Lab Project 1
Do microRNA mutations affect *Drosophila* NMJ development?
If so, when?

Instructors: Dr. David Van Vactor
Dr. Lauren Dembeck

Schedule

July 26, Wednesday

Lab rotations "Introduction to *Drosophila* Embryo Dissection"
9:00-10:30, 13:00-14:30, 14:30-16:00
Hands-on training.

July 27, Thursday

13:00 – 14:30 "Dissection and Immunohistochemistry of the *Drosophila* Larva"
Preparing larva dissections and primary antibody staining.

14:30 – 17:30 "Embryo Dissection, Anatomy, and an Introduction to DIC Microscopy"
Dissecting and imaging fixed mutant and wild-type embryos.

July 28, Friday

13:00 – 15:30 "Immunohistochemistry of the *Drosophila* Larva Part 2"
Pelt washes and antibody staining (concurrent embryo dissections).
15:30 – 17:30 "Preparing larval pelts for imaging"
Pelt washes, mounting, and clearing (concurrent DIC imaging of embryos).

July 29, Saturday

12:10 – 13:00 "Introduction to IMARIS and IMARIS tool kits" (Lunch Demonstration)
13:30 – 15:30 "Introduction to super-resolution microscopy part 1"
Students will split into two groups, one group to use N-SIM and the other STED.
15:30 – 17:45 "Introduction to super-resolution microscopy part 2"
The two student groups will switch and train on the other microscope.

July 30, Sunday

13:00 – 18:00 "Image acquisition and analysis"
Work with BitPlane specialists to learn the IMARIS software for NMJ analysis.
Image acquisition of embryo fillet with Olympus Bx51. Students will split into two groups.
Collect more images from all microscopy setups in preparation for final analyses and presentation.

Do microRNA mutations affect *Drosophila* NMJ development?

MicroRNAs are small, single strand RNA molecules (~22 nucleotides) that regulate post-transcriptional gene expression [1]. MicroRNAs are required for many key biological processes such as cell differentiation and apoptosis. They have also been implicated in the differentiation of the nervous system in model organisms and their dysregulation has been associated with neurological diseases in humans [2,3]. We have selected five strains of *Drosophila melanogaster* that are homozygous for microRNA deletions to analyze in our lab project [4]. These lines have been identified as having abnormal gross NMJ morphology at the late third instar in addition to abnormal larval crawling behavior phenotypes and/or adult flight.

In developmental studies one key experiment is to determine at what time point do aberrant phenotypes arise. In this rotation, students will analyze embryos and 3rd instar larvae to address this question for the five microRNA deletions.

Embryos: Students will dissect and analyze late stage 17 *D. melanogaster* embryos (Figure 1) for motor axon and early NMJ phenotypes. Due to time constraints, the embryos were fixed and stained for two set of antibodies prior to the start of the course. The 1D4 antibody labels the axons of motor neurons in the embryo [5].

	Primary antibody	**Secondary antibody**
1.	1D4 anti-Fasciclin II (Fas2; generated in mouse)	anti-mouse conjugated with horseradish peroxidase (HRP) then treated with 3,3'-Diaminobenzidine (DAB) leading to visible, light brown staining

Students will image these embryos using differential interference contrast (DIC) microscopy. In wild-type embryos, the neuromuscular junction is composed of five motor nerves that innervate the ventral and dorsal muscles of each abdominal hemisegment. We will assess developmental defects by scoring embryos for the percentage of hemisegments with guidance defects for each motor nerve. Particular attention to axon trajectory and motor terminal morphology will be focused on intersegmenal nerve b (ISNb). Alternatively, depending on the phenotypes observed, we will assess developmental defects by scoring embryos for each or a particular motor nerve for the presence or absence of guidance defects in hemisegments A2-A7, where motor neurons and muscles are identical (Figure 2 and 3). At the late 3rd instar, we will confirm that the mutants display gross NMJ phenotypes by counting boutons, and we will ask the question of whether sites of synaptic neurotransmitter release (so-called active zones) are abnormal using a specific AZ marker (mab-nc82 against bruchpilot, Brp).

Embryos and larvae: In addition to dissecting and imaging embryos, students will have hands-on experience dissecting, fixing, and staining *Drosophila* 3rd instar larvae for nc82 (a synaptic marker) and HRP (labels all neurons) paired with fluorescent secondary antibodies [6,7].

	Primary antibody	Secondary antibody
2.	nc82 anti-Bruchpilot (*Brp*; generated in mouse)	anti-mouse conjugated Dylight488 (green)
	Anti-HRP (generated in rabbit)	anti-rabbit conjugated Dylight549 (yellow)

Students will work with Nikon and Leica microscopy specialists to image the NMJ of these fluorescently stained embryo and larval fillets using two super-resolution microscopy systems. We will assess gross morphology of the nerves in the larval preps such as the number of branches and the cumulative branch length, as well as, synaptic bouton count and volume. For this phenotypic analysis, students will work with specialists from BitPlane using the IMARIS software.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. Nature Publishing Group; 1998;391: 806–811. doi:10.1038/35888
2. Davis GM, Haas MA, Pocock R. MicroRNAs: Not "Fine-Tuners" but Key Regulators of Neuronal Development and Function. Front Neurol. Frontiers Media SA; 2015;6: 245. doi:10.3389/fneur.2015.00245
3. Loya CM, Lu CS, Van Vactor D, Fulga TA. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Methods. Nature Publishing Group; 2009;6: 897–903. doi:10.1038/nmeth.1402
4. Chen Y-W, Song S, Weng R, Verma P, Kugler J-M, Buescher M, et al. Systematic study of Drosophila microRNA functions using a collection of targeted knockout mutations. Dev Cell. 2014;31: 784–800. doi:10.1016/j.devcel.2014.11.029
5. Van Vactor D, Sink H, Fambrough D, Tsoo R, Goodman CS. Genes that control neuromuscular specificity in Drosophila. Cell. 1993;73: 1137–1153. doi:10.1016/0092-8674(93)90643-5
6. Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, et al. Bruchpilot, a Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of Synaptic Active Zones in Drosophila. Neuron. 2006;49: 833–844. doi:10.1016/j.neuron.2006.02.008
7. Fabini G, Freilinger A, Altmann F, Wilson IB. Identification of core alpha 1,3-fucosylated glycans and cloning of the requisite fucosyltransferase cDNA from Drosophila melanogaster. Potential basis of the neural anti-horseadish peroxidase epitope. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2001;276: 28058–67. doi:10.1074/jbc.M100573200
8. Meyer F, Aberle H. At the next stop sign turn right: the metalloprotease Tolloid-related 1 controls defasciculation of motor axons in Drosophila. Development. 2006;133. Available: http://dev.biologists.org/content/133/20/4035

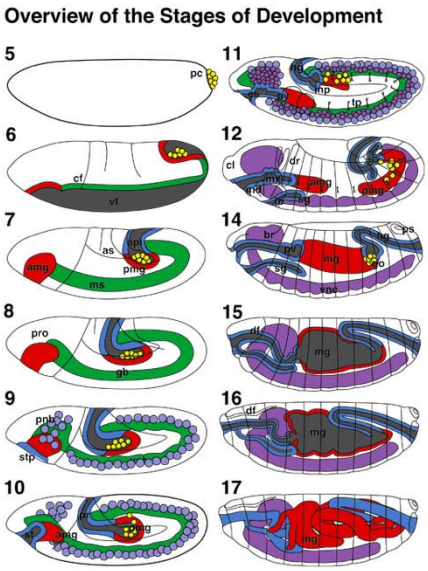

Figure 1. Stages of Embryonic Development pg. 52 in Atlas of *Drosophila* Development by Volker Hartenstein. All embryos are in lateral view (anterior to the left). Endoderm, midgut; mesoderm; central nervous system; foregut, hindgut and pole cells in yellow. (amg) (Anterior midgut rudiment; (br) brain; (cf) cephalic furrow; (cl) clypeolabrum; (df) dorsal fold; (dr) dorsal ridge; (es) esophagus; (gb) germ band; (go) gonads; (hg) hindgut; (lb) labial bud; (md) mandibular bud; (mg) midgut; (mg) Malpighian tubules; (mx) maxillary bud; (pc) pole cells; (pmg) posterior midgut rudiment; (pnb) procephalic neuroblasts; (pro) procephalon; (ps) posterior spiracle; (po) proventriculus; (sg) salivary gland; (stp) stomodeal plate; (st) stomodeum; (tp) tracheal pits; (vf) ventral furrow; (vnb) ventral neuroblasts; (vnc) ventral nerve

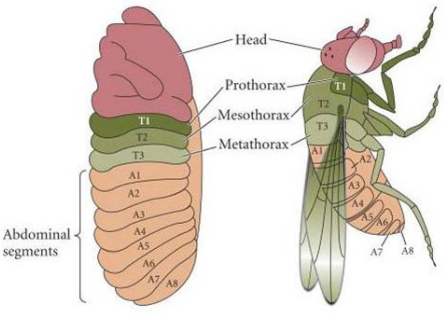

Figure 2. Comparison of the *D. melanogaster* embryo segmentation with that of the adult fly. Thoracic (T1-T3) and abdominal segments (A1-A8) are indicated. Taken from Developmental Biology, Sinauer Associates, 2000.

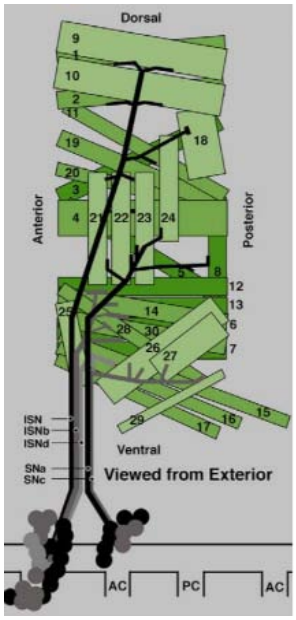

Figure 3. Schematic of the innervation pattern of *D. melanogaster* musculature of an embryonic hemisegment at late stage 17. Taken from Figure 1 in [8].

Immunohistochemistry and mounting of *Drosophila* embryo

L. Dembeck modified from Cecilia Lu

Materials:

100 mm petri dishes with apple or grape juice agar (Flystuff Grape Agar Powder Premix Packets)
Embryo collection cages (http://flystuff.com/drosophila-products/cages/)
Fine paint brush
Yeast paste - Dry baker's yeast mixed with DI water to the consistency of peanut butter
Cell strainers with nylon mesh (40µm)
100 mL graduated cylindar
Plastic funnel
PBST waster container
Bleach waste container
2 glass or hard plastic dishes with cover (1)
10 µl pipette tips
1.5 mL microfuge tubes
Rocker/shaker platform
rotating shaker
Paper towels

Reagents:

Sodium Hypochlorite (bleach)
Phosphate Buffer, pH7.2-7.4 (PBS)
Phosphate Buffer with 0.1% Triton X-100 (PBST)
Heat-inactivated normal goat serum (HIGS)
4% Paraformaldehyde (PFA)*
Methanol (MeOH)*
n-Heptane*
Thermo Scientific Ultra-sensitive ABC Peroxidase Staining Kit (32052)
Vector Laboratories DAB Peroxidase Substrate Kit (SK-4100)
*Hazardous chemicals

Protocol:

Fixation, Methanol Devitellination, and Storage

1. Collect 18-21 hr old embryos on apple or grape juice agar plates with fresh yeast paste at 25°C.

2. Rinse embryos from the agar with 1xPBST in a squirt bottle, brush them gently with the paint brush, and transfer them into a strainer using the paint brush. The strainer fits neatly into the top of the graduated cylinder (empty as needed) to collect the waste rinse. Repeat for all egg collections.

3. Place the strainers into a glass dish. Remove yeast and dechorionate embryos by covering the embryos with 25% Chlorax bleach (1:4 dilution from sodium hypochlorite solution) for 3 min; thoroughly stir embryos with bleach using a 10 µl pipette tip and gently swirl the strainers periodically.

4. Wash embryos by squirting 1x PBST over a funnel into a bleach waste container, especially along the edges of the strainer for at least three times; absorb carry-over 1x PBST from washes with paper towels.

5. Place the strainers into a clean glass dish. Immerse embryos in 10 ml n-heptane and add 10 ml 4% paraformaldehyde (PFA) to the strainers, mix by pipetting up and down the solutions outside of the strainers to make fixative emulsion*. *The emulsion will form two layers: the lower (aqueous) layer is fixative, the upper layer is heptane, and the embryos will sink to be at the interface of the two layers. The heptane will become saturated with fixative allowing the fixative to penetrate the hydrophobic vitelline membrane surrounding the embryos.

6. Cover the dish and agitate embryos in fixative emulsion for 20 min on a platform shaker with gentle rotation.

   **********The following steps (7-8) must be performed inside of a fume hood**********

7. To stop fixation, remove the lower phase of the fixation mix by blotting dry on paper towels. Discard the PFA/heptane emulsion. Place the strainers back into the dish and add 15ml heptane followed by 5ml of methanol to split open the vitelline membrane by pipetting up and down (8-10 full draws of 500 µl) with an Eppendorf pipette (blue tip). Fixed embryos will sink to the bottom of the strainer.

8. Move the strainers to paper towels and then discard the heptane and methanol mixture. Return the strainers to the dish. Wash embryos three times with methanol then transfer the embryos in methanol to 1.5 mL microfuge tubes for storage at 20°C until rehydration and blocking.

Rehydration and Blocking

9. Remove excess MeOH. Rehydrate the embryos with 2x 5 min washes in 1x PBST followed by 30 min at room temperature. All washes should be done with gentle shaking on a rocking shaker.

10. Incubate in blocking solution, 1 mL of 1x PBST with 5% heat-inactivated normal goat serum (HIGS), on a rotating shaker for 30 min at room temperature.

Primary and Secondary Antibody Incubation Procedure

11. Remove blocking solution and add the appropriate amount of primary antibody diluted in blocking solution to reach the desired final concentration (final volume 1 mL).

12. Mix embryos with the primary antibody solution in a rocking shaker. Depending on the quality and source of the primary antibody, incubations can last from 2h at room temperature (high affinity antibodies) to overnight at $4^{o}C$ (low affinity antibodies). Overnight incubation at $4^{o}C$ aides the perfusion of the antibody.

13. Wash 3x 5 min washes in 1x PBST followed by 30 min at room temperature. All washes should be done with gentle shaking on a platform shaker.

14. Add fluorophor-conjugated (or Thermo Scientific biotinylated; mixed according to kit instructions) secondary antibody diluted in blocking solution and incubate for 1hr at room temperature on a rocking platform.

15. Wash 3x 5 min washes in 1x PBST followed by 30 min at room temperature. All washes should be done with gentle shaking on a rocking shaker.

16. Incubate fluorescently stained embryos in anti-fade agent or 50% and then 70% glycerol in PBS until they sink to the bottom of the microcentrifuge tube (about 30 min). Fluorescently stained embryos can be stored in anti-fade agent at 4°C.
17. For embryos to be stained with DAB: Incubate in Thermo Scientific ABC Solution (mixed according to kit instructions) for 60 min at room temperature on a rocking shaker.

18. Wash 3x 5 min washes in 1x PBST followed by 30 min at room temperature. All washes should be done with gentle shaking on a rocking shaker.

19. Mix DAB substrate working solution according to the kit instructions. Add 300-500 µl of solution to each tube of embryos. Incubate at room temperature on a rocking shaker for 20 minutes.

20. Wash 3x 5 min washes in 1x PBST followed by 30 min at room temperature. All washes should be done with gentle shaking on a rocking shaker.

21. Incubate in 50% glycerol/PBS for at least 30 mins at 4°C then replace the solution with 70% glycerol/PBS for storage at 4°C.

Dissection and mounting

Version 1 – Embryo fillets

22. Allow embryos to warm to room temperature. Place 25 µl into a glass depression slide and examine under a dissecting microscope. Select embryos of the desired developmental stage with a tungsten loop and transfer to a glass slide.

23. Use two sharpen tungsten needles to fillet each fixed embryo in a small droplet of anti-fade agent or 70% glycerol.

24. Carefully remove from the slide the waste tissue cut from the embryo fillet with triangles of Whatman filter paper.

25. Add a droplet of anti-fade agent or 70% glycerol as mounting media to the side of the embryo fillets. Then gently place a coverslip over the prep allowing the droplet to “seal” the coverslip to the slide.

26. Place small droplets of fingernail polish on each corner of the coverslip. Wait 30 seconds. Then use fingernail polish to seal the remaining edges of the coverslip.

27. Glue down with a droplet of fingernail polish two 18x18 mm #1 thickness coverslips as bridges to keep embryos from being crushed by the coverslip that will go on top. Alternatively, place two layers of double-sided Scotch tape on each side of the oriented embryos lined up in a row.

28. Add additional anti-fade agent or 70% glycerol and place a single 18x18 mm #1 thickness coverslip over the embryos. Remove any excess mounting media by wicking it away with a Whatman filter paper triangle.

29. Flatten the embryos further to achieve single focal plane by pressing gently on the top cover slip. To save slide for future viewing, seal all edges with clear nail polish as described above.

Immunohistochemistry and mounting of *Drosophila* larva

OIST Van Vactor lab (SMA team) protocol

Reagents:

PBS
PBST (PBS with 0.1% Triton X-100)
4% Paraformaldehyde (PFA)
Normal Donkey Serum (DNS)
FlourSave Antifade Reagent (Calbiochem, 345789)
Antibodies

Materials :

Sylgaard dissection plate
Small dissection pins
Forceps : 2 pairs
Vannas spring scissors
4-well plate
Transfer pipet
Glass microscope slides
Glass cover slips
Platform shaker

Protocol:

1. Dissect and pin third instar larvae in ice-cold PBS on the dissection plate.
2. Fixation: Fix in 4% formaldehyde drop (500 µl) for 5 minutes.
3. Rinse with PBS twice (500 µl).
4. Blocking: incubate samples in 4-well plate in 5% DNS-PBST (500 µl) for 1 hour at room temperature.
5. Prepare primary antibody solution: Dilute the appropriate amount of primary antibody in blocking solution to reach the desired final concentration (final volume 500 µl).
6. Replace blocking solution with 500 µl of primary antibody solution.
7. Incubate overnight at 4°C on the shaker with gentle agitation.
8. Remove antibodies and rinse samples with PBST twice.
9. Wash samples with PBST for 6 times (10 min/each) on the shaker.
10. During final wash, prepare secondary antibody solution: Dilute the appropriate amount of secondary antibody in blocking solution to reach the desired final concentration (final volume 500 µl).
11. Replace PBST with 500 µl of secondary antibody solution.
12. Incubate 2 hours at room temperature on the shaker.
13. Rinse samples with PBST twice.
14. Wash samples with PBST 6 times (10 min/each) on the shaker.
15. Rinse samples with PBS.
16. Transfer samples on the slide glass inner side up.
17. Cut off the anterior side and posterior side.
18. Drop anti-fade reagent on the samples (20 µl – 40 µl).
19. Place cover glass over samples.
20. Leave the prepared slides overnight at 4°C.

Seal with nail polish.