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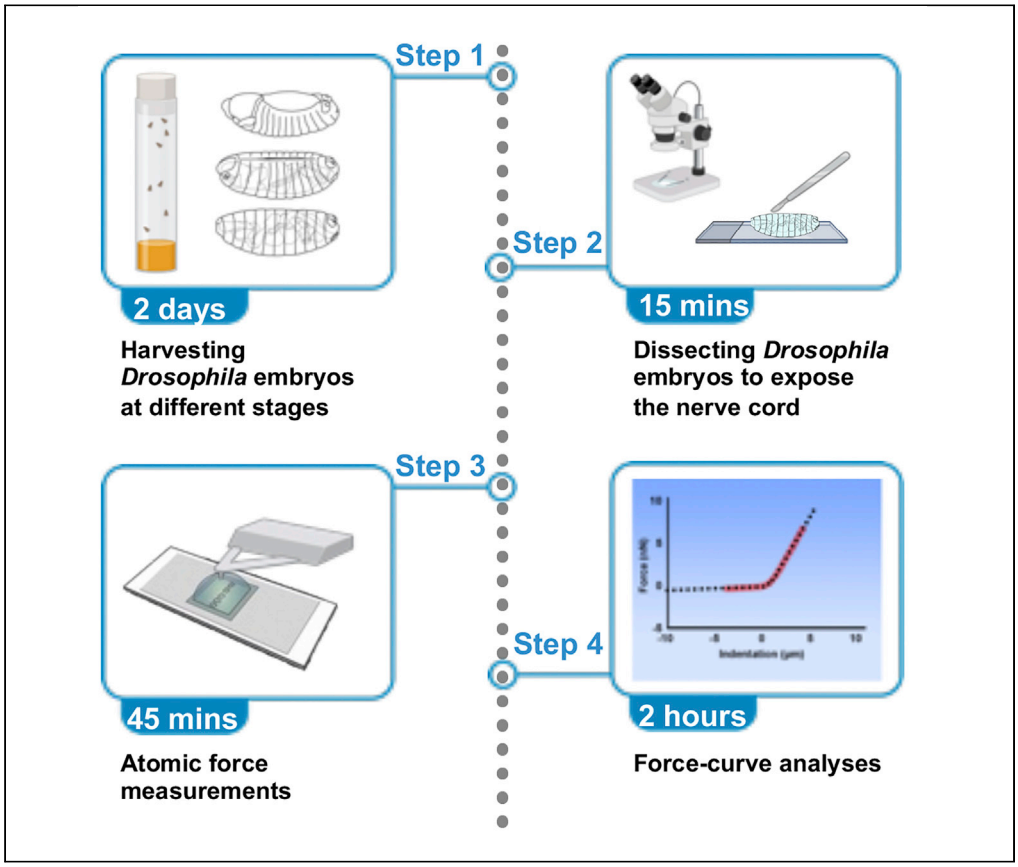
Measuring ventral nerve cord stiffness in live flat-dissected *Drosophila* embryos by atomic force microscopy



Drosophila is an amenable system for addressing the mechanics of morphogenesis. We describe a workflow for characterizing the mechanical properties of its ventral nerve cord (VNC), at different developmental stages, in live, flat-dissected embryos employing atomic force microscopy (AFM). AFM is performed with spherical probes, and stiffness (Young's modulus) is calculated by fitting force curves with Hertz's contact model.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

This protocol allows CNS stiffness measurements by AFM on intact, non-fixed tissue

Live, flat-dissected embryonic CNS can be prepared at different developmental stages

AFM on soft tissues is achieved employing spherical probes

Young's modulus is calculated by fitting the force curve with Hertz's contact model

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Measuring ventral nerve cord stiffness in live flat-dissected *Drosophila* embryos by atomic force microscopy

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SUMMARY

***Drosophila* is an amenable system for addressing the mechanics of morphogenesis. We describe a workflow for characterizing the mechanical properties of its ventral nerve cord (VNC), at different developmental stages, in live, flat-dissected embryos employing atomic force microscopy (AFM). AFM is performed with spherical probes, and stiffness (Young's modulus) is calculated by fitting force curves with Hertz's contact model.**

For complete details on the use and execution of this protocol, please refer to Karkali et al. (2022).

BEFORE YOU BEGIN

Timing: 3 days

In this protocol we describe the steps and discuss the main critical points for measuring the mechanics of the *Drosophila* embryonic VNC, in live dissected embryos. For simplicity, this protocol describes embryo collection and staging from a genetically homogeneous population (wild type strain *w[1118]*), cultured at 25°C. Alterations in fly culture conditions to serve experimental needs or discrimination between siblings, based on fluorescent markers, can be also applied. AFM is then employed to measure the viscoelastic properties of the tissue, which is very soft, with high spatial and force resolution. Importantly, this protocol can be employed on other *Drosophila* tissues or on highly soft biological samples of distinct origins. In any scenario, sample mounting and AFM tip selection are the critical issues to consider for obtaining high-quality and reliable results.

1. Embryo collection cage set up and flies' acclimatization.
   a. At least 2 days prior to experiment, transfer young, 3–5 days old, adult flies to embryo collection cages.
      i. Seal the cages with a 55 mm apple juice –agar plate carrying 0.5 g of yeast paste.

Note: Yeast paste serves as food source for the flies. The 2 days period proposed corresponds to the minimum adaptation time required for the flies to overcome the stress induced due to habitat change. The average number of flies introduced in a single cage is between 50 and 100 adults. Over-crowding the cage could stress the flies and influence embryo deposition.

b. Maintain flies in embryo collection cages at optimal culture conditions (Temperature: 25°C, Humidity: 60%, 12 h light / darkness cycle).
c. Exchange the food plate daily to avoid chamber contamination with 1st instar larvae, hatching after 24 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
European bacteriological agar	Condalab	Cat#1800
D(-)-Fructose	(BioChemica) PanReac AppliChem ITW Reagents	Cat#A3688
D(+)-Glucose anhydrous	(BioChemica) PanReac AppliChem ITW Reagents	Cat#A1422
Propionic acid 99%	PanReac AppliChem ITW Reagents	Cat#161810.1611
Agarose D1 Low EEO	Condalab	Cat#8010
Di-sodium hydrogen phosphate (Na_2HPO_4)	Merck	Cat#106585
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2\ H_2O$)	Merck	Cat#106342
Sodium hypochlorite (NaClO) - Bleach	Merck	Cat#105610
Experimental models: Organisms/strains		
Species: *Drosophila melanogaster* Strain: *w[1118]* Egg-laying cages: 3–5 days old adult flies of both sexes Embryos for dissections: stage 16	Bloomington *Drosophila* Stock Center	Cat#3605
Software and algorithms		
MATLAB	MathWorks	RRID: SCR-001622
Other		
Apple juice	N/A	N/A
Dry powdered yeast	N/A	N/A
Embryo collection cage (fits 60-mm Petri dish)	Genesee Scientific (Flystuff)	Cat#59-100
Falcon Cell Strainers, Sterile, Corning (100 µm)	VWR	Cat#21008-950
Golden Nylon round paint brush Size 6 diameter 5 mm × length 18 mm	OMNI	Cat#GNR1#6
Sterilin Standard 90 mm Petri dish	Thermo Fisher Scientific	Cat#11389273
55 mm × 14 mm Petri dish	Deltalab	Cat#2002001
Dumont #5 Forceps	Fine Science Tools	Cat#11252-30
Double sided tissue tape	Superior Link Marketing Pte Ltd	N/A
Thermo Scientific X50 SuperFrost Microscope slides, ISO8037/I, White 26 × 76 × 1 mm	Thermo Fisher Scientific	Cat#17234884
Advanced PAP pen	Merck	Cat# Z377821-1EA
Capillary glass tube with flame polished ends. Borosilicate thin wall with filament OD=1 mm; ID=0.78; mm length=10 cm	Warner Instruments	Cat# G100TF-4
Masterflex Tygon Microbore Tubing I.D. 0.76 mm; O.D. 2.29 mm; wall thickness: 0.8 mm	Thermo Fisher Scientific	Cat#15283647
Zoom Stereomicroscope, Nikon SMZ645	Nikon	N/A
Inverted microscope, Nikon TE2000	Nikon	N/A
NanoWizard 4XP BioScience	Bruker	N/A
Spherical AFM probes. k = 0.01 N/m polystyrene R = 10 µm	Novascan Technologies	N/A
Sutter Instrument P-87 Flaming Brown Micro-pipette Puller	Sutter Instrument	N/A

Note: Equivalent products provided by other commercial houses can substitute reagents and equipment used in this protocol.

MATERIALS AND EQUIPMENT

- Microscopy equipment:

Drosophila embryo handling and flat-prep dissections were done using a Nikon SMZ645 Zoom Stereomicroscope.

AFM was coupled to a Nikon Eclipse TE2000 inverted microscope, equipped with a CFI Plan Fluor 10× / N.A. 0.3 and a CFI Plan Fluor 40× / N.A. 0.75 objectives.

- Atomic Force Microscope:

The measurements were performed using a custom-built AFM. A detailed protocol of the setup can be found in (Rico Camps, 2006). Yet, the majority of commercial AFM systems allow for the mechanical characterization of biological samples. We suggest employing the Bruker NanoWizard 4 XP BioScience, although it should be stressed that several equally excellent options are available.

- Micro-pipette Puller:

The glass needles used in embryo dissections were made employing a Sutter Instrument P-87 Flaming Brown Micro-pipette Puller using the following settings: Heat: 395; Pull 0; Vel 50; Time 50; Pressure 500.

- Recipe for preparing apple juice – agar embryo collection plates:

Reagent	Concentration	Amount
Bacteriological agar	2% w/v	20 g
(D-)-Fructose	1% w/v	10 g
(D+)-Glucose anhydrous	2% w/v	20 g
Apple Juice	50% v/v	500 mL
ddH_2O	N/A	500 mL
Propionic acid	0.5% v/v	2.5 mL
Total	**N/A**	**1 L**

* Plates are stored sealed in a plastic bag, at 4°C for up to 1 month.

- 0.1 M Sörensen phosphate buffer.

Reagent	Final concentration	Amount
Na_2HPO_4	72 mM	1.03 g
$NaH_2PO_4.2\ H_2O$	28 mM	0.44 g
ddH_2O	N/A	100 mL
Total	**N/A**	**100 mL**

* Store at 22°C for as long as pH is 7.2.

STEP-BY-STEP METHOD DETAILS

Experimental material preparation

Timing: 1–5 h

Timing: 4 h (for step 1)

Timing: 1 h (for step 2)

Timing: 1.5 h (for step 3)

Timing: 30 min (for step 4)

1. Prepare apple juice - agar plates for embryo collection (recipe can be found in materials and equipment section 4).
   a. Dissolve agar in water, in 1 L Pyrex glass conical flask and autoclave (15 min exposure at 121°C and 1 bar pressure) to ensure full agar dissolution.
   b. Dissolve all sugars in apple juice, in a 1 L glass beaker, by means of a laboratory magnetic stirrer.
   c. Add the autoclaved agar solution gradually to the sugars - juice mix under constant stirring.
   d. Add 2.5 mL of propionic acid to the homogenized mix and keep stirring.
   e. Evenly distribute the mix in 55 mm Petri dishes and allow agar to solidify at room temperature (22°C) for at least 2 h.
   f. Store the apple juice – agar plates sealed in a plastic bag at 4°C for up to 1 month.

   Note: Propionic acid serves as antifungal and conservation agent, while it acidifies the mix to mimic the smell of rotten fruit.

2. Prepare 0.1 M Sörensen phosphate buffer (buffer contents are listed in materials and equipment section 5).
   a. To facilitate salts dissolution, heat the mix in a microwave for 1 min.
   b. Fully dissolve the salts by stirring, using a magnetic laboratory stirrer.
   c. Allow the solution to cool at room temperature (22°C) before using.
   d. Adjust pH to 7.2, if needed.
   e. Store buffer at room temperature (22°C) for as long as pH remains 7.2.
3. Prepare agarose plates for handling dechorionated embryos.
   a. Dissolve 1 g of agarose in 100 mL of dH_2O.
   b. Bring the mix to ebullition by heating it in a microwave oven, for approximately 2 min.
   c. Distribute agarose solution evenly on 55 mm Petri dishes.
   d. Let it solidify at room temperature (22°C) for at least 1 h.
   e. Store agarose plates, sealed in a plastic bag, at 4°C for up to 2 months.
4. Generate glass needles for embryo dissection.
   a. Pull borosilicate thin wall capillary glass tubes, with filament and flame polished ends, using a Flaming Brown P87 needle puller (for settings see materials and equipment section 3).

Collection and processing of precisely timed embryos

Timing: 2 days

VNC stiffness is sensitive to the developmental stage of the embryo; therefore, assuring that AFM measurements are performed on precisely timed dissected embryos is crucial for data coherence

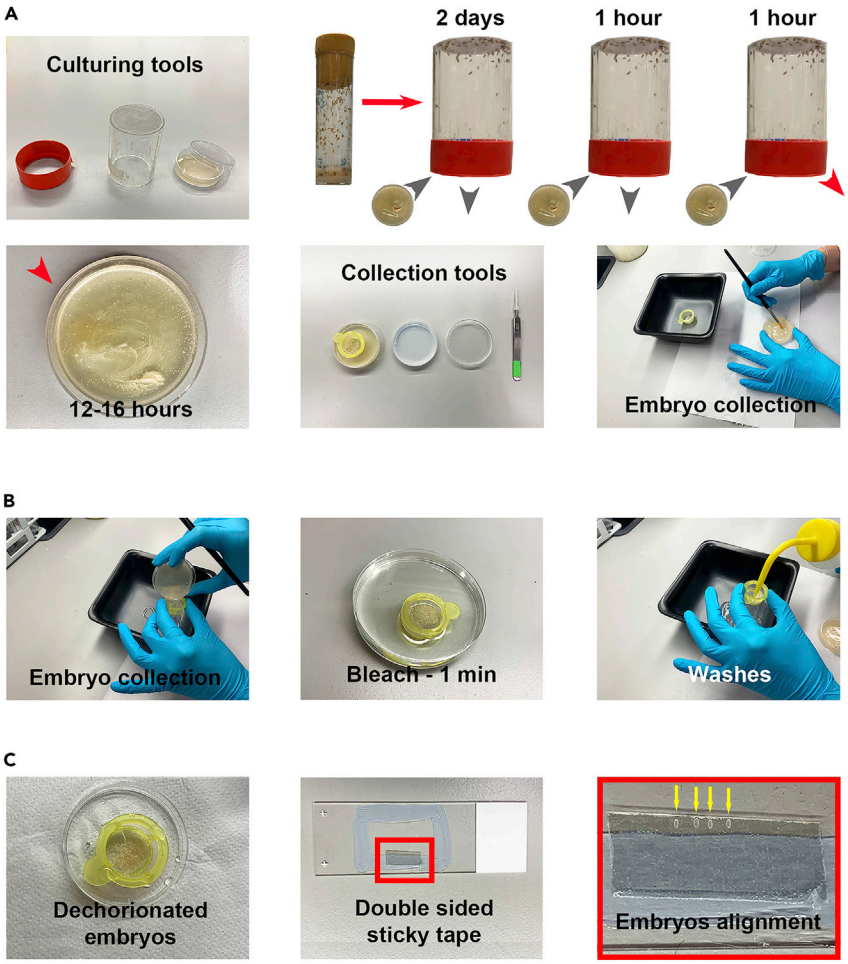

Figure 1. Collection and preparation of timed embryos for flat dissections.

Visual guide summarizing the steps required for collecting, dechorionating and mounting timed *Drosophila* embryos.

(A) Parental flies are transferred from culture vials to embryo collection chambers and are allowed to acclimatize for a minimum of 2 days. Culture synchronization is achieved by changing the apple juice-agar plate sealing the chamber every 1 h. Embryos laid within the first 1 h period are discarded (gray arrowheads), while those laid in subsequent rounds are aged (red arrowheads) until they reach the required developmental stage (in this case 12 h at 25°C to reach early stage 16).

(B) For harvesting the embryos, the apple juice-agar plate is filled with distilled water and embryos are detached from the surface using a brush. Suspension passes through a strainer and the embryos retained are dechorionated by submerging in bleach for 1 min.

(C) Dechorionated embryos are thoroughly rinsed with distilled water and aligned with their dorsal side up on a piece of double sided sticky tape placed on the dissection slide.

and comparison. This step refers to the synchronization of the fly culture for collecting embryos of the same developmental stage, with a maximum of 1 h difference (Figure 1).

5. Synchronization of the fly culture.
   a. Bring 4 fresh apple juice-agar plates to room temperature and add 0.5 g of yeast paste to each one of them.
   b. Exchange the juice-agar plate sealing the embryo collection cage to a fresh one.

   Note: In this step, the flies presented with fresh food source deposit all embryos that could possibly be retained due to environmental stress.

c. After 1 h, repeat step 5b and discard the previous plate.

Note: This point corresponds to collection time zero given that all embryos deposited from this point onward will be of the same developmental age.

d. After 1 h, exchange the juice-agar plate as in 5b.
e. Maintain the plate containing the embryos deposited during this time period (0–1 h After Egg Laying (AEL)) at 25°C for a total of 12 h. Troubleshooting 1 and 2.

Note: The incubation time indicated is for obtaining stage 16 embryos. Stage 16, or 3-part gut stage, lasts from 13–16 h AEL at 25°C, therefore the proposed 12 h incubation of the collected embryos should yield embryos at the beginning of this stage. It is worth mentioning that embryo dissections and AFM measurements of the VNC can be done from stage 13 till early stage 17. For obtaining stage 13 embryos the incubation time is reduced to 10 h, while for stage 17 is increased to 15 h, respectively. In addition, considering development is delayed at 18°C and accelerated at 29°C, the hours AEL corresponding to stage 16 should be adjusted based on the following equations: t (at 18°C) = 1,82*t (at 25°C) and t (at 29°C) = 0,86*t (at 25°C) (Kuntz and Eisen, 2014).

f. Repeat step 5d for a second collection round.

Note: Considering that AFM measurements on the VNC of a single embryo require approximately 1 h, it is advisable to perform several collection rounds to ensure availability of precisely age matching embryos over an extended period of time. This step can be repeated for as many times as required.

6. Dechorionation: remove embryos' chorion with bleach prior to dissection. Troubleshooting 3.
   a. Detach stage 16 embryos from the juice-agar plate.
      i. Use a squeeze bottle to fill the juice-agar plate with dH_2O.
      ii. With a soft painting brush, dissolve the yeast paste added in step 5a, and gently remove the embryos from the agar surface, while swirling the water.
      iii. Wet a cell strainer through running water, under the tap.
      iv. Decant the water containing the floating embryos to the cell strainer.

   Note: Water shall pass freely through the strainer, while embryos are retained.

      v. Repeat steps i, ii and iv, at least two more times, or until all embryos are transferred from the juice-agar plate to the strainer.
      vi. Wash embryos in the strainer with dH_2O to remove any remaining yeast paste.
   b. Dechorionate embryos with bleach.
      i. Fill a 90 mm Petri dish with bleach (10 mL approximately or until the filling line) and submerge the strainer containing the embryos.

Note: Introduce cell strainer in bleach in an angle to avoid air bubbles formed underneath.

⚠ CRITICAL: Incubate embryos in bleach for 1 min. Gently swirl the Petri containing the bleach to ensure embryos remain in suspension.

Note: Dechorionation can be monitored under a dissecting scope. A small air bubble is formed at the anterior tip of each embryo, as the air trapped between the chorion and the vitelline membrane is released.

⚠ CRITICAL: After dechorionation, rinse the embryos extensively (for at least 10 s) with running water under the tap.

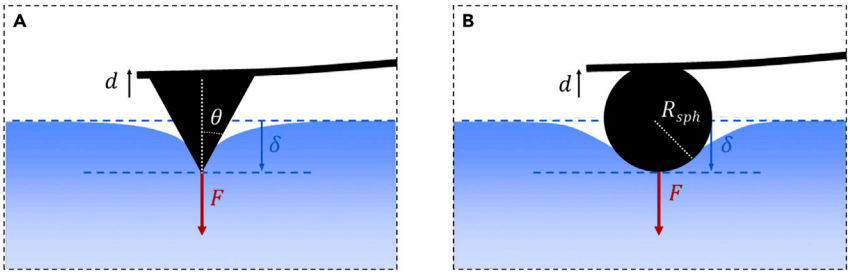

Figure 2. Four-sided pyramidal and spherical tips.
Commercially available pyramidal tips with high lateral resolution introduce high strains to the sample due to its small contact tip-sample area. Spherical tips produce low strains and are suitable for very soft and fragile samples, as the *Drosophila* embryo VNC. In the A and B panels are shown the differences in indentation that pyramidal and spherical tips attached to a cantilever (black) exert over a flat substrate (blue). *d* - Cantilever deflection; *δ* - Indentation depth; *θ* - Half-opening angle of pyramid tips; *Rsph* - Radius of spherical tips; *F* - Deflection force.

Note: Take special care not to overflow the strainer. Dechorionated embryos form clusters, since the exposed vitelline membrane is hydrophobic.

c. Transfer dechorionated embryos on a 55 mm Petri dish containing solid 1% agarose in dH_2O.
   i. Dry meticulously the exterior surface of strainer with paper.
   ii. Under a dissecting scope, transfer the embryos from the strainer to the agarose surface, using forceps.

Note: Embryo clusters formed upon dechorionation, stick easily to the metal forceps facilitating the transfer.

   iii. Distribute embryos evenly on the agarose surface and phenotypically cross check that embryos are on the right developmental stage (3-part gut).

AFM tip selection

Timing: 30 min

AFM uses a flexible cantilever with nanometer or micrometer sized tip at its end to probe the mechanical properties of a sample. A high-precision positioning system (usually a piezoelectric system) allows for the displacement of the cantilever and for changing the relative position between the tip and the sample. The cantilever bends when local interaction between the tip and the sample occurs in the range of pN and nN. For small cantilever bending, the cantilever-tip behaves as an ideal spring, enabling computing the deflection force (F) using Hooke's law ($F=k \cdot d$), where k is the spring constant of the cantilever-tip and d the cantilever bending or deflection. The deflection is quantified by measuring the photodiode voltage signal generated by a laser focalized on top of the cantilever. The tip-sample interaction produces a deformation or indentation δ to the sample (Figure 2).

7. Optimal cantilever-tip selection depends on the type of sample and measurement (Table 1), while three main parameters need to be taken into account:
   a. Cantilever spring constant, k: based on Hooke's law a large k will lead to very low deflections, lowering system's sensitivity. Therefore, the closer k is to the local sample stiffness the higher the sensitivity in the measurements of F. In our experience, cantilevers with $k \sim 0.01–0.1$ N/m are a suitable starting point for very soft biological samples as the *Drosophila* VNC (Alcaraz et al., 2018).

Table 1. Cantilever-tip selection for different biological samples.

	Cantilever spring constant, k (N/m)		Tip geometry		Tip material	
	< 0.1	≥ 0.1	4-side pyramid	Spherical	Polystyrene / Glass	Silicon nitride
Extracellular matrix (Andreu et al., 2014; Jorba et al., 2019)	x	x	x	x	x	x
Cells (Alcaraz et al., 2003; Oliveira et al., 2019)	x		x	x	x	x
Very soft tissues (brain, *Drosophila* embryo) (Jorba et al., 2017a; Karkali et al., 2022)	x			x	x	

⚠ CRITICAL: Tip geometry: while measuring soft and fragile samples it is important to take into account the pressure applied to the samples. The contact area between the tip and the sample depends on the tip geometry. Standard commercial tip geometries used in AFM setups can be four-sided pyramids and spheres. Based on Hertz's model (Jorba et al., 2017b), the radius (a) of the projected tip-sample contact area ($A=\pi \cdot a^2$) are:

$$a = \frac{\delta \cdot tan\theta}{\sqrt{2}} \quad \text{(Equation 1)}$$

$$a = \left(R_{sph}\delta\right)^{1/2} \quad \text{(Equation 2)}$$

For a four-sided pyramidal tip (Equation 1), θ corresponds to the half-opening angle of the pyramid, while for spherical tips; R_{sph} represents the radius of the sphere (Equation 2) (Figure 2). As expected, the pyramidal tip has a lower contact area for the same δ (or F), thus, increasing the strains applied to the sample compared to a spherical tip. To evaluate the viscosity/rigidity of the extracellular matrix or cells in culture, pyramidal and spherical tips have been used indistinctively. For very soft samples, such as the *Drosophila* VNC, selecting a spherical tip to generate small strains is advisable to avoid damaging the sample while measuring. In this protocol, after several tests with spherical tips of different diameters, those with a relatively large radius of 10 μm were selected.

b. Tip material: usually commercial tips can be made of glass, polystyrene and silicon nitride depending on the tip geometry and manufacturer. Usually, glass and polystyrene tips show lower adhesion to the sample, helping to obtain reliable force curves. In our case, we employed polystyrene tips.

Embryo flat-prep dissection for AFM measurements on the VNC

Timing: 10–15 min for 4 embryos

In this step the dissection of live stage 16 embryos is described. This process requires practice since it is crucial that the VNC is not damaged or altered in any way during dissection. Also, it is worth mentioning that since AFM measurements are performed on non-fixed tissue, handling time cannot exceed 45 min (Figure 3).

8. Prepare the dissection slide.
   a. Place a Super Frost glass slide, with its positively charged surface facing up, under a dissecting scope (preferentially one equipped with an underneath light source).
   b. Create an approximately 3 cm × 1.5 cm rectangular hydrophobic barrier, at the center of the slide, with a PAP pen or nail polish.
   c. Allow it to air dry for a few minutes.

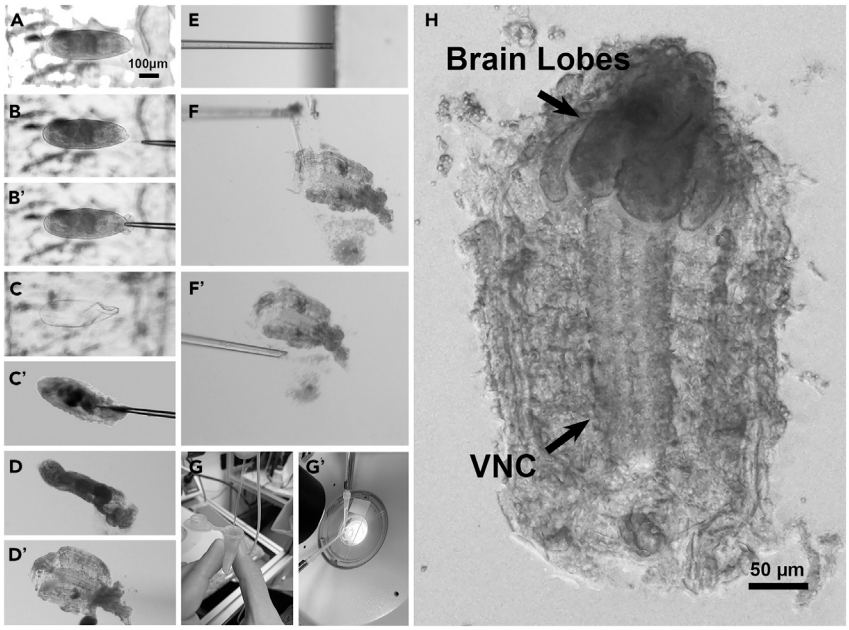

Figure 3. Live *Drosophila* embryo flat-prep dissection.

Photos captured at key points of the embryo dissection process. Dechorionated embryos of the appropriate stage are aligned with their dorsal side up on a piece of double sided sticky tape (see also Figure 1), anterior is to the right (A) and covered with 0.1 M Sörensen buffer. A pulled glass needle is used for manually devitellinizing the embryos. The needle pierces the embryo anteriorly, in a flat angle, and a shallow incision is made towards the posterior tip of the embryo (B and B'). Staying within the buffer, the embryo is dragged towards the center of the dissection area and is stuck on the glass, while the vitelline membrane remains on the tape (C and C'). The incision made is used for fully dissecting the embryo and big part of the intestine is removed (D and D'). After dissecting all embryos on slide, the tip of the needle is broken in a controlled manner to achieve a wider opening, which will serve the cleaning of the dissections (E). Intestines and embryo debris are removed from the preparation by mouth suction (F and F'). Dissections are completely flattened by blowing fresh buffer on their surface (G) and the complete buffer volume on slide is exchanged prior to AFM measurements (G'). At the end of the process, both brain lobes and VNC should be fully exposed, while the lateral body wall should be intact and flattened on the slide (H). Scale bar is 50 µm.

d. Cut an approximately 1 cm × 0.5 cm piece of double-sided adhesive tape using scissors.
   i. Utilizing forceps carefully place it on one of the long sides of the hydrophobic barrier created in step 8b.
   ii. Remove the paper that protects the upper side of the tape, without touching the surface of the glass slide.

9. Transfer selected embryos from the 1% agarose containing Petri to the dissection slide.
   a. Using forceps, group 4 embryos and transfer them on the surface of the double-sided tape pasted on the dissection slide.
   b. Carefully disperse and orient the embryos on the double-sided adhesive tape by gently rolling them with the forceps.
   c. Place embryos dorsal side up with their posterior tip pointing towards the center of the slide.
   d. Using a plastic Pasteur pipette fill the area framed by the hydrophobic barrier with 0.1 M Sörensen buffer.
   e. Ensure that the embryos are fully covered by buffer.

⚠ CRITICAL: During this step, take special care to avoid buffer from overflowing the barrier or air bubbles from forming between the embryos and the double-sided adhesive tape.

10. Manually devitellinize the embryos on the slide.

a. With a pulled glass needle pierce the vitelline membrane and enter embryo in the space between the brain lobes.

⚠ CRITICAL: Steadily drag the needle towards the posterior tip of the embryo, at a flat angle.

Note: It is important to perform the incision at the dorsal surface of the embryo, without disturbing ventral structures.

b. With the needle still in contact with the posterior part of the embryo, and while staying within the buffer volume, drag the embryo away from the vitelline membrane.

Note: The vitelline membrane should remain stuck on the double-sided tape.

c. Gently, press down the embryo at the center of the slide and retract the needle.

11. Dissect the embryos on the slide.
    a. Complete the incision initiated in step 10a to fully open the anterior and posterior tips of the embryo.
    b. Carefully, lift out the gut to reveal the VNC underneath it.

Note: Steps 10 and 11 are repeated for all 4 embryos on the slide.

12. Complete flat-preparation of dissected embryos.
    a. Using forceps, break the fine tip of the glass needle to generate an opening of a diameter corresponding roughly to 1/3 of the embryo.
    b. Attach the blunt end of the needle to one end of a 0.5 m piece of Masterflex Tygon tubing.
    c. Place a yellow tip at the free end of the Masterflex Tygon tubing.

Note: The yellow tip serves as a mouthpiece.

d. Add 0.5 mL of 0.1 M Sörensen buffer to a 2 mL plastic tube.
e. Submerge the tip of the glass needle in the buffer.
f. Apply mouth suction to gently fill up the needle with buffer, until approximately 1/3 of its length is full.

⚠ CRITICAL: It is important to avoid forming air bubbles that could block the needle.

g. Without releasing pressure at the mouthpiece, remove through aspiration the guts and other debris from the preparation.

Note: The buffer containing debris should never pass to the tubing.

h. When approximately the full length of the needle is filled with buffer and embryonic material, blow out on a tissue paper to completely empty the needle.
i. Fill 2/3 of the glass needle with 0.1 M Sörensen buffer, similarly to step 12f.
j. Blow a stream of buffer on each embryo to flatten it down.

⚠ CRITICAL: Be aware of the buffer level in the needle, since blowing air on the dissected embryos would destroy the preparation. Repeat as required. By the end of this step, the lateral epidermis should be attached to the slide surface and the VNC should be fully exposed, without nicks.

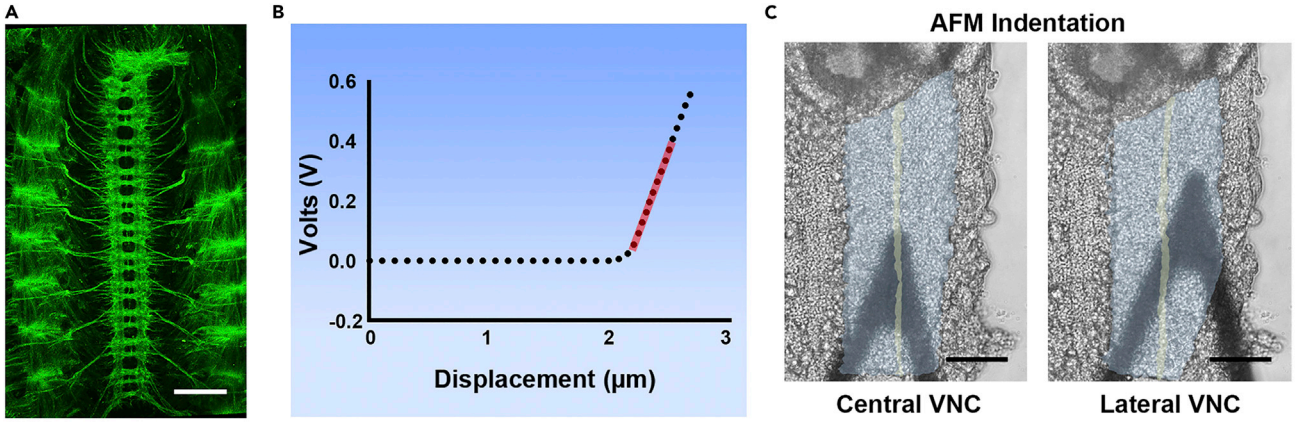

Figure 4. AFM indentation of neural tissue.

(A) Dissected flat-preparation of the VNC, of a stage 16 wild type embryo. GFP (green) labels Tubulin expression. Anterior (brain lobes) is up. Scale bar is 30 µm.

(B) Representative linear Voltage displacement curve (dotted line) measured when the AFM tip contacts the glass slide surface. A linear fit (red line) allows calculating the photosensitivity, s (1.08±0.05 V/nm).

(C) Phase contrast images of a dissected *Drosophila* embryo indented with the AFM cantilever in central (left) and lateral positions. The VNC is false colored in pale blue and the midline in yellow. Scale bar is 30 µm.

Note: Step 12 could be performed employing a syringe. In this case a 1 mL syringe substitutes the mouthpiece at the free end of the tubing. Holding the syringe with the non-dominant hand, intend to control the level of buffer in the needle in order to gently aspirate embryonic debris and flatten dissected embryos, as described in steps 12g and 12j. This alternative is more challenging; mouth suction is preferred and the 0.5 m tubing length ensures no accidental liquid ingestion.

13. Exchange buffer in the preparation prior to AFM.
    a. Using two p200 micropipettes gradually dispense fresh buffer on the preparation, while simultaneously removing the equivalent volume from it.
    b. Repeat step 13a at least three times, to ensure that buffer in the preparation is fresh.

⚠ CRITICAL: Irrespective of the efficiency in removing embryonic material and cellular debris by mouth suction in step 12g, it is important to exchange the buffer in the preparation before proceeding to the AFM. Avoid abrupt volume changes that could disturb the embryos and destroy the preparation.

AFM measurements

Timing: 30–45 min (per embryo)

Below we describe the steps required for measuring the elastic moduli of the *Drosophila* embryonic VNC via nano-indentation, using a custom-built AFM (Rico Camps, 2006). The AFM is coupled to an inverted microscope (Nikon TE2000) to allow for the correct tip localization at the measurement site of the embryo. The embryos can be visualized by phase contrast microscopy or by epi-fluorescence microscopy, in case fluorescent markers are expressed in the tissue of interest (Figure 4).

14. Mount the AFM cantilever using the liquid AFM tip holder.
15. Place the dissected *Drosophila* embryos preparation (from step 13) on the AFM stage.

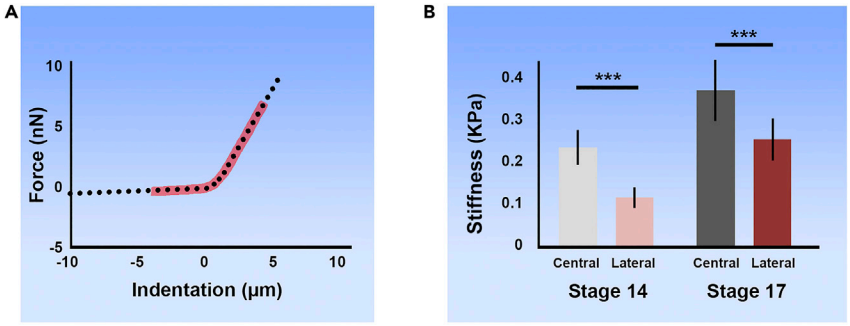

Figure 5. VNC stiffness measurements by AFM.

(A) Representative force curve (dotted line) fit to a Hertz's model (red line) for a spherical probe.

(B) Tissue stiffness (E) measured by AFM in dissected VNCs at early stages (13–14) and late stages (16–17) from WT embryos. Bars denote mean (Average) ± SD (Standard Deviation) values measured at the ventral midline (grey) and at lateral cortex areas (brown). n (number of measurements) = 15 in all cases. *** p value < 0.001.

16. Introduce the AFM cantilever in the liquid and calibrate k using the thermal noise fluctuations method (Schillers et al., 2017).

Note: For calibration, the cantilever should be 200–300 μm away from the sample.

17. Define a clean (sample-free) area on the glass and approach the tip to engage with the surface.
18. Calibrate the photosensitivity of the photodiode by contacting the tip against the glass surface.

Note: A linear relationship (force - deflection curve) between the voltage measured with the AFM photodiode and the AFM tip displacement (V - displacement) needs to be observed since the tip will not deform the glass (Figure 4). Troubleshooting 4.

19. Place the tip on top of the *Drosophila* VNC and measure 5 force-curves per location at the midline or at lateral cortex regions (Figure 4). Measure different points along the antero-posterior axis of the *Drosophila* VNC using an amplitude of 20 μm and velocity of 5 μm/s. Troubleshooting 5.

Note: Low approaching speeds (< 5–10 μm/s) should be used for the measurement in order to minimize viscous cantilever friction with either the surrounding liquid or the intrinsic sample viscoelasticity.

20. Discard and/or repeat measurements, if the force curves show unclear approaches or retraction curves.
21. Repeat the previous steps for the different *Drosophila* embryos.

AFM data analysis

Timing: 2 h per embryo

Figure 5 shows a representative force curve recorded from the embryonic VNC samples using the custom-built AFM in our lab and a spherical tip attached to a cantilever of k=0.01 N/m (Jorba et al., 2017b). The non-linear shape of the curve is due to the increase of tip-sample contact area as indentation increases (Equation 2).

22. Force recording. The force recorded when approaching the sample surface is zero until reaching surface contact, at the so-called contact point. From then on, F increases monotonically as

sample indentation increases. The elastic properties of the sample are characterized by an effective Young's modulus (E) computed by fitting a contact model according to tip-sample geometry. For a spherical probe indenting a flat surface of an elastic body the force curve is described by Hertz's contact model (Johnson and Johnson, 1987; Jorba et al., 2017b).

$$F = \frac{4 \cdot E \cdot R_{sph}^{1/2}}{3(1 - \nu^2)} \delta^{3/2} \quad \text{(Equation 3)}$$

Where ν represents the Poisson's ratio. Since ν is challenging to determine, biological samples like cells and tissues are considered incompressible materials (ν=0.5). The E is determined by fitting Equation 3 by setting the contact point as a fitting variable.

⚠ CRITICAL: The effect of indentation on E can be calculated by fitting the force curve with Hertz's contact model using Equation 3 at different indentation depths (Gavara, 2016; Rico et al., 2005). For the different embryo stages and positions we employed between 300 and 1,000 nm.

Note: Commercial AFMs come with analysis software packages. The user can perform the analysis of the force curves using these packages. Alternative published scripts like Atomic J are available (Hermanowicz et al., 2014). In our case, an in-house MATLAB based software was utilized to calculate the effective Young's modulus.

23. Perform statistical analyses using any statistics software, in our case SigmaPlot 13.0 software (SigmaPlot Software). Data quantification procedures have been extensively detailed in previous studies (Jorba et al., 2017b; Rico et al., 2005).

EXPECTED OUTCOMES

With the current protocol, users will be able to flat-dissect living *Drosophila* embryos and quantify VNC tissue rigidity/viscosity (stiffness) by AFM.

The protocol was employed to calculate the effective Young's modulus of the VNC of embryos of different stages, on different positions of the neuromere. The Young's modulus is a mechanical property that describes the viscosity/rigidity (stiffness) of a solid material. Applying different indentation depths to the cantilever we observed a large variability in effective Young's modulus at indentations <1,000 nm, mainly due to contact point indetermination. At larger indentations, different E values are retrieved for central (where most axons locate) and lateral regions of the VNC (where most cell bodies are present). Further, different E values were retrieved from young and old specimens, which eventually provided information on the local diversity and morphogenetic maturation of the tissue (Figure 5). Beyond 3,000 nm tissue rigidity measurements become stable revealing the material properties of the VNC (On average, about 100 pascals at the lateral domain and 250 pascals at the central domain of the VNC at stages 13 - 14, and 200 and 350 pascals respectively at stages 16 - 17). This increase in rigidity will probably be linked to the full ensheathing of the VNC by glia, at late stages.

Besides discriminating differences in viscosity at distinct positions within the VNC, or related to developmental age, AFM could also provide information on tissue complexity. While indenting, the strains generated by AFM can penetrate up to 10 times the indentation depth (Gavara, 2016) and, therefore, the forces measured with AFM are influenced by the different tissue types at different depths of the sample. At intermediate indentation depths a gradual increase in viscosity measurements was observed for all conditions except at the central positions of stage 17 embryos, where retrieved rigidity values were constant beyond 1,500 nm indentation depth.

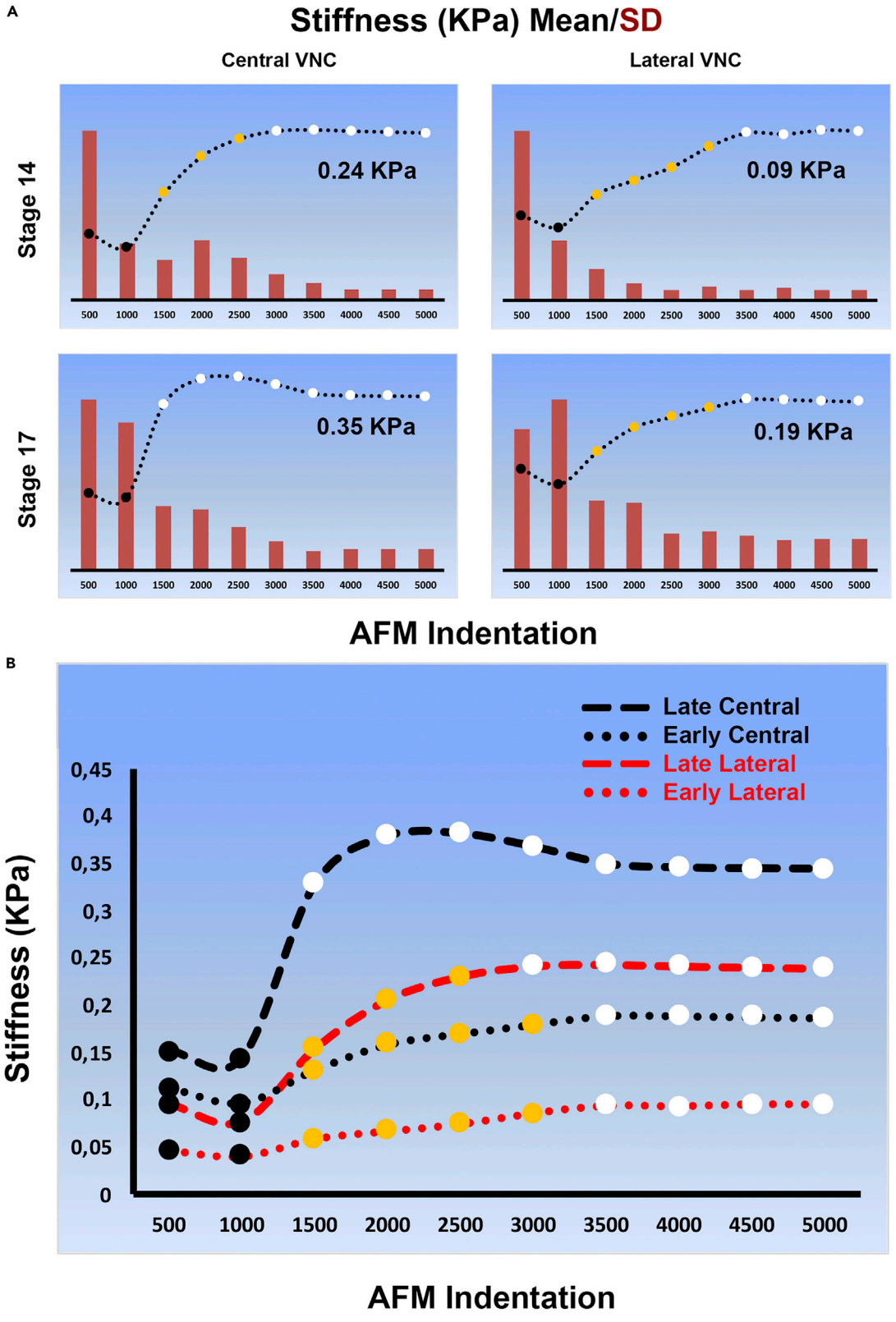
A
Stiffness (KPa) Mean/SD
Central VNC
Lateral VNC
Stage 14
0.24 KPa
0.09 KPa
Stage 17
0.35 KPa
0.19 KPa
500 1000 1500 2000 2500 3000 3500 4000 4500 5000
AFM Indentation
B
Late Central
Early Central
Late Lateral
Early Lateral
Stiffness (KPa)
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
500 1000 1500 2000 2500 3000 3500 4000 4500 5000
AFM Indentation

Figure 6. Tissue heterogeneities revealed by AFM.

(A) Graphic representation of viscosity/rigidity (stiffness) values evaluated at different indentation depths, at different positions (central vs lateral), for embryos at different developmental stages. At low indentation depths, normalized values for standard deviation are high (red histograms) and KPa values are negligible (black dots). A different gradual increase in viscosity was observed for the distinct conditions reaching in all cases a plateau (white dots – stable rigidity) at different depths. The intermediate character (yellow dots) of the AFM viscosity measurements denotes the dynamics of the process of organization of the glia, at the surface of the VNC.

(B) Comparison of viscosity/rigidity at different indentation depths. The central domain of late embryos, with a fully structured glia/neural cord, reaches stable rigidity (high – 350 pascals) at low depth (discontinuous black line). In early embryos, however, the glia layer is not fully established, and this domain is much softer (190 pascals) (discontinuous red line). Intermediate mixed values are observed up to a 3,000 nm depth. At lateral positions, the tissue is softer denoting a less rigid underlying composition (mostly cell bodies instead of structured axons) and a thinner overlaying glia, with intermediate viscosity values, present to indentation depth up to 3,500 nm. Again, at early stages (dotted red line) the tissue was softer than at late ones (dotted black line).

This inhomogeneous character of the AFM viscosity measurements at different time points and positions denotes the dynamics of the process of organization of the glia at the surface of the VNC. The contribution of the soft underlying neural substrate to rigidity measurement is more perceptible in those places and times in which the external glia layer is under construction or just thinner (Figure 6).

LIMITATIONS

Limitation 1

There are no data comparing the mechanical properties of the embryonic VNC between different *Drosophila* wild type strains. We anticipate differences will be minimal. In this protocol we used the *w[1118]* strain as a control, which represents the genetic background of numerous transgenic lines. Either way, as in all experiments, results should be compared to those obtained from the corresponding genetic controls (measurements performed on the progeny of crosses between each experimental strain and the wild type).

Limitation 2

An obvious limitation of this protocol relies on the anatomy of the embryos at different developmental stages. Dissections can be performed as early as stage 13, after germ band retraction, up to late stage 16, before the onset of cuticle deposition (Rickert et al., 2013). Therefore, the investigation of tissue mechanical properties by AFM, in live dissected embryos, can be performed only within the aforementioned time window. Regarding CNS, its formation starts at embryonic stage 9 with the delamination of the neuroblasts and ends at stage 17, when full VNC condensation and functional maturation of the system occur (Crews, 2019). It becomes obvious that VNC stiffness cannot be quantified using this protocol at early stages of CNS formation. In contrast, VNC elastic properties could be potentially assessed after embryogenesis by applying larva dissection protocols (Brent et al., 2009), which are challenging due to the small size of the larvae and their motility.

Limitation 3

One critical aspect for understanding the mechanical properties of the embryonic CNS is spatial resolution. In this study, we used a spherical bead, with a radius of 10 μm, in order to diminish the strains applied to the embryos and avoid tissue adhesiveness. Using Equation 2 with an indentation of 100 nm, the contact radius tip-sample is approximately 3 μm. Therefore, the lateral resolution of our measurement was limited to around 6–10 μm. Taking into account the small dimensions of the *Drosophila* VNC (total width 75 μm), this resolution is low. However, higher resolutions can be achieved by selecting smaller tip radius, always taking into account that smaller tips will produce higher force in the sample, increasing the possibility of damaging it.

Limitation 4

This protocol requires delicate albeit fast handling of the sample, given that tissues are not fixed. In addition, fine tuning of the AFM and positioning of the cantilever along the specimen can also result time consuming. Overall experimentation time, in order to ensure tissue integrity, cannot exceed 1 h

per embryo. It is conceivable that this protocol is not suitable for high throughput analysis, due to the delicate nature of the measurements.

TROUBLESHOOTING

Problem 1

Flies in the embryo collection chambers do not deposit embryos or embryos deposited are unfertilized (collection and processing of precisely timed embryos, steps 5d and 5e).

Potential solution

This problem can arise from parental flies being under environmental stress or by the apple juice - agar Petri dish being cold. Optimize fly culture conditions according to guidelines. Avoid cleaning embryo collection chambers with scented soap. In case the problem persists, it is advisable to revise fly stocks and if necessary re-establish transgenic fly lines after crossing to a wild type stock, to remove genetic modifiers.

Problem 2

Embryos in synchronized collection are not of the desired developmental stage (collection and processing of precisely timed embryos, steps 5b–5d).

Potential solution

Flies in embryo collection chambers are stressed and have withheld embryos or developmental temperature is not properly controlled. Optimize fly culture conditions according to guidelines and ensure that temperature in the fly-chamber is properly adjusted and stable.

Problem 3

Embryos are not properly dechorionated. If the juice-agar matrix has not been prepared properly to ensure full dissolution of the agar or in cases where detachment of the embryos from the agar surface has been abrupt, small fragments of agar enter the strainer along with the embryos. In such a scenario the embryos are not in direct contact with bleach and they are not successfully dechorionated in the limited time exposed (collection and processing of precisely timed embryos, steps 6a and 6b).

Potential solution

Using forceps remove the visible parts of agar from the strainer and pass most of the embryos in a new strainer by washing them off with distilled water. Make sure that the strainer enters the bleach solution in an angle and no air bubbles are formed underneath. Extend the dechorionation time by no more than 30 s, with constant swirling of the embryos in bleach.

Problem 4

Force-deflection curve on the glass does not follow a straight line. The tip might be damaged or carries debris from previous measurements (AFM measurements, step 18).

Potential solution

Clean the tip with UV-ozone and/or piranha solution (Sirghi et al., 2006).

Problem 5

Force-deflection curve on the sample does not show a clear non-contact and contact region (AFM measurements, step 19).

Potential solution

The measured amplitude is not large enough to overcome the adhesion of the sample to the tip. Consider increasing the amplitude, while maintaining the velocity constant.

RESOURCE AVAILABILITY

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

K.K. carried out the experimental procedures and performed image analyses. I.J. performed the AFM analyses and quantifications. K.K. and I.J. wrote the manuscript with inputs of D.N. and E.M.-B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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