Chapter 20

Live Staining of *Drosophila* Embryos with RPTP Fusion Proteins to Detect and Characterize Expression of Cell-Surface RPTP Ligands

Namrata Bali, Hyung-Kook (Peter) Lee, and Kai Zinn

Abstract

The activity and/or localization of receptor tyrosine kinases and phosphatases are controlled by binding to cell-surface or secreted ligands. Identification of ligands for receptor tyrosine phosphatases (RPTPs) is essential for understanding their in vivo functions during development and disease. Here we describe a novel in vivo method to identify ligands and binding partners for RPTPs by staining live-dissected *Drosophila* embryos. Live dissected embryos are incubated with RPTP fusion proteins to detect ligand binding in embryos. This method can be streamlined to perform large-scale screens for ligands as well as to search for embryonic phenotypes.

Key words Receptor tyrosine phosphatases, Live dissection, Fusion proteins, Ligand screening, Schneider cells

1 Introduction

Receptor tyrosine phosphatases (RPTPs) are transmembrane receptor proteins that reverse reactions catalyzed by tyrosine kinases. Phosphotyrosine signaling is essential for cell-cell communication in all metazoans [1]. We know a great deal about interactions of ligands with receptor tyrosine kinases, but relatively little about ligands for RPTPs. For example, of the six *Drosophila* RPTPs (*Ptp10D*, *Ptp69D*, *Ptp99A*, *Ptp4E*, *PTP52F*, and *Lar*) ligands and/or co-receptors have only been described for Ptp10D and Lar [2–4].

The first reported screen for *Drosophila* RPTP ligands was conducted by our group in 2002–2005 using live embryo staining with RPTP fusion proteins. This was a screen of a genome-wide collection of deletion mutations, called deficiencies (*Dfs*). It identified a deletion that eliminated a portion of the staining pattern observed with Lar fusion proteins, and we found that the responsible gene within the deletion was *Syndecan* (*Sdc*), which encodes

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a heparan sulfate proteoglycan [2]. This screen did not identify ligands for other RPTPs. Data from the ectopic expression screen described below suggested that this is because each RPTP binds to multiple ligands that are expressed in overlapping patterns.

More recently, we conducted an ectopic expression screen for RPTP ligands using live embryo staining and identified an in vivo ligand for Ptp10D, Stranded at second (Sas). This screen also identified several other candidate Ptp10D ligands [4], in addition to candidate ligands for Lar, Ptp69D, and Ptp99A (unpublished results). In the ectopic expression screen, 311 fly lines with "UAS"-containing insertion elements ("EP-like elements") upstream of genes encoding cell surface and secreted (CSS) proteins were screened by driving ectopic expression of each individual CSS protein using a pancellular (Tubulin-GAL4) driver. The available EP insertions covered 30–40 % of all CSS proteins, and the screen identified more than 20 candidate RPTP ligands, implying that there might be 50–60 proteins encoded in the genome that can bind to these four RPTPs in this assay. Our data also suggest that new ligands for vertebrate RPTPs can be identified by characterizing ligands that bind to their *Drosophila* orthologs (unpublished results).

For all of these screens, the extracellular domain (XCD) of an RPTP (or other cell surface protein) was fused to multimeric human placental alkaline phosphatase (AP) constructs to create RPTP-AP fusion proteins. These were expressed using baculovirus or *Drosophila* Schneider 2 cell (S2) systems, and the supernatant from infected or transfected cells was used directly for binding to live-dissected embryos. Dissected, unfixed embryos must be used because the fusion proteins cannot penetrate the vitelline membrane, and fixation destroys binding. Results from such screens can be confirmed by performing "reverse-binding" experiments. In these experiments, the RPTP is ectopically expressed in embryos using the GAL4 system and the embryos are incubated with candidate CSS ligand-AP fusion proteins to detect ectopic binding [4]. If specific binding patterns are observed in both the forward and reverse directions, this proves that the CSS protein can bind to the RPTP. However, it does not show that it can bind without additional cofactors that might be expressed in the embryo. For Sdc and Sas, we were able to show that they could bind to Lar and Ptp10D, respectively, in the absence of additional cofactors by performing in vitro binding studies [2, 4].

Here, we describe in detail live embryo dissections in *Drosophila* as a technique to detect ligands for RPTPs and other orphan receptors. Briefly, stage 16 *Drosophila* embryos are collected and dechorionated, followed by dissection with a glass needle on a slide (*see* Fig. 1 for staging criteria). Dissected embryos are then incubated with dimeric or pentameric RPTP-AP fusion proteins (or other CSS-AP fusion proteins), followed by fixation and anti-AP antibody staining to visualize bound AP fusion proteins. If a strong staining pattern such as those shown in Fig. 2 and in refs. 2, 4, 5 is

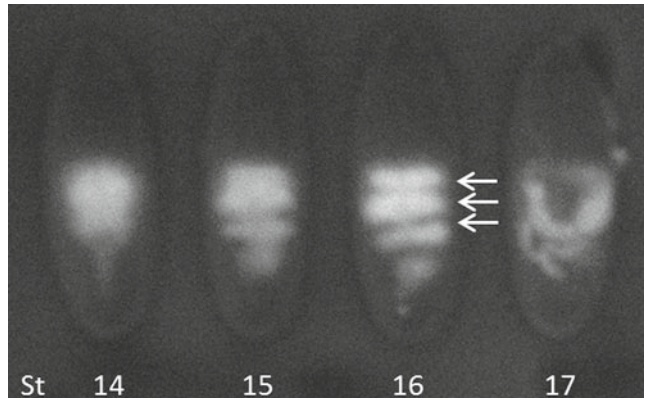

Fig. 1 Gut morphology in different stages of *Drosophila* embryos. The gut appears like a blob at early stage 13–14. A fissure appears in the gut and it starts to separate into bands at early stage 15. By stage 16, the gut has clearly divided into three distinct bands (*arrows*). This is the ideal stage for dissection. As the embryos age to stage 17–18, the gut forms spirals and the embryos can no longer be properly dissected

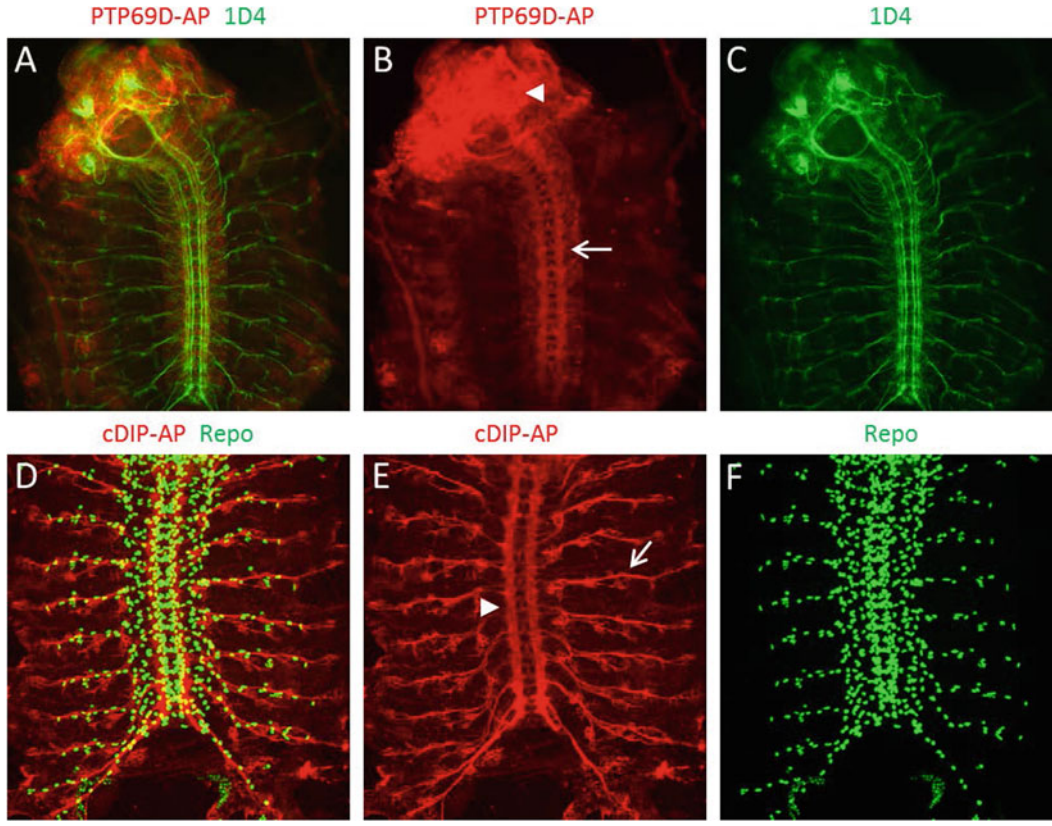

Fig. 2 Dissected and stained *Drosophila* stage 16 embryos. (**a**) A live dissected stage 16 embryo incubated with PTP69D-AP fusion protein and stained with anti-AP (*red*, **b**) and mAb 1D4 (*green*, **c**) antibodies. PTP69D-AP fusion protein binds specifically to longitudinal and commissural axon tracts in the ventral nerve cord (*arrow* in **b**) and also shows strong binding in the brain (*arrowhead* in **b**). (**d**) A live dissected stage 16 embryo incubated with cDIP-AP fusion protein and stained with anti-AP (*red*, **e**) and anti-Repo (*green*, **f**). cDIP is a cell surface protein and shows binding to CNS axons (*arrowhead* in **e**) and motor axons (*arrow* in **e**)

observed, this can be used as the basis for a *Df*, ectopic expression, or candidate gene screen to molecularly identify the responsible ligand(s). In the protocol below, we describe methods for expressing pentamerized AP fusion proteins in S2 cells, which is the easiest method. If adequate expression levels cannot be obtained in this system, it may be necessary to express the fusion proteins using baculovirus systems. The protocol for live embryo dissection and staining can be streamlined for many other applications including screening for embryonic phenotypes [5]. This live dissection technique is much faster than fixed embryo dissections for the purpose of screening for phenotypes as fixed embryo dissections take much longer than live dissections. Moreover, AP fusion protein staining can also be performed on third instar larvae to study ligand binding patterns of CSS proteins at later developmental stages (*see* **Note 1**).

2 Materials

1. Egg collection chambers: Arrange five 50 ml conical tubes upside down on a 100 × 15 mm plastic petri dish and glue them together using silicon rubber adhesive sealant. Then, glue the lid of the petri dish to the conical ends of the tubes to create a 'Five-barrel' chamber. Make holes in each tube using a hot needle for air circulation (*see* **Note 2**).
2. Egg collection plates: Make 1% grape agar gel by first mixing 50 g Bacto-Agar in 1.5 L distilled water. Secondly, add 177 ml frozen grape juice concentrate (*see* **Note 3**) and 25 g sucrose to 500 ml water. Autoclave both solutions separately for 45 min. Remove both solutions from autoclave and cool to approximately 60 °C. Mix the Bacto-Agar solution with the grape juice solution. Add 30 ml 10% Nipagin (*p*-hydroxybenzoic acid methyl ester) and 0.4 g Streptomycin sulfate to the mixed solution. Pour 10–11 ml of grape agar gel in each 100 × 15 ml plastic petri dish and let solidify (*see* **Note 4**). Store upside down sealed at 4 °C.
3. AP fusion proteins: Clone the extracellular domains (XCD) of desired proteins into an expression vector containing a pentamerization sequence and a human placental alkaline phosphatase (AP) tag [6]. Plasmid containing the XCD domain fused to AP tag can be transfected into Schneider cells (S2 cells). We use the Effectene transfection reagent to routinely transfect DNA into S2 cells with good yields of fusion protein in the supernatant. Collect the supernatant from transfected cells 2–3 days after transfection and concentrate the 1× fusion proteins up to 5× using Amicon Ultra-15 Centrifugal Filter Units. Store the fusion proteins at −80 °C for long term storage and at 4 °C for immediate use.

4. Dry yeast.
5. Superfrost Plus Micro slides.
6. Double-sided scotch tape.
7. Hydrophobic barrier pen.
8. Pulled glass needle for dissection: Pre-pulled glass microcapillary needles are available commercially. Alternately, glass capillary tubes can be bought and pulled individually. The glass capillary tubes have a 1 mm outer diameter and 0.6 mm internal diameter.
9. Phosphate buffered saline (PBS, 10×): Add 80 g NaCl, 25.6 g $Na_2HPO_4{\cdot}7H_2O$, 2 g KCl, and 2 g KH_2PO_4 to 800 ml water. Bring final volume up to 1 L with water and adjust pH to 7.4. Autoclave for 45 min. Store at room temperature.
10. PBT: PBS containing 0.05% Triton X-100 and 1% bovine serum albumin (BSA) (*see* **Note 5**).
11. 20% paraformaldehyde.
12. Fix solution: 4% paraformaldehyde in 1× PBS (*see* **Note 6**).
13. Normal goat serum.
14. Blocking buffer: PBT containing 5% normal goat serum (*see* **Note 7**). Store at 4 °C.
15. Rabbit anti-human placental alkaline phosphatase antibody.
16. Goat anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 568.
17. Antifade mounting medium.
18. Rectangular glass coverslips: #1 thickness, 22 mm × 40 mm.
19. Clear nail polish.

3 Methods

All procedures are carried out at room temperature unless specified otherwise. Ref. [7] contains a video demonstration of the live embryo dissection process and staining of embryos.

3.1 Preparation of Flies and Embryo Collection

1. Set up a cross using >50 virgins and >20 males and transfer the flies to one chamber of the five-barrel chamber. Label each barrel carefully with the correct genotype of the cross. Egg collection plates are used as the lid of the five-barrel chamber. Put a small amount of yeast paste at five spots on an egg collection plate in the center of each chamber. Cover the five-barrel chamber with the egg collection plate and gently press down. Tape the chamber and grape plate together using lab labeling tape. Flies in the five-barrel chamber should be incubated at 18 °C to prolong lifespan (*see* **Note 8**).

2. Use a fresh egg collection plate for each embryo collection. Put a very small amount of yeast paste on an egg collection plate in the center of each chamber. While holding the old plate on top of chamber carefully, gently tap the whole chamber down to tap down the flies. Tap flies down a few times and replace the old plate with the new egg collection plate.
3. Incubate the flies at room temperature for 2–4 h to allow sufficient embryos to accumulate on the plate. Replace the egg collection plate with a new one and return flies to 18 °C. Place a Whatman filter paper inside the lid of the grape plate and add enough water to completely soak it. Cover the egg collection plate with the lid and incubate at 18 °C overnight.
4. Change the grape plate of the chamber every day even if not collecting embryos (*see* **Note 9**).

3.2 Aging Embryos, Sorting, and Staging

1. Embryos are best suited for live dissections at stage 16 (Fig. 1) as the CNS can be fully visualized at that stage. It becomes harder to dissect stage 17 embryos as their outer chorion becomes tougher and we have found that stage 17 embryos do not stick well to the glass slide. Incubate embryos at 18 °C for ~22 h after collection to get stage 15 embryos. For studies using the UAS/GAL4 system, incubate embryos at 18 °C for ~20 h. Transfer embryos to a 29 °C incubator for 1–2 h to activate the UAS/GAL4 system.
2. GFP balancers are used to differentiate between different genotypes in a line or a cross. We use CyO arm-GFP as the second chromosome balancer, TM3 arm-GFP as third chromosome balancer and FM7 Kr-GAL4, UAS-GFP as the X chromosome balancer. GFP negative embryos are used to select homozygotes. GFP sorting is done under a GFP dissecting microscope where embryos are sorted and dechorionated at the same time.
3. Embryos are sorted using gut morphology around stages 14–16. The gut has autofluorescence under the GFP microscope and is visible as white and opaque under normal white light if using a regular dissecting microscope. At stage 16 the gut appears as three distinct bands. At earlier stages the gut looks like a single blob. This is closer to stage 14. As aging progresses from stage 14 to 16, the gut can be seen separating into bands. At stage 15 the gut has started to divide into bands, but the bands are not completely separate from each other. It is best to sort embryos and dechorionate them between stages 14 and 15, so that as soon as they reach the three band gut stage (stage 16) they are ready for dissections (Fig. 1) (*see* **Note 10**).

3.3 Embryo Dechorionation and Preparation for Dissection

1. Stick a long piece of double-sided sticky tape on a regular glass slide and place small pieces of grape plate agar slabs beside the sticky tape. Dechorionated embryos are placed on the agar slab for aging.

2. Transfer the collected embryos from the egg collection plate to the sticky tape on the slide using a fine paint brush. Wet the brush a little and spread the embryos evenly across the sticky tape. Break up any large embryo clusters gently using the brush.
3. Dechorionate the embryos on the double sided tape by gently rolling them on the tape using a blunted dissecting needle (*see* **Note 11**). Pick up the dechorionated embryos with the needle and place them gently on the agar slab beside the tape.
4. Dechorionate ~100 embryos so that there will be enough embryos of the right age at the time of dissection. Sort the dechorionated embryos according to age and place stage 16 embryos on a new agar slab. Place the embryos dorsal side down against the agar slab with their posterior ends facing toward you. Arrange the embryos in a row of five to ten embryos per genotype (*see* **Note 12**).
5. Prepare a super-frost plus slide for live dissection as follows. Cut a small piece of double-sided sticky tape (the size of the tape should be sufficient to accommodate the rows of embryos). Place the piece of sticky tape near one end of the super-frost plus slide.
6. Draw a rectangle around the sticky tape using a wax pen. We have found the ImmEdge hydrophobic barrier pen to be best suited for detergent-based washes during the immunohistochemistry steps. The rectangle should be big enough to allow dissection of all embryos on the agar slab. Sometimes a slightly larger rectangle can be drawn to allow more space option for embryos that do not stick very well.
7. Transfer the aligned embryos from the agar slab to the piece of sticky tape on the super-frost plus slide by gently lowering the tape on the slide over the embryos on the agar slab. The embryos now will be dorsal side up on the slide.
8. Add cold 1× PBS on top of the embryos. The wax rectangle will help to retain the PBS within the boundaries of the wax seal.

3.4 Embryo Live Dissection: See Ref. [7] for a Video Demonstration of the Live Embryo Dissection Process

1. A pulled glass dissection needle is used for the actual dissections. Make a superficial cut in the embryo along the dorsal midline starting from the posterior end of the embryo going all the way to the anterior end. This will rip open the interior vitelline membrane.
2. Poke the anterior end of the embryo with the needle and gently lift it up, keeping the embryo submerged in the PBS at all times. Transfer the embryo to one corner of the slide surface. A stage 16 embryo should stick to the slide surface easily. Using this same technique transfer all embryos from the tape to the slide surface in the same order as on the tape. Take extra caution to keep embryos arranged properly in neat rows to avoid confusion about the genotype of the embryos later on.

3. Once all embryos have been transferred to the slide surface, start dissecting the embryos one by one using the same glass needle. The cut made in the embryo in **step 1** should also cut open the embryo to separate the two sides of the body wall. However, this is not always the case. In this scenario, make another shallow cut along the dorsal midline starting at the posterior end. The two ends of the body wall should now be separate.
4. Gently paste the body wall flaps onto the slide on both sides. Make sure to not stretch the body wall or remove material from the surface. The best way to ensure least damage to the embryo is by only touching the anterior and posterior ends of the body walls and only at the dorsal-most edges since motor axons do not extend this far dorsally.
5. Fillet all embryos in this manner while leaving the gut intact on top of the ventral nerve cord. Once all embryos have been "filleted", begin to remove the gut from all embryos one by one (*see* **Note 13**). Make a cut where the hindgut is attached to the body wall. The gut can now be removed from the embryo in one of two ways. The gut can either be completely removed from the embryo by cutting the foregut which lies under the brain lobes, which would result in a free-floating gut which can be transferred back to the sticky tape to get it out of the way of other embryos. Alternately the gut can be simply displaced beside the embryo by pulling it away from the embryo toward the anterior end and sticking it on the slide. The foregut will stay attached to the embryo but this will not interfere with the staining of the embryos.

3.5 Fixation and Immunohistochemistry

1. After dissection, embryos are incubated with AP fusion proteins for 2 h. This is done before fixation. We typically use AP fusion proteins at 5× concentration if made using transfected Schneider (S2) cells. However the ideal concentration for optimal signal to noise ratio of the fusion protein binding signal should be titrated and optimized for individual AP fusion proteins. Weakly expressed fusion proteins will need to be used at a higher concentration (~5×) compared to strongly expressed ones.
2. Remove the sticky tape from the slide using a blunt-ended forceps and discard. Remove as much PBS as possible using a P200 pipette tip (*see* **Note 14**). Add 150–200 μl AP fusion protein, depending upon the size of the rectangle containing the dissected embryos.
3. Incubate at room temperature in a covered and humidified chamber for 2 h.
4. Prepare fresh fix solution (4% PFA in PBS) and replace the AP fusion protein with the fix. Wash using the fix five times, using

1 ml fix per wash to a total of 5 ml fix per slide. Use a pipette tip fitted at the end of a vacuum to remove solutions from the slide. A waste receptacle is attached to the end of the vacuum to collect solutions as they are removed from the slide. Fix embryos for 30 min. Again, take care to not expose the embryos to the meniscus of solutions. Keep embryos submerged in solution at all times.

5. Remove fix solution and replace with PBS. Wash three times with PBS, using 1 ml PBS per wash. Let the embryos sit in PBS for 5 min.
6. Replace PBS with PBT. Wash three times, using 1 ml per wash. Let embryos sit in PBT 5 min.
7. Once the embryos have come into contact with detergent in the PBT, embryos become less sensitive to the meniscus and solutions containing detergent (PBT, Block and antibody solutions) can be removed from the slide by blotting off with a Kimwipes. Place a folded Kimwipes at the edge of the wax seal and gently tip the slide over the Kimwipes. This will remove most of the solution from the slide and minimize carryover of solutions from a previous step.
8. Replace PBT with Block, by removing the PBT as described in **step 7**. Use ~200 μl Block per slide. Incubate in Block at least 30 min, up to 1 h. Additional blocking over an h does not seem to enhance signal to noise ratio.
9. Replace Block with desired primary antibody in Block solution. Remove Block using Kimwipes method (*see* **step 7**) and add ~200 μl primary antibody solution. Incubate in primary antibody overnight at 4 °C.
10. Next day, wash three times with PBT. Each wash should include four changes of PBT, 1 ml per change for a total of 4 ml per wash.
11. Replace last wash solution with secondary antibody in Block. Add ~200 μl secondary antibody solution and incubate at room temperature for 2 h.
12. Wash three times with PBT, similar to **step 10**.
13. Replace the last PBT wash with PBS. Wash with PBS two times, each wash with four changes of PBS, 1 ml per change. Incubate in the last PBS wash for 10 min.
14. Remove as much PBS as possible using a P200 pipette tip. Add ~40 μl Vectashield mounting medium for fluorescence around all embryos. Gently place a #1 glass coverslip over the embryos making sure there are no bubbles over the embryos. The Vectashield should diffuse evenly and cover the entire area of the coverslip. Place one drop of clear nail polish at each end of the coverslip and let dry in the dark for ~10 min (*see* **Note 15**).

15. Once the nail polish drops have dried, swipe with the clear nail polish around all edges of the coverslip, sealing the coverslip in place.
16. Store sealed slides horizontally at 4 °C.

4 Notes

1. In order to study ligand binding patterns in third instar larvae, dissect and fillet the larvae as described in [8]. Following the dissections, incubate the dissected larvae in AP fusion proteins. The concentration of the AP fusion proteins used will need to be optimized for each individual protein. Incubate larvae in AP fusion proteins for 2 h, followed by fixation and routine immunohistochemistry procedures.
2. Each chamber of the five-barrel chamber can hold up to 300 flies without overcrowding. However, if more flies are needed for an experiment, a larger plastic container can be used in place of the five-barrel chamber. Individual embryo collection cages are available commercially in three different sizes from flystuff.com (Catalog No: 59-100—59-106, Flystuff.com, San Diego, CA, USA). Small petri dishes of comparable size are also available from the same supplier, which can be filled with the grape juice solution to make appropriate sized egg collection plates.
3. Frozen grape juice can be bought at any grocery store and stored at −20 °C until used. We prefer to buy the juice in cardboard cans vs. tin cans as the juice is thawed in the can itself. Place the frozen cardboard container in a microwave and heat at 30 s to 1 min intervals until fully thawed. Measure out the thawed juice using a measuring cylinder.
4. Since the grape juice solution is a 1% agar solution, it will start to solidify as it cools in the flask. To minimize this, keep the solution submerged in a 55–60 °C water bath while you pipette out the solution into petri dishes. It may be useful to use an automatic solution dispenser to help make this process faster.
5. First add Triton X-100 to 1× PBS and mix thoroughly until fully dissolved. Then add BSA and mix thoroughly. This solution should be filtered using a bottle-top vacuum filter of pore size 0.45 μm. Once filtered, store PBT at 4 °C. Do not leave PBT at room temperature for extended periods of time. Filtered PBT can be used for up to 6 months if stored properly at 4 °C.
6. Fix solution should be made fresh just prior to use.
7. Thaw and aliquot the 10 ml normal goat serum received from the company into 0.5 ml aliquots in 15 ml conical tubes.

Freeze the conical tube aliquots at −20 °C until used. To make Blocking solution, thaw an aliquot of serum and add 9.5 ml PBT. Gently mix and store at 4 °C.

8. Keep the chamber containing flies with the egg collection plate end elevated at ~30–45° angle. We use a piece of Styrofoam to make a "pillow" to keep the grape plate end of the chamber elevated. This helps in the embryos being deposited mostly on the plate and not on the interior walls of the chamber.
9. Changing the grape plate every day is essential as the grape solution provides moisture to the flies and the agar tends to dry out after 1 day. If the plate is not changed daily, large numbers of embryos begin developing on the agar resulting in crawling larvae in the chamber. Moreover, the grape agar tends to fall down into the chamber if it dries out.
10. Place the slide containing dechorionated embryos on the agar slab in a humidified chamber such as a petri dish with a soaked Whatman filter paper to prevent the embryos from getting dried out.
11. After dechorionation, the embryos will stick more strongly to the needle than to other embryos or to the removed chorion. But they will still stick strongly to the sticky tape. To pick up the embryos effectively with the needle, roll the embryos either on top of each other or on top of their removed chorions. Embryos lying on the sticky tape may be difficult to pick up.
12. Do not put more than ~50 embryos on one slide for dissection, as the yolk released from the gut during dissections makes it difficult for later embryos to stick to the slide.
13. The gut can be removed right after dissecting each embryo as well. However, in this case it might be difficult for later-dissected embryos to stick well to the glass slide as the yolk released from the gut during gut-removal makes the PBS fatty. This can become a problem if the embryos have already been transferred to the slide surface, as the body walls of the embryos will not stick to the slide.
14. Take extra care to not remove too much PBS from the slide, as removing too much will expose the dissected embryos to the PBS meniscus. If this happens, the dissections are ruined as the embryos "explode" towards the meniscus. Some become detached from the slide surface and some lose the ventral nerve cord.
15. Once the coverslip has been placed over the embryos, make sure to not move the coverslip even a little bit. Any movement of the coverslip over the embryos will move the ventral nerve cord from its normal position and will ruin the dissections.

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