment model for studying questions in cell biology[4,5]and development[6-9]. *C. elegans* is amendable to genetic manipulation by forward genetics (based on random mutagenesis[10,11]) and reverse genetics to target specific genes (based on RNAi-mediated interference and targeted mutagenesis[12-15]). In addition, transgenic animals can be readily created to express fluorescently tagged proteins or reporters[16,17]. These traits combine to make it easy to identify the genetic pathways regulating fundamental cellular and developmental processes *in vivo*[18-21]. In this protocol we present methods for live imaging of *C. elegans* embryos using DIC optics or GFP fluorescence on a compound epifluorescent microscope. We demonstrate the ease with which readily available microscopes, typically used for fixed sample imaging, can also be applied for time-lapse analysis using open-source software to automate the imaging process.

Protocol

Worm Preparation

1. Transfer L4 larval worms to appropriate plates 18-24hrs prior to imaging[22]. This will ensure you have egg-bearing adults for an ample supply of early embryos.
2. Prepare tubes of Vaseline and Agarose. We prepare several 15ml falcon tubes by melting Vaseline in a beaker in the microwave and dispensing into 4-5 mL aliquot per tube. Similarly melt 2-3% (w/v) Agarose in Buffer (M9 or Egg Buffer) and aliquot 4-5 mL into falcon tubes. Be careful not to boil to excess in order to minimize evaporation. On day of imaging, melt 1 tube of Vaseline in a 65-70°C heat block and melt 1 tube of agarose in the microwave, again be careful to minimize boiling over. Store the tubes in the heat block to keep the Vaseline and the Agarose in the molten state.
3. Make Agarose pad. Position a new microscopy slide between 2 guide slides, which are slides covered by a single layer of common laboratory tape adhered to the top and bottom of each guide slide. Use a Pasteur pipette with end broken off to place 2-3 drops of melted Agarose on the new slide. Cover with a 2nd slide perpendicular to the original slide, so that it covers and helps to spread the Agarose to create a thin square pad. The 2nd slide should rest above the tape on the guide slides. The Agarose concentration may be increased to 5% for thicker pads.
4. Using a dissecting scope and a platinum wire "pick", transfer 2 adult worms to a 9-12μL drop of M9 or Egg Buffer on an 18 mm x 18 mm coverslip.
5. Cut worms open with a needle (we use 27G1/2 needles) or scalpel to release embryos. Try to cut in the middle of the worm.
6. Lower Agarose pad (with the agar side down) onto the 18x18 mm coverslip containing the embryos. Trim agar extending from the edge of the cover slip with a razor blade.
7. Seal Coverslip by using a paintbrush or thin wooden stick to apply the melted Vaseline to all four edges of the coverslip.

Alternative to agar mounts are hanging drops[23] if embryos are susceptible to pressure (i.e. if the egg shell does not form properly). Imaging can also be performed *in utero* to follow fertilization or early events such as meiosis. Worms should be anesthetized with Levamisole[24]. In order to increase the number of embryos from a particular stage of development, several worms may be dissected at one time and the embryos positioned together gently using a fine pipette or an eyelash brush or by mouth pipetting.

4D Nomarski DIC (Differential interference contrast) Movies

8. Turn on the Olympus BX61 microscope. Because the camera is sensitive to EM waves emitted by other devices it is turned on last, the mercury bulb (if necessary for GFP or fluorescence) first. Start Micro-Manager once everything is on. Choose the appropriate configuration file for DIC or fluorescent imaging. The major difference is that the DIC file does not include control of fluorescent shutter. Each file also selects the appropriate filter cube and camera gain setting.
9. Find embryos on the microscope using 10x objective. Look near worm bodies for groups of young embryos in order to get multiple embryos within the imaging field. Avoid embryos inside the worm for best images.
10. Using the software switch to a higher magnification (40x to 100x) and resolution (N.A. 0.9 to 1.4) objective for imaging.
11. Adjust Nomarski DIC Optics (The following web sites contain helpful explanation of DIC optics and the terminology;
    http://www.olympusmicro.com/primer/techniques/dic/dichome.html
    http://microscopy.berkeley.edu/Resources/instruction/DIC.html)
    1. On our Olympus BX61 the important components are:
        1. Polarizer - slider below the condenser

     2. Condenser Wollaston Prism - turret on the condenser, just below the stage.
     3. Objective Wollaston Prism - slider below the filter cubes
     4. 2nd Polarizer (Analyzer) - filter cube in the DIC position

2. Switch Filter Cube to DIC to position the Analyzer (2nd polarizer) in the light path (software should do this on start up).
3. Make sure that the two polarizers are properly aligned (crossed). When both Wollaston prisms are out of the light path the field of view should be dark. If necessary, rotate the polarizer below the condenser until this is correct.
4. Insert the objective (upper) Wollaston Prism into the light path.
5. Rotate the turret on the condenser to select the Wollaston prism that matches the objective you are using.
6. Adjust slider below this turret to match NA of lens.
7. Focus the condenser for optimal Koehler illumination.
8. Adjust the upper Wollaston Prism to get optimal contrast by rotating the knob on the slider.
9. Other settings controlled by software startup include camera gain (set to 0) and switch to saving files as 8-bit not 16-bit tiff files.

12. Adjust light level to give nice image. (usually use 4.7 Volts) (controlled via software). We prefer to illuminate the sample so that the brightest pixels are just below saturation levels.
Good DIC optics results in an image with a 3-dimensional look where the yolk granules in the embryo stand out clearly. Alternatively this can be described as if the light illuminating the sample appears to shine from an angle so that one side of the sample (or features in the sample) is lit brightly and the opposite side is in shadows and appears darker.
13. Select Region Of Interest (ROI) to include the embryos you want to image, click set ROI button to reduce window.
14. Open the multi-dimensional acquisition window. Select Z-stacks (slices) and Time Points.
15. Use microscope focus knob to find the bottom of the embryo (last focal plane with some yolk granules in focus). Click on Select bottom. Repeat to identify and set the top of the embryo. We usually use 1 micron step size and end up with ~20 focal planes. We generally collect multiple focal planes at each time point because the embryos and cells are relatively thick. In addition especially when cells begin to divide in different orientations they can displace other cells around in the embryo moving them in or out of the original focal plane. If your microscope does not have a focus motor you can manually adjust the focus to follow the cell or process you are interested.
16. Input number of time points and time interval needed. We usually use 15 seconds interval and set a max of 500 time points. We start with more time points than we'll need and stop the acquisition when desired. If you are imaging multiple focal planes, make sure there is enough time to acquire all of the focal planes in the interval between time points.
17. Select or create a location to save the data. Make sure that software is set to display last image only. Give the file a name and click Acquire. Monitor the automated image acquisition for the first few time points to ensure it is operating properly.
18. Click stop when you wish to stop the acquisition, switch to 10x objective and prepare next slide.
19. When done imaging remove DIC components from the light path. Clean oil off objective if necessary. Turn everything off starting with the camera.

GFP Movies

20. Start microscope and find embryos as above. Use configuration file that includes software control of fluorescent shutter.
21. Make sure that the upper DIC prism (slider) is not in the light path.
22. Switch Filter Cube to FITC/GFP (automatically set on startup)
23. Change Camera gain to 255 and image file to 16-bit Tiff (automatically set on startup with this configuration file).
24. Turn off white light.
25. Click Live Imaging to preview image. Work quickly to minimize light exposure. Alternatively use Snap Image to collect single image.
26. Adjust Mercury bulb power/neutral density filters, exposure length to get a good image. In general, you should use minimum light reaching the sample to acquire the data you want to reduce photodamage to the cell and photobleaching of the fluorescence signals.
27. Set up time interval, number of time points and number of focal planes. We often use 5-10 second intervals and 1-3 focal plane.
28. Select and set parameters for image acquisition as described earlier in Steps 13-19.

Analyzing movies

29. Data will be saved to folder with the name you entered and contain a series of tiff files for each image of the 4D stack, a text file and an xml file containing metadata such as exposure levels and time intervals.
30. The tiff images can be opened by ImageJ via two methods. The first (method a) is the simplest and will also read the metadata of each file. The second (method b) is able to use virtual memory to open files that are larger than the amount of memory allocated to ImageJ, but it does not include the metadata.
    1. Using Micro-Manager Plugin
       1. Micro-Manager is built on and uses ImageJ (it installs a new version of ImageJ which will be different from any other version that you may have previously installed) and includes a plugin to open data. Alternatively you can copy the content of the Micro-Manager-1.3/plugins folder to the plugins folder for another version of ImageJ.
       2. Go to Plugins menu, select Micro-Manager and then Open Micro-Manager File.
       3. Select the folder containing the data you wish to view and click ok.
       4. Play through the Z and T dimensions.

    2. Using LOCI plugin to open movies with BioFormats Importer.
       1. Transfer the xml and txt files from the folder containing the tiff files, and rename the two files to match the name of the data set.
       2. Within ImageJ, go to the Plugins pull down menu, select LOCI and then either Data Browser or Bio-Formats Importer.
       3. Find the folder with your tiff stacks. Click on the first tiff file, then open.
       4. View stack with Hyperstack. Under Dataset organization: Select Group Files with Similar names, Swap dimensions and Open all series. Under Memory management: Select: Use virtual stack (especially for large data sets). Click ok
       5. The plugin will determine the dimensions of the data set (# of time points and focal planes) and display the range between a set of <> brackets. Click ok or alter the number of time points and/or focal planes to open.
       6. Confirm which dimension corresponds to each series.
       7. Play through the Z and T dimensions.

Representative Results
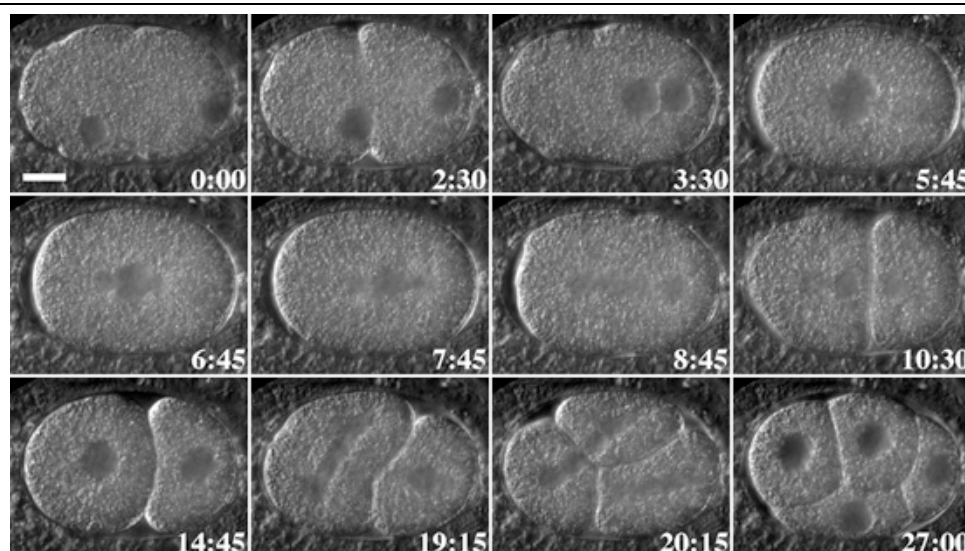
Figure 1. Stills from a Nomarski DIC movie illustrating normal cellular events during early *C. elegans* embryo development: pronuclear migration (0-5:45), asymmetric first division (6:45-14:45) and asynchronous second division (14:45-27:00). Data was collected with a 60x 1.35 NA lens. 20 focal planes at 1 micron intervals were collected every 15 seconds with 40 μs exposure. Images were rotated so that posterior is to the right and ventral is down. Scale Bar represent 10 microns.
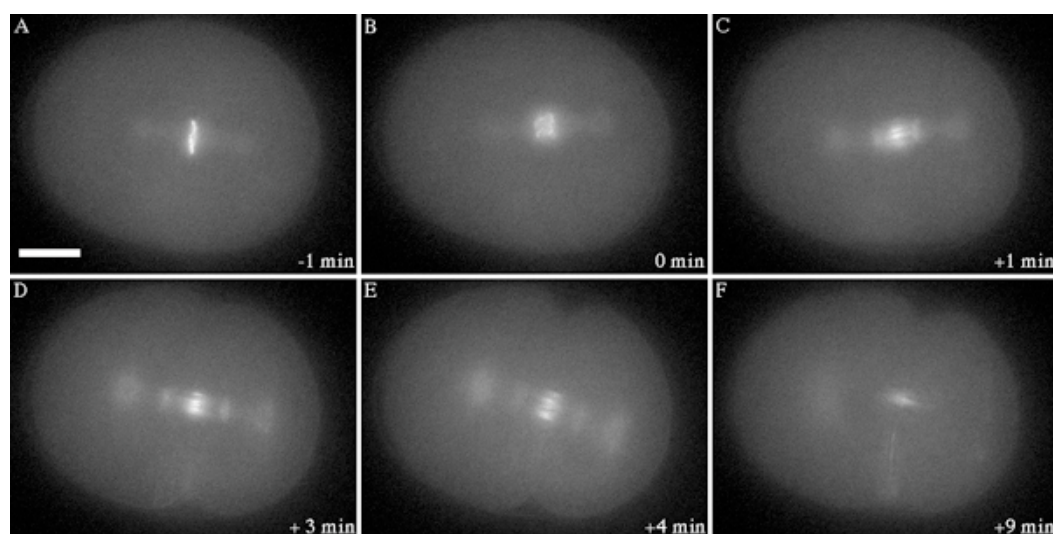
Figure 2. Stills from a fluorescent movie illustrating the dynamic movements of a GFP-labeled subunit of the chromosome passenger complex (AIR-2) present on chromosomes from metaphase to early anaphase (A to C) before appearing on the spindle midzone in anaphase until cytokinesis completes (C to F). Data was collected with a 60x 1.35 NA lens. A single focal plane was collected every 10 seconds with 50 μsec exposure. Images were rotated so that posterior is to the right. Scale Bar represent 10 microns.

Movie 1 Nomarski Movie of Wild-Type Embryo.
Movie corresponding to Figure 1. Posterior is to the bottom right and ventral is to the lower left. Shows a single focal plane of original data set. Image was collected every 15 seconds and play back is set at 14 frames per second. Click here to watch Video

Movie 2 Fluorescent Movie of Wild-Type embryo expressing GFP::AIR-2.
Movie corresponding to Figure 2. Posterior is to the upper right. Movie was collected as a single focal plane. Image was collected every 10 seconds and play back is set at 14 frames per second. Click here to watch Video

Disclosures

No conflicts of interest declared.

Discussion

A major consideration for live time-lapse imaging is to preserve the integrity and viability of the cell and the organism. DIC microscopy provides the benefit that the sample is not exposed to ultraviolet light and excessive heating from the lighting source. DIC microscopy is well suited to detect cell migration and cell shape changes such as cytokinesis and to identify some subcellular structures, such as the mitotic spindle and nuclear membrane. Fluorescence imaging of reporter proteins complements DIC microscopy by identifying additional subcellular compartments and allowing visualization of specific proteins. To mitigate the hazards of phototoxicity and damage to the embryo during fluorescence imaging, we usually increase the digital gain of the camera to offset the reduced UV light intensity and duration of illumination. For faint fluorescent transgenic reporters, deconvolution algorithms to reassign out-of-focus light or deblurring algorithms to reduce background can improve the quality of the captured images. While advanced microscopes such as confocal or multiphoton systems are sometimes necessary, we have found that many fluorescent reporters can be imaged using this epifluorescent microscope setup, yielding high quality images.

Micro-Manager (http://www.micro-manager.org), in addition to being free, saves files directly to tiff format instead of proprietary file formats of many commercial software packages. This significantly simplifies the analyses of our data by eliminating the file conversion step needed to read the image files in ImageJ, Photoshop and other image editing software.

Acknowledgements

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