Video Article

Preparing Developing Peripheral Olfactory Tissue for Molecular and Immunohistochemical Analysis in *Drosophila*

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Abstract

The olfactory system of *Drosophila* is a widely used system in developmental neurobiology, systems neuroscience, as well as neurophysiology, behavior, and behavioral evolution. *Drosophila* olfactory tissues house the olfactory receptor neurons (ORNs) that detect volatile chemical cues in addition to hydro- and thermo-sensory neurons. In this protocol, we describe the dissection of developing peripheral olfactory tissue of the adult *Drosophila* species. We first describe how to stage and age *Drosophila* larvae, followed by the dissection of the antennal disc from early pupal stages, followed by the dissection of the antennae from mid-pupal stages and adults. We also show methods where preparations can be utilized in molecular techniques, such as the RNA extraction for qRT-PCR, RNAseq, or immunohistochemistry. These methods can also be applied to other *Drosophila* species after species-specific pupal development times are determined, and respective stages are calculated for appropriate aging.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57716/>

Introduction

The olfactory system of *Drosophila* is a widely used system in developmental neurobiology, systems neuroscience, as well as neurophysiology, behavior studies, and behavioral evolution^{1,2,3,4}. *Drosophila* olfactory tissues house the olfactory receptor neurons (ORNs) that detect volatile chemical cues in addition to hydro- and thermo-sensory neurons^{1,5,6}. The overall goal of this manuscript is to demonstrate how to stage and dissect developing pupal and adult olfactory tissue in *Drosophila* species. The rationale for this technique is to generate samples from different pupal stages for molecular and developmental analyses of the peripheral olfactory system, such as RNAseq and quantitative RT-PCR, in addition to *in vivo* tissue labeling techniques. This technique can be especially useful to identify dynamics of transcriptional profiles in the developing adult olfactory system from non-*melanogaster* *Drosophila* species, which do not have all the transgenic reagents for cell type specific labeling and analyses. In this manuscript, we demonstrate how to dissect antennal discs and antennae from pupal and adult *Drosophila* species. First, we show how to identify *Drosophila* 0 h of puparium formation (APF, white pupae or prepupae) for developmental staging and species-specific aging. Next, we show how to dissect antennal discs and the third antennal segment; specifically, how to dissociate them from the eye disc and second antennal segment for the tissue purity required for RNAseq or RT-PCRs. The stages in *D. melanogaster* described in this protocol roughly correspond to the pre-patterned antennal disc (prepupae), the selection of precursors from the antennal disc (8 h APF), the start of the terminal differentiation for ORNs (40 h APF), and adult antenna. Finally, we give examples for a quantitative RT-PCR from adult antennae isolated using the method, and for *in vivo* immunohistochemistry. This method can be used to generate samples for an RNA/DNA analysis and for immunohistochemistry on developing peripheral olfactory tissues from different *Drosophila* species.

Protocol

The following protocol is consistent with the ethics guidelines established by the Duke University Research Ethics Committee.

1. Tissue Preparation and Developmental Staging of *Drosophila melanogaster* Pupa

1. First, identify how many flies will be necessary for the dissection. For RT-PCR and RNAseq, collect 100 - 200 antennal discs and antennae from individuals which are necessary at prepupal, 8 h APF, 40 h APF, and adult stages. For immunohistochemistry, dissect 10 - 20 individual.
2. Clean all materials, including dissection pads and forceps, using wipes with 70% EtOH. If the dissected material is to be used for RNA extractions, clean everything with RNase neutralizers and make all solutions using either nuclease-free or diethyl pyrocarbonate (DEPC) treated water.

NOTE: This protocol uses silicone dissection pads instead of glass for the dissections. Silicone pads are friendly to ultra-fine forceps and promote fast and high-quality dissections.

3. Collect pupa at the 0 h APF/prepupal stage.
 1. To ensure the most precise staging, monitor the larvae at 30 min to 1 h intervals.
NOTE: Wandering third instar larvae in a moderately crowded bottle will likely pupate within a few hours.
 2. Once the larvae become immobile and are surrounded by a very light (almost white) colored pupal case, transfer them into a small 2-in Petri dish with a moist filter paper.
 3. Continue for 1 - 2 h, occasionally monitoring, identifying, and transferring prepupae.
NOTE: Alternatively, clear a bottle of all pupae and allow the larvae to develop for 2 h. All pupae collected after 2 h will be within a 0 - 2 h APF range.
4. Immediately move to step 3.1 to dissect the prepupal antennal disc for the 0 h APF stage. If the prepupa needs to be aged, collect them into a dish, cover the dish and place a paraffin film around the edges to inhibit dryness, and place the Petri dish at 25 °C.
NOTE: The pupae can now be aged to eclosion.
5. For pupae collected in a 2 h range, age the prepupae for 2 h less than the desired age in order to ensure that the pupae do not overage.
6. Use ultra-fine forceps (Dumont 55) as the tissue is very small and fragile.

2. Tissue Preparation and Developmental Staging of Pupa from Other *Drosophila* Species

1. To determine the length of the pupal development in other *Drosophila* species, place the samples at the optimum temperature, record the dates when prepupae are observed, sort them into a fresh vial, and record the number of days from prepupa to adulthood.
2. Record the ratio of time for pupal development for non-*melanogaster* species and for *melanogaster*. Multiply the ratio of the developmental time by the number of hours used for staging *melanogaster* to obtain the number of hours to age the non-*melanogaster* species.

3. Dissection of *D. melanogaster* Pupal Antennal Disc

Note: All the aging times and dissection protocols are described for *Drosophila melanogaster*. For all other species, a modification of the aging protocol based on species-specific developmental time points will be required, as described above.

1. For pupal antennal disc dissections, collect 50 - 100 0 - 2 h or 6 - 8 h old pupae using a spatula and place them on a dissection pad.
2. Pipette 150 µL of RNA isolation solution into an RNase free 1.5-mL microfuge tube using a 200-µL pipette and place the tube on ice.
3. Dissect the pupae in 150 - 200 µL of 1x PBS (phosphate buffered saline, nuclease-free). Place multiple droplets of PBS (150 - 200 µL per droplet) on the dissection pad.
4. On the dissection pad, place the 0 - 2 h or 6 - 8 h aged pupae (one pupa per drop) to be dissected in one of the 1x PBS droplets. Use forceps in one hand to stabilize the body.
5. For the 0 - 2 h aged pupae, using forceps in the other hand, hold on to the most anterior part (the mouth hooks) of the prepupa and pull it out to expose the brains and the imaginal discs attached to the anterior pupal case.
6. For the 8 h APF pupae, first gently peel off the pupal case from the most anterior quarter of the pupa, exposing the head within the membrane sack that surrounds the body.
NOTE: The body, at this stage, looks like a degenerating larva.
7. Remove the head at the neck joint.
NOTE: This ensures that the antennal discs will not be lost, which at this stage are connected primarily to the optic lobes of the brain.
8. Transfer the tissue with the brains and the imaginal discs to another 1x PBS droplet using forceps or a 20-µL pipette.
9. Identify the eye-antennal disc connected to the brain at the optic lobes. Using forceps, disconnect the eye-antennal disc from the brain and the pupal case. Transfer it to a new 1x PBS droplet using forceps or a 20-µL pipette.
NOTE: The prepupal eye-antennal disc is a flat tissue. However, by the 8 h pupae, the eye discs are rotated cupping the antennal discs, which sits anterior to the central brain.
10. Using forceps, cut the tissue at the site of connection between the eye and the antennal disc. Use a 20-µL pipette to gently transfer the dissected tissue into the RNA isolation solution reagent. Minimize the amount of liquid transferred by pipetting an amount as small as possible.
11. Repeat until all pupae have been dissected.

4. Dissection of *D. melanogaster* Mid-pupal and Adult Antennae

Note: By 40 h APF, the majority of the metamorphosis is complete, and the adult structures are becoming apparent, but are still white and nondescript. At this developmental time point, the adult antenna has taken its final shape yet still is transparent and the majority of the olfactory receptors, except for a few, have not started expression.

1. For pupal antennal dissections, collect 50 - 100 prepupae as described in step 1 and age them for 40 h at 25 °C.
2. Pipet 150 µL of RNA isolation solution into an RNase free 1.5-mL microcentrifuge tube using a 200-µL pipet and place the tube on ice.
3. On the dissection pad, place the pupa to be dissected into one of the 1x PBS droplets. Use forceps in one hand to stabilize the body. Using forceps in the other hand, gently peel off the pupal case from the most anterior quarter of the pupa, exposing the head within the membrane sack that surrounds the body.
4. Using forceps, carefully cut off the head from the rest of the pupal body by holding the body with one set of forceps and ripping the head off at the neck joint with another set of forceps. Gently transfer the head into another 1x PBS droplet using forceps. Pipette up and down to clean out the contents of the head (the brain, etc.) to obtain a clear membrane that used to surround the developing *Drosophila* head.
NOTE: At 40 - 50 h APF, the antennae are connected to the anterior-most section of the membrane. They will become apparent for dissection when the contents of the head are cleared with pipetting.
5. Using forceps, disconnect the pupal antennae from the membrane. Disconnect the 2nd segment of the antenna from the 3rd using forceps to slice at the segmental joint, as only the 3rd segment will house the olfactory receptor neurons.

6. Use a 20- μ L pipette to gently transfer the dissected tissue into the RNA isolation solution reagent. Minimize the amount of liquid transferred by pipetting an amount as small as possible. Repeat until all pupae have been dissected.
7. For adult antennal dissections, collect approximately 50 adults and age them for a week post-eclosion.
NOTE: The expression of olfactory receptor genes and other genes peak a week after eclosion. The adults are aged for a week to ensure biologically relevant genes with low abundance transcripts to rise above the detection threshold.
8. For RNA extractions, dissect flies while they are still alive on a CO₂ pad. Treat the CO₂ pad with RNase inhibitors. For confocal imaging, dissect the flies on a dissection pad in 1x PBS or PBS + 0.2% Triton.
9. First, put the adults to sleep on the CO₂ station pad under the dissection scope. On the CO₂ station pad, using forceps in one hand to stabilize the body, use forceps in the other hand to gently pull/pinch out the third antennal segment from the second.
10. Transfer the antennae into an RNA isolation solution, using forceps to place the antennae directly into the liquid. Repeat until all adults have been dissected.
NOTE: Ensure that the antennae are submerged in RNA isolation solution. The antennae may become stuck on the side of the tube. This will cause an increased degradation of the RNA, reducing RNA quality and quantity.
11. Directly proceed to the RNA extraction or place the tube at -80 °C for long-term storage.

5. RNA Isolation from Dissected Tissues

1. Remove all samples from the -80 °C storage and thaw them on ice.
2. Briefly spin (5 - 6 s, ~6,000 x g) each sample to collect the material at the bottom of the tube.
3. Grind each sample using an autoclaved plastic pestle and an electric motor for 10 s on ice.
4. Repeat steps 5.2 and 5.3 4.
5. Perform the RNA extraction using commercially available kits and following the manufacturer's protocols for an optimal yield.
6. Perform qRT-PCR using commercially available reagents and specific primers against genes of interest and following the manufacturer's protocols.
 1. Carry out all PCR reactions using the following conditions: 95 °C denaturation for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 60 °C for 30 s.
NOTE: Primer pairs for *DIP* and *dpr* genes are listed in **Table 1**, and primers for olfactory receptor genes are listed in Hueston CE *et al.*⁸.

6. Fixation of the Pupal Antennal Discs and Antennae for Immunohistochemistry

NOTE: Fix the tissues in batches from 10 - 20 individuals to ensure the tissues are fixed for the same amount of time. Minimize the amount of time the samples stay in PBT (PBS with detergent) prior to transferring them to a fixative to maximize the integrity of the tissues.

1. Collect the dissected antennal discs or pupal antennae in a 200- μ L PCR tube containing 200 μ L of 0.2% PBT on ice to maintain tissue integrity, and to avoid the sample from sticking to the wall of 200- μ L PCR tubes which will lead to an incomplete fixation.
Note: Pupal antennae and antennal discs can be difficult to see as they are quite translucent. It is advisable to use a dissection scope for all washing steps to prevent any loss of tissue. Alternatively, 96-well Terasaki plates may be used for the fixation and staining to best track the tissue.
2. Fix the dissected antennal disc and antennal samples from pupae in approximately 200 μ L of 4% PFA (Paraformaldehyde) for 30 min at room temperature. For this and subsequent steps, keep the tubes with the samples on a nutator to properly mix the samples in the solution. Progress to step 6.7.
3. For the adult antenna, dissect the entire head first and fix it in approximately 200 μ L of 4% PFA (paraformaldehyde) for 1 h at room temperature.
4. Wash 3x with 200 μ L of 0.2% PBT for 10 min each. Keep the samples on the nutator during the incubation.
5. Go back to the dissection microscope for a finer dissection of the second antennal segment from the fixed heads. Using forceps to stabilize the head, gently pull/pinch out the third antennal segment from the second.
6. Transfer the dissected third antennal segments into approximately 200 μ L of 4% PFA (paraformaldehyde) and fix them for 30 min at room temperature.
7. Wash 3x with 200 μ L of 0.2% PBT for 10 min each. Keep the samples on the nutator during the incubation.
8. Store the samples at 4 °C or continue directly with whole mount immunohistochemistry.
Note: The efficiency of staining might change when the antibody is being used. This method should be appropriate for less abundant protein tags or weaker antibodies. However, at times, it might require an optimization of the protocol (either the identity or amount of the fixative or the time of fixation).

Representative Results

The dissected developing olfactory tissue can be used for RNA extraction followed by RT-PCR to assess gene expression or can be taken through immunohistochemistry protocols to determine its expression pattern, and the sub-cellular localization of genes of interest. In this section, we present representative results from either protocol. **Figure 1** is the representative quantitative RT-PCR showing the transcription of cell surface receptor and olfactory receptor genes in wild-type adult antennae. **Figure 2** shows the 6 - 8 h and 12 h APF antennal disc stainings on Rn-EGFP transgenic flies using anti-GFP (1:1000) and anti-lamin antibodies (1:100) (**Figure 2A** and **2B**). Rn-EGFP labels particular regions within the antennal disc during early pupal stages. We also show 40 h APF pupal antennal anti-GFP antibody staining of *Or67d-GAL4 UAS-CD8GFP* transgenic flies, and adult antennal anti-elav (1:100) antibody staining of ORNs (**Figure 2**). Other examples can also be found in the literature^{7,8,9,10}.

Figure 1. Quantitative RT-PCR from wild-type adult antennal RNA samples for different genes. These panels show quantitative RT-PCR results for (A) *DIP-alpha*, *DIP-eta*, *dpr5*, *dpr8*, *kug*, *beatIIIb* (for primer pairs see Table 1), and (B) olfactory and ionotropic receptor (*Or/Ir*) genes from adult antennal RNA samples. The error bars show a standard error of mean (SEM). *Or/Ir* genes are low abundance RNAs that can still be detected by qRT-PCR. The expression of each gene was analyzed in triplicate. Ct values were used to calculate the dilution factors for each gene based upon standard curves created for each gene. The dilution factors were then normalized for each gene to the average expression of all genes measured as described before⁸. [Please click here to view a larger version of this figure.](#)

Figure 2. Immunohistochemistry using different antibodies on developing olfactory tissues. These panels show anti-GFP (green) and anti-lamin (red) antibody stainings on (A) 6 h APF pupal antennal discs and (B) 12 h APF pupal antennal discs. (C) This panel shows a 40 h APF antenna from *Or67d GAL4 UAS-CD8GFP* transgenics stained with rabbit anti-GFP (green) antibodies. (D) This panel shows adult antennae stained with anti-CD2 antibodies, which label *Or47b-CD2* positive ORNs (magenta). [Please click here to view a larger version of this figure.](#)

Forward primer	Reverse primer	Length
CGGAGAGGAGGTTATGAGCA	GGAAGTGCACAACCTAGCGTT	143
CGCGAATCGTTGTGTCAGTA	TGTCGATAGCGTGAACCTGA	129
AGGAGTGGACGTTGAGTTACA	CAATCATGTCCTCGTGACCG	146
TAGAGTCCGAGCAGCAGATG	GTCAGAATTCGAGTTGTCCGC	104
GGCGCTAAGTAGCAGGACG	GGCCAAGTCTGTTTGTGAGG	215
TAAGACCCTAGCGCCCTCTT	AGGGAGATGCGCTTTGAGAC	129

Table 1. List of PCR primers used in qRT-PCR.

Discussion

This protocol is a useful resource for labs interested in identifying the genetic and transcriptional programs operating in developing *Drosophila* olfactory tissue, as well as a comparative analysis of these programs across *Drosophila* species. It is especially useful if systems-level transcriptional profiles from different developmental stages are needed as a primer for future studies. The protocol described here demonstrates how to stage and isolate developing olfactory tissue from *Drosophila* to obtain samples from four key stages of olfactory system development from pre-patterning to terminal differentiation, to be used in molecular and immune-histological studies. Dissected *Drosophila* antennal discs and antennae at different pupal stages can be used to identify gene expression profiles during the olfactory system development at the systems level by RNA-seq, or at the level of individual genes by quantitative RT-PCR. Samples can also be used for immunohistochemistry and *in situ* hybridization to identify *in vivo* patterns of tissue-specific gene expression. An additional advantage of this protocol is that dissected samples can be further dissociated using standard methods, and FAC-sorted to obtain a purified cellular population (or single cells) for cell type-specific molecular analyses, such as quantitative RT-PCR or RNAseq.

Even though the protocol demonstrates dissections of antennal discs and antennae from pupal (at 0 h/prepupa, 8 h, and 40 h APF) and adult stages, it can be adapted to other pupal time points. It is also worth mentioning that learning these dissections takes time and practice. An expert should be able to dissect 50 samples an hour. It is important to not overage the pupae. Therefore, an hour before beginning dissection,

ensure that no pupae are older than the desired age. Dissections can take time to learn, so a period of practice for each stage is required prior to starting experiments. Dissections become more difficult from 9 - 12 h APF due to morphological changes. It is possible to sort pupae by age when collecting prepupae (by body color, with darker colors being older). Initially sorting prepupae allows for dissection in order of age to prevent overaging.

The protocol described above can be applied to other *Drosophila* species. The timing for the pupal dissections of other species can be based both on the ratio of the pupal development of a given species at 25 °C to the *D. melanogaster* and on comparative observations of antennae/antennal disc morphology during dissections, with a priority given to morphological considerations to be as identical as possible. For *Drosophila melanogaster*, the pupal stage takes approximately 4 days. *D. sechellia* and *D. erecta* pupal developmental staging is similar to *D. melanogaster*; however, *D. virilis* pupal development is 1.3x longer^{11,12}. When other species are prepared for dissection, the staging should be based on both the timing of the pupal period and comparative observations of stage-specific antennae/antennal disc morphology, with the morphological similarity being the highest priority. First, identify the optimum handling temperature for the species you are interested in. Many species grow well at 25 °C, but detailed information on maintenance conditions can be obtained from the UC San Diego *Drosophila* species stock center.

The most critical steps in the protocol are the correct staging and proper dissection of the tissue of interest in adequate numbers from different species. Once these are in place, the protocol provides an efficient method of tissue collection for such analyses and can be practically adapted to any developing imaginal disc tissue during the pupal stages in any *Drosophila* species.

One limitation of this protocol is that it does not provide cell type-specific samples. A more detailed understanding of cellular function and morphology requires cell type-specific profiling of transcription at a systems level, in addition to standard molecular procedures such as quantitative RT-PCR or immunohistochemistry. Cell type-specific profiling has been difficult, especially given the lack of cell type-specific reporter transgenics in non-*melanogaster* species. With the current advances in cellular profiling, transgenics, and CRISPR-Cas9 mediated genome editing in non-*melanogaster* species, it is now possible to label and sort out single cells of interest to profile transcription¹³.

Disclosures

The authors have nothing to disclose.

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