nature protocols

https://doi.org/10.1038/s41596-023-00907-7

Protocol

Check for updates

Experimental modulation of physiological force application on leg joint neurons in intact *Drosophila melanogaster*

Max Buhlan, Dmitrij Ljaschenko, Nicole Scholz & Tobias Langenhan

Abstract

The study of how mechanical forces affect biological events in living tissue is important for the understanding of a multitude of physiogical and pathophysiological phenomena. However, these investigations are often impeded by insufficient knowledge about force parameters, inadequate experimental administration of force stimuli and lack of noninvasive means to record their molecular and cellular effects. We therefore introduced a procedure to study the impact of force stimulation on adhesion G-protein-coupled receptor dissociation in mechanosensory neurons. Here, we detail a procedure to harness the mechanical force spectrum that emerges during the natural flexion-extension cycle of the femorotibial joint of adult fruit flies (*Drosophila melanogaster*). Mechanical load generated during the joint's motion is transmitted to specialized mechanosensory neurons residing close to the joint axis, which serve as proprioceptive sensors in the peripheral nervous system of the animal. Temporary immobilization of the joint by a restraint made of a human hair allows for the observation of transgenic mechanosensitive reporters by using fluorescent readout in the neurons before, during and after cessation of mechanical stimulation. The assay harnesses physiologically adequate stimuli for joint flexion and extension, can be conducted noninvasively in live specimens and is compatible with various transgenic reporter systems beyond the initially conceived strategy and mechanobiological hypotheses tested. The application of the protocol requires knowledge in *Drosophila* genetics, husbandry and fluorescence imaging and micromanipulation skills. The experimental procedure can be completed in 10 h and requires an additional 30 min in advance for fly fixation and leg immobilization. The apple agar cooking and heptane glue preparation requires a maximum of 30 min on the day before the experiment is conducted.

Key points

- This protocol describes an assay to study molecular mechanosensation, in which mechanical forces cause stimulation of mechanosensitive target molecules in proprioceptive neurons. Transgenic reporters of force stimulation or neuronal activity can be coupled to binary expression systems such as the GAL4/upstream activating sequence (UAS), LexA/lexAop and QF/Q upstream activating sequence (QUAS).
- The approach provides a noninvasive and imaging-based approach to manipulating joint motion (i.e., mechanical force generation) and measuring the molecular effects caused by this motion in vivo.

Key references

Scholz, N. et al. *Nature* **615**, 945–953 (2023): https://doi.org/10.1038/s41586-023-05802-5

Rudolf Schönheimer Institute of Biochemistry, Division of General Biochemistry, Medical Faculty, Leipzig University, Leipzig, Germany. e-mail: scholzlab@gmail.com; tobias.langenhan@gmail.com

Introduction

Mechanical stimuli convey vital information for the functions of cells pertaining to their development, proliferation, metabolism and movement, and serve as a source of sensory information to the nervous system. Consequently, failing mechanical signal transduction has emerged as a driver of many disease processes[1]. Mechanical stimulation is perceived through a multitude of transmembrane and intracellular molecules that include ion channels, metabotropic receptors such as integrins, G-protein-coupled receptors (GPCRs), cytoskeletal proteins and transcription factors[2–7]. However, studying how these mechanosensors are activated through physiologically relevant stimuli (i.e., stimuli mimicking adequate forces to which cells are naturally exposed) and the assessment of their cellular consequences in an experimental setting can be confounded and, thus, be difficult to interpret. Foremost, this is due to the lack of knowledge of the quantitative characteristics of most physiological stimuli (such as magnitude, vector, loading rate, sense, frequency and duration), which are incompletely defined or entirely unknown as of yet. Accordingly, their experimental emulation cannot succeed. Second, controlled stimulus delivery requires direct force transmission to the biological material, which often is not readily feasible without invasive measures such as surgical preparation to expose the targeted tissue to the mechanostimulation device. Unfortunately, such intervention often alters the mechanical environment of the target cell and its cellular responses.

Development of the method

To circumnavigate these problems, we established an in vivo assay designed to study the effects of force application to mechanosensitive molecules and transgenic mechanosensors in *Drosophila melanogaster*[8]. We have used the protocol to analyze the dissociation of the adhesion-type GPCR- calcium-independent receptor of α-latrotoxin (Cirl; also known as latrophilin 1), which is required for mechanosensory neuron function[9–11], through a novel molecular N-terminal fragment release sensor (NRS) that we transgenically expressed in experimental animals (referred to as 'Cirl-NRS').

As a main mechanical element in the protocol, a restraint made of a human hair is glued to the tarsus of one of the metathoracic (most posterior) legs to immobilize its movement. Consequently, the naturally occuring motion of each joint of the experimental leg is suppressed to a minimum, and force transmission to proprioceptive neurons, which monitor the parameters of each individual joint such as load, velocity and frequency, is stinted. Because of the physiological extension-flexion motion cycle of the joint, the proprioceptive neurons serve as a natural biomechanical device and have been previously used to define the role of genes involved in mechanosensing[12]. In the protocol, mechanosensitive molecules that are endogenously or transgenically expressed within the neurons are subjected to adequate force stimuli. The setup, therefore, allows the study of mechano-biochemical signal conversion in a physiological, noninvasive manner.

Through the immobilization approach, mechanically inflicted molecular responses of mechanosensitive molecules or transgenic sensors can be experimentally controlled and visualized in these specialized mechanosensory neurons. The experimental cell and mechanosensitive reporters can be assayed for activation of mechanical responses (e.g., ion conductance, metabotropic signaling and biochemical processing) at different time points of interest (i.e., the beginning, the end and, by cutting the hair restraint, after the immobilization period).

In the current experiment, Cirl-NRS activity stimulates the release of a membrane-attached transcription factor, which initiates expression of the bipartite, two-color transcriptional reporter TransTimer[13] commensurate with mechanical stimulation of the molecular NRS dissociation/force sensor[8]. Reporter signals can be obtained through fluorescence imaging of leg joint neurons in live animals at any point during the procedure, which can be performed for up to 10 h. The protocol requires an upright fluorescent-light microscope equipped with a digital camera and suitable imaging software.

Overview of the procedure

The workflow can be understood as a timeline of the manual tasks of force application on the fly leg. This protocol should offer basic instruction on the micromanipulation, enabling the experimenter to adapt the procedure to address different mechanobiological questions (Fig. 1a,b); that is, the impact of the procedure can be assessed by different readouts. In the current case, effects of the differential force regimes on mechanosensory neurons were evaluated through fluorescent activity of a transgenic molecular reporter (Cirl-NRS) that responds to mechanical stimulation, and by video recordings to extract motion parameters of leg movement. In both cases, female flies are treated with the same conditions as described in 'Fly fixation and leg immobilization' (Steps 1–10). Eight-minute videos are recorded at three time points: $t = 0$ h (directly before joint immobilization, representing active movement before manipulation), $t = 5$ h (right after the end of the immobilization interval, representing reduced activity in the femorotibial joint) and $t = 10$ h (at the end of the re-mobilization interval, assessing whether the leg is still capable of moving after leg bending). The video recordings are performed separately from the fluorescent imaging experiments.

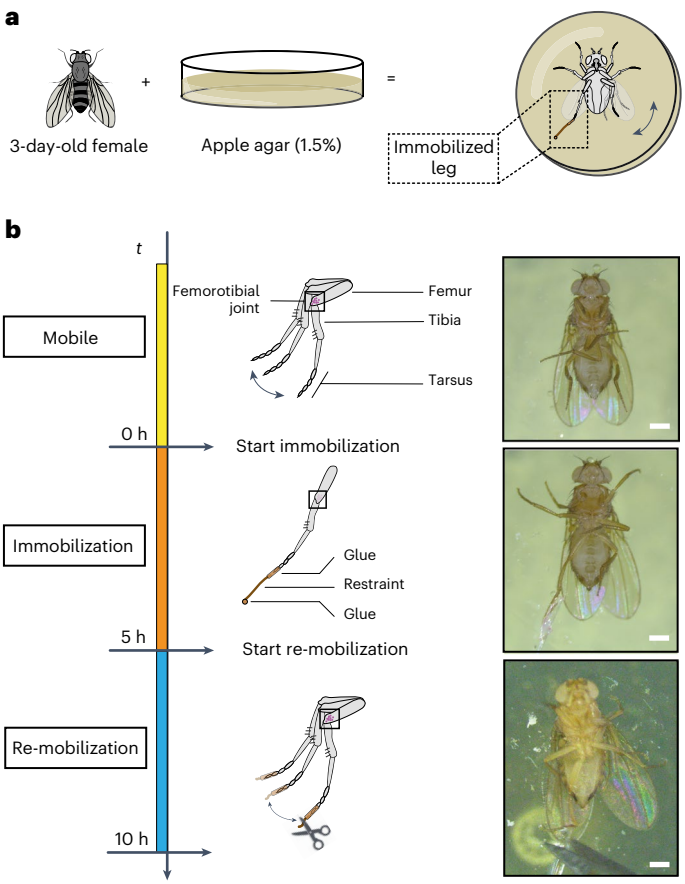

Fig. 1 | General workflow of the fly and leg immobilization/re-mobilization procedure. a, Preparation procedure for leg fixation. A 3- to 5-day-old female fly is fixed on its back and wings to apple agar by using a custom-made heptane glue mixture. All legs can move freely to ensure the least experimental interference in the physiological leg/joint motion of the living fly. **b**, Experiment intervals. Mobile interval ($<t = 0$ h): both metathoracic legs (posterior leg pair) can move freely while the fly is fixed on the apple agar up until $t = 0$ h. The fly's right metathoracic leg is then fixed to a hair restraint and straightened to reduce the flexion-extension angle of the femorotibial joint. Immediately after the leg immobilization, the first transgenic reporter value, which represents the mobile interval, is recorded. Immobilization interval ($t = 0$–5 h): at the end of the immobilization interval, while the metathoracic leg is still fixed, the second reporter signal is acquired. Re-mobilization interval ($t = 5$–10 h): the hair restraint is cut near the tarsus of the immobilized leg. This is followed by another 5 h of spontaneous leg movements. Finally, the third signal-acquisition round documents sensor activity achieved through the re-mobilization interval. Scale bars, 0.5 mm.

Applications and advantages of the method

The presented approach can be adopted for any questions pertaining to the measurement of mechanical force administration to molecules that may exhibit mechanosensory properties including ion channels, integrins, GPCRs, talins, nuclear pore complex components and transcription factors. Importantly, the approach allows the study of mechano-biochemical signal conversion in an experimental setting in which physiologically relevant mechanical stimuli can be controlled by the experimenter. In addition, the method of leg fixation can be combined with protocols for quantitative fluorescence-based readouts that are noninvasive, thereby minimizing disturbances of the mechanical environment of the experimental system. Leg movement in our setting is spontaneous and will not exceed the natural range of joint motion, confining mechanical stimulation to the neurons to natural limits (similar to a hinge). The joint motion is defined as the rotational movement of the tibia about the transverse axis of the femorotibial joint relative to the femur in the metathoracic leg. We chose the most posterior leg pair (metathoracic legs) for our approach, because it offers the best accessibility for the mechanical restraint procedure. We assume that the other leg pairs (prothoracic and mesothoracic) can also principally be investigated. This opens the possibility of studying differences in mechanical stimuli that are effected on proprioceptive neurons located in different joints along the anteroposterior axis of the animals.

An advantage of the approach concerns its recording of repeated measures of the same variable (reporter activity in the same cell upon different mechanical stimulation parameters), which is beneficial in separating signal from noise, attaining higher statistical power. In addition, mechanosensory neurons in the nonimmobilized legs of the same animal can serve as an independent control for mechanostimulation, receptor protein expression and reporter activity.

Furthermore, the presented protocol can be combined with a versatile set of optical readouts. Transgenic reporters of neuronal activity or force application can couple mechanostimulation of the assay's proprioceptive neurons to binary expression systems such as the popular GAL4/upstream activating sequence (UAS), LexA/lexAop and QF/Q upstream activating sequence (QUAS) transcription factor/transcription factor recognition site pairs. This suggests that in addition to NRS activity, multiple other mechano-dependent cellular molecular readouts can be evaluated through the wealth of transgenic sensor molecules developed for or adapted to investigations in *Drosophila*[14,15]. These encompass the impact of mechanical stimulation on metabotropic pathways downstream of G-protein-coupled signaling (e.g., cAMP-EPAC interaction[16,17]), on voltage-sensitive ion channels in the central nervous system[18,19] and on intracellular calcium levels by transgenic integrators such as CaMPARI[20]. Furthermore, the application of this method to assess subcellular mechanosensor activity states can generate detailed information about the interaction of cytoskeletal components with the extracellular matrix (e.g., by using a tension sensor module (TSMod))[21,22].

Limitations of the method

The protocol is limited to the study of proprioceptive neurons, in which physiologically adequate mechanical stimulation causes the activation of a mechanosensitive target molecule. Short-lived, instantaneous signals are not suitable for detecting motor activity by following the presented protocol. Force stimulation and acquisition cannot be conducted at the same time point because of movement artefacts. Consequently, a binary reporter system that combines a stable expression profile and dynamic variability within a few hours is required. Optimally, the fluorophore unites the dualism between a medium-lived and long-lived signal, which allows the visualization of dynamic fluctuations objectively. Although the sample is alive for the whole experimental period (e.g., 10 h), a potential limitation in the technical setup is the rigidity of the microscope. Focusing the leg joint requires a high range of z movement, because the anesthetized fly could adjourn in variable positions. However, the emitted photon count must be high enough to be clearly detectable by the optical aperture.

Expertise needed

All steps of the experiment can be accomplished by a single experimenter. The method requires knowledge in *Drosophila* genetics, husbandry and fluorescence imaging and preparation skills.

Experimental design

The experiment is divided into three intervals: mobile, immobilization and re-mobilization, where fixation of the leg with a restraint marks the division between the mobile and immobilization intervals. Data collection starts immediately after leg fixation to obtain measurement of the mechanical reporter signal at the end of the mobile interval. After a defined immobilization interval (5 h in the NRS experiments) but before the restraint is cut, the second measurement is taken, representing the mechanoresponse of the cell during the immobilization interval. After restraint removal, a re-mobilization interval can be added, but the procedure should not exceed 10 h starting with leg immobilization. The experiment should be started in the morning hours in a well-illuminated room, which ensures that flies are completely awake and have already moved in the vial for several hours before the immobilization procedure to generate sufficient mechanical stimulation to the proprioceptive leg neurons and of the mechanosensitive molecules expressed within them.

To adapt the experimental design of this study to your own setup requires an upright fluorescent-light microscope with a camera connected to suitable imaging software (e.g., Leica DM6B and an sCMOS (scientific complementary metal–oxide–semiconductor) camera (Leica DFC9000GTC) connected to LAS.X software). Working with adult *Drosophila* requires anesthesia using CO_2. All steps of the experimental approach can be conducted at room temperature. Other than the immobilized (i.e., restrained) metathoracic leg, the fly can move all other legs spontaneously, providing convenient in-animal control for mechanostimulation of proprioceptive neurons through the leg movements and for the overall viability of the animal throughout the experiment.

For a reporter of mechanical activation of the NRS sensor, we used an advanced dual-color fluorescent transcriptional timer (TransTimer)[13], which can be potentially applied to a wide range of other biomechanical studies. The TransTimer reporter is composed of a fast-folding, destabilized GFP (dGFP) and a stable, slow-folding, long-lived RFP. dGFP levels reflect dynamic changes in transgene activation, while RFP serves as a stable expression and motion control protein for dGFP signal normalization throughout the experiment. We used TransTimer for recording Cirl-NRS dissociation in vivo. The chimeric Cirl-NRS sensor protein is located at the plasma membrane of mechanosensitive neurons in the femorotibial joint. Upon mechanical force through flexion and extension, the N-terminal fragment of Cirl (Cirl-NTF) dissociates from its juxta- and transmembrane segment (JTS; derived from the Notch receptor), leading to the release of a transcription factor component (e.g., LexA, GAL4 or QF2) fused to the intracellular C terminus of the JTS. This is effected by proteolytic processing of the JTS through the matrix metalloprotease ADAM and the γ-secretase complex. After Cirl-NRS dissociation, the transcription factor component translocates into the nucleus and activates expression of its target, the TransTimer transgene, leading to dGFP and RFP production. Although dGFP matures within a few minutes after transcriptional activation, RFP requires 90 min before optical detection through fluorescence imaging is possible. Upon cessation of TransTimer transcription, dGFP degrades 10 times faster than RFP, rendering dGFP fluorescence dynamics a proxy for mechanically inflicted Cirl-NRS activity (i.e., Cirl-NRS dissociation).

Device calibration

Acquisition settings can be different between microscope providers. This assay was conducted by using a Leica DM6B upright microscope embedded in LAS.X software. Generally, the Fluorescence Intensity Manager and the exposure time should be chosen to be suitable to the fluorescence intensity emitted by the sample. Settings used in this protocol are provided below at the appropriate sections.

Device maintenance

No special maintenance is needed besides standard servicing of the microscope setup.

Number of animals and controls

Depending on the properties of the respective reporter signals studied, the minimum sample size number required to detect an effect on this reporter of a given size can be estimated by a

power calculation. In the current example[8], we used a total number of 16 three- to five-day-old female flies for the experiments. The right metathoracic leg was immobilized and thus served as the experimental leg, and the contralateral left metathoracic leg acted as a nonimmobilized control within each analyzed fly. Nonsynonymous leg pairings (e.g., immobilized metathoracic versus mobile mesothoracic leg) should be avoided because of the different anatomical and force parameters by which each leg joint type is characterized.

Materials

Biological materials

Flies of the required genotype carrying the respective mechanosensitive reporter transgene(s). Animals depicted in this protocol in Figs. 1, 2 and 3g,l,m were w^{1118} flies (Bloomington Drosophila Stock Center, stock no. 5905), and those depicted in Fig. 3h–k were *Cirl-NRS-Gal4* > *UAS-TransTimer*[8].

Reagents

Apple agar

- Agarose for gel electrophoresis (LE agarose, Biozym)
- Clear apple juice (commercially available)

Heptane glue mixture

- n-Heptane (Merck)
- TESA 4124 sticky tape
  ▲ **CRITICAL** This type of sticky tape does not contain toxic and harmful detergents, thus preventing tissue damage during the fixation procedure.

Restraint

- A 3–4-cm-long piece of human hair
  ▲ **CRITICAL** The diameter of the hair should be smaller than the leg diameter.

Equipment

- Laboratory gloves (latex or nitril)
- Laboratory coat
- CO_2 access
  ▲ **CAUTION** Anesthesia of *Drosophila* should be conducted in an appropriate laboratory setting to limit the CO_2 exposure of the experimenter.
- Petri dishes (65 or 15 mm; Greiner Labortechnik)
- Erlenmeyer flask (500 ml)
- Microwave (min. 700 W)
- Glass vial equipped with well-closing shutter, >20 ml
- Laboratory pipette (1,000 µl; Eppendorf)
- Anatomical tweezers
- HulaMixer sample mixer (Thermo Fisher Scientific)
- Fine paint brush (grade: 0)
- Dissection scissors
- Dissection tweezers (grade: 3)
- MZ10F microscope (Leica) connected to a MC190 high-definition camera (Leica) or similar microscope
- Leica DM6B upright microscope equipped with a 10× 0.32 numerical aperture dry objective and an sCMOS camera (Leica DFC9000GTC) or similar microscope. The presented method for experimental joint control can be easily combined with other, more-elaborate optical readout methods like confocal microscopy.
- Imaging software (e.g., LAS.X or similar products)

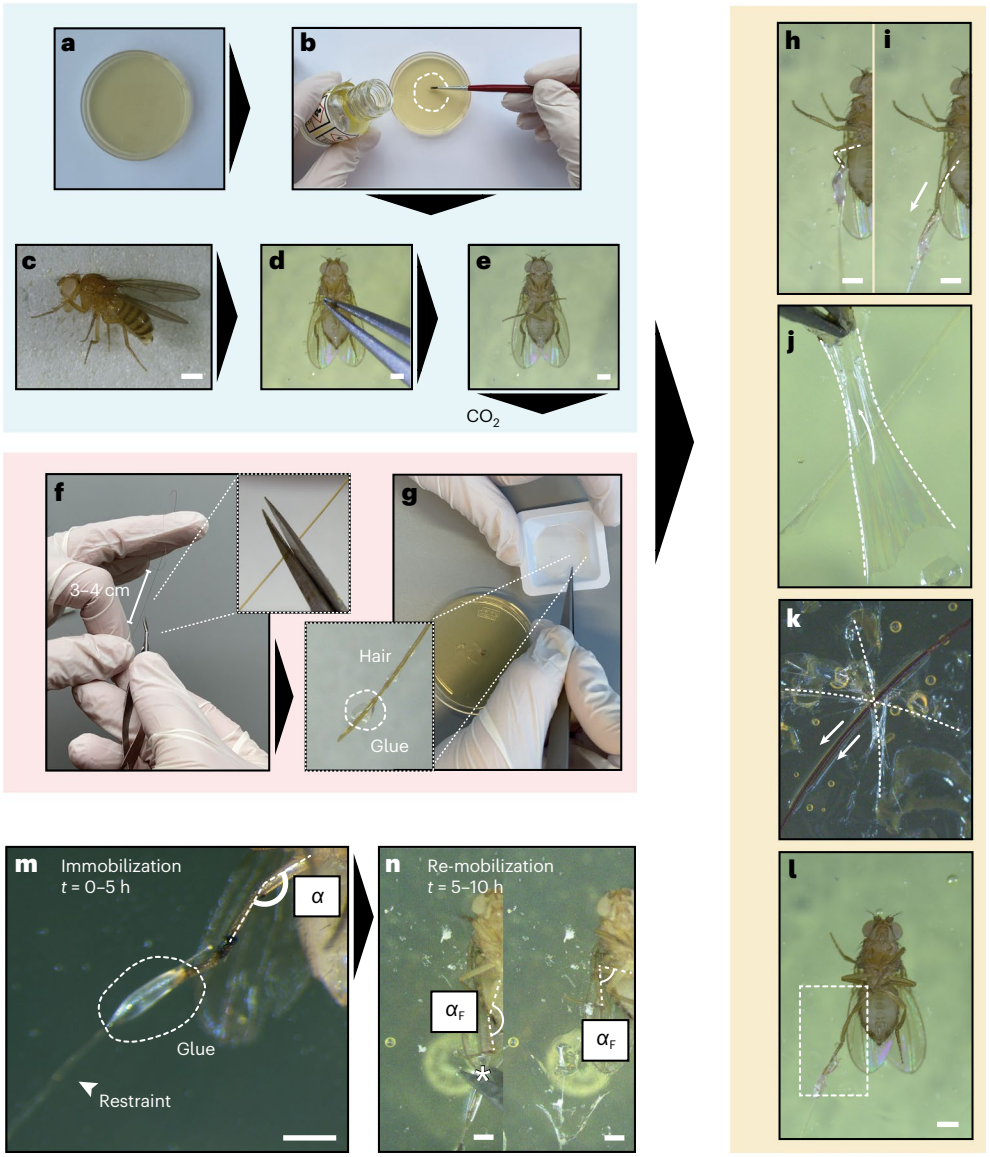

Fig. 2 | Detailed workflow of the leg immobilization procedure. a, A 65-mm Petri dish filled with apple agar (Step 3 in Reagent setup, Apple agar (1.5%, wt/vol)). **b**, Heptane glue mixture is applied to the agar surface (Step 1 in the Procedure). **c**,**d**, An anesthetized female fly (**c**) is fixed on its back and wings to the glue mixture spot by using dissection tweezers holding the mesothoracic legs (Step 3) (**d**). **e**, The final position of the fixed fly. **f**, Hair trimming by using dissection scissors. Inset: detail of trimming (Step 5 in the Procedure). **g**, Heptane glue mixture is applied to the hair restraint by immersing it in the glue. Insert: droplets of heptane glue mixture (dashed circle) attached to the restraint (Step 5). **h**,**i**, Successful hair-tarsus connection. The leg can still flex and extend, and the leg can be straightened by gently pulling at the restraint (the arrow indicates the pulling direction) (Steps 7 and 9). **j**, Hair fixation by tying it to the agar support through threads of heptane glue mixture (arrow) (Steps 9 and 10). **k**, glue threads are applied from both sides in a cross-wise fashion (dashed lines). The hair is retracted by the threads away from the body (arrows) (Step 10). **l**, The final prepared sample (immobilized hair-tarsus connection in the dashed box). **m**, Enlarged image of the hair-tarsus connection. The hair restraint (arrowhead) is connected to the tarsus by a glue mixture droplet (dashed circle). The flexion-extension angle α in the femorotibial joint is depicted. **n**, The re-mobilization process: the immobilized and re-mobilized leg is distinguishable by large and small femorotibial joint angles during a flexion phase (α_F) before (left) and after (right) re-mobilization. The hair-tarsus connection is indicated by an asterisk (Step 17). Scale bars, 0.5 mm.

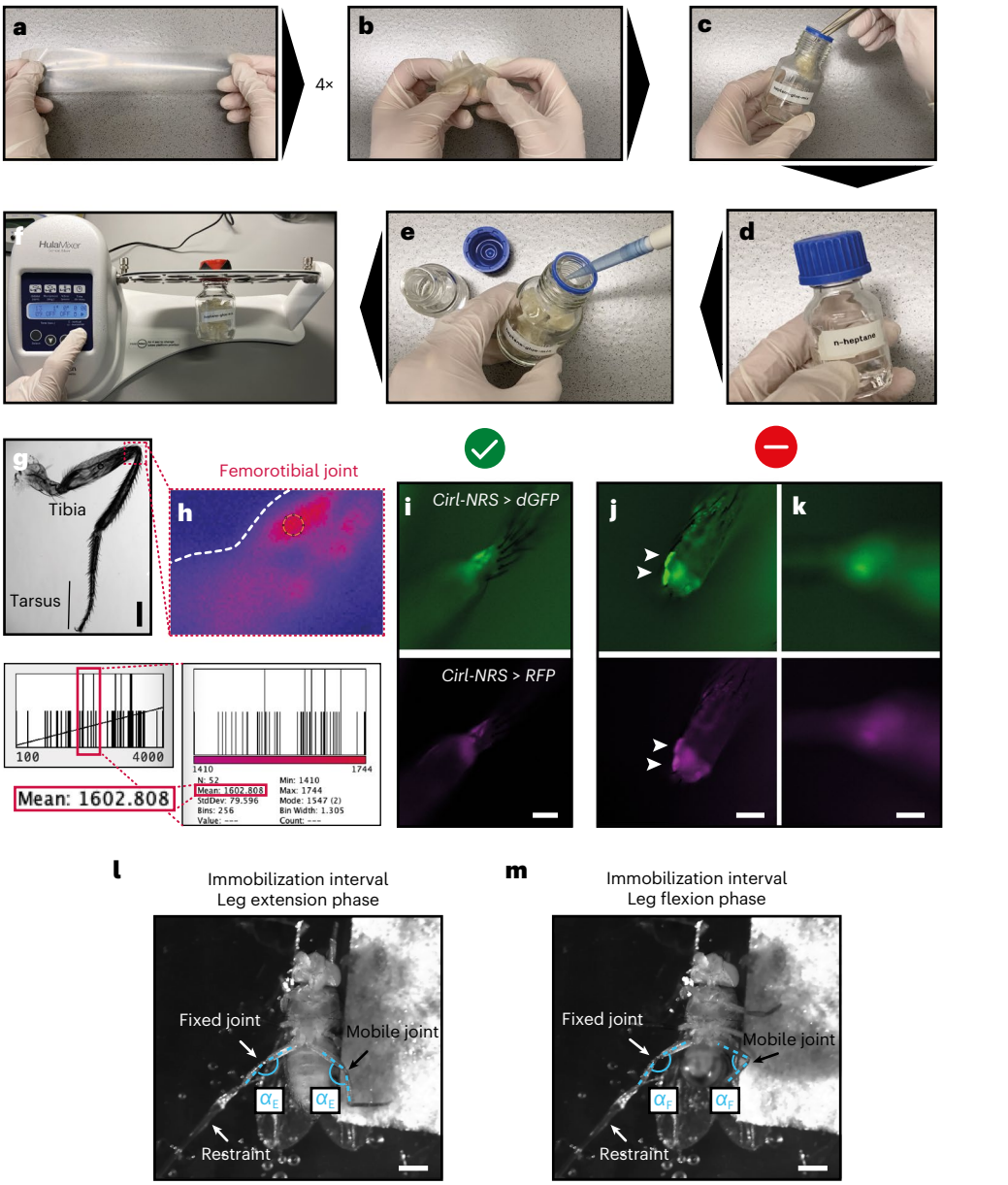

Fig. 3 | Preparation of heptane glue mixture and TransTimer intensity analysis for quantification of mechanosensitive reporter responses. a,**b**, Sticky tape (TESA 4124) is cut into four 15–20-cm-long pieces, which are folded into balls (step 1 in Reagent setup, Custom-made heptane glue mixture). **c**–**e**, A 100-ml glass vial is filled with TESA balls by using tweezers. n-Heptane is added, and the lid is closed (steps 2 and 3 in Reagent setup, Custom-made heptane glue mixture). **f**, The heptane glue mixture is rotated at 13 rpm overnight. The TESA balls are discarded the next morning (steps 4 and 5 in Reagent setup, Custom-made heptane glue mixture). **g**, Metathoracic leg with flexed femorotibial joint (red dashed rectangle) that is imaged in **h**–**k**. Scale bar, 200 µm. **h**, Image displayed in the *Fire* look-up table. The ROI (5.2 µm in diameter) is positioned at the brightest area of TransTimer fluorescence at the femorotibial joint (the dashed line indicates the leg surface). Application window for 'Brightness/Contrast' (left, below), where minimum and maximum intensities are set to range between 100 and 4,000. Histogram of pixel intensities within the ROI (right, below): the mean pixel intensity is documented for further statistical analysis. The panel represents typical data collection applied in ref. 8. Mechanical forces stimulate Cirl-NRS (Fig. 2h). **i**, Typical fluorescent images of the TransTimer reporter showing distinct dGFP and RFP signals at the femorotibial joint neurons of the metathoracic leg. **j**, The joint region is contaminated by heptane glue mixture, which is visible as a strongly auto-fluorescent profile (arrowheads). **k**, When the joint moves out of focus, it appears blurred. Scale bars (**i**–**k**), 50 µm. **l** and **m**, Comparison of the movement range in the femorotibial joint between immobilized and mobile legs. While the hair restraint prevents the femorotibial joint from flexion, the control (mobile) leg shows a wide physiological range of joint movement between extension (α_E) and flexion (α_F) phases. Data are displayed in figure 2f of ref. 8. Scale bars (**l** and **m**), 0.5 mm.

Reagent setup

Custom-made heptane glue mixture

Purpose: Adhesive substance for fly fixation and fixation of the leg restraint. Preparation is adapted from ref. 23.

Preparation (20 min, overnight): Glue should be prepared ≥1 day in advance and stored in a tightly sealed glass vial. The glue can be stored for several weeks at room temperature.
▲ **CAUTION** Do not use plastic Falcon tubes, to avoid vial damage and evaporation.
n-Heptane should be handled exclusively under the fume hood and while wearing gloves and a laboratory coat.

1. Cut TESA 4124 into four pieces of 15–20 cm (Fig. 3a) and roll it up into small balls (Fig. 3b).
2. Place the balls in the vial by using anatomical tweezers (Fig. 3c).
3. Add 10 ml of n-heptane into the glass vial (Fig. 3d,e).
   ▲ **CAUTION** Perform under a fume hood.
4. After closing the vial, incubate the heptane-sticky tape mixture on the HulaMixer overnight at room temperature (Fig. 3f) at 13 rpm.
5. Remove the sticky tape balls from the vial by using anatomical tweezers and dispose of them. The heptane glue mixture is ready to use.
   ▲ **CAUTION** Perform under a fume hood.

Apple agar (1.5%, wt/vol)

Purpose: Apple agar improves viability of the animals during the experiment.

Preparation (30 min): Apple agar can be prepared several days in advance of the experiment and kept at 4 °C. It should not be stored for >1 week.

1. Dose clear apple juice with 1.5% (wt/vol) agarose in an Erlenmeyer flask.
2. Cook the suspension three times at 700 W in a kitchen microwave.
   ▲ **CAUTION** Boiling delay can cause injuries.
   ▲ **CRITICAL** The solution has to be clear without any streaks or air bubbles to guarantee a flat surface for fly fixation.
3. Fill the Petri dishes with the apple agar solution when it is still hot.
   ▲ **CAUTION** The use of heat protection for your hands is required.
4. Cool the Petri dishes down to room temperature before storing them at 4 °C.
   ▲ **CAUTION** The Petri dishes should not be closed completely, to prevent mold formation upon water condensation.

Equipment setup

Instructions and settings for fluorescence image acquisition

Note that the following details pertain to the setup used by the authors and may differ significantly for other setups. Leica LAS.X software was used for imaging fluorescent signals. For dGFP imaging, a filter cube with the following parameters (in nanometers) was used: excitation, 450–490; dichroic, 495; emission, 500–550. For RFP imaging, the following parameters (in nanometers) were used: excitation, 540–580; dichroic, 585; emission, 592–668. All microscope settings should remain static over all three acquisition intervals of the experiment. To keep sample bleaching to a minimum, the fluorescence of the light-emitting diode light source Sola light engine (Lumencor) was reduced to ≤30% by using the Leica Fluorescence Intensity Manager. Exposure of 40 ms per channel resulted in absolute intensities <4,000 a.u. (out of the 65,536, i.e., the intensity range of the 16-bit camera) for both channels. Testing the appropriate microscope settings before the start of the experiment is recommended. During the acquisition, two channel images focusing on the femorotibial joint of the immobilized leg and the contralateral leg (mobile control) are recorded. Flies should be anesthetized by CO_2 before each acquisition step.

Procedure

Fly fixation and leg immobilization

● **TIMING 20–40 min to prepare five samples**

1. Use a paintbrush to cover a 1-cm-wide spot of the apple agar with a thin layer of heptane glue mixture (Fig. 2a,b). Evaporation of the heptane occurs in ~10 s.
   ▲ **CAUTION** Perform this step under a fume hood.
   ◆ **TROUBLESHOOTING**
2. Start CO_2 anesthesia.
   ▲ **CRITICAL** Anesthesia suppresses the leg movements of the fly for 10–20 s. The anesthetic effect is completely reversible upon stopping of the CO_2 exposure within 1–2 min.
3. Place anesthetized female flies back-down (i.e., on their back and wings) on the heptane glue mixture spot by using a pair of spring steel tweezers (Fig. 2c–e). Hold the fly at the mesothoracic (middle pair) legs.
   ▲ **CRITICAL** Fly handling should be conducted as gently as possible. Ensure that the flies can move all legs after body fixation.
4. Stop CO_2 anesthesia.
5. Prepare a 3–4-cm strand of straight hair (Fig. 2f) and dip one end into a puddle of the heptane glue mixture by using dissection tweezers to hold the hair steady (Fig. 2g).
   ▲ **CRITICAL** Human hair should be as straight as possible and at least as thin as the *Drosophila* leg itself.
6. Start CO_2 anesthesia.
7. Gently attach the sticky end of the hair to the tarsal part of the right leg. After a few seconds, the glue will be dry, and the hair will be strongly affixed to the tarsus (Fig. 2h).
   ▲ **CRITICAL** Handle as fast and precisely as possible to guarantee that the heptane does not evaporate before the hair is placed accurately at the tarsus (complete Steps 3 and 4 in <10 s).
   ◆ **TROUBLESHOOTING**
8. Stop CO_2 anesthesia.
9. Once the hair-tarsus connection is firm, stretch the leg by pulling the hair at its free end straight away from the body (Fig. 2i). The free hair end should then be directly fixed on top of the heptane glue mixture–covered apple agar.
10. Use the tweezers to grab a layer of the solidifying glue on the agar adjacent to the hair and pull a film of glue across the fixed hair at a skewed angle (Fig. 2j). Repeat this step on the opposite side of the hair so that the hair is pinned down by a cross-wise fetter consisting of the two glue threads (Fig. 2k).
    ▲ **CRITICAL** Reduce the movement range in the femorotibial joint to a maximum of 20° by gently pulling at the hair. The cross-wise fetter is necessary for firm and sustained leg fixation during the immobilization interval.
    ◆ **TROUBLESHOOTING**
11. The sample is ready for acquisition (Fig. 2l,m).

Acquisition of the TransTimer reporter in neurons of the femorotibial joint

● **TIMING 10 h**

12. Perform CO_2 anaesthesia at $t = 0$ h and quickly transfer the anesthetized animal to the microscope.
13. Start microscopic acquisition of fluorescent signals at the immobilized joint immediately after anesthesia-induced cessation of fly movements. Record the control mobile leg joint after completing the procedure for the immobilized leg. The acquired datasets represent the mobile interval.
    ▲ **CRITICAL** CO_2 anesthesia is conducted to eliminate leg movement.
14. Stop CO_2 anesthesia. Let flies resume motor activity for 5 h (immobilization interval).

■ **PAUSE POINT** Incubate the flies at room temperature for 5 h. Perform this step during the morning hours in a well-illuminated room to ensure high activity and mobility of the flies.

15. Perform CO_2 anesthesia at $t = 5$ h and quickly transfer the anesthetized animal to the microscope.
16. Acquire data for the immobilization interval of the immobilized leg followed by the mobile leg under the same conditions as described in Step 13.
17. For re-mobilization of the immobilized leg, the restraint (human hair) is cut as close to the tarsus as possible.
    ▲ **CAUTION** Avoid tarsus amputation, to keep the animals intact and viable. Use a stereo microscope (e.g., Leica MZ 10 F) to ensure a precise cut.
18. Stop CO_2 anesthesia. Allow previously immobilized and mobile metathoracic legs to resume movement at the metathoracic joint at physiological angles for another 5 h (re-mobilization interval) (Fig. 2n).
    ■ **PAUSE POINT** Incubate the flies at room temperature for 5 h. Perform this step in a well-illuminated room to ensure high activity and mobility of the flies.
19. Perform CO_2 anesthesia at $t = 10$ h and quickly transfer the anesthetized animal to the microscope.
20. Acquire data for the re-mobilization interval of the previously immobilized leg and the mobile leg under the same conditions as described in Step 13.
21. Save the data and export them to the analysis software (e.g., ImageJ Fiji).
22. Discard the flies.

Analysis of acquired images of the TransTimer reporter in the femorotibial joint

● **TIMING 15–30 min for five samples**

23. Import the raw data into ImageJ (Fiji).
24. Adjust brightness equally to every image dataset. In our case, the displayed intensities were set to 100–4,000 a.u. via the following workflow: Image → Adjust → Brightness/Contrast → Set: 100 (Minimum)-4000 (Maximum) (Fig. 3h).
25. The two fluorescent channels are split via the following: Image → Color → Split channels.
26. Apply the color mode Fire: LUT mode → Fire.
    ▲ **CAUTION** Use a lookup table with a strong contrast.
27. Place a circular region of interest at different positions within the area of visibly highest intensity.
28. Use the histogram tool to find the position with the highest mean pixel intensity for dGFP and RFP channels: *Analyze* → *Histogram* → *Mean* (Fig. 3h).
29. Document the mean pixel intensitiy of the brightest area (Fig. 3h, 'Mean').
30. Depending on the experimental design, statistical comparison of normally distributed samples between two groups can be conducted by a paired *t* test, and comparison of normally distributed samples between more than two groups can be conducted by a repeated one-way ANOVA with Geisser-Greenhouse correction followed by Tukey's multiple comparisons test.
31. Depending on the experimental design, statistical comparison of non-normally distributed samples between two groups can be conducted by a Wilcoxon matched-pairs signed rank test, and comparison of normally distributed samples between more than two groups can be conducted by a Friedman test followed by Dunn's multiple comparisons test.
32. The normalized ratio of dGFP to RFP can be graphed in a scatter plot.

Quantification of leg movement in the femorotibial joint

● **TIMING 10 h**

▲ **CRITICAL** Record immobilized and mobile control legs in the same video. Record flies for 8 min under each experimental condition at a frame rate of 30 Hz in MP4 format.

33. Start CO_2 anesthesia.

34. For the fixation of the fly, follow the steps described in Fly fixation and leg immobilization (Steps 1–4).
35. Stop CO_2 anesthesia.
    ▲ **CAUTION** As opposed to the acquisition of fluorescent imaging data for the mobile interval, both metathoracic legs are still mobile at this point, to assess whether the fly is capable of moving them both.
    ▲ **CRITICAL** Allow the flies to fully recover from the CO_2 anesthesia, which takes ~2 min.
36. For the mobile interval (t = 0 h), start video acquisition directly before leg bending occurs.
37. Start CO_2 anesthesia.
38. *Leg immobilization*. As described in 'Fly fixation and leg immobilization' (Steps 5–10), fix the metathoracic right leg in a straight position.
39. Stop CO_2 anesthesia.
    ■ **PAUSE POINT** Incubate the flies at room temperature for 5 h. Perform these steps during the morning hours in a well-illuminated room to ensure high activity and mobility of the flies.
40. *Immobilization interval* (t = 5 h). Conduct acquisition under the same microscope conditions as described in Step 36 at the end of the leg fixation interval.
41. Start CO_2 anesthesia.
42. *Re-mobilization of immobilized leg*. Cut the restraint (human hair) as near to the tarsus as possible. Both metathoracic legs should reach the physiological angle of movement again.
    ▲ **CAUTION** Make sure not to amputate the tarsus. Use of a binocular microscope (e.g., Leica MZ 10 F) and CO_2 anesthesia can help to make a precise cut.
43. Stop CO_2 anesthesia.
    ■ **PAUSE POINT** Incubate the flies at room temperature for 5 h. Perform this step in a well-illuminated room to ensure high activity and mobility of the flies.
44. *Re-mobilization interval* (t = 10 h). Conduct acquisition under the same conditions as described in Step 36 at the re-mobilization interval.
45. Save the data in MP4 format and exported them to the analyzing software.
46. Discard the flies.

Measurement of femorotibial joint angles

● **TIMING 15–30 min for five samples**

47. Convert videotaped movie files (frame rate of 30 Hz in MP4) to 1 Hz and export as a TIFF movie in Adobe Photoshop.
48. Open the TIFF data set in ImageJ/Fiji.
49. Locate time periods of spontaneous bursts of leg movements in each movie, which occur ~1/min and usually last for <10 s (ref. 8).
50. The 'Angle tool' in Fiji is used to determine the smallest and largest angles between the proximo-distal femur and tibia axes at the femorotibial joint. The measurement is conducted during every burst of leg movement activity by pressing Cmd + M. The difference between the largest and smallest angle values at each movement burst is described as '$\alpha_{Extension} - \alpha_{Flexion}$' for the immobilized and mobile leg (Fig. 3l,m).
    ▲ **CAUTION** When joint angles are measured with a single camera orientation, rotation of the leg or subtle leg misalignment with respect to the camera position may cause small errors in angle determination.
51. In Prism, calculate mean values for mobile ($\Delta\alpha_{mobile}$) and immobilized ($\Delta\alpha_{immobile}$) legs during the mobile, immobilization and re-mobilization intervals for time points t = 0, 5 and 10 h, respectively.
52. Mean $\Delta\alpha_{mobile}$ and $\Delta\alpha_{immobile}$ can be visualized with a polar plot approach in which mobile, immobilization and re-mobilization interval values from an individual fly leg are plotted on the same radius of the graph[8].

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
1 and 7	The fly body is partially or completely submerged in the heptane glue mixture. The fly dies during the fixation procedure	The heptane glue mixture is too liquid at the time of fixation	Evaporation of heptane should be completed before the fly is attached to apple agar
	Fly legs get inadvertently glued to the apple agar during the fly fixation procedure	The heptane glue mixture spot is too large Legs get stuck to the glue surface during spontaneous movements	Place a sheet of paper or foil on both sides of the fly body to avoid legs getting glued to the agar surface (Fig. 3l,m)
10	Micromanipulation with risk of leg amputation	Too strong of a pull on the leg	Increase routine in micromanipulation

Timing

Steps 1–11: 20–40 min for five samples
Steps 12–22: 10 h
Steps 23–32: 15–30 min for five samples
Steps 33–46: 10 h
Steps 47–52: 15–30 min for five samples

Anticipated results

Figure 3i shows typical fluorescent mechano-dependent Cirl-NRS-controled TransTimer signals in the femorotibial joint. The main limitations during the experiment are caused by contamination by the heptane glue mixture (Fig. 3j) or blurred images (Fig. 3k).

This contamination can be prevented by sparing the joint region when covering the apple agar surface with heptane glue. If some glue reaches the desired joint region, the experimenter should discard the sample. The glue contamination imposes as a bright auto-fluorescent signal, which does not show the same dynamic fluorescence changes as those produced by the functional reporters used. To avoid unintended fixation of the mobile control leg to the sticky surface of the apple agar, a small copy paper cutout (~2 × 1 cm) can be placed on the control leg side next to the fly atop the fixed wing (Fig. 3l,m; right side). Furthermore, a small magnification of the upright microscope setting (e.g., 10× objective) is recommended to avoid movement artefacts manifesting in blurriness during the exposure time.

It is recommended to anesthetize the animals close to the imaging microscope station, extending the time available for leg movement acquisition. The experimenter should exclude blurred images without clearly distinguishable structures (Fig. 3k).

Two main failures in fly handling can seriously injure flies. First, if the heptane solvent has not completely evaporated upon fly mounting, its fluidity will remain high so that the animal will drown immediately. The sample has to be discarded. To prevent this, use a thin monolayer of heptane glue, which is sufficient to mount and fix the animal. Second, a small movement amplitude in the immobilized leg in Steps 14 and 15 (<20° in the femorotibial joint) is always observable, based on our experience. Therefore, if the experimenter notices a completely

motionless leg during the immobilization interval, this indicates that the leg was injured or even amputated. Discard the animal. Limb injury can be avoided by gentle manipulation (i.e., by not grasping the leg directly with the tweezers in Steps 7–10).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data for Figs. 1–3 are available in Figshare with the identifier https://doi.org/10.6084/m9.figshare.24024480 (ref. 24).

Received: 22 May 2023; Accepted: 25 August 2023;
Published online: 09 November 2023

References

1. Hoffman, B. D., Grashoff, C. & Schwartz, M. A. Dynamic molecular processes mediate cellular mechanotransduction. *Nature* **475**, 316–323 (2011).
2. Hudspeth, A. J. Integrating the active process of hair cells with cochlear function. *Nat. Rev. Neurosci.* **15**, 600–614 (2014).
3. Ranade, S. S., Syeda, R. & Patapoutian, A. Mechanically activated ion channels. *Neuron* **87**, 1162–1179 (2015).
4. Langenhan, T., Piao, X. & Monk, K. R. Adhesion G protein-coupled receptors in nervous system development and disease. *Nat. Rev. Neurosci.* **17**, 550–561 (2016).
5. Scholz, N., Monk, K. R., Kittel, R. J. & Langenhan, T. Adhesion GPCRs as a putative class of metabotropic mechanosensors. *Handb. Exp. Pharmacol.* **234**, 221–247 (2016).
6. Wolfenson, H., Yang, B. & Sheetz, M. P. Steps in mechanotransduction pathways that control cell morphology. *Annu. Rev. Physiol.* **81**, 1–21 (2017).
7. Mammoto, A., Mammoto, T. & Ingber, D. E. Mechanosensitive mechanisms in transcriptional regulation. *J. Cell Sci.* **125**, 3061–3073 (2012).
8. Scholz, N. et al. Molecular sensing of mechano- and ligand-dependent adhesion GPCR dissociation. *Nature* **615**, 945–953 (2023).
9. Scholz, N. et al. The adhesion GPCR Latrophilin/CIRL shapes mechanosensation. *Cell Rep.* **11**, 866–874 (2015).
10. Dannhäuser, S. et al. Antinociceptive modulation by the adhesion GPCR CIRL promotes mechanosensory signal discrimination. *Elife* **9**, e56738 (2020).
11. Scholz, N. et al. Mechano-dependent signaling by Latrophilin/CIRL quenches cAMP in proprioceptive neurons. *Elife* **6**, e28360 (2017).
12. Desai, B. S., Chadha, A. & Cook, B. The stum gene is essential for mechanical sensing in proprioceptive neurons. *Science* **343**, 1256–1259 (2014).
13. He, L., Binari, R., Huang, J., Falo-Sanjuan, J. & Perrimon, N. In vivo study of gene expression with an enhanced dual-color fluorescent transcriptional timer. *Elife* **8**, e46181 (2019).
14. Simpson, J. H. & Looger, L. L. Functional imaging and optogenetics in *Drosophila*. *Genetics* **208**, 1291–1309 (2018).
15. Luo, L., Callaway, E. M. & Svoboda, K. Genetic dissection of neural circuits: a decade of progress. *Neuron* **98**, 256–281 (2018).
16. Bos, J. L. Epac: a new cAMP target and new avenues in cAMP research. *Nat. Rev. Mol. Cell Biol.* **4**, 733–738 (2003).
17. Robichaux, W. G. & Cheng, X. Intracellular cAMP sensor EPAC: physiology, pathophysiology, and therapeutics development. *Physiol. Rev.* **98**, 919–1053 (2018).
18. Yang, H. H. & St-Pierre, F. Genetically encoded voltage indicators: opportunities and challenges. *J. Neurosci.* **36**, 9977–9989 (2016).
19. Bando, Y., Sakamoto, M., Kim, S., Ayzenshtat, I. & Yuste, R. Comparative evaluation of genetically encoded voltage indicators. *Cell Rep.* **26**, 802–813.e4 (2019).
20. Fosque, B. F. et al. Labeling of active neural circuits in vivo with designed calcium integrators. *Science* **347**, 755–760 (2015).
21. Grashoff, C. et al. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* **466**, 263–266 (2010).
22. Krieg, M., Dunn, A. R. & Goodman, M. B. Mechanical control of the sense of touch by β-spectrin. *Nat. Cell Biol.* **16**, 224–233 (2014).
23. Schmied, C. & Tomancak, P. Sample reparation and mounting of Drosophila embryos for multiview light sheet microscopy. *Methods Mol. Biol.* **1478**, 189–202 (2016).
24. Buhlan, M., Ljaschenko, D., Scholz, N. & Langenhan, T. *Figshare Raw Image Data*. Available at https://doi.org/10.6084/m9.figshare.24024480

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft to N.S. and T.L. through FOR2149, project numbers 265903901 (project P01) and 265996823 (project P03) and through CRC 1423, project number 421152132 (projects A06 and B06), and by a junior research grant from the Faculty of Medicine, Leipzig University to N.S. M.B. is funded by the Studienstiftung des deutschen Volkes. Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. The authors thank the Tomancak group at the MPI-CBG Dresden for suggestions regarding fly immobilization.

Author contributions

M.B. designed, conducted and analyzed the experiments and wrote the manuscript. D.L. initiated and analyzed the experiments. N.S. initiated and designed the experiments and wrote the manuscript. T.L. initiated, designed and analyzed the experiments and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-023-00907-7.

Correspondence and requests for materials should be addressed to Nicole Scholz or Tobias Langenhan.

Peer review information *Nature Protocols* thanks Dimitris Placantonakis and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Related links

Key references using this protocol
Scholz, N. et al. *Nature* **615**, 945–953 (2023): https://doi.org/10.1038/s41586-023-05802-5

nature portfolio

Corresponding author(s): N. Scholz, T. Langenhan

Last updated by author(s): 2023-08-23

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study.
For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Leica Application Suite X 3.7.4.23463, Adobe Photoshop v22.4.2
Data analysis	ImageJ2/Fiji v2-3-0/1.53q; Prism 9.3.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Source data for Figs. 1-3 are available in Figshare with the identifier doi:10.6084/m9.figshare.24024480 (ref. 24).

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

◉ Life sciences ○ Behavioural & social sciences ○ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	N/A
Data exclusions	No data were systematically excluded.
Replication	Examples where attempts of replication were not successful due to technical problems are showcased in Fig. 3. All other attempts of replication
Randomization	N/A
Blinding	N/A

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	
Research sample	
Sampling strategy	
Data collection	
Timing	
Data exclusions	
Non-participation	
Randomization	

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	
Research sample	
Sampling strategy	
Data collection	
Timing and spatial scale	
Data exclusions	
Reproducibility	
Randomization	

Blinding

Did the study involve field work? ○ Yes ○ No

Field work, collection and transport

Field conditions

Location

Access & import/export

Disturbance

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
○	○ Antibodies
○	○ Eukaryotic cell lines
○	○ Palaeontology and archaeology
○	● Animals and other organisms
○	○ Clinical data
○	○ Dual use research of concern

Methods

n/a	Involved in the study
○	○ ChIP-seq
○	○ Flow cytometry
○	○ MRI-based neuroimaging

Antibodies

Antibodies used

Validation

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

Authentication

Mycoplasma contamination

Commonly misidentified lines
(See ICLAC register)

Palaeontology and Archaeology

Specimen provenance

Specimen deposition

Dating methods

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	3-5 d old female adults of Drosophila melanogaster were used in this study:
Wild animals	N/A
Reporting on sex	Only female flies were used in this protocol study for clarity of presentation, but the protocol also works efficiently with males. Females
Field-collected samples	N/A
Ethics oversight	No ethical approval or guidance was required for this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	
Study protocol	
Data collection	
Outcomes	

Dual use research of concern

Policy information about dual use research of concern

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No	Yes	
○	○	Public health
○	○	National security
○	○	Crops and/or livestock
○	○	Ecosystems
○	○	Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes	
○	○	Demonstrate how to render a vaccine ineffective
○	○	Confer resistance to therapeutically useful antibiotics or antiviral agents
○	○	Enhance the virulence of a pathogen or render a nonpathogen virulent
○	○	Increase transmissibility of a pathogen
○	○	Alter the host range of a pathogen
○	○	Enable evasion of diagnostic/detection modalities
○	○	Enable the weaponization of a biological agent or toxin
○	○	Any other potentially harmful combination of experiments and agents

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links *May remain private before publication.*	
Files in database submission	
Genome browser session (e.g. UCSC)	

Methodology

Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Instrument

Software

Cell population abundance

Gating strategy

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI ○ Used ○ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis: ○ Whole brain ○ ROI-based ○ Both

Statistic type for inference (See Eklund et al. 2016)

Correction

Models & analysis

n/a | Involved in the study

- Functional and/or effective connectivity
- Graph analysis
- Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis