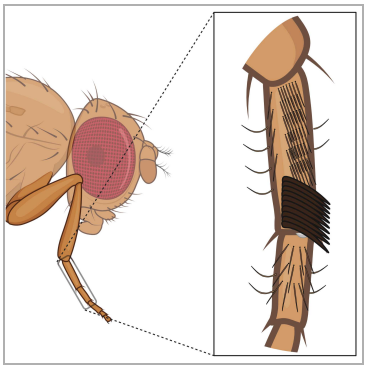

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Single-cell dissociation of Drosophila melanogaster pupal tarsi V.1

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ABSTRACT

This protocol outlines a step-by-step guide to generating single-cell suspensions of *Drosophila melanogaster* pupal tarsi for use in 10x single-cell transcriptome profiling. This protocol has been successfully used on male legs between 16h and 30h after puparium formation.

From an input of 65-70 first tarsal segments, we typically generate a suspension with the following metrics:

Expected volume of suspension generated: ~65ul
Expected cell concentration of suspension generated: ~1000-2000 cells/ul
Expected cell viability of suspension generated: ~98%

Keywords: single-cell; tissue dissociation; scRNA-seq; Drosophila; tarsus; tarsi; pupa; pupal dissection; puparium; leg development; sensory organ; cuticle-associated cells

Collecting, sexing, and ageing pupae

35m

1 Collect white prepupae. Individuals should meet the P1 aging criteria laid out by Bainbridge and Bownes (1981): the pupae should be white or cream coloured, have stopped moving completely, and display everted anterior spiracles. 20m

> **Note**
>
> Collect pupae *n* -1 hours before starting pupal de-casing, where *n* is the desired developmental timepoint for sequencing. Pupae are removed from the pupal case 1 hour before the leg dissection, hence the '-1'.

> **Note**
>
> Helpful, high-quality images of pupae at the correct stage can be found in Chyb and Gompel's 'Atlas of *Drosophila* morphology' (https://www.sciencedirect.com/book/9780123846884/atlas-of-drosophila-morphology).

> **Note**
>
> We aim to dissect 65-70 legs for a single-cell suspension. Collecting 40-50 pupae provides a buffer that allows for some failed dissections.

2 Identify individuals of the correct sex. Place white prepupae in a glass well with water under a dissection microscope. At this developmental stage the male gonads are visible on either side of the posterior ~1/3rd of the pupae. 10m

3 Fold a kimwipe in half and then in half again a further two times. Place inside a Petri dish and wet the kimwipe with 500 µL of water. Then transfer the pupae from the glass well to the kimwipe using a paintbrush. Cover the Petri dish and move to an incubator set to 25 °C. 5m

Note

We have found that this volume of water is optimal. Too much and the pupal case softens and is hard to cut; too little and pupal development appears to be compromised. Beyond 30h dissections (36h and 40h), we have found 750 µL to be optimal.

De-casing pupae

45m

4 At *n* - 1 hours, remove the Petri dish from the incubator. Using a wet paintbrush, gently remove the pupae from the by now dry kimwipe. Wetting the paintbrush helps to loosen the pupae from the wipe. Transfer the pupae to a dry kimwipe and gently pat them down to further dry them. Then transfer dorsal side up to a piece of sticky tape (sticky side up) that is secured to a flat surface (*e.g.* a piece of plastic or microscope slide). 5m

Note

Transfer multiple pupae at a time to give them an opportunity to continue to dry while on the tape. This makes for easier de-casing.

5 Using forceps, gently remove the pupal casing. Begin by removing the operculum and then cut laterally down the side of the case, working from anterior to posterior and gently peeling away the cut casing to expose the pupa. Then transfer the de-cased pupa to a kimwipe soaked through with water in a Petri dish. 40m

Note

O'Connor *et al.* (2022) and Psathaki and Paulula (2022) both give helpful visual guides to performing this procedure.

Note

Line pupae dorsal-side down (to avoid water-logging the legs) on the kimwipe in the order in which they were de-cased.

Dissecting legs

1h 30m

6 Add 100 µL of Dulbecco's PBS (DPBS) to a glass well on ice under a dissection scope. 1m

Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8537**

7 Use forceps to transfer several pupae, starting with the first that were de-cased, to the same tape-covered surface used for the de-casing. Place pupae ventral side up (*i.e.* legs facing up). 3m

8 For each pupa, begin by pinching the base of the abdomen to release fluid. This prevents the release of large amounts of fluid while pinching the leg, which interferes with the dissection. Gently press on the head to push a small amount of fluid out. 1m

9 To dissect forelegs, pull the second and third legs away from the body such that they point ~90° away from the body. This exposes the foreleg tarsus. The tibia/tarsus joint should be visible approximately in line with or just below the base of where the leg connects to the thorax (directly below the mouthparts). 2m

Note

The tibia/tarsus joint in the second leg can be exposed by pulling away the third leg following the same approach above. The joint is a little more distal with respect to the thorax than the homologous joint in the foreleg and falls approximately in line with the position of the first/second tarsal joint in the foreleg. The third leg tibia/tarsus joint is a little more distal again.

10 Using forceps, pinch just proximally to the joint and pull away from the body of the pupa. The tarsus should come away. 2m

Note

At 16h after puparium formation, the actual tibia/tarsus joint seems to sit a little more proximally than the joint-like indentation that's visible in the cuticle. At this timepoint, the pinch should be made a little higher up to account for this. The pinch will need to be made more proximally if targeting tibia at any timepoint.

Note

If targeting the lower tarsal segments or pretarsus, it is still easiest to make the pinch at the tibia/tarsus joint but adjust the position of the slice in step 12.

11 Place the tarsus down on the tape and cover with 10 µL of DPBS. 1m

12 Using a Micro Knife, slice at approximately the midsection of the tarsal segment adjacent to the segments that are being targeted *i.e.,* if targeting the first tarsal segment slice at the midsection of the second tarsal segment. A slice can be made on both the proximal and distal side of the targeted region to isolate a subset of segments. After making the slice(s), use forceps to gently ease the targeted region out from the pupal cuticle. 1m

Equipment

Micro Knives - Plastic Handle	NAME
Ultra-thin dissecting blade	TYPE
Fine Science Tools	BRAND
10318-14	SKU
https://www.finescience.com/en-US/Products/Scalpels-Blades/Micro-Knives/Micro-Knives-Plastic-Handle/10318-14	LINK

Note

Generally, the definition of the different tarsal segments is improved at later timepoints.

13 Using a freshly BSA-coated 10ul tip, pipette the first tarsal segment and transfer to the well of DPBS on ice. 1m

Note

BSA-coat a tip by pipetting up and down a solution of 1% BSA in DPBS 10 times.

14 Repeat until the well contains 65-70 dissected tarsal segments. Two trained personnel working simultaneously can generally complete this within 01:30:00 1h 30m

Performing the dissociation 52m 30s

15 Using a 10ul tip, remove all of the DPBS from the well. Avoid removing dissected tissue. 3m

16 Add 100 µL of dissociation buffer, which consists of 10x TrypLE with a final concentration of [M] 2 mg/mL collagenase. 2m

TrypLE™ Select Enzyme (10X), no phenol red **Thermo Fisher Catalog #A1217701**
Collagenase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C0130**

Note

The buffer can be premade and stored in individual aliquots at -20 °C.

17 Cover the well with Nunc Aluminium Seal Tape and submerge inside of a bead bath at 37 °C for 00:35:00. 35m

Equipment

Lab Armor Beads NAME

Non-uniform metal beads TYPE

Gibco BRAND

A1254301 SKU

https://www.thermofisher.com/order/catalog/product/A1254301 LINK

Equipment

Auminum Seal NAME

Auminum Seal TYPE

Nunc BRAND

232698 SKU

https://www.thermofisher.com/order/catalog/product/232698 LINK

18 Remove from the bead bath and then remove ~ 85 µL of dissociation buffer using a 10ul tip. 3m

Note

The buffer removal is a balancing act. In theory, leaving too much buffer could interfere with the reagents used during 10x library preparation. However, after removing the first ~60ul it can be hard to remove more without losing tissue. Take this step slowly and move the tip to different regions of the well to avoid losing or disrupting the tissue.

19 Add 50 µL of room temperature DPBS to the well. 2m

20 Pipette the solution up and down 20x using a widebore, BSA-coated, lowbind tip. Aim for ~1.5 pipettes up and down per second, moving the tip around in the well to ensure an even dissociation throughout the suspension. Avoid introducing bubbles into the suspension. Use a 200ul pipette set to 40ul. 15s

Equipment

ART 200ul Wide Bore Filtered Pipette Tips	NAME
Pipette tips	TYPE
ART	BRAND
2069G	SKU
https://www.thermofisher.com/order/catalog/product/2069G	LINK

Note

BSA-coat the tip as in step 13.

21 Pipette the solution up and down a further 20x using a BSA-coated, flame-rounded 200ul tip. Again, make sure to move the tip around in the well. 15s

Note

Prepare tips ahead of time by quickly passing them near a flame. Check under a microscope that the tip walls at the end are not rough but smooth and slightly rounded.

22 Slowly pipette the solution up and down a further three times using the same flame-rounded tip before taking up 40 µL and transferring to a 2mL low-bind Eppendorf tube on ice. 1m

23 Add a further 20 µL of DPBS to the well, pipette up and down three times using the flame- 1m

rounded tip, and add 25 µL to the same Eppendorf on ice.

24 Preview 10 µL of suspensions using a hemocytometer or automated cell counter. 5m
Concentrations and singlet rates should be checked against the requirements of the single-cell technology being used.