Expansion Microscopy Protocol for Drosophila Tissues

BACKGROUND: This protocol is based off of methods used in the publication “Superresolution Imaging of Drosophila Tissues Using Expansion Microscopy” and is adapted from the protocol used in “Expansion microscopy with conventional antibodies and fluorescent proteins” (Chozinski et al., 2016). The following is meant for brain, embryo, and larval Drosophila tissues only. Figures are provided to aid in visualizing the key steps in the procedure.

REAGENTS OR SUPPLIES TO OBTAIN OR PREPARE AHEAD OF TIME (see Supplementary Table for a list of vendors and item numbers):

1. Sylgard 184 Silicone Elastomer Kit (For dissection pads and Polydimethylsiloxane (PDMS) wells)
   a. Sylgard 184 Silicone Elastomer Base
   b. Sylgard 184 Silicone Elastomer Curing Agent
   c. Store at RT
2. Grape juice agar plates (for embryo collection)
   a. Store at 4 °C
3. Dissection tools: Minutien insect pins, forceps, vannas scissors
4. 50% bleach
5. Paraformaldehyde solution (4% in PBS; methanol-free)
6. N-heptane (for embryos)
7. Methanol (for embryos)
8. Poly-L-lysine Solution (0.1% (w/v) in water)
9. Ammonium Persulfate (APS is a salt and initiates polymerization)
   a. Store at 4 °C
10. Tetramethylethylenediamine (TEMED is a liquid and catalyzes polymerization)
    a. Store at 4 °C
11. 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO is a solid and inhibits polymerization so that gelation starts after reagents permeate sample)
    a. Store at 4 °C
12. Methacrylic Acid-NHS (MA-NHS is a solid and is used to link proteins to the gel)
    a. Store the powder at 4 °C. KEEP AWAY FROM WATER.
    b. As with all water-sensitive reagents stored below room temperature, allow bottle to warm to room temperature before opening to avoid condensation of water.
13. 40% Acrylamide (w/v) (Acrylamide is a liquid solution and is a monomer of the hydrogel.)
    a. Store at 4 °C
14. 2% Bisacrylamide (w/v) (Bisacrylamide is a liquid solution and is a hydrogel crosslinker.)
    a. Store at 4 °C
15. Sodium Acrylate (SA is an ionic monomer for the hydrogel.)
    a. Store in desiccator at RT.
16. Sodium Chloride (Salt)
17. 1x PBS Buffer pH 7.0
a. Prepared by diluting 10x PBS Buffer pH 7.0 with deionized water.

18. 1x PBS buffer pH ~6
   a. Made by dissolving 86.8 mol% KH$_2$PO$_4$ and 13.2 mol% K$_2$HPO$_4$ in deionized water.

19. 10x TAE Buffer (Tris base, acetic acid, and EDTA)

20. 8M Guanidine-HCl (component of proteinase digestion buffer)

21. 1x HBSS with 0.01M CaCl$_2$ and 0.01M MgCl$_2$ (used as collagenase digestion buffer)
   a. Add 0.01M CaCl$_2$ and 0.01M MgCl$_2$ to 1x HBSS w/o calcium, magnesium, and phenol red to make this buffer solution.

22. Proteinase K (Digestion enzyme, 600-800 Units/mL in 50% glycerol.)
   a. Store at -20 °C

23. Chitinase (Enzyme digestion of chitin-rich cuticle, 25 Units.)
   a. Store at -20 °C

24. Collagenase Type I (Digestion enzyme for collagen, 1 g.)
   a. Store at 2-8 °C

25. #1.5 Coverglass (24mm x 50 mm) is convenient to be used as a substrate. Coverglass is convenient as a substrate for two reasons. First, it is useful for performing pre-expansion imaging with short-working-distance microscope objective lenses. Second, excess coverglass can be removed by cutting the coverglass with a diamond knife so it may be digested in a small well that conserves reagents.

26. 8mm Disposable Biopsy Punch

27. Multiwell plate or similar for digestion. 12-well plates are convenient.

**STOCK SOLUTIONS**

1. Formaldehyde: 4% in PBS
   a. Prepare from 8% stock sealed under inert gas
   b. Store at 4 °C for 1-2 days

2. APS: 10% (w/w) in water
   a. Store at -20 °C for up to 1 week

3. TEMED: 10% (v/v) in water
   a. Store at -20 °C for up to 1 week

4. TEMPO: 1% (w/w) in water
   a. Prepare freshly, within a few hours of use

5. MA-NHS: 1 M in anhydrous DMSO
   a. Store at -20 °C
   b. Keep away from water

6. Monomer Solution. Final concentrations are listed. Recipe achieves ~4x expansion. Bolded quantities in brackets, below, are for 10 mL of monomer solution.
   a. 2 M NaCl [$1.17$ g]
   b. 8.625% (w/v) Sodium Acrylate [$0.863$ g]
   c. 2.5% (w/v) Acrylamide [$0.625$ mL]
   d. 0.15% (w/v) Bisacrylamide [$0.75$ mL]
   e. 10x PBS (concentrated PBS stock), pH 7.4 [$1$ mL]
   f. Add sufficient water to reach a final volume of 10 mL.
   g. Store at 4 °C for up to two weeks.

7. Proteinase digestion Buffer. Bolded quantities in brackets, below, are for 10 mL of solution.
a. 10x TAE Buffer [1 mL]
b. 0.8 M Guanidine-HCl [1 mL]
c. 0.5% Triton [0.25 mL]
d. Add sufficient deionized water to reach a final volume of 10 mL.
e. Store at 4 °C. Solution should be stable but we usually consume within a week.

8. Chitinase stock solution: 5 Units/mL in PBS pH 6.0
   a. Aliquot 200 µL of stock solution into 1.5 mL Eppendorf tubes.
   b. Freeze and store aliquots at -20 °C. Frozen aliquots should be stable for at least 1 month.

9. Collagenase stock solution: 5 mg/mL in 1x HBSS with 0.01M CaCl₂ and 0.01M MgCl₂
   a. Aliquot 200 µL of stock solution into 1.5 mL Eppendorf tubes.
   b. Freeze and store aliquots at -20 °C. Frozen aliquots should be stable for at least 1 month.

10. Grape juice agar plates: quantities listed are for 1 L of grape juice agar
   a. 750 mL water
   b. 250 mL grape juice
   c. 30 g agar
   d. 10 g sucrose
   e. 20 mL EtOH
   f. 10 mL Acetic Acid
   g. Dissolve agar in water/grape juice mixture by heating, add remaining ingredients, mix and dispense to petri dishes.
   h. Prior to use, score the surface of the plates with forces and add a dollop of yeast paste to facilitate egg laying.

11. Embryo fixative
12. Tissue Fixative

PRE-EXPANSION PREPARATION

1. Tissue isolation/fixation
   a. Embryos.
      i. Collect Drosophila embryos on a yeasted grape juice agar plate for the appropriate length of time and age as needed.
      ii. Harvest embryos, dechorionate in 50% bleach for ~2 minutes, and rinse thoroughly with water
      iii. Using a paintbrush or spatula, transfer embryos to a glass vial containing embryo fixative (equal volumes of heptane and 4% formaldehyde). Fix with vigorous shaking at room temperature for 10-15 minutes.
      iv. Allow heptane/formaldehyde phases to separate (embryos will settle at the interface) remove the upper phase (formaldehyde) and add one volume of methanol.
      v. Devitellinize embryos by shaking the methanol/heptane mixture for ~30 seconds. Remove heptane phase, rinse embryos in methanol, and transfer embryos to a 1.7 mL tube using a Pasteur pipette.
      vi. Wash embryos 3 x in 1 mL PBS + 0.1% Triton-X100 and proceed with immunostaining.
   b. Larval/Adult tissue
i. Dissect larvae/adults. “Butterfly” preparations are compatible with staining of body wall tissue or removal of other tissues of interest. Pin larvae (or adult abdomen) at head and tail in a Sylgard dish filled with PBS, fillet along the long axis using vannas scissors, and pin tissue flaps. Remove excess tissue as needed. For more details on fillet preparations see Parton et al., 2010.

ii. Fix tissue with 4% Formaldehyde (or other fixative; see supplemental table 1 for details) in PBS with gentle shaking for 15-30 minutes.

iii. Remove fixative and rinse tissue extensively with PBS.

iv. Transfer tissue to 1.7 mL eppendorf tube, wash 3x with PBS (5 min each) to remove residual fixative, and proceed with immunostaining.

2. Immunostaining. Details of immunostaining protocols are available in the materials and methods section of the main text and the accompanying Supplemental Table S1.

3. Prepare a lysine-coated coverglass by applying 10 µL of the stock poly-L-lysine solution to a clean, sterilized 24 mm x 50 mm coverglass and spread out the liquid to cover the region of the surface to be used. Allow the lysine solution to air dry. The lysine-coated coverglass is used to ensure the tissue stays intact on the coverglass.
   a. Apply the poly-L-lysine solution to the coverglass by pipetting 10 µL and spreading it evenly throughout the coverglass.
   b. If different size of coverglass is used, adjust the volume so that the poly-L-lysine solution thoroughly covers the surface of the coverglass.

4. Prepare PDMS (polydimethylsiloxane) wells for convenient solution exchange during post-stain treatment and gelation step. Quantities in brackets, below, are for 10 g. (See Figure 1.)
   a. Mix 91% Sylgard Silicone Elastomer Base [9.1 g] and 9% Sylgard Silicone Elastomer Curing Agent [0.9 g] in a 50 mL Falcon centrifuge tube. Make sure the ingredients are thoroughly mixed.
   b. Centrifuge the mixed solution at about 500 g for 3 min.
   c. Pour the PDMS solution into a 3.5’’ diameter petri dish. Place the petri dish on a 55 °C hot plate and allow the PDMS to solidify overnight.
      i. If small bubbles form during the process, remove them by gently blowing air with an air hose.
      d. Cut off a rectangular piece using a razor blade and punch out 1-3 holes (roughly matched to the size of the rectangular piece) using an 8 mm biopsy punch or similar. Make sure that the bottom of the PDMS is clean and kept untouched.
   e. This procedure produces ~1 mm thick wells. (See Figure 1.)

5. Place the cut-out PDMS wells onto the lysine-coated coverglass. If necessary, press on the PDMS wells to make sure there are no bubbles or gaps between them and the coverglass.

6. Place the tissues inside each PDMS well as shown in Figure 2. Make sure the tissues are flat on the surface of the coverglass.
   a. For brain and embryo tissues
      i. Transfer the tissue with forceps or glass pipets to the center of the well.
   b. For larval tissues
      i. Transfer the tissue to the coverglass with the cuticle side facing down. This step is important because it allows for more efficient cuticle (chitin) digestion.
c. Remove excess water with Kim wipe and allow the tissue to “stick” to the coverglass.

**POST-STAIN TREATMENT**

1. After preparing and immunostaining the sample (or after expression of FP), treat with 1 mM MA-NHS in PBS (freshly diluted from the concentrated DMSO stock). Because NHS groups can rapidly hydrolyze in aqueous solutions, do not make the NHS solution in PBS until just before you are ready to treat your sample.
   a. Depending on how your tissue was fixed, you may need to alter the concentration of MA-NHS.
2. Allow the sample to react for 1 h at room temperature.
3. Wash the sample 2-3 times with several volumes of PBS. Make sure not to disturb the sample.

**GELATION**

1. Incubate the tissue in monomer solution for 30-45 min at 4 °C prior to gelation to allow monomer to penetrate the whole tissue. **NOTE: the monomer solution here DOES NOT contain APS, TEMED, or TEMPO.**
2. Remove excess monomer solution using a micropipette. Ensure tissue is flat against the glass and not disturbed during the process.
3. Prepare the gelation solution in a separate tube. Quantities in brackets, below, are for a 50 μL volume which is well-suited to 8 mm PDMS wells. Note that APS should always be added last to the gelation solution tube, right before adding the solution to the specimen.
   a. 0.2% TEMED [1 μL of 10% solution]
   b. 0.01% TEMPO [0.5 μL of 1% solution]
   c. 95% monomer solution [~48 μL of monomer stock]
   d. 0.2% APS [1 μL of 10% solution]
4. Cover the tissue with the gelation solution without disturbing it. Avoid letting the tissue fold over or float up into the gel.
5. Place a drop of leftover gelation solution on top of the coverglass as a “test” gel to see when the polymerization is complete.
6. Allow the sample to gel at 37 °C for 1.5-2 h.

**DIGESTION**

1. Remove the PDMS wells without lifting the gel off the coverglass (Figure 2). Do not remove the gels from the coverglass at this stage because they may tear.
   a. Note: the sample will slightly expand if it is gelled for too long. While this is fine in most cases, try to avoid this.
2. Cut away excess gel from around the tissue (Your sample will be cleared after expansion and will be difficult to find in a lot of excess gel.) Use a diamond knife or razor blade to score the bottom coverglass near to the tissue and then break away excess coverglass so that the coverglass and gel will fit into the digestion buffer well. (Figure 2).
3. Place the gel sitting on the coverglass in a suitably sized well, e.g., a well of a 12-well plate.
4. Different digestion steps are required depending on the type of the tissue:
   a. For brain and embryo tissues
      i. Add proteinase digestion buffer with ~8 Units/mL of proteinase K to the sample. Make sure to cover the sample completely and allow to digest for 1 h at 37 °C.
   b. For larval tissues
      i. Dilute chitinase stock solution to 1 Unit/mL in 1x PBS 6.0 and add to the sample. Make sure to cover the sample completely and allow to digest for ~4 days at 37 °C.
      ii. Wash the sample 2-3 times with several volumes of PBS.
      iii. (For ECM/larval body wall samples only) Dilute collagenase stock solution to 1 mg/mL with 1x HBSS (w/ 0.01 M CaCl2 and 0.01 M MgCl2) and add to the sample. Digest the sample overnight at 37 °C.
         1. Then wash the sample 2-3 times with PBS.
      iv. Digest the gel with Proteinase K by following the procedure in (a).

EXPANSION

1. Remove the gel from digestion buffer and place in DI water to expand. Anticipate the size of the expanded gel (~32 mm for 8 mm PDMS wells) and use a suitably sized container such as a 3.5” petri dish.
2. Exchange water as needed until fully expanded (typically 2-3 exchanges). The refractive index of the gel is nearly identical to that of water so you will not easily see the gel. Be careful not to pour out or aspirate the gel. Typical expansion times are 1-2 hours, total, with exchanges every ~30 min (Figure 3). The specimen should expand ~4× compared to the initial sample before gelation. If desired, the expansion factor can be tuned by adding more or less bisacrylamide crosslinker, where more bisacrylamide leads to less expansion and vice versa.
3. Thinner gels will expand relatively quickly and may only need one water exchange.

SAMPLE HANDLING TIPS

- Removing expanded gels from petri dishes (or handling them in general) can be difficult. A large rectangular coverglass (~1” x 2”) is probably the best tool to use but other flat objects or spatulas may also work well. Place the coverglass short edge against the petri dish surface and tilt the dish to allow the gel to gently slide onto the coverglass. Cut away excess gel if necessary and remove as much water as possible before picking up the gel. (Figure 3)
- Try to gently wick away excess water before imaging using a Kim wipe. The gels will otherwise slide around during imaging. Be sure not to touch the side where the sample is facing (usually the bottom) as doing so would disturb the sample.
- If the gels fold over onto themselves after removing excess water, try to use a fine tip paintbrush and gently poke the edges of the gel back until the sample is back in its original shape.
- Illuminating the specimen from below with a flashlight and observing against a dark background are helpful when trying to locate your tissue within the gel. Look for a small amount of scattering.
- It is highly recommended to use a poly-L-lysine coated coverglass for post-ExM imaging because it will firmly hold the gel in place and reduce sample drift during acquisition. If there are bubbles present underneath the gel, pop them by simply pressing down the gel.

Figure 1. Preparation of PDMS wells. Sylgard silicone elastomer base and curing agent mixture A) before and B) after centrifugation to remove bubbles. C) PDMS solution poured into a 3.5” diameter petri dish and placed on a 55 °C hot plate. D) Rectangular PDMS block cut out with a razor blade. E) 8 mm wells created in rectangular PMDS block using disposable 8mm biopsy punch. F) PDMS wells are detached and stored in a clean petri dish until use.
Figure 2. A) Mounted *Drosophila* larval tissues (small opaque rectangle) inside 8 mm PDMS wells. Specimen B) after polymerizing hydrogel within the PDMS wells and C) after removal of PDMS well, leaving behind 8 mm hydrogel “pucks” containing *Drosophila* tissues. D) Individual hydrogel puck after trimming coverglass substrate with glass cutter or razor blade.

Figure 3. A) Trimmed coverglass specimen is placed in a well of a 12-well plate and digested. B) Specimen after digestion. C) Specimen after expansion. D) Excess hydrogel is trimmed away from the specimen. E) Removal of expanded, trimmed specimen using a disposable spatula. F) Mounted specimen on poly-L-lysine coated coverglass.

REFERENCES

Chozinski, T. J., Halpern, A. R., Okawa, H., Kim, H.-J., Tremel, G. J., Wong, R. O. L., and Vaughan, J. C. (2016). Expansion microscopy with conventional antibodies and fluorescent proteins. Nat. Methods 13, 485–488.

Parton, R. M., Vallés, A. M., Dobbie, I. M., and Davis, I. (2010). *Drosophila* larval fillet preparation and imaging of neurons. Cold Spring Harb. Protoc. 2010, pdb.prot5405.
## Sources for reagents used in this protocol

| Reagent                                                                 | Vendor                      | Product #       |
|------------------------------------------------------------------------|----------------------------|----------------|
| Sylgard 184 Silicone Elastomer Kit                                      | Dow Corning                | N/A            |
| Minutien Insect Pins                                                    | Fine Science Tools          | 26002-10       |
| Vannas Scissors                                                        | World Precision Instruments | 501778         |
| 8% paraformaldehyde solution                                            | Electron Microscopy Sciences | 157-8          |
| n-heptane                                                              | Thermo Scientific           | BP1115500      |
| methanol                                                               | Thermo Scientific           | BP1105         |
| Poly-L-lysine Solution (0.1% (w/v) in water)                           | Sigma-Aldrich               | P8920          |
| Ammonium Persulfate                                                    | Thermo Scientific           | 17874          |
| Tetramethylethylenediamine                                             | Bio-Rad                     | 1610800        |
| 2,2,6,6-Tetramethylpiperidine-1-oxyl                                    | Sigma-Aldrich               | 176141         |
| Methacrylic Acid-NHS                                                   | Sigma-Aldrich               | 730300         |
| 40% Acrylamide (w/v)                                                   | Bio-Rad                     | 1610140        |
| 2% Bisacrylamide (w/v)                                                  | Bio-Rad                     | 1610142        |
| Sodium Acrylate                                                        | Sigma-Aldrich               | 408220         |
| Sodium Chloride, Granular                                              | Avantor                     | 7713           |
| 10x PBS pH 7.0                                                         | Thermo Scientific           | BP39920        |
| Potassium Phosphate Monobasic                                          | Thermo Scientific           | P285500        |
| Potassium Phosphate Dibasic                                            | Thermo Scientific           | P290500        |
| 10x TAE Buffer                                                         | Thermo Scientific           | 148698         |
| Guanidine-HCl                                                          | Thermo Scientific           | 101905         |
| 1x HBSS w/o Calcium, Magnesium, and Phenol Red                         | Corning                     | 16115011       |
| Proteinase K                                                           | Thermo Scientific           | EO0491         |
| Chitinase                                                              | Sigma-Aldrich               | C6137          |
| Collagenase Type I                                                      | Gibco                       | 17100017       |
| #1.5 Microscope Cover Glass                                            | Thermo Scientific           | 12-544E        |
| 8mm Disposable Biopsy Punch                                            | Acuderm                     | P850           |
| 12-Well Plate with Lid                                                 | CytoOne                     | CC7672-7512    |
Supplemental Figure S1. (A-C) Correlative imaging and distortion analysis of *Drosophila* tissues imaged by confocal microscopy before and after expansion. *Drosophila* (A) embryo immunostained for Futsch, (B) larval brain immunostained for FasII, and (C) larval body wall expressing C4da neuron-specific marker ppk-mCD8-GFP (labeling C4da dendrites) and immunostained for GFP. Distortion analyses (right panels of A-C) show root mean-squared error (RMSE, black line) plus or minus standard deviation (gray lines) over a range of length scales. (D) DAPI-stained *Drosophila* larval body wall imaged by widefield microscopy before and after attempted expansion; without chitinase treatment, the body wall specimen detached from the hydrogel, showing substantial distortion and tearing (arrows). Scale bars, 10 μm (A-C), 500 μm (D). All distances and scale bars refer to pre-expansion dimensions.
Supplemental Figure S2. Distortion analysis. (A-E) Comparison of pre- and post-expansion confocal images of larval body wall fillets immunostained for Bruchpilot. (A) Overlay of pre-expansion (magenta) and post-expansion (green) images after registration by similarity transform (a rigid transformation including scaling, rotation, and translation). (B) Overlay of post-expansion image before (magenta) and after (green) application of a B-spline registration (non-rigid transformation) to warp the post-expansion image to optimally align it with the pre-expansion data. Yellow arrows show the direction of the relative magnitude (scaled up by a factor of 3.5 for visibility) of the local deformation applied to align the pre- and post-expansion images. (C) Zoomed-in view of the boxed region in (B). (D) Schematic of correlation analysis procedure. The value $m$ represents the distance between points a and b in the pre-B-spline registration image (magenta) while $m'$ is the distance between points a’ and b’ in the post-B-spline-registration image (green). Root mean-square (RMS) error plots were generated by calculating the difference between $m$ and $m'$ as a function of distance $m$ for many sets of points throughout the image (shaded areas represent standard deviation). Note that (E) was calculated by performing distortion analysis in three dimensions. Scale bars: 5 μm (A,B) and 1 μm (C). (F-G) Distortion
Supplemental Figure S3. Related to Figure 2. (A-B) Maximum intensity projections of epithelial cells immunostained for acetylated tubulin (acTub) in unexpanded third instar larval body walls. (B) Zoomed-in view of single focal plane image corresponding to boxed regions in (A). (C) Line profile from B (arb., arbitrary units). (D) Analysis of microtubule widths.
Supplemental Figure S4. Related to Figure 3. (A-B) Representative structured illumination microscopy (SIM) images of unexpanded Brp-labeled active zones in fillet preparation of third instar larval body wall (A) and zoomed-in view (B). The resolution achieved by SIM, which was performed on a Nikon N-SIM microscope, was intermediate between that of confocal microscopy and confocal microscopy of expanded specimens. (C-D) Characterization of multiple Brp ring-containing active zones at the adult CM9 neuromuscular junction (NMJ). (C) Distribution of multiple Brp ring-containing active zones in 10 day, 30 day, and 65 day adult CM9 NMJs stained with an anti-Brp antibody and imaged using ExM. (D) Scatter plot showing the size of individual Brp rings in 65 day adult active zones containing one (singles), two (doubles), or three to five (multiples) Brp rings. Points mark individual measurements and bars mark mean values. *p<0.05, one-way ANOVA with a post-hoc Dunnett’s test.
**Sample** | **Fixation** | **Washing and blocking** | **Primary antibodies** | **Incubation time** | **Secondary antibodies** | **Incubation time** | **MA-NHS treatment**
---|---|---|---|---|---|---|---
embryos | Dechorionate 4 min in 50% bleach | wash with PBST block with 5% NGS in PBST | Mouse anti-Futsch, clone 22c10 (DSHB, 1:100) | 4°C overnight | Alexa Fluor 488 (Goat anti-Mouse, Thermofisher A31561, 1:100) | 20-25°C 6 h | 1mM, 1 h
larval VNC | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | 1D4 (Mouse, DSHB, 1:20) | 4°C overnight | Alexa488 (Goat anti-Mouse, Thermofisher A31561, 1:100) | 20-25°C 6 h | 1mM, 1 h
larval VNC | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Rabbit anti-dsRed (Clonetech #632496, 1:50) | 4°C overnight | ATTO 565 (Donkey anti-Rabbit, Vaughan lab, 1:10) | 20-25°C 6 h | 1mM, 1 h
larval body wall: C4da neurons | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Mouse anti-GFP, clone 3E6 (Invitrogen #A11120, 1:100) | 4°C overnight | Mouse, Thermo Fisher A31561, 1:100 | 20-25°C 2-4 h | 1mM, 1 h
larval body wall: mitochondria | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Mouse anti-GFP, clone 3E6 (Invitrogen #A11120, 1:100) | 4°C overnight | Mouse, Thermo Fisher A31561, 1:100 | 20-25°C 2-4 h | 1mM, 1 h
larval body wall: microtubules | PEM/0.5% Triton X-100, 30 s PEM/4% PFA + 0.5% Triton X-100, 15 min PEM/0.5% Triton X-100, 30 s | wash with PBST block with 5% NGS in PBST | Mouse anti-acetylated-tubulin, clone 6-11B-1 (Sigma #T7451, 1:200) | 4°C overnight | Alexa Fluor 488 (Goat anti-Mouse, Thermo Fisher A31561, 1:100) | 20-25°C 2-4 h | 1mM, 1 h
larval body wall: nuclei | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Hoechst 33258 (Thermo Fisher, 1:1000) | | | 20-25°C 2-4 h | 1mM, 1 h
larval/adult body wall: NMJ active zones | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Mouse anti-Bruchpilot (Brp), clone nd32 (DSHB, 1:50) | 4°C overnight | Mouse, Thermo Fisher A31561, 1:100 | 20-25°C 6 h | 1mM, 1 h
larval body wall: ECM | 4% PFA 30 min | wash with PBST block with 5% NGS in PBST | Rabbit anti-Perlecan (courtesy of Stefan Baumgartner,1:1000 ) | 4°C overnight | ATTO 565 (Donkey anti-Rabbit, Vaughan lab, 1:10) | 20-25°C 2-4 h | 1mM, 1 h

**Abbreviations:** ECM, extracellular matrix; MA-NHS, methacrylic acid N-hydroxysuccinimidyl ester; NGS, Normal Goat Serum (Jackson Labs); NMJ, neuromuscular junction; PBST, PBS + 0.3% Triton X-100; PEM, 80 mM PIPES pH 6.9, 2 mM MgCl2 and 0.5 mM EGTA; PFA, EM-grade Paraformaldehyde (EMS); VNC, ventral nerve cord.