Video Article

Live Dissection of *Drosophila* Embryos: Streamlined Methods for Screening Mutant Collections by Antibody Staining

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Abstract

Drosophila embryos between stages 14 and 17 of embryonic development can be readily dissected to generate "fillet" preparations. In these preparations, the central nervous system runs down the middle, and is flanked by the body walls. Many different phenotypes have been examined using such preparations. In most cases, the fillets were generated by dissection of antibody-stained fixed whole-mount embryos. These "fixed dissections" have some disadvantages, however. They are time-consuming to execute, and it is difficult to sort mutant (GFP-negative) embryos from stocks in which mutations are maintained over GFP balancer chromosomes. Since 2002, our group has been conducting deficiency and ectopic expression screens to identify ligands for orphan receptors. In order to do this, we developed streamlined protocols for live embryo dissection and antibody staining of collections containing hundreds of balanced lines. We have concluded that it is considerably more efficient to examine phenotypes in large collections of stocks by live dissection than by fixed dissection. Using the protocol described here, a single trained individual can screen up to 10 lines per day for phenotypes, examining 4-7 mutant embryos from each line under a compound microscope. This allows the identification of mutations conferring subtle, low-penetrance phenotypes, since up to 70 hemisegments per line are scored at high magnification with a 40X water-immersion lens.

Video Link

The video component of this article can be found at https://www.jove.com/video/1647/

Protocol

Introduction

Drosophila embryos between stages 14 and 17 of embryonic development can be readily dissected to generate "fillet" preparations. In these preparations, the central nervous system (CNS) runs down the middle, and is flanked by the body walls. The gut is removed. When stained with antibodies, fillets allow much better visualization of CNS and body wall structures (*e.g.* motor axons, muscles, peripheral sensory (PNS) neurons, tracheae) than do whole-mount embryos, because there is no tissue intervening between the preparation and the coverslip, and because fillets are flat, allowing structures that extend across the body wall to be visualized in a single focal plane. Many different phenotypes have been examined using such preparations. In most cases, fillets are generated by dissection of fixed, antibody-stained whole-mount embryos. These fixed preparations are generated by the following steps: 1) chorion removal with bleach; 2) fixation with paraformaldehyde/heptane; 3) vitelline membrane removal with methanol; 4) antibody staining using immunohistochemistry or immunofluorescence; 5) clearing in glycerol; 6) dissection with tungsten needles. Detailed protocols for staining these "fixed dissections" are provided in ref. [1].

Fixed dissections have some disadvantages, however. First, it is often difficult to sort fixed, stained mutant (GFP-negative) embryos from stocks or crosses in which mutations are balanced over GFP balancers, even when anti-GFP is used for detection. This is due to a variety of factors, including maternal expression of GFP. For example, we have found that it is almost impossible to sort fixed, stained homozygous mutant embryos from balanced third chromosome stocks using either actin-GFP or armadillo (arm)-GFP balancers. Second, it is quite time-consuming to generate high-quality fixed dissections. 10-15 per hour is about as fast as most people can do this. Third, some antibodies do not stain well in fixed dissections, either because the antibody epitopes are sensitive to fix, or because an antibody that stains both internal and external structures is "soaked up" by the external structures and does not penetrate to internal structures (*e.g.* antibodies against fasciclin III (Fas3)). Fourth, live staining with receptor fusion proteins to detect ligand expression cannot be done on fixed preparations.

Since 2002, our group has been conducting deficiency (Df) and ectopic expression screens to identify RPTP ligands. In order to do this, we developed streamlined protocols for live embryo dissection and staining of collections containing hundreds of balanced lines. Staining for orphan receptor ligands with receptor fusion proteins is a specialized application that is not employed by many groups. However, many groups do use antibody staining of fillets to visualize embryonic phenotypes. Through our development of these methods, we have concluded that it is considerably more efficient to examine phenotypes in large collections of stocks by live dissection than by fixed dissection. We have used

live dissection to characterize motor axon, CNS, and muscle phenotypes in more than 600 Dfs, and have also characterized nervous system phenotypes produced by ectopic expression of more than 400 different cell surface and secreted proteins (A.W. *et al.* in preparation; H-K. L. *et al*., in preparation).

The live dissection protocols we have used have evolved over the years. One of the authors (K.Z.) was first introduced to live dissection more than 20 years ago by Nipam Patel, who was then a graduate student in Corey Goodman's lab. In more recent years, we used this method to stain the CNS with anti-Fas3 [2], but employed fixed dissections for all other experiments. In 1999, Aloisia Schmid, then a postdoc in our group, introduced us to live dissection with glass needles, which she used to examine phenotypes in double stranded RNA-injected embryos[3, 4]. When we began doing the RPTP ligand screens a few years later, we modified her protocols to allow rapid analysis of large collections of stocks maintained over GFP balancers. We found that GFP-negative embryos can be easily sorted from GFP-balanced stocks even when there is substantial maternal GFP expression (*e.g.* for the TM3armGFP balancer), because the embryos are sorted live and subtle differences in GFP expression can be readily detected. Our successful Df screen for a Lar ligand is described in[5].

Using our current protocols, a single trained individual can screen 5-10 lines per day for phenotypes, examining 4-7 mutant embryos from each line under a compound microscope. After selecting and arraying the embryos, it takes about one hour to dissect 50 embryos. This method allows us to identify mutations conferring subtle, low-penetrance phenotypes, since up to 70 hemisegments per line are scored at high magnification with a 40X water-immersion lens. Such phenotypes would be difficult or impossible to detect in screens of stained whole-mount embryos under a dissecting microscope.

We have defined a Df kit for phenotypic screening that contains about 250 lines and represents about half of the genome (A.W. *et al*., in preparation). The development of this kit was begun as part of our screen of the Bloomington Df kit as it existed in 2002-2003 for genes required for RPTP ligand synthesis[5]. In the course of this screen, we replaced Bloomington kit Dfs for which Df/Df homozygotes did not develop a CNS (and therefore could not be screened for CNS ligand expression) with smaller Dfs, preferably molecularly mapped, that had better development.

Df/Df homozygotes from lines in our phenotypic screening kit have normal overall morphologies at stage 16, allowing an investigator to systematically screen for genes affecting development of the CNS, the PNS, the muscle fibers, the tracheal network, and many other tissues. Any structure, expression pattern, or subcellular localization pattern that is visualizable with a specific antibody or GFP marker at stages 14-16 can be screened for phenotypes using this kit. This means that, using the protocol described here, one person spending about half of his/her time on this project could systematically screen half of all *Drosophila* genes for any desired embryonic phenotype within six months to one year.

A. *Drosophila* embryo collection

1. Prepare "Five-Barrel" egg collection chambers

These chambers save time and effort when examining a large number of fly lines.

1. Arrange five 50mL Conical Tubes upside down on a bottom part of a 100 x 15 mm plastic Petri dish and glue the tubes together using silicon rubber adhesive sealant.
2. Once the glue is hardened, glue the bottom part of the Petri dish onto the conical ends of the tubes. Five conical tubes will stand inside of the Petri dish.
3. Using a hot needle, make holes in the tubes for air circulation.

2. Prepare egg collection plates

1. Pour about 7mL of grape agar gel (1%) in each 100 x 15 mm plastic Petri dish. The ideal thickness of the gel is about 3-4 mm to seal each conical tube and to provide moisture for 24 hours. If the gel is too thick, it may fall down when we change the plate.
2. After cool off the gel plates, cover and put these in the original plastic Petri dish bag. Seal the bag tightly and store at 4°C.

3. Prepare flies

1. In order to obtain enough eggs for easy selection of embryos of the correct stage, we try to put >50 virgins into a cross, together with >20 males.
2. If a cross is to be screened, mix the male and female flies and keep in a fly food vial with lots of dry yeast pellets on the bottom and a thin paper strip (10 X 100 mm, folded in half) planted in the middle of the food. The paper strip will provide more surface area and keep the flies from excessive moisture.
3. Keep the vials with flies at RT for at least 3 days.
4. If a stock from a bottle is to be screened, simply transfer flies into the collection chamber.

4. Transfer flies to Five-barrel egg collection chambers.

1. Label the each barrel of egg collection chambers.
2. Put yeast paste on an egg collection plate for each barrel.
3. Prepare 20 inch long labeling tape to hold the chamber and the plate together. Fold 10 mm of each end for easy plate change.
4. Inject CO_2 gas in the fly vials and transfer the flies into the labeled barrels.
5. Cover the chamber with the egg collection plate and press down.
6. Tape around the chamber and the plate together starting from the middle of agar plate. The tape will overlap on the egg collection plate.

5. Collect embryos

1. Prepare egg collection plate: Put yeast paste on an egg collection plate for each barrel.
2. Tap down the flies in egg collection chambers and remove the tape around the chambers (Hold the old plate tightly).

3. Tap down the flies again and replace the old plate with new one.
4. Collect embryos in slanted position for 2-4 hours.
5. Replace the egg collection plate at desired time.

6. Age embryos

Place the egg collection plate upside down on a covered Petri plate with wet 90 mm circle filter paper and keep at the desired temperature. For dissections of stage 16 embryos from balanced mutant lines, we typically do a collection in the late morning, then incubate the embryos for >22 hr at 19° C. For gain-of-function studies using the UAS/GAL4 system, we collect embryos at RT in the afternoon and incubate for 16 hr at 19°C. Next day we transfer the embryos to a 29°C incubator to activate the UAS/GAL4 system for 1 or 2 hr. After this amount of time, the embryos are mostly stage 15, and this allows enough time to sort embryos for GFP expression and line them up, so that they will be at stage 16 when the dissection is done.

7. Staging embryos and sorting for GFP expression.

To discriminate the genotypes of embryos, we use GFP balancers. We examine dechorionated embryos on double-stick tape (see next section) under an Olympus GFP dissecting scope, and we select them for age and GFP expression at the same time.

a) GFP sorting:

1. The 2nd chromosome balancer we use (CyOarmGFP) has GFP driven by the armadillo promoter, which is expressed throughout the ectoderm. CyOarmGFP heterozygotes are olive green, and CyOarmGFP homozygotes are bright green. In a CyoarmGFP stock, the embryos that lack the balancer are easily distinguished, because they have almost no GFP expression (they look like embryos from a stock with no GFP balancer).
2. The 3rd chromosome balancer (TM3armGFP) is harder to use for sorting, because it has more maternally driven GFP expression. As a result, the embryos are divided into more and less green categories. With some practice, it is usually straightforward to sort the less and more green embryos from each other, but it is also necessary to resort them by clustering them and picking out the occasional embryos that are more green than the others (TM3armGFP heterozygotes) after transfer to the agar plate. Otherwise, there are sure to be some embryos that are of the incorrect genotype.
3. The X balancer is FM7 Kr-GAL4, UAS-GFP. This is expressed in a tissue-specific pattern. The patterns that are relevant to sorting at stages 15-16 are the amnioserosa and two points of expression in the head, which are the Bolwig's organ cells. Unfortunately, at the stage where one wants to sort embryos, the amnioserosa is fading and the Bolwig's organs have not yet begun to glow. It is thus necessary to look very carefully at the embryos on the tape, often rolling them over, to score them for GFP, and it is essential to resort them on the agar plate. Usually we do this twice: once immediately after moving all the embryos from the tape to the plate, and again after aging to the appropriate stage, just before lining them up in preparation for dissection. Sometimes, we also re-examine the slide under the GFP scope after transfer of the embryos to the PBS pool (see below), because visualization of GFP is better under these conditions. Then, we can destroy embryos that have GFP just before beginning the dissection.

b) Staging

The primary tool for staging embryos around st. 14-16 is gut morphology. The gut glows with autofluorescence under the GFP scope. Prior to attempting to stage embryos, one should consult reference books or sites that have pictures and diagrams of embryos (*e.g.* the Hartenstein and Campos-Ortega green book), so that one understands what they should look like. The ideal stage for dissection is when the gut has divided into three bands. Prior to that, the gut appears as a single blob. One often sorts embryos at the blob stage and then ages them until they reach the best stage for dissection. After the three-band stage, the gut begins to develop diagonal bands near the tail, and then coils, and the embryo becomes clearer. Within this period, the embryo becomes difficult to dissect because it won't stick onto the glass slide. If one wants to dissect late stage 16/early stage 17 embryos, it is necessary to dissect many embryos and evaluate which gut morphologies are associated with embryos that stick or do not stick. This varies somewhat between genotypes as well.

B. *Drosophila* embryo live dissection

1. Embryo dechorionation
    1. Prepare double-sided sticky tape and grape plate slab on a slide
    2. Transfer the aged embryos from the collection plate to the double-sided sticky tape using a wet paintbrush. Spread embryos evenly.
    3. Dechorionate the embryos on the double-sided sticky tape by rolling them across it gently with a blunted dissecting needle. After dechorionation, the embryos will stick more strongly to the end of the needle than to other embryos or to the chorion, but not as strongly as to the tape; thus one rolls the embryos on top of the chorion or onto other embryos, then lifts it off and transfers it to the grape plate slab. Genotyping and staging (see above for detailed description) is done during the process of dechorionation and transfer to the needle.
    4. After resorting for genotype and age, move the embryos to a smaller rectangular slab of agar, of sufficient width to allow lining up of 10-15 embryos in a row. Align the dechorionated embryos on this slab: dorsal side down against the agar slab and posterior ends facing toward you. We normally make rows of 5-10 embryos.

2. Prepare a slide and transfer embryos for live dissection
    1. Cut a small rectangular piece of double-sided sticky tape and place it near one end of Super Frost/Plus slide (Fisher Scientific, Cat#12-550-15).
    2. Draw a rectangle (30-40 mm long, and the full width of the slide) around the tape with a wax pen, so that there is ~3 mm of space between the wax and tape (Super HT Pap Pen, www.rpicorp.com) and transfer the embryos from the grape plate slab on to the tape, by inverting the slide and gently lowering it onto the embryos, so that the tape comes into contact with the lined-up embryos (done

under a dissecting scope). The embryos should be aligned so that the posterior end is toward you. They will now be dorsal side up on the slide.
3. Immediately add 1X PBS (Gibco recipe) over the embryos to fill the wax rectangle.

3. Embryo live dissection
   1. Using a glass dissection needle (made from an injection needle), cut along the dorsal midline, starting from the posterior end of the embryo.
   2. Poke the anterior end of the embryo, lift up and transfer the embryo to the slide until it sticks. Keep embryos in the buffer. Make an orderly line of embryos on the slide that matches each row on the agar slab. Organize the rows so that they will all fit on the slide.
   3. There are a variety of possible dissection sequences that can be used in generating fillets. People typically develop their own optimized procedures through doing the dissections themselves. The video demonstrates a sequence in which a small cut is made at the posterior end of the embryo to separate the two sides of the body wall from each other. If desired, one can also break the midgut/hindgut connection with this cut, and displace the hindgut out onto the slide at this time (in the video, the hindgut displacement is done later). Then, cuts are made on each side just posterior to the brain lobes in order to separate the body walls from the brain. Depending on the age of the embryo, one may also need to make a shallow cut down the dorsal midline to sever the amnioserosal connection between the body walls.
   4. Then, gently paste down the flaps of body wall onto the slide, avoiding stretching them or removing material from their surface. This is best done by only allowing the needle to contact the anterior and posterior corners at the dorsal edges of the body wall. The motor axons do not extend this far dorsally, so in the best case this will produce an embryo with completely intact ISN branches. In some cases the ISN will be truncated, even with the best technique, because the embryo does not always separate exactly along the dorsal midline. If done correctly, this method will preserve the body wall structures in segments T3-A6 (approximately).
   5. One can leave the midgut on top of the embryo, and then remove it from all dissected embryos after finishing all the dissections. This is done by poking the gut, displacing it away from the embryo, and stretching it to break its connection to the foregut, which lies under the brain lobes. Or, one can remove the gut from each dissection as one completes it. With mutants that have abnormal gut development, it is an advantage to remove all the guts at once after finishing the dissections, because released yolk from the gut can make it more difficult to stick later-transferred embryos to the slide after transfer from the doublestick tape. It is sometimes helpful to transfer the embryos starting at the bottom of the slide (toward you) rather than at the top, as yolk tends to spread toward you due to the typical movements of the needle.

4. Fixation and immunofluorescence/immunohistochemistry
   1. At this point, one can either fix the embryos immediately, if one is staining with antibodies, or one can remove the PBS and replace it with fusion protein supernatant, if one is doing a live staining experiment to detect ligand expression. This aspect of the experiment is described in detail in the Methods and Supplementary Methods sections of ref. [5].
   2. Here we describe the fixation protocol, which is done either immediately or after fusion protein staining. Using two Pasteur pipettes, replace the PBS with 5% paraformaldehyde in PBS (**EMS,** Cat. #15713-S, www.emsdiasum.com). Exchange in fix solution three times, which involves using ~6 ml of fix solution, since a Pasteur pipette holds 1.5-2 ml of solution. Fix for 45'.
   3. Remove fix solution, replace with PBS (3 changes), then with PBT (PBS + 0.1% Triton X-100 + 1 mg/ml Fraction V BSA; 3 changes), then replace PBT with ~200 µl block solution (PBT + 5% heat-treated normal goat serum), then with the desired primary antibody in block solution. It is very important to not allow the meniscus to contact the embryos, particularly while they are in PBS, as this will destroy the dissections. After detergent is added the embryos become less sensitive to the meniscus. Thus, one can replace the PBT with block by tipping the slide and blotting off most of the solution by touching a Kimwipe briefly to the corner of the area enclosed by the wax. The same method can be used to replace the block with anti body solution. This minimizes carryover of solutions.
   4. Incubate overnight at 4°C, then wash 3X with PBT (>15 minutes/wash) in a tray on a rocking platform (make sure there is enough PBT so that the slide is always completely immersed during rocking), reblock as described above, add 200 µl secondary antibody, incubate 2 hr. at RT, rewash with PBT. Check staining (if green fluorescence is used) if desired under the GFP dissecting scope.
   5. Wash 2X with PBS (5'), then add 500 µl 70% glycerol/1X PBS (made by mixing 35 ml glycerol, 5 ml 10X PBS, and 10 ml water in a 50 ml tube), and clear overnight at 4°C (or for 2 hr at RT). Remove glycerol and put on a #1 coverslip.

Discussion

We hope that this video demonstration has displayed our methods for live embryo dissection and staining in sufficient detail that they can now be readily executed by any person who has some experience in *Drosophila* genetics and embryology. Of course, considerable practice will still be required, primarily for the dissections themselves. In our experience, every person who learns the live dissection methods will create a subtly different dissection protocol that allows him/her to most rapidly generate high-quality arrayed dissections. This process requires months for full development. However, most people can generate high-quality dissections after a few weeks of practice.

Although we favor the use of live dissections for screening, they cannot replace fixed dissections, because fixed dissection protocols can be applied to a wider range of embryonic stages. For example, in the analysis of our Df kit for motor axon guidance phenotypes, we found that the details of motor axon branches, including filopodia, can be much better visualized by immunofluorescent staining of live fillets than by conventional horseradish peroxidase (HRP) immunohistochemical visualization of fixed fillets (for examples of high-quality HRP staining, see the primer of motor axon guidance on our lab web page). However, embryos older than the beginning of stage 17 will not stick to SuperFrost Plus slides due to their development of cuticle. Thus, for quantitative analysis of phenotypes for late-developing motor axon branches such as the SNa, we still rely on fixed dissections (A.W. *et al*., in preparation).

At present, these methods have only been applied to a few problems by our group. These include the identification and phenotypic analysis of new genes involved in CNS and motor axon guidance, the identification of orphan receptor ligands, and the identification of genes required for synthesis of epitopes recognized by specific mAbs. However, many other applications can be envisioned, especially when these methods are combined with the analysis of our Df kit and ectopic expression collection, or of collections generated by other investigators. For example, it

should be possible to systematically screen for genes required for synthesis, cellular expression pattern, or subcellular localization of any protein that can be detected by a high-quality antibody or a GFP reporter.

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