Video Article

Microinjection Techniques for Studying Mitosis in the *Drosophila melanogaster* Syncytial Embryo

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Abstract

This protocol describes the use of the *Drosophila melanogaster* syncytial embryo for studying mitosis[1]. *Drosophila* has useful genetics with a sequenced genome, and it can be easily maintained and manipulated. Many mitotic mutants exist, and transgenic flies expressing functional fluorescently (e.g. GFP) - tagged mitotic proteins have been and are being generated. Targeted gene expression is possible using the GAL4/UAS system[2].

The *Drosophila* early embryo carries out multiple mitoses very rapidly (cell cycle duration, ≈10 min). It is well suited for imaging mitosis, because during cycles 10-13, nuclei divide rapidly and synchronously without intervening cytokinesis at the surface of the embryo in a single monolayer just underneath the cortex. These rapidly dividing nuclei probably use the same mitotic machinery as other cells, but they are optimized for speed; the checkpoint is generally believed to not be stringent, allowing the study of mitotic proteins whose absence would cause cell cycle arrest in cells with a strong checkpoint. Embryos expressing GFP labeled proteins or microinjected with fluorescently labeled proteins can be easily imaged to follow live dynamics (Fig. 1). In addition, embryos can be microinjected with function-blocking antibodies or inhibitors of specific proteins to study the effect of the loss or perturbation of their function[3]. These reagents can diffuse throughout the embryo, reaching many spindles to produce a gradient of concentration of inhibitor, which in turn results in a gradient of defects comparable to an allelic series of mutants. Ideally, if the target protein is fluorescently labeled, the gradient of inhibition can be directly visualized[4]. It is assumed that the strongest phenotype is comparable to the null phenotype, although it is hard to formally exclude the possibility that the antibodies may have dominant effects in rare instances, so rigorous controls and cautious interpretation must be applied. Further away from the injection site, protein function is only partially lost allowing other functions of the target protein to become evident.

Video Link

The video component of this article can be found at http://www.jove.com/video/1382/

Protocol

Recipes:

Grape Juice plates:

- 5.5g bacto agar
- 14.5 g dextrose or glucose
- 7.15 g sucrose
- 45 ml grape juice concentrate (100% juice).
- 204.5 ml H_20
- 625 µl 10N NaOH

Mix all ingredients and microwave until boiling.

Add 2.8 ml acid mix (acid mix: 20.9 ml propionic acid, 2.1 ml phosphoric acid, 27 ml H_20)

Mix and pour onto 35 mm Petri dishes. Let solidify at room temperature for one or two days. If plates are not going to be used soon, seal with parafilm and keep at 4°C (allow to equilibrate to RT before using).

Yeast paste:

Dissolve about one teaspoon of yeast (Sigma YSC2, yeast from Saccharomyces cerevisiae type II) in water to form a thick paste. Place a small amount of this on each grape juice plate just prior to use.

G-PEM buffer:

- 80 mM Na-PIPES, pH 6.9
- 1 mM $MgCl_2$
- 1 mM EGTA
- 1 mM GTP

Injection buffer:

- 150 mM K-Aspartate
- 10 mM K-phosphate
- 20 mM imidazole, pH 7.2

Heptane glue:

Unroll double sticky tape and place in a 100ml bottle, add about 50ml heptane, seal the bottle, and rock for several days. When using, add heptane if the glue is too thick.

Dehydration chamber:

Take a 100 mm Petri dish, put one part of a 35 mm dish inside to make a "table" and add Drierite (anhydrous calcium sulfate) around it so that the height of Drierite is no higher than the "table" and cover. The coverslip with embryos will be placed on this "table" for dehydration before injection. It is a good idea to use at least some indicating Drierite and change it when it has changed color.

Protocol:

This protocol can be used for injection of virtually any soluble reagent into the Drosophila syncytial embryo: For example, either a fluorescent protein for observation or a target protein inhibitor or both.

Getting everything ready:

1.- 2 to 3 days before experiment, make grape juice plates and set up lay cage: Take a plastic bottle (6 oz, round bottom from Applied Scientific Drosophila), cut two small holes (about $1cm^2$) on opposite sides of the bottle and fill with a cotton piece (this will allow air to flow), tap the flies into the bottle and cover with a grape juice plate. Keep at 25°C (or room temperature), flies should lay embryos for about 10 days.

2.- At least one day before the experiment pull needles for injection, we use a Kopf Needle/Pipette puller model 720 and thin walled glass filaments (World Precision Instruments tw100f). For injection of fluorescent tubulin, keep needles at 4°C to prevent temperature-induced polymerization in the needle.

Tubulin preparation:

Note: All work with tubulin should be done at 4°C to prevent polymerization.

1. Take labeled tubulin out of the freezer and put it on ice for 5 to 15 minutes.
2. Dilute with G-PEM buffer (4 to 10 fold depending on the intensity required).
3. Centrifuge at 13k rpm at 4°C for 10-15 minutes.
4. Load 1 - 2 µl into needle.

Antibody or chemical inhibitor:

Prepare needles as for tubulin, using an appropriate concentration (this may need to be tested). The antibody can be in injection buffer, G-PEM buffer or PBS. If another buffer is needed, this buffer will need to be injected as a control to make sure this buffer causes no damage on its own.

Note: Inhibitor and fluorescent tubulin can be mixed and injected together if the concentrations allow it. Otherwise, inject the fluorescent tubulin and wait 5 to 10 minutes before injecting the inhibitor. When performing a double injection, start with more embryos, since fewer embryos will recover normally.

Embryo collection:

1. Put a new grape juice plate on the lay cage.
2. Remove this plate after one hour, this is the first collection of the day and often is not very good. It can be discarded.
3. Change the plate every hour to keep collecting embryos for one hour each.

Coverslip preparation:

1. Put a 50 x 22mm coverslip on one side of a microscope slide. Tape the four corners to the slide so it doesn't move.
2. Using a cotton tipped applicator, put one layer of heptane glue in one line on the coverslip (the glue should not be viscous and should dry in a few seconds, otherwise add more heptane).

3. Put a piece of double sticky tape on the slide toward the side of the coverslip.

Embryo preparation:

Note: Embryos should be imaged about 2 hours after start of collection, so begin the following steps allowing sufficient time to finish them within this time-frame.

1. With a moistened brush carefully pick up the embryos from the grape juice plate and place on double sticky tape on slide.
2. Using the outer part of the tweezers, roll the embryos over the double sticky tape until the chorion (the outer membrane) breaks open.
3. Pick up the embryo by gently rolling it over the chorion so it sticks to the tweezer and place it on the heptane glue on the coverslip with the long side of the embryo parallel to the long side of the coverslip. Set up 10 to 20 embryos in one row.
4. Remove the coverslip and place it in the dehydration chamber for 3 to 8 minutes (this depends on the humidity of the room and the amount to be injected).
5. Place on a metal chamber with vacuum grease.
6. Cover the embryos with halocarbon oil 700 to avoid further dehydration. Embryos are now ready for injection.

Embryo injection:

1. Find the embryos under a 16x objective.
2. Move the embryos away, and find the needle without moving the focal plane.
3. Center the needle and move it up without moving it in x or y direction.
4. If the needle is not open, put the edge of the coverslip in the field of view, but not where the needle will be, and lower the needle to the same focal plane. Very carefully move the coverslip until it hits the needle and gently breaks it open. If the needle is open, go to step 5. (Needles can be opened with hydrofluoric acid before filling).
5. Put the embryos in view (but not where the needle will be), lower the needle into the oil and make sure you obtain nice liquid drops from the needle.
6. Carefully but steadily move the embryo into the needle and inject a drop into the embryo and move the embryo away. (Or follow the instructions of your injection apparatus).
7. After all the embryos have been injected, they are ready for observation on a confocal microscope.

Imaging:

Image the embryos on a confocal microscope with a 60 or 100X objective:

Notes:

1. The time interval on a time lapse series should be at most a few seconds, since mitosis in the embryo is very fast, depending on the exact process to be studied.
2. A 3D series or a single plane can be acquired depending on the process of interest.
3. For fast-acting inhibitors, the transfer from injection microscope to imaging microscope has to be fast.

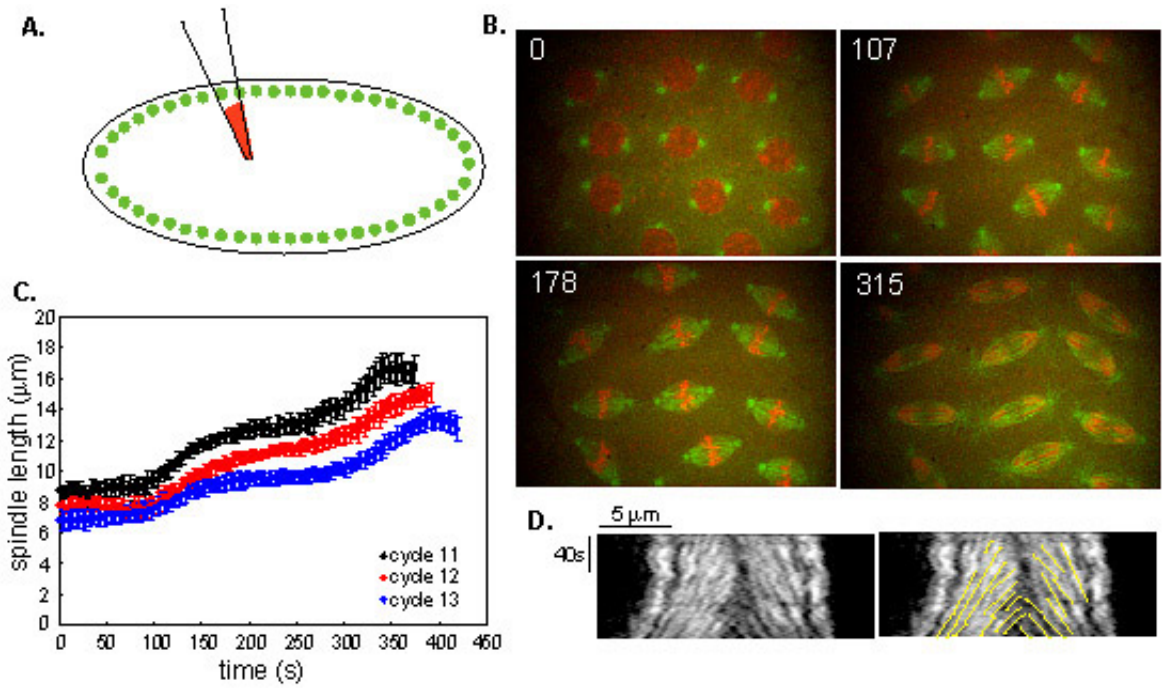

Figure 1: Study of mitosis in the *Drosophila* syncytial embryo. Nuclei in the early embryo undergo rapid divisions without cytokinesis, during cycles 10 thru 13 nuclei form a monolayer at the cortex. **A**. Schematic of early embryo with nuclei at cortex. Embryo can express GFP and/

or RFP labeled proteins and can be microinjected with other labeled proteins and/or inhibitors. **B.** Time lapse imaging of embryo expressing GFP-tubulin and RFP-histone. **C.** Plot of pole-pole separation for cycles 11, 12 and 13 in a wild type embryo. Spindle length exhibits periods of isometric length and periods of elongation, which are very consistent and reproducible. **D.** Dynamics of spindle microtubules. Injection of low concentration of rhodamine tubulin leads to speckles formed of a few tubulin subunits, which can be used to study microtubule dynamics.

Discussion

This protocol is relatively straightforward, however, each step requires practice to make sure the embryos are not damaged. Careful control experiments always need to be done to ensure that reliable results are being obtained. One good way to begin this is by microinjecting buffer or rhodamine tubulin into control embryos expressing GFP-tubulin to make sure that mitosis proceeds normally through all cycles at the embryo surface (cycles 10 through 13). In control embryos you should not observe any physical connections between spindles which are often the result of too much dehydration, so lower the dehydration time. When embryos are damaged, nuclear fallout is often observed, free centrosomes or uneven spacing of nuclei or spindles are indicative of damage. Plots of pole-pole distance are very reproducible and are a very reliable measure of “success”, in control embryos they should look like those shown in figure 1.

We have used this protocol to study many aspects of spindle assembly, maintenance and elongation using monoclonal, peptide or polyclonal antibodies raised against the protein of interest [1,5,6,7,8,9,10,11,12,13]. Specific protein inhibitors or other chemicals affecting the protein of interest can also be microinjected and their effects observed and quantitatively measured. To assess the effect of a particular inhibitor, we compare spindle length after inhibition to that observed in control embryos. We can also look at tubulin dynamics by using Fluorescent Speckle Microscopy[14] after microinjection of a low concentration of fluorescent tubulin (Fig. 1d).

We generally collect for one hour and let embryos mature for one more hour before observation, this means that embryos are between 1 and 2 hours old when observed. If the flies are laying well and many embryos are collected in one hour, then the collection time may be shortened so that more synchronously-dividing embryos are obtained. If the timing of inhibition is critical or of interest to the study, the inhibitor can be injected under confocal observation. This is harder, but it is possible nonetheless.

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