Chapter 3

Visualizing the Dynamics of Cell Division by Live Imaging *Drosophila* Larval Brain Squashes

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Abstract

The dramatic changes of subcellular structures during mitosis are best visualized by live imaging. In general, live imaging requires the expression of proteins of interest fused to fluorophores and a model system amenable to live microscopy. *Drosophila melanogaster* is an attractive model in which to perform live imaging because of the numerous transgenic stocks bearing fluorescently tagged transgenes as well as the ability to precisely manipulate gene expression. Traditionally, the early *Drosophila* embryo has been used for live fluorescent analysis of mitotic events such as spindle formation and chromosome segregation. More recent studies demonstrate that the *Drosophila* third instar neuroblasts have a number of properties that make them well suited for live analysis: (1) neuroblasts are distinct cells surrounded by plasma membranes; (2) neuroblasts undergo a complete cell cycle, consisting of G1, S, G2, and M phases; and (3) neuroblasts gene expression is not influenced by maternal load, and so the genetics are therefore relatively more simple. Finally, the *Drosophila* neuroblast is arguably the best system for live imaging asymmetric stem cell divisions. Here, we detail a method for live imaging *Drosophila* larval neuroblasts.

Key words *Drosophila*, Neuroblast, Mitosis, Live imaging, Division, Stem cell

1 Introduction

Some of the most striking changes in cell architecture occur when a cell divides. The nuclear envelope breaks down and reforms, the mitotic spindle assembles and disassembles, duplicated sister chromatids separate, and the cell is cleaved into two. Live imaging dividing cells has resulted in a quantum step forward in our understanding of how these events occur [1]. For example, live microscopy has revealed how spindle dynamics control chromosome movement [2, 3] and how distinct processes govern cytokinesis [4]. In particular, the development of fluorescence microscopy has contributed to understanding the molecular basis for large-scale mitotic events [5–7]. However, a number of key requirements must be considered when selecting a good candidate tissue for live fluorescence microscopy. Subcellular structures should be

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visualized by easily introduced fluorescently tagged proteins, samples must be easily acquired and able to withstand the rigors of fluorescent microscopy, and genetic manipulation of the sample should be straightforward to allow for experimentation.

Given these criteria, *Drosophila melanogaster* is an excellent system for live imaging. Thousands of fly stocks expressing fluorescently tagged proteins and RNAi are already widely available from centers such as the Bloomington Drosophila Stock Center and the Vienna Drosophila Resource Center. In addition, the GAL4/UAS system facilitates the expression of fluorescently tagged proteins (and RNAi knockdowns) specifically in the larval neuroblasts and numerous other tissues [8]. Because *Drosophila* are easy to grow and have a rapid generation time, sample tissue is continually available. In addition, a variety of *Drosophila* tissues are fairly resistant to the stresses of live imaging. Because of these characteristics, the *Drosophila* cell biologist can choose to use one of several tissues to study cell division: early embryos [9, 10], male germ stem cells [11], oocytes [12], the midgut [13], wing disk [14], cultured cells [15], or larval neuroblasts [16].

Here, we present a detailed description of how to live image dividing larval neuroblasts, which possess a number of the advantages of the early embryo and cultured cells without several of their disadvantages. For example, like early embryos, larval neuroblasts exist in an extant tissue (the brain) and therefore represent a more in vivo context for a dividing cell than cultured cells. In addition, like cultured cells, neuroblasts are individual cells surrounded by a plasma membrane that undergo a full cell cycle, unlike the syncytial embryo. Furthermore, the introduction of fluorescently tagged proteins and/or mutant alleles of genes of interest can be easily achieved through crossing schemes as opposed to transfection, and yet larval neuroblasts bypass any potential complications caused by the contribution of maternally loaded RNA and protein. In addition, neuroblasts provide an opportunity to study the dynamics of asymmetric stem cell divisions with one daughter undergoing self-renewal and the other differentiation. We and others have used live imaging dividing larval neuroblasts as presented here, first developed in [17], to study aspects of chromosome segregation [18, 19], spindle dynamics [20, 21], nuclear envelope reassembly [22–24], and cleavage furrow formation [25, 26].

2 Materials

2.1 Growing Larvae

1. Bottles for growing *Drosophila*.
2. Standard brown fly food: molasses, agar, cornmeal, yeast, methylparaben, ethanol, diH_2O, and propionic acid.

2.2 Dissecting

1. 1× PBS. We make a 10x PBS stock solution and dilute to 1× when ready for dissecting and imaging. 10× PBS: 40 g of NaCl, 7.2 g of Na_2HPO_4, 1 g of KCl, and 1.2 g of KH_2PO_4 to 500 mL of diH_2O. Stir until all components are fully dissolved and filter through a 0.2 μm filter. 10× PBS can be stored at room temperature.
2. A pair of ultrafine tip forceps.
3. A dissecting dish.
4. A dissecting microscope.

2.3 Squashing

1. Microscope slides.
2. Appropriate square coverslips for the microscope you will be using.
3. Disposable wipes.
4. Halocarbon oil 700.

2.4 Imaging

1. Fluorescent microscope. We primarily use one of two microscopes for imaging mitotic neuroblasts: a widefield microscope or a spinning disk confocal microscope.

3 Methods

3.1 Growing Larvae

1. Set up cross in large *Drosophila* bottles. Crosses should be at least 7 × 7 to ensure an adequate number of larvae for filming.
2. We recommend growing crosses at 25 °C in a 12 h light-dark cycle.

3.2 Dissecting

1. Fill your dissecting dish with 1× PBS. 200–500 μL is a sufficient volume for the three-depression spot plates we use.
2. Gently pick a crawling third instar larva. Avoid larvae that seem lethargic or close to pupation.
3. Place larva in the PBS-filled dissecting dish.
4. Using the forceps in your nondominant hand, gently grab the larva in the area around its mouthparts (*see* Fig. 1). Press the larva to the bottom of the dissecting dish so as to pin it in 3D space.
   (a) Once larvae are grasped, they usually begin to thrash. Pinning the head makes the following incision easier to perform.
5. While pinning the larva to the bottom of the dish, use the second pair of forceps in your dominant hand and create an incision in the larva about one third to halfway of the larva's

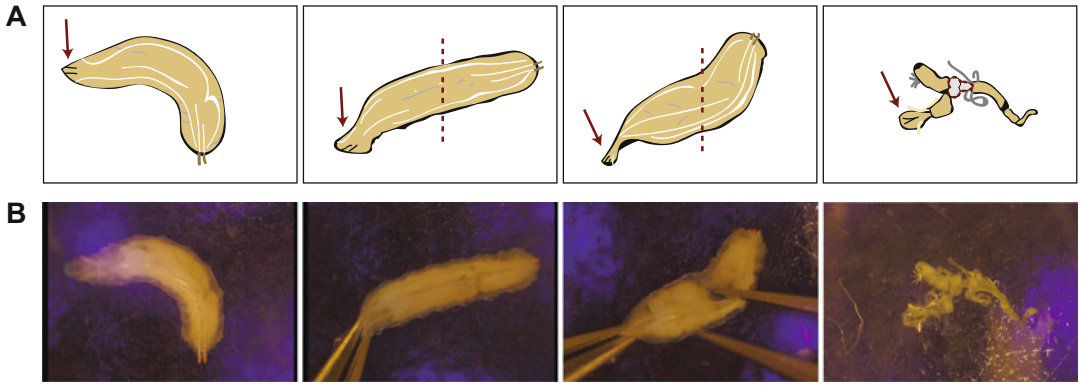

Fig. 1 Initial cut during third instar larval brain dissection. (**a**–**b**) Diagram (**a**) and images (**b**) showing the initial cut of the dissection. First, hold the mouthparts (arrow) with the forceps in your nondominant hand. Next, cut about halfway along the body of the larva (dashed line). Sometimes cutting with open forceps is easiest. The brain generally stays attached to the anterior segment of the carcass. The brain is encircled in a dashed line. The rest of the tissue must be cleaned away

body length posterior to the mouthparts (*see* Fig. 1). In one motion, create the incision and pull the resulting posterior segment of the larva away.

(a) This movement is best made by starting with the forceps open: create the incision with one blade of the forceps, quickly close the forceps, and then pull the posterior segment of the carcass away.

(b) Performing this movement quickly and with confidence will help ensure the dissection is clean and the brain is undamaged during this step.

6. Usually, the brain remains attached to the anterior segment of the larval carcass (*see* Fig. 1).

(a) The brain is recognizable as two connected opaque spheres with a cylinder of tissue extending from between them (the brainstem).

(b) Sometimes, the brain will be hidden in the carcass after this first incision. If this is the case, gently pick away at the external tissue of the carcass until the brain is visible.

7. Clean the brain of non-brain tissue (*see* Fig. 2).

(a) We recommend continuing to pin the tissue by the mouthparts to the bottom of the dissecting dish with your nondominant hand and tearing excess tissue with the forceps in your dominant hand.

(b) Avoid touching the lobes of the brain. If you must, grasp the brain by the brainstem as if it were a handle.

(c) If the lobes are damaged, discard the brain, pick another larva, and start again.

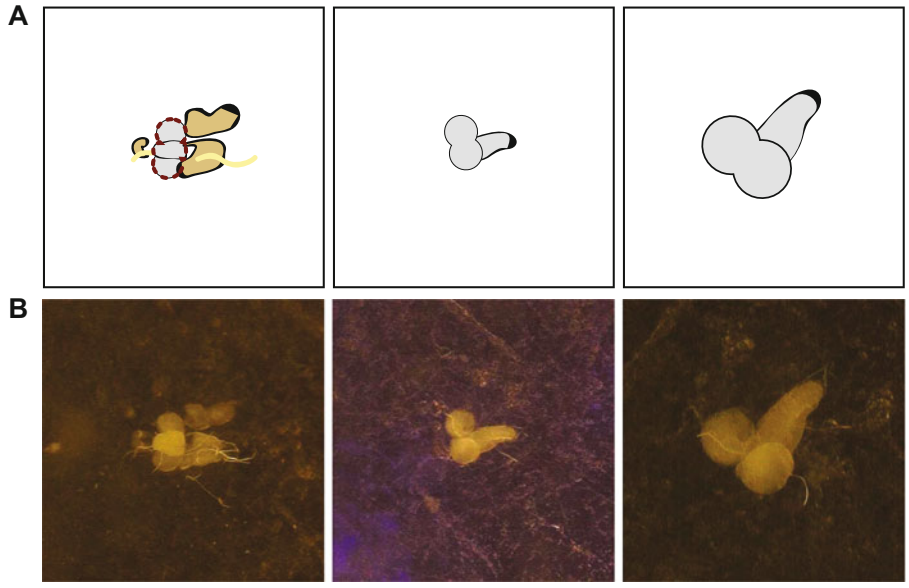

Fig. 2 Cleaning the brain from surrounding tissue. (**a**–**b**) Diagram (**a**) and images (**b**) showing before and after cleaning the brain. The brain is encircled in a dashed line. The rest of the tissue must be cleaned away. The cleaned brain should have no excess tissue on it, but nerve fibers are acceptable

(d) You will want your brain to be as clean as possible. However, removing the brainstem or the axon bundles projecting from the brain is not required.

3.3 Squashing

1. Pipette 15–20 μL of 1× PBS onto microscope slide (we recommend starting with 17 μL).
2. Transfer the brain from the dissecting dish to the middle of the PBS drop on the slide.
   (a) Move the brain by grasping the brainstem with the forceps.
3. Use a disposable wipe to dust off the appropriate coverslip for your microscope.
4. Carefully place the cleaned coverslip for your microscope over the top of the PBS drop.
   (a) Try to avoid introducing air bubbles into the PBS drop when placing the coverslip onto the top.
   (b) If there is an air bubble that presses up against the brain, discard the slide, pick a new larva, and start again.
   (c) We recommend placing one side of the coverslip down first and then slowly lowering the other side of the coverslip until the coverslip is flush with the slide.

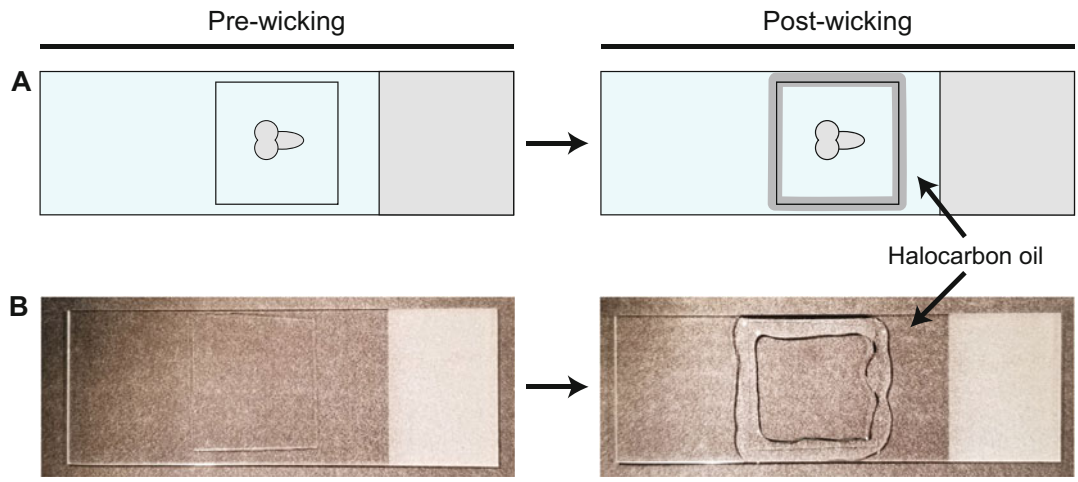

Fig. 3 Gentle squashing of the brain through wicking. (**a**–**b**) Diagram (**a**) and images (**b**) showing before and after a gentle brain squash. The brain is placed in a drop of PBS between a cleaned slide and coverslip. After wicking away some of the PBS from each edge of the coverslip with a Kimwipes, apply halocarbon oil around all the edges of the coverslip. Completely covering the edges will help prevent brain movement during imaging

5. At this point, the PBS drop will have spread to the edges of the coverslip. Use a disposable wipe, and wick away PBS from all four sides of the coverslip.
   (a) Hold the edge of the disposable wipe to the edge of the coverslip for about 2–3 s per side, but avoid over-squashing the brain (*see* **Note 1**).
6. The brain will now be gently squashed. If you hold the slide up to the light, you should see the lobes spread out.
7. Add halocarbon oil to the perimeter of the coverslip (*see* Fig. 3).
   (a) Make sure that no edges of the coverslip are exposed (*see* **Note 2**), but avoid using excess halocarbon oil, as the halocarbon oil will expand, and you do not want to mix the halocarbon oil with the immersion oil used for the objective of your microscope.

3.4 Imaging

1. Larval neuroblasts can be filmed at room temperature.
2. Film neuroblast divisions with a 100× objective.
3. Find the focal plane of the squashed brain.
4. After the gentle squashing, the neuroblasts will be pushed to the edge of the brain, so move the stage until the edge of the brain is in the field of view.
5. Search around the edge of the brain for a mitotic neuroblast.
   (a) This can be done in two ways: by using transmitted light (search for cells that have the nuclear envelope broken down) or by using a fluorophore-tagged protein that

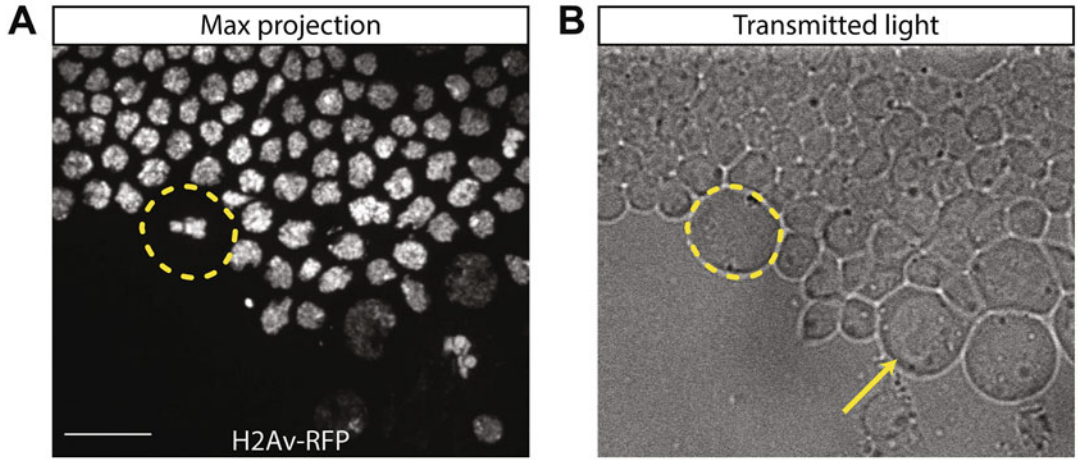

Fig. 4 Identifying mitotic neuroblasts. (**a**–**b**) Images showing the same neuroblast in metaphase using fluorescence (**a**) or transmitted light (**b**). Dashed lines mark the outline of the cell to be imaged. (**a**) H2Av-RFP (gray) marks chromosomes and can be used to identify cells in metaphase by observing sister chromatids align on the metaphase plate. Note how neuroblasts can be identified as large cells due to the space surrounding the chromosomes. (**b**) Transmitted light easily shows which cells are neuroblasts based on size: look for large cells surrounded by smaller cells. Mitotic cells can be identified by the absence of a nuclear envelope. The arrow marks a comparative neuroblast in interphase (the ridge of the nuclear envelope indicates this cell is not in mitosis). Imaging was performed on a spinning disk confocal microscope. Scale bar is 10 μm

exhibits a stereotyped behavior during mitosis (e.g., we image neuroblasts expressing the tagged histone variant H2Av-RFP to mark chromosomes and search for cells in which the chromosomes are aligned on the metaphase plate) (*see* Fig. 4).

(b) Neuroblasts are relatively large cells that undergo an asymmetric division to produce one self-renewed neuroblast and a smaller ganglion mother cell. When searching the edge of the brain for decent cells to image, look for large, spherical cells that are surrounded by smaller cells (*see* **Note 3**; Fig. 4).

6. When setting the parameters for your movie, make sure your Z-stack takes in the entire neuroblast cell.
   (a) We usually film with Z-stacks that are 7–10 μm in depth with a step size of 0.5 μm (widefield microscopy) or 0.36 μm (spinning disk confocal microscopy).
7. The interval between successive timepoints is dependent on the microscope you are using and how many fluorophores you are filming.
   (a) Typically, we image all neuroblast divisions with 20 s timepoints when we perform widefield microscopy.

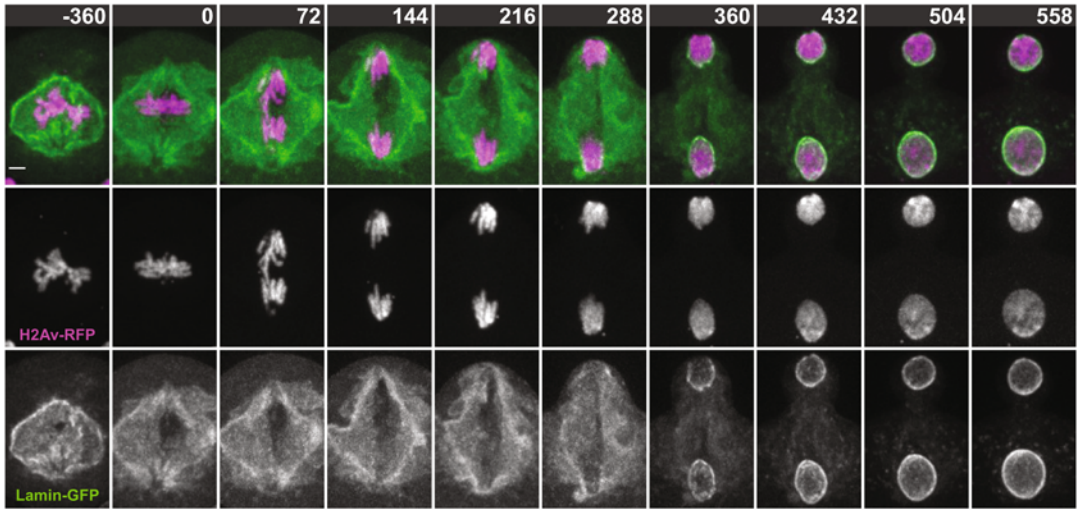

Fig. 5 Typical division of a neuroblast cell. Stills from a movie of mitotic neuroblast expressing H2Av-RFP (magenta) to mark chromosomes and Lamin-GFP (green) to mark the nuclear envelope. Note the asymmetric division to create a neuroblast (bottom daughter) and a ganglion mother cell (top daughter). Time is written in seconds after anaphase. Imaging was performed on a spinning disk confocal microscope. Scale bar is 2 μm

(b) We image neuroblasts expressing one fluorophore at 8 s intervals and neuroblasts expressing two fluorophores at 18 s intervals when we perform spinning disk confocal microscopy.

8. Film for 20–25 min. This will provide sufficient time for a metaphase cell to enter into anaphase and progress through telophase and into the subsequent cell cycle.
9. Brains can be imaged for up to 1 h.
10. An example of a typical neuroblast division can be seen in Fig. 5.

4 Notes

1. If the brain is under-squashed, it will be difficult to film cells on the edge of the brain as the cells will be stacked on top of one another. If the brain is over-squashed, cells will arrest in metaphase and not divide. If the brain is obviously under-squashed or over-squashed, discard the slide and start again. If the cell hasn't begun anaphase after 10 min of metaphase, end the video and select a different cell. If multiple neuroblasts are arrested in metaphase, discard the slide and perform another dissection and squash.
2. It is imperative to make sure the halocarbon oil overlaps all the edges of the coverslip. If there is an edge that is uncovered, some of the PBS from the squash may evaporate during

filming. This will lead to the cell moving significantly laterally during the filming and potentially even out of frame. If you notice this occurring, discard the slide and start again.

3. Neuroblasts that are directly on the edge of the brain squash (i.e., not completely surrounded by other cells) are the best candidates for filming. Additionally, avoid filming neuroblasts if there is a bright spot of fluorescence in an adjacent cell.

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