STAR Protocols

Protocol

A thermogenetics protocol for detecting gap junction channels in *Drosophila* egg chambers

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HIGHLIGHTS

Functional gap junction channels can be detected using a "thermogenetics" approach

The thermosensitive cation channel TrpA1-A is expressed in the germline using Gal4

The fluorescent [Ca^{2+}] sensor (GCaMP6s) is expressed in follicle cells using LexA

A TRPA1-A dependent cation influx can be detected upon temperature increase

In this protocol, we took a "thermogenetics" approach to detect functional gap junction channels between cells in *Drosophila* egg chambers. We expressed the thermosensitive cation channel TrpA1-A in the germline using Gal4, and a fluorescent [Ca^{2+}] sensor (GCaMP6), in all follicle cells using the LexA/LexAop system. If gap junctions connect germ cells and follicle cells, we expect a temperature-dependent TRPA1-A-dependent cation influx into the germline from the culture medium to result in a GCaMP signal in follicle cells.

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A thermogenetics protocol for detecting gap junction channels in *Drosophila* egg chambers

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SUMMARY

In this protocol, we took a ''thermogenetics'' approach to detect functional gap junction channels between cells in *Drosophila* egg chambers. We expressed the thermosensitive cation channel TrpA1-A in the germline using Gal4, and a fluorescent $[Ca^{2+}]$ sensor (GCaMP6), in all follicle cells using the LexA/LexAop system. If gap junctions connect germ cells and follicle cells, we expect a temperature-dependent TRPA1-A-dependent cation influx into the germline from the culture medium to result in a GCaMP signal in follicle cells.
For complete details on the use and execution of this protocol, please refer to Miao et al. (2020).

BEFORE YOU BEGIN

Gap junctions are constitutively open channels allowing passage of molecules <1–3 kDa between cells. The proteins capable of forming gap junctions - connexins in vertebrates and innexins in invertebrates – exhibit both channel-dependent and channel-independent functions. It is therefore important to establish where functional channels exist in tissues. Historically, identifying functional gap junctions between cells has relied upon dye injection and transfer, which is severely limited by physical access to the cells of interest. Therefore, in many biological processes where gap junction proteins are implicated, it has not been possible to assess where open channels exist. Optogenetic excitation of the light-sensitive ion channel Channelrhodopsin, combined with GCaMP Ca^{2+} sensors, has revolutionized neurobiology by allowing investigators to manipulate and monitor ion fluxes with light. To date, such studies have been limited to excitable cells, which amplify the signal. A further constraint of optogenetics is that a two-photon microscope is typically required for precise photo-activation. An alternative to optogenetics is ''thermogenetics,'' which employs temperature-sensitive ion channels, such as Transient Receptor Potential (Trp) channels, instead of Channelrhodopsin. An advantage of thermogenetics is that Trp channel ion fluxes are >10× the than Channelrhodopsin. A further difference is that a temperature shift activates the channel rather than photons. We wished to determine where in *Drosophila* egg chambers functional gap junction channels exist. So, we developed a thermogenetics approach and established that this technique can be used in non-excitable cells. We expressed the thermosensitive cation channel TrpA1-A in the germline using Gal4, and a fluorescent $[Ca^{2+}]$ sensor (GCaMP6s), in all follicle cells using the LexA/LexAop system. If gap junctions connect germ cells and follicle cells, we expect a temperature-dependent and TRPA1-A-dependent cation influx into the germline from the culture medium to result in an increase in GCaMP fluorescence in follicle cells. This approach may be useful for mapping patterns of gap junction connectivity in other tissues and organisms though challenges exist, including the need to express the channel and sensor in different cell types and the limited signal-to-noise ratio in non-excitable cells.

Fly genetics

⊙ Timing: 4 weeks

1. To start a thermogenetics experiment, first set up crosses to produce female flies with the appropriate genotypes.
   a. Collect 20 females of LexA-OP-GCaMP6s/Cyo; MAT-α-Gal4/Tm6B in 2–4 days.
   b. Divide 20 females in two vials, 10 females each vial. Cross the females with 3–5 male flies. In control group, use FC-lexA/Tm6B males; in experimental group, use UAS-TrpA1/Cyo; FC-lexA/Tm6B males.
   c. Culture the flies in 18°C. Transfer the flies to a new vial after 10 days. Each cross can flip once. The progeny adults take 4 weeks to come out.

   Note: To reduce the background signal of GCaMP, we culture the flies at 18°C.

   Note: To reduce background, we keep Gal4/UAS and LexA/LexA-OP lines apart in different lines and combine the elements by doing a cross.

   Note: To obtain healthy progeny, we only transfer the parent flies once to avoid using older parents.

Collecting and fattening female flies

⊙ Timing: 4 days

2. Collect 4–6 MAT-α-Gal4/+; LexA-OP-GCaMP6s/FC-LexA females in control group and MAT-α-Gal4/UAS-TrpA1; LexA-OP-GCaMP6s/FC-LexA in experimental group in 2 days.
3. Fatten the females with 2 males in a vial with a little bit (roughly 1–2 g) yeast paste for 2 days at 18°C.

   Note: We find mated females fatten better than unmated females. So, 2 males of any genotype are added.

   Note: We add about 1–2 g yeast paste per vial for fattening. To make yeast paste, we add water to dry yeast powder (dry yeast powder: water= 2:1).

   ⚠ CRITICAL: The fattening time affects the quality of ovary a lot. We fatten the flies 2 days at 18°C with freshly made yeast paste (Figure 1A).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Carbenoxolone	Sigma-Aldrich	Cat#C4790
Insulin	Sigma-Aldrich	Cat# I1882-100MG
Experimental models: organisms/strains		
D. melanogaster: slbo-Gal4	(Rørth et al., 1998)	FBtp0002716
D. melanogaster: w[1118]	Bloomington *Drosophila* Stock Center	RRID:BDSC_3605 FBst0003605

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: w[*]; P{w[+mC]=matα4-GAL-VP16}V37	Bloomington _Drosophila_ Stock Center	BDSC_7063 FBst0007063
D. melanogaster: FC-LexA	Laboratory of Xiaobo Wang, University of Toulouse	N/A
D. melanogaster: [1118]; P{y[+t7.7] w[+mC]=13XLexAop2-IVS-GCaMP6s-SV40}su(Hw)attP5	Bloomington _Drosophila_ Stock Center	BDSC_44589 FBst0044589
D. melanogaster: w[*]; P{y[+t7.7] w[+mC]=UAS-TrpA1(B).K}attP16	Bloomington _Drosophila_ Stock Center	BDSC_26263 FBst0026263
Software and algorithms		
FIJI	PMID: 22743772	https://fiji.sc/
Adobe Illustrator CS6	Adobe.com	N/A
Prism 6 GraphPad	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Other		
Schneider's _Drosophila_ medium	Life Technologies	Cat# 21720-024
Fetal bovine serum	Sigma-Aldrich	Cat# F4135
Halocarbon oil 27	Sigma-Aldrich	Cat# H8773
2-well concavity slide	Electron Microscopy Sciences	Cat# 50-949-550
Lumox dish 50 mm	Sarstedt	Cat# 94.6077.410
Eppendorf tube	Eppendorf	Cat# 22363611
Dumont #5 forceps	Fine Science Tools	Cat# 11251-30
Coverslips, size: 22 × 22 mm	Fisher	Cat# 12541-B
Coverslips, size: 40 × 22 mm	Fisher	Cat# 12544-B
Dissecting microscope	n/a	n/a
Zeiss LSM 780 microscope	Zeiss	LSM 780

MATERIALS AND EQUIPMENT

Dissecting medium		
Reagent	Final concentration (mM or μM)	Amount
Schneider's _Drosophila_ medium	n/a	40 mL
Fetal bovine serum	n/a	10 mL
Total	n/a	50 mL

⚠ CRITICAL: Adjust the pH to 6.85–6.95, which is required for normal border cell migration (Prasad et al., 2007). Aliquot to 1 mL/tube and store in 4°C.

Insulin solution		
Reagent	Final concentration (mM or μM)	Amount
Insulin	10 mg/mL	10 mg
Concentrated HCl	n/a	1 μL
ddH$_2$O	n/a	1 mL
Total	n/a	1 mL

⚠ CRITICAL: Aliquot to 4 μL/tube in 200 μL EP tubes and store in −80°C. The egg chambers maintain normal development with insulin in the culture medium (Prasad et al., 2007).
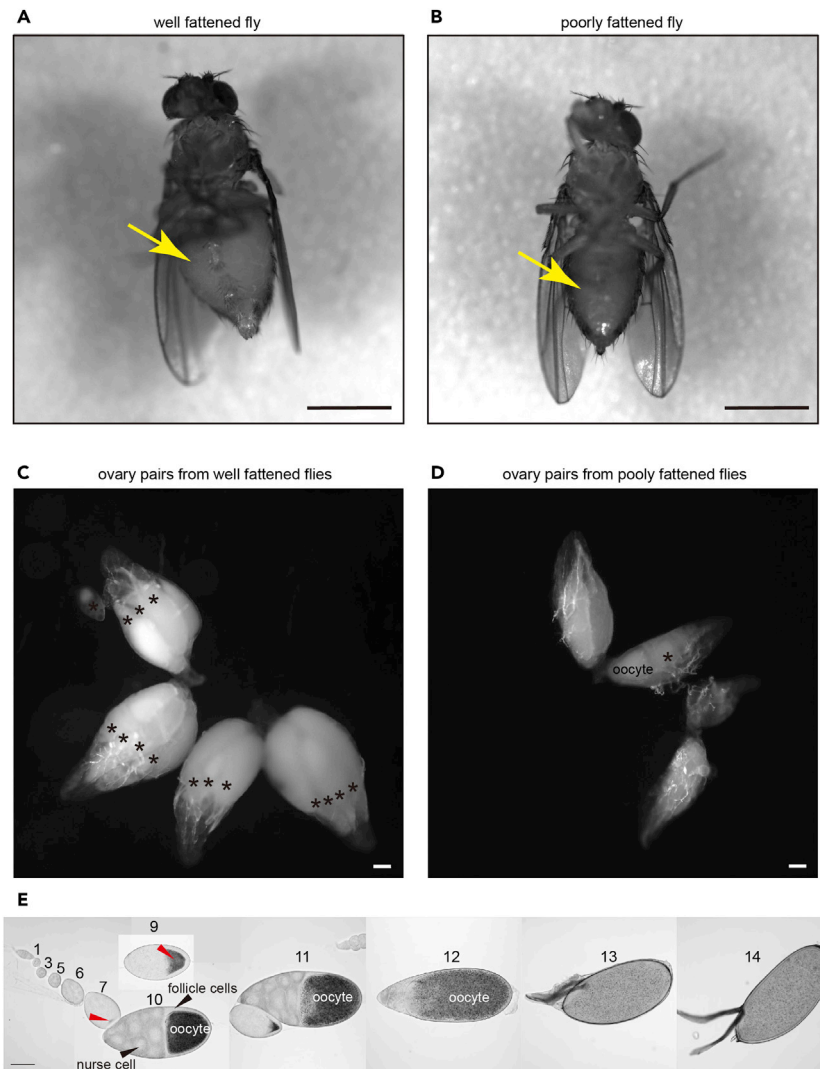
Figure 1. Assessing ovarian fattening and developmental stages

(A) A well-fattened female fly.

(B) A poorly fattened female fly. Yellow arrows indicate the abdomen. Scale bar, 1 mm.

(C) Two ovary pairs from well-fattened flies with numerous stage 10 egg chambers.

(D) Two ovary pairs from poorly fattened flies with few stage 10 egg chambers. Asterisks label stage 10 egg chambers.

(E) Stages of egg chamber development. Red arrowheads indicate the oocyte. Scale bar, 100 μm.

STEP-BY-STEP METHOD DETAILS

Fly ovary dissection

⏱ Timing: 15 min

This step describes the dissection of fly ovaries and the collection of egg chambers at the appropriate stage for live imaging.

1. Prepare 18°C fresh dissecting medium. In a 500 μL Eppendorf tube, add 100 μL medium in the tube.

2. Dissect fly ovaries and collect stage 10 egg chambers. Troubleshooting 1

a. Dissect up to 6 flies, pull the ovaries out, and remove the surrounding tissues. Carefully pull out the ovarioles from the muscle sheath and remove the late stage egg chambers (stage 12–14).

b. Transfer the egg chambers to the Eppendorf tube with medium and wash the egg chambers with 200 μL dissecting medium twice.

3. In a new 200 μL Eppendorf tube, add 4 μL insulin solution to 196 μL dissecting medium. Remove the wash medium in the 500 μL Eppendorf tube and add 200 μL medium with insulin to it.

Note: Use fresh medium (less than one month) for live imaging.

Note: Use ovaries from well-fattened females (Figure 1C) to ensure egg chamber health.

Note: To identify different stage egg chambers using a dissecting microscope, we focus on the overall size of the egg chamber and the relative volume of the oocyte (yolk) as a proportion of the whole egg chamber. Generally, yolk accumulates in the oocyte from stage 7. Stage 8 egg chambers are typically 200 μm long. By stage 9, the oocyte occupies ~1/3 the volume of whole egg chamber volume and $\frac{1}{2}$ by stage 10. During stage 11, the nurse cells transfer the majority of their cytoplasmic contents into the oocyte so that at stage 12, the oocyte occupies the majority of the egg chamber volume. The dorsal appendages become visible in stage 13 and fully elongate in stage 14 (Figure 1E).

Sample mounting

⊙ Timing: 5 min

Samples are carefully mounted to the dish for live imaging.

4. Use the handle of forceps to break one 22 × 22 mm coverslip to two equal pieces by pushing hard in the center of the coverslip on a hard surface (Figure 2A). Add two small drops of medium on the dish and carefully place the coverslip pieces on the Lumox dish (Figures 2B and 2C).

5. Transfer samples in 90 μL medium to the center of two coverslip pieces (Figure 2D). Then cover the sample with a 22 × 40 mm coverslip (Figure 2E). Remove the excessive medium (Figure 2F).

6. Add small droplets (less than 50 μL) of halocarbon oil 27 carefully to the rims of coverslip (Figure 2G) and let them spread to all the sides (Figure 2H).

⚠ CRITICAL: Be sure the coverslip pieces do not have any small glass fragments, which can lead to uneven mounting. Mounting should avoid bubbles and too much medium/halocarbon oil, which can cause the egg chambers to shift during imaging.

Imaging acquisition setup and live imaging

⊙ Timing: 30–45 min

7. Mount the Lumox dish to the microscope stage. On the Zeiss 780 microscope, mount the Lumox dish onto the motorized stage with temperature control module. Cover with the lip of temperature control module. Then close the image air chamber.

Note: We suggest changing the normal stage to motorized stage with temperature control module before opening the Zen software.

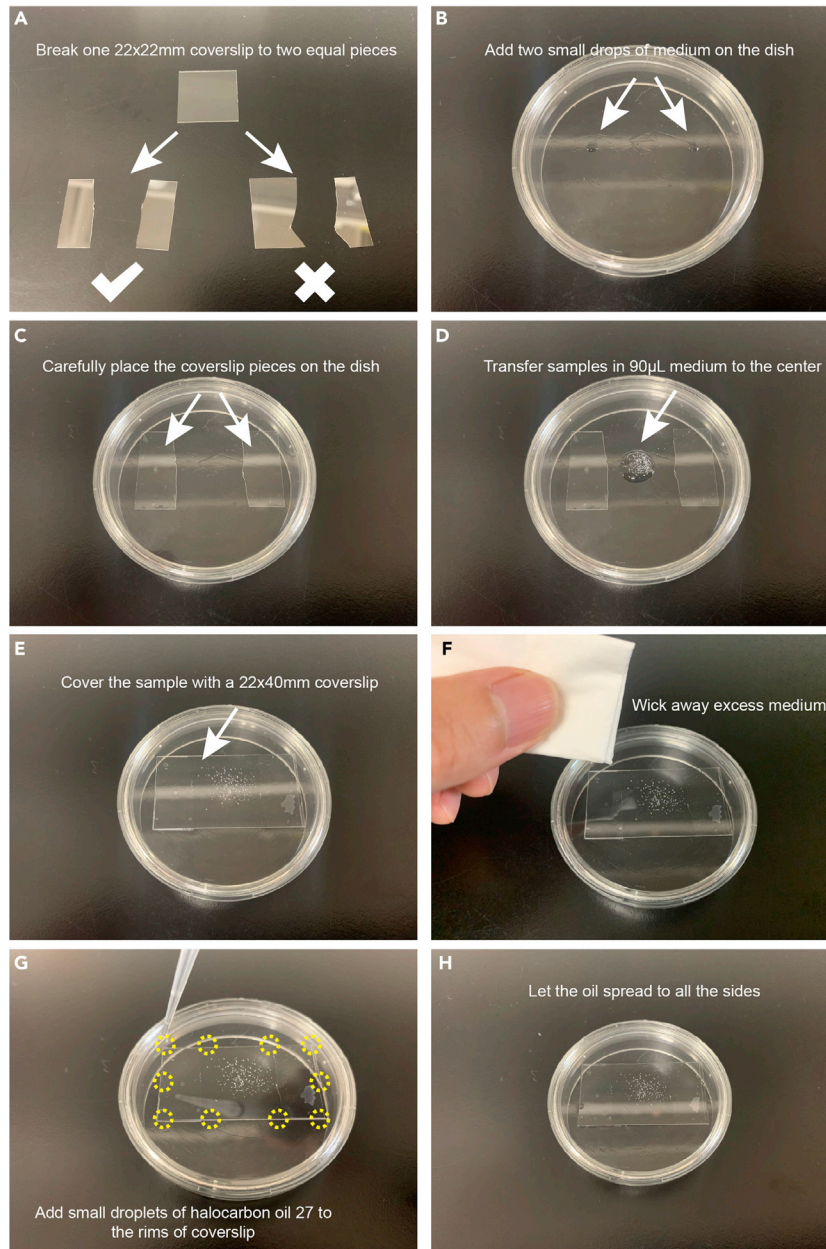*Note:* We image the control group first.

Figure 2. Sample mounting

(A) Break one 22 × 22 mm coverslip into two equal pieces as shown on the left.

(B) Add two small drops of medium onto the dish.

(C) Carefully place the coverslip pieces on the Lumox dish.

(D) Transfer samples in 90 μL medium to the center of the two coverslip pieces.

(E) Cover the sample with a 22 × 40 mm coverslip.

(F) Carefully wick away the excess medium with a tissue.

(G) Add small droplets of halocarbon oil 27 carefully to the rims of coverslip. Yellow circles show the positions to add oil.

(H) Let the oil spread to seal the coverslip.

8. Open temperature control module and set the temperature to 23°C. On Zeiss 780 microscope, open Zen software, select "temperature" control module and set the temperature to 23°C.

9. Switch to 20× objective lens. On Zeiss 780 microscope, select "Position" in the "Acquisition" mode then switch to "Locate" mode.
10. Select stage 10 egg chambers. On Zeiss 780 microscope, click "GFP" filter. Use microscope eyepiece to adjust focal plane and select stage 10 egg chambers by clicking "Add" in the "position" box. Select up to 10 egg chambers.

    Note: We describe the details for the Zeiss 780 confocal microscope and associated Zen software. Laser power, "gain," and other details will vary for different manufacturers so some empirical parameter determinations will be necessary.

    Note: To image stage 10A egg chambers, we select those in which border cells contact the oocyte but are not yet in contact with centripetal cells.

11. Adjust the 488 nm laser parameters. On the Zeiss 780 microscope, switch back to "Acquisition" mode and set up imaging parameters. Use a 488 nm argon laser for excitation and acquire GFP images using channel 1 (500–550 nm). Adjust the laser power (2% laser power in our experiment) and the pinhole size (2 Airy units in our experiment). Channel gains should be lower than 800.
12. Adjust imaging parameters. On Zeiss 780 microscope, use frame size "512 × 512" pixels. Use averaging to 8 times for maximum signal-to-noise ratio. Use maximum speed.
13. Move to the first egg chamber. On Zeiss 780 microscope, click "Move to" in the "Position" box to move the position to the first egg chamber. Click "Live," adjust focus to the mid-plane of the border cells. Rotate crop box to make the egg chamber posterior side facing right side. Troubleshooting 2
14. Acquire single section image in 23°C. Troubleshooting 3
15. Move to the next egg chamber and repeat steps 12 and 13. On Zeiss 780 microscope, click "Move to" in the "Position" box to move the position to the next egg chamber.

    Note: To best image the border cells, carefully adjust the focus and acquire the images one by one manually.

    Note: If the orientation of the egg chamber happens to be with the dorsal side closest to the objective lens, the border cells will be closer as well and the signal will be stronger. It is not possible to control egg chamber orientation, and this assessment can only be made by focusing up and down to determine the position of the border cell cluster relative to the objective.

16. After imaging all egg chambers at 23°C, return to the position of the first egg chamber. Set the temperature in the "temperature" module to 32°C. Wait for the air temperature to stabilize at 32°C. In our experiments, it typically takes 10 min.
17. Repeat steps 12–14 to acquire the same single section image in 32°C.

    ⚠ CRITICAL: Use the lowest laser power and exposure time that allow visualization of the sample. Exposing the egg chamber too long or using high laser power can cause photobleaching. Do not use too high channel gains which can induce strong background.

Image analysis

    ⓘ Timing: 30 min

18. Remove image background.
    a. Open the image in FIJI.
    b. Measure background in a nurse cell region using a "Rectangular selection" box without selecting the follicle cells
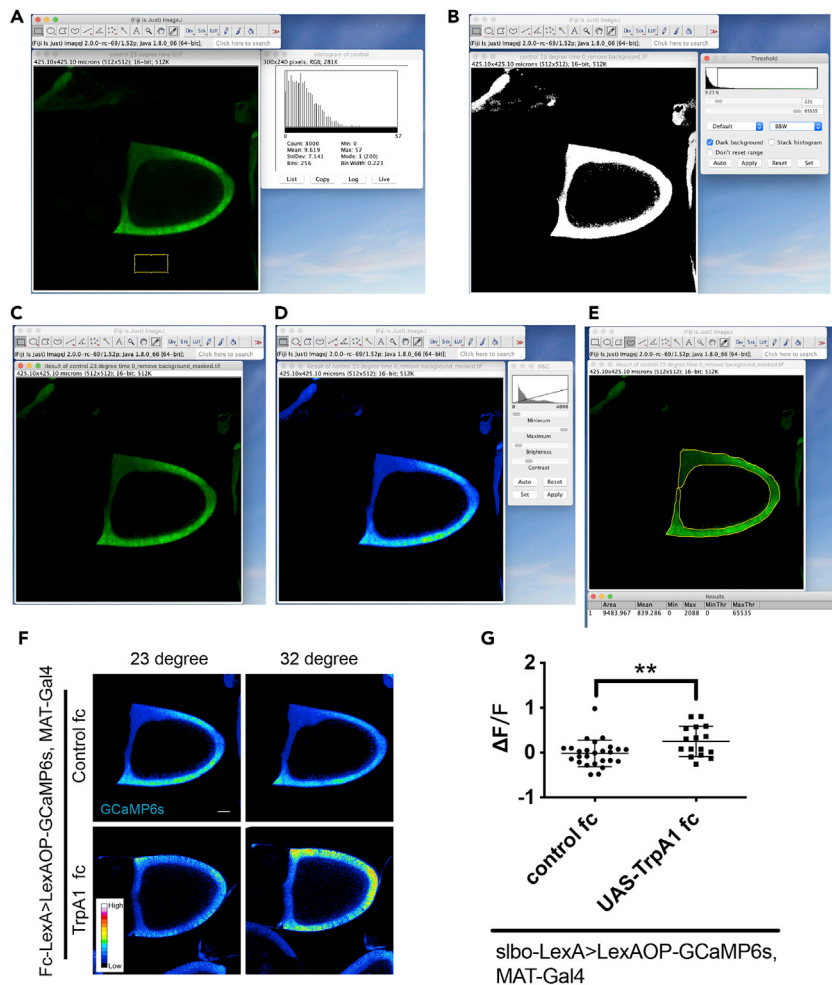
Figure 3. Data analysis

(A) Use FIJI to remove image background.

(B) Use FIJI to generate mask image.

(C) Use FIJI to generate adjusted image.

(D) Choose pseudo color and adjust image for display.

(E) Use the adjusted image to measure the intensity in follicle cells.

(F) Examples show GCaMP signal in control and TrpA1 egg chambers.

(G) Quantification of the level of GCaMP6s in thermogenetics experiments in follicle cells (fc). **p < 0.01, unpaired Student's t test (two-tailed).

c. Use "Analyze->Histogram" to obtain the background mean and standard deviation (Figure 3A).

d. Calculate background value as "mean+3 × StdDev." Select the entire image. Subtract background value using "Process->Math->Subtract" function.

e. Save the image as "file name_remove background"

19. Generate mask image.

    a. Use "Image->Duplicate" function to duplicate the image.

    b. In the duplicated image, use "Image->Adjust->Threshold" to create binary mask. Choose "Dark background" to select the bright signal. Use the default algorithm (Figure 3B).

    c. Use "Process->Math->Divide" to divide mask by 255 to create a 0/1 binary mask image.

    d. Save the mask as "file name_mask"

20. Generate adjusted image and display.

   a. Multiply the mask with image using "Process->Image Calculator" to obtain a new image. Save the image as "file name_remove background_masked" (Figure 3C).

   b. Use "Image->Duplicate" function to duplicate the image.

   c. In the duplicated image, use "Image->Lookup Tables" to change the lookup table which can visualize the dynamic range in different color coding. In our experiment, we used "16 colors" (Figure 3D).

   d. Use "Image->Adjust->Brightness/Contrast," adjust the brightness/contrast to best capture the dynamic range of the image.

   e. Save the 32-bit image in tiff format. Convert image to RGB image and save for display.

Note: The display image will NOT change the actual value of each pixel.

21. Use the "file name_remove background_masked" to measure the intensity in follicle cells (Figure 3E).

   a. Use "Freehand selections" function to select the ROIs of follicle cell.

   b. Use "Analyze->Measure" function to measure the intensity.

   c. Export the data and use the "Mean" intensity for further analysis.

22. Summarize and plot the result.

   a. Changes in GCaMP6s intensity are calculated and expressed as $\Delta F/F_0$. $\Delta F = F_1 - F_0$, F_1 is the GCaMP6s intensity after temperature shift to 32°C for 10 min, F_0 is the GCaMP6s intensity in 23°C before temperature shift.

   b. Import ΔF in control and experimental group in Prism 6 GraphPad. Plot the data and perform statistical analysis (Figures 3F and 3G).

EXPECTED OUTCOMES

TrpA1-A is a temperature-sensitive cation channel that allows Na+ and Ca^{2+} into cells at >27°C (Viswanath et al., 2003). In this thermogenetics protocol, we express UAS-TrpA1 in the germline with MAT-α-Gal4 and GCaMP6s in follicle cells with FC-LexA. We grow the flies at 18°C, dissect egg chambers, and mount them for live imaging on Zeiss 780 confocal microscope equipped with an environmental chamber. We then measure baseline GCaMP fluorescence in the outer layer of the follicle cells and border cells at 23°C and shift the temperature to 32°C and measure the change in fluorescence.

Gap junction proteins form constitutively open channels in neighboring cells that allow small molecules <1–3 kDa to pass (Krysko et al., 2005; Skerrett and Williams, 2017). Since gap junction channels have been reported to exist between the oocyte and follicle cells based on dye transfer (Bohrmann and Zimmermann, 2008), we expect a temperature-dependent rise in GCaMP signal in MAT-α-Gal4>TrpA1 expressing LexA-OP-GCaMP6s under the control of a follicle cell LexA driver. In control egg chambers lacking TrpA1 expression, no increase in GCaMP fluorescence occurred upon temperature shift.

LIMITATIONS

The primary advantage of this method over dye transfer is that no injection is necessary. Therefore in principle this method could be used for cells and tissues that are inaccessible to injection. However, the fold change ΔF is only about 20%.

The spatial resolution of thermogenetics is limited by the enhancers/promoters driving TrpA1 and GCaMP expression unless one makes new transgenes expressing TrpA1 and/or GCaMP directly under the control of desired enhancer/promoters. An optogenetics approach, i.e., expressing Channelrhodopsin in some cells and GCaMP in others and stimulating cation influx with light would provide higher spatial resolution. However, thermogenetics is more sensitive than optogenetics because the conductance of Trp channels is ~10–20× higher than the conductance of

Channelrhodopsins. In addition, activating Channelrhodopsin deep inside tissue would likely require 2-photon excitation. Nevertheless these approaches would greatly enhance the study of gap junction proteins and aid in discriminating between their channel-dependent and channel-independent functions.

TROUBLESHOOTING
Problem 1
Too few stage 10 egg chambers in the ovaries (related to step 2).

Potential solution
The quality of ovary largely depends on the fattening time. In our hand, fattening the flies in 18°C for 2 days with fresh made yeast paste gives best result.

Problem 2
Egg chambers move during imaging (related to step 13).

Potential solution
Mount the sample carefully to avoid this problem. Uneven mounting, bubbles or too much medium/ halocarbon oil can cause this problem. Do not mount too many egg chambers on one dish and do not select too many egg chambers in one experiment.

Problem 3
The GCaMP signal is too weak (related to step 14).

Potential solution
When border cells complete their migration at stage 10A, they dock to anterior dorsal side of oocyte membrane. So, when dorsal or dorsal lateral side of the egg chamber faces the objective during imaging, the border cells are closer to the objective, hence it gives better signal. When the ventral or ventral lateral side faces the objective, the border cells are away from the objective, the signal hence much weaker. We found the orientation of egg chambers significantly affects the border cell GCaMP signal. Since the border cells associate with the dorsal/anterior aspect of the oocyte, and interact first with a dorsal centripetal cell, we restrict the analysis to those egg chambers that happen to be oriented with the dorsal side closest to the objective lens. We exclude egg chambers oriented with the ventral side closest to the objective lens. We also suggest not use high laser power or long exposures to prevent photobleaching.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Denise Montell (dmontell@ucsb.edu).

Materials availability
This study did not generate new unique reagents

Data and code availability
This study did not generate new datasets or codes.

AUTHOR CONTRIBUTIONS

G.M. developed the protocol, designed and carried out the experiments, and wrote the manuscript. D.M. conceived of the approach and experimental design, secured funding, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bohrmann, J., and Zimmermann, J. (2008). Gap junctions in the ovary of *Drosophila melanogaster*: localization of innexins 1, 2, 3 and 4 and evidence for intercellular communication via innexin-2 containing channels. BMC Dev. Biol. *8*, 111.

Krysko, D.V., Leybaert, L., Vandenabeele, P., and D'Herde, K. (2005). Gap junctions and the propagation of cell survival and cell death signals. Apoptosis *10*, 459–469.

Miao, G., Godt, D., and Montell, D.J. (2020). Integration of migratory cells into a new site in vivo requires channel-independent functions of innexins on microtubules. Dev. Cell *54*, 501–515.e9.

Prasad, M., Jang, A.C.-C., Starz-Gaiano, M., Melani, M., and Montell, D.J. (2007). A protocol for culturing *Drosophila melanogaster* stage 9 egg chambers for live imaging. Nat. Protoc. *2*, 2467–2473.

Rørth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G.M., Weigmann, K., Milàn, M., Benes, V., Ansorge, W., and Cohen, S.M. (1998). Systematic gain-of-function genetics in Drosophila. Development *125*, 1049–1057.

Skerrett, I.M., and Williams, J.B. (2017). A structural and functional comparison of gap junction channels composed of connexins and innexins. Dev. Neurobiol. *77*, 522–547.

Viswanath, V., Story, G.M., Peier, A.M., Petrus, M.J., Lee, V.M., Hwang, S.W., Patapoutian, A., and Tim, J. (2003). Opposite thermosensor in fruitfly and mouse. Nature *423*, 822–823.