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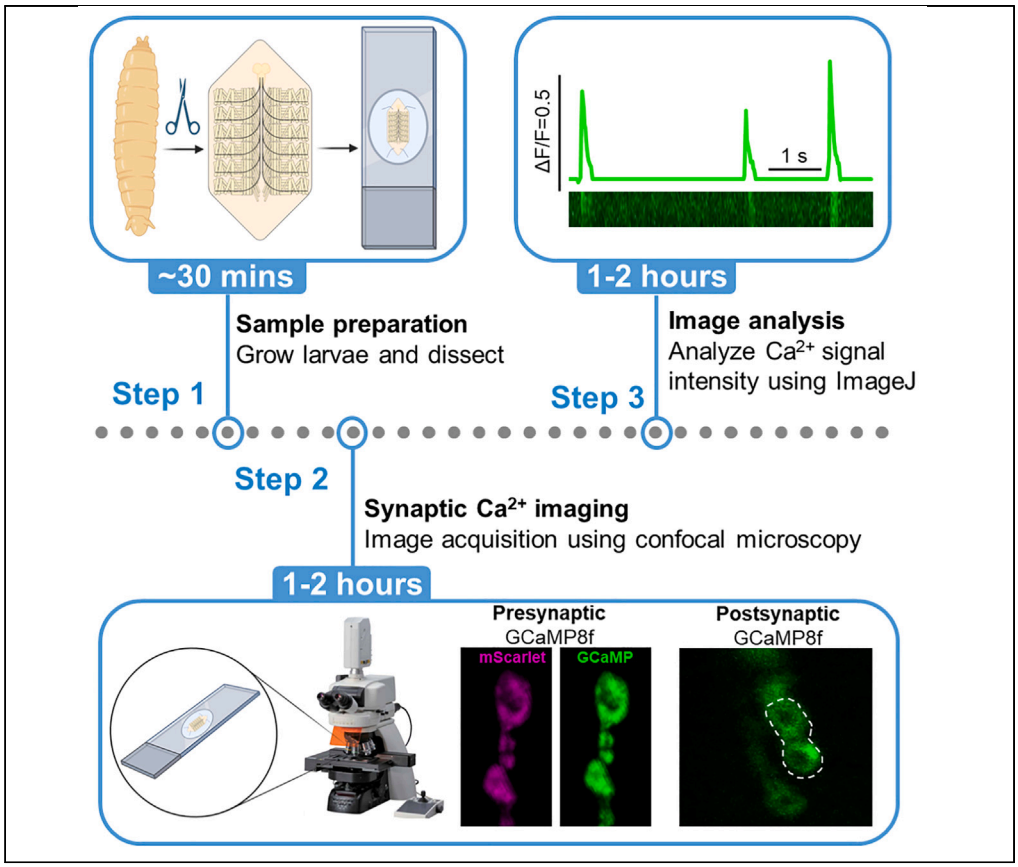
Protocol

Ca^{2+} imaging of synaptic compartments using subcellularly targeted GCaMP8f in *Drosophila*



GCaMP8f is a sensitive genetically encoded Ca^{2+} indicator that enables imaging of neuronal activity. Here, we present a protocol to perform Ca^{2+} imaging of the *Drosophila* neuromuscular junction using GCaMP8f targeted to pre- or postsynaptic compartments. We describe ratiometric Ca^{2+} imaging using GCaMP8f fused to mScarlet and synaptotagmin that reveals Ca^{2+} dynamics at presynaptic terminals. We then detail "quantal" imaging of miniature transmission events using GCaMP8f targeted to postsynaptic compartments by fusion to a PDZ-binding motif.

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

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Highlights

Ca^{2+} imaging to assess single-action-potential presynaptic signals at the fly NMJ

Image and quantify "quantal" postsynaptic Ca^{2+} signals at the *Drosophila* NMJ

Sub-synaptically targeted GCaMP8 indicators for ratiometric and quantitative imaging

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Ca^{2+} imaging of synaptic compartments using subcellularly targeted GCaMP8f in *Drosophila*

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SUMMARY

GCaMP8f is a sensitive genetically encoded Ca^{2+} indicator that enables imaging of neuronal activity. Here, we present a protocol to perform Ca^{2+} imaging of the *Drosophila* neuromuscular junction using GCaMP8f targeted to pre- or postsynaptic compartments. We describe ratiometric Ca^{2+} imaging using GCaMP8f fused to mScarlet and synaptotagmin that reveals Ca^{2+} dynamics at presynaptic terminals. We then detail "quantal" imaging of miniature transmission events using GCaMP8f targeted to postsynaptic compartments by fusion to a PDZ-binding motif.

For complete details on the use and execution of this protocol, please refer to Li et al.,[1] Han et al.,[2] Perry et al.,[3] and Han et al.[4]

BEFORE YOU BEGIN

This protocol describes presynaptic Ca^{2+} imaging using Syt::mScarlet::GCaMP8f (**mScar8f**)[1] and postsynaptic Ca^{2+} imaging using **SynapGCaMP8f**[2] (Figure 1). This approach can also be adapted for performing Ca^{2+} imaging using other genetically-encoded Ca^{2+} indicators.

This protocol provides detailed instructions for conducting Ca^{2+} imaging using the latest and most sensitive genetically-encoded Ca^{2+} indicator, GCaMP8. We describe imaging using a standard confocal microscope with line scan acquisition, and also highlight the advantages of rapid area scan imaging using a resonant scanner.

Drosophila husbandry

Timing: 4–6 days larval development

1. Establish the *Drosophila* stocks needed for imaging.
   a. For presynaptic imaging, cross the mScar8f reporter, located on the third chromosome, with an appropriate neuronal GAL4 driver for expression. This transgene can be combined with other transgenes and/or mutations of interest.
   b. For postsynaptic imaging, choose SynapGCaMP8f that is located on either the second or third chromosome and is constitutively expressed in muscle under the control of the MHC promoter.[2,5] This transgene can be combined with other transgenes and/or mutations of interest.
   c. Perform electrophysiological recordings to confirm that baseline transmission in the final stock(s) are not perturbed by inclusion of the GCaMP8f reporters; expression of these reporters should not impact transmission.
2. Grow larvae.

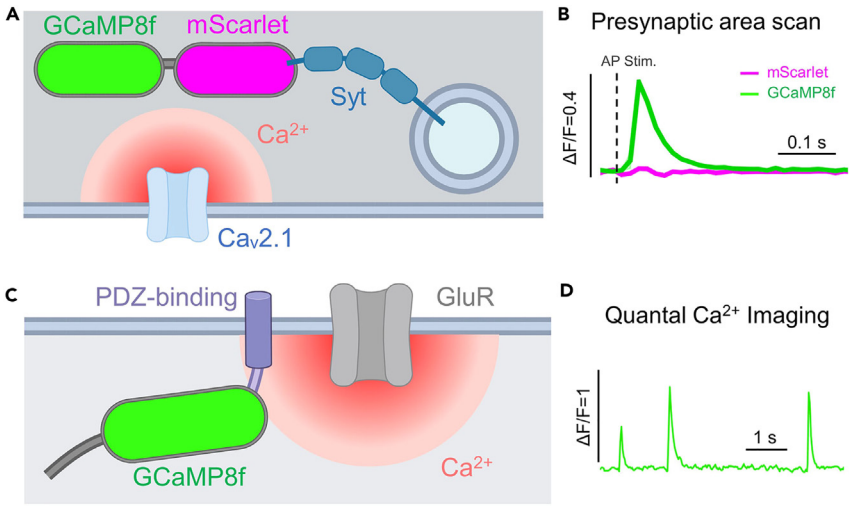

Figure 1. Schematic and representative Ca^{2+} signals of pre- and post-synaptically targeted GCaMP8f

(A) Schematic of a ratiometric indicator (mScarlet::GCaMP98f) localized to presynaptic terminals through fusion with the synaptic vesicle protein Synaptotagmin (Syt::mScarlet::GCaMP8f; mScar8f).

(B) Representative traces of the presynaptic mScar8f signals acquired by area scan. Scale bars: ΔF/F = 0.4; 0.1 s.

(C) Schematic of SynapGCaMP8f localized to the postsynaptic density via binding to a PDZ motif.

(D) Representative traces of spontaneous ("quantal") postsynaptic Ca^{2+} signals. Scale bars: ΔF/F = 1; 1 s.

a. Grow fly stocks at 25°C in vials with standard food.
b. Select wandering third-instar larvae from the vials (~5 days old).

Pull electrodes for sharp electrode recordings and nerve stimulation

Timing: 10–15 min

3. Pull sharp glass recording electrodes.
   a. Securely attach a TW120F-4 glass capillary on the glass puller (Sutter Instrument Co. Model P-97) and set the program as follows: HEAT = 520°C, PULL = 110, VEL (velocity) = 80, TIME = 250.

   Note: The filament for each puller has its own unique ramp value; adjust the puller program accordingly.

   b. Fill the glass electrode with 3M KCl solution using a long & thin plastic syringe. The filament inside the glass capillary will guide the KCl solution to the tip of the glass pipette. Gently tap the glass pipette to remove residual air bubbles in the electrode tip.
4. Pull glass suction electrode for nerve stimulation.
   a. Securely attach a TW120-4 glass capillary on the glass puller (Sutter Instrument Co. Model P-97) and set the program as follows: HEAT = 520°C, PULL = 110, VEL (velocity) = 80, TIME = 250.
   b. Polish the rough electrode end using a micro forge fire polisher. Gently heat and melt the tip opening to slowly shrink it until an opening width of ~5–10 μm is reached.
   c. Fill the suction electrode with HL-3 solution. Store HL-3 solution at 4°C and put on ice during the experiment.

Prepare the sample for imaging

Timing: 20–30 min per larvae

5. Dissect the larvae using a stereo microscope.
   a. Place the third-instar larva onto a glass slide with a circular concave Sylgard pad in the center. Add 500 mL HL-3 to bathe the entire preparation.
   b. Pin the larvae dorsal side up with a right-angled incision.
   c. Use dissecting micro scissors to cut open the larva along the midline of the tracheal tube from the dorsal side. Complete the larval dissection 20°C (see Imlach, McCabe 2009[6] for detailed protocol).
   d. Gently complete the larval dissection. Secure the four corners of the larvae onto the Sylgard pad using four right-angled pins; be cautious not to stretch the larval musculature.
   e. Carefully remove the internal tissues, including the trachea, with forceps and use dissection scissors to disconnect the internal tissues from the body wall. This step should remove all the internal fat and gut tissue and expose an intact ventral nerve cord (VNC) connected to the larval body wall with motor nerves.
   f. Carefully cut the motor nerve close to the VNC end and leave the floating nerves.
   g. Remove the HL-3 saline with a pipette and replace with new HL-3 containing the preferred Ca^{2+} concentration. Here we use HL-3 with 1.5 mM Ca^{2+} unless otherwise indicated.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
KCl	Sigma	Cat#P9333
NaCl	Sigma	Cat#S7653
$MgCl_2 \cdot 6H_2O$	Sigma	Cat#M2670
$NaHCO_3$	Sigma	Cat#S5761
Sucrose	Fisher Scientific	Cat#S233-500
D-(+)-trehalose dihydrate	Sigma	Cat#T9531
HEPES	Sigma	Cat#H3375
$CaCl_2 \cdot 2H_2O$	Sigma	Cat#C5080
4-inch thin wall glass capillaries 1.2 OD/0.9ID	World Precision Instruments	TW120-4
4-inch thin wall glass capillaries 1.2 OD/0.9ID w/ filaments	World Precision Instruments	TW120F-4
Glass puller	Sutter Instrument Co.	Model P-97
Microforge	Narishige	MF-830
Orthodontic wax	Amazon	B09KBPZQCP
Non-drying modeling clay	Amazon	B0C5W5YPHL
Sylgard	Dow	DC4019862
Experimental models: Organisms/strains		
SynapGCaMP8f	Han et al.[2]	N/A
UAS-Syt::mScarlet::GCaMP8f (mScar8f)	Li et al.[1]	N/A
Software and algorithms		
ImageJ	https://imagej.net/	1.8.0
NIS-Elements software	Nikon Instruments	5.41.02 (Build 1711)
Excel	Microsoft	2021
Mini Analysis	Synaptosoft	6.0.7
Other		
Nikon Eclipse Ni-E upright microscope	Nikon Instruments	Eclipse Ni-E
Nikon A1R confocal	Nikon Instruments	A1R
7×-45× Stereo microscope	AmScope	SM 1-B

MATERIALS AND EQUIPMENT

HL-3 Saline (1 L)		
Reagent	Final concentration	Amount
NaCl	70 mM	4.09 g
KCl	5 mM	0.37 g
$MgCl_2$	10 mM	4.07 g
$NaHCO_3$	10 mM	0.84 g
Sucrose	115 mM	39.4 g
Trehalose	5 mM	1.89 g
HEPES	5 mM	1.19 g
ddH_2O	N/A	Fill up to 1 L
Total	**N/A**	**1 L**

Note: Store at 4°C for up to one month, pH = 7.2.

STEP-BY-STEP METHOD DETAILS

Part 1. Presynaptic Ca^{2+} imaging using Syt::mScarlet::GCaMP8f (mScar8f)

Timing: 30 min per larvae

This section describes the steps necessary to acquire presynaptic Ca^{2+} signals.

1. Mount the dissected larvae on the confocal microscope.
   a. Fix the suction electrode to the imaging slide with orthodontic wax and clay of proper size (Figures 2A–2C). Orthodontic wax provides solid support while the modeling clay offers optimal flexibility.
   b. Place the slide on the confocal stage and secure it with stage clips. Insert the suction electrode beneath the objective for stimulation and submerge the grounding wire in the imaging solution. Secure the position of electrode wires to the confocal stage with modeling clay (Figure 2C).
   c. Gently move the suction electrode under the microscope objective to the center of abdominal segments A2/A3 and ~100 μm above the muscle (Figure 2D). The suction electrode tip should be in close proximity to the nerve innervating the A3 muscle fibers.
   d. Suck the nerve by applying a brief negative pressure to the suction electrode using a 1 mL syringe via 2 mm outer diameter/1 mm inner diameter silicon tubing (Figure 2A).
2. Time-lapse imaging.
   a. Move the muscle of interest (here we use muscle 6 of the A3 segment) to the center of view by moving the confocal stage under a low magnification (Nikon Microscope Lens Plan Apo 10X 0.3 NA DIC L) air objective coupled with diascopic light.
      i. Switch to a 60X 1.0 NA (Nikon CFI Apo NIR 60X 1.0 NA W) water immersion objective.
      ii. Adjust the focus under wide-field illumination using excitation light appropriate for red fluorophores to locate the mScarlet signal at motor neuron terminals.

   Note: Make sure to submerge the 60× objective in the imaging solution, otherwise add more solution between the lens and imaging sample.

   Note: All imaging procedures are performed at 20°C.

   b. Switch the emission output to confocal detector. Turn on the FITC (488 nm excitation) channel for GCaMP8f, and TRITC (561 nm excitation) for mScarlet.

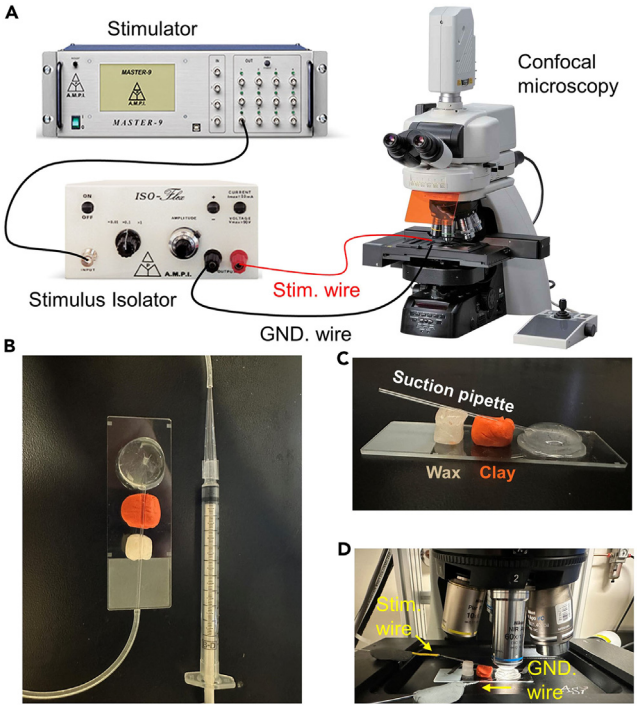

Figure 2. Set up of resonant scanning microscope and stimulation programmer with Drosophila larval preparation

(A) Example of hardware setup for confocal Ca^{2+} imaging paired with electrophysiological stimulation of motor neurons.

(B) Top view of imaging chamber and nerve suction electrode and syringe.

(C) Side view of the imaging chamber. A combination of orthodontic wax and modeling clay provides optimal support and flexibility for the suction electrode.

(D) Side view of imaging chamber under the confocal objective. Note the ground wire (GND) is submerged in the recording solution and stimulation (stim) wire is inserted into the suction electrode, each of which is fixed to the confocal stage with modeling clay.

i. For GCaMP8f imaging, the estimated rise time constant is 25 ms (equivalent to 20 Hz). Nyquist' theorem dictates a minimum sampling rate of 40 Hz, and a higher sampling rate yields a higher temporal resolution at the cost of the signal to noise ratio.

ii. For fast area scanning: To achieve a sufficient sampling frequency with a confocal microscope, use the resonant scanning mode if available on the microscope to acquire area scan data.

Note: For the Nikon A1R confocal system we use, change the "averaging" setting to "no averaging", the "channel" mode to "simultaneous", and make the scan area size 256 × 128. Go *to A1plus Pad> Averaging Speed> 1, Channel mode> 1, Scanning> Resonant, Settings> Scanning direction> bidirectional.* Using these settings, we achieve 59.6 frames-per-second (fps) for presynaptic Ca^{2+} imaging (Figure 3A). These settings can be modified to obtain a desired sampling rate if a different system is used.

iii. To acquire line scan data: If the confocal microscope is not equipped with a resonant scanner, then use line scanning under Galvano scan mode. Switch the scan area to single line mode (128×1 px) and draw a line across the desired synaptic bouton.

Note: Here, we use the Galvano scan mode with no averaging and the fastest dwell time to achieve a maximum scan speed of 521 fps for best assessment of calcium signal dynamics (Figure 3B). Changing parameter such as the "averaging" and "dwell time" settings can increase the signal to noise ratio but may yield a lower temporal resolution.

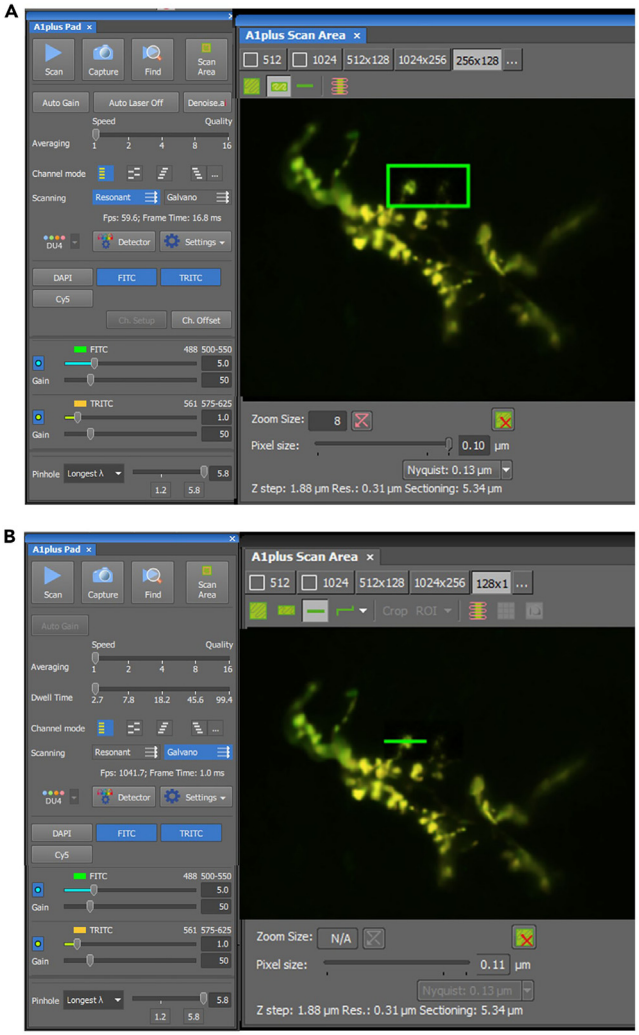

Figure 3. Confocal microscopy acquisition settings using Nikon NIS Elements

(A) Software setup for resonant area scan imaging. NIS Elements (5.41.02) is shown here. Software interface will vary depending on company and version.

(B) Software setup for line scan imaging.

c. To stimulate motor neurons, use an electrophysiological stimulation system to program a 1-ms stimulus duration at a frequency of 1 Hz. Gradually increase the stimulation strength until flashing of the GCaMP8f signal is observed.

Note: Our system uses a pulse stimulator coupled with the stimulation isolator (Iso-Flex) for stimulation control. Alternatively, control the stimulation using a computer and the associated digidata board.

d. Begin time-lapse imaging for 30 s and save the file for later analysis (Methods video S1).

⚠ CRITICAL: Presynaptic GCaMP8f signals can be weak and may not be detectable under lower extracellular Ca^{2+} concentrations (e.g., 0.4 mM). In most cases, 1.5 mM is sufficient for Ca^{2+} detection; however, we recommend acquiring the image first and performing fluorescent intensity analysis to determine if there is a response. If there is no or low responses, increase the Ca^{2+} concentration accordingly.

Part 2. Quantal Ca^{2+} imaging using SynapGCaMP8f

Timing: 1 h per larvae

This section describes the steps necessary to acquire postsynaptic Ca^{2+} signals.

3. Locate the target region under the confocal microscope.
   a. Place the prepared sample in the center of the confocal stage as described in **A1a,b** above. Performed all imaging procedures at 20°C.
   b. Use diascopic light or fluorescence light to identify the NMJ target region of the sample (segment A3, muscle 6) and center the field of view.
4. **Image acquisition** (troubleshooting 1).
   a. Open the NIS Elements acquisition software.
   b. Turn off the diascopic light and turn on the FITC (488 nm excitation) channel coupled with the 435–460 nm excitation filter.
   c. Turn on only the FITC channel, leaving the other channels off.
   d. Use live scanning to preview the image.
   e. Turn the fine adjust knob to focus on the NMJ GCaMP8f signal.
   f. Set the pinhole of the FITC (488 nm excitation) channel to 3 AU (set *pinhole> longest λ > 3 AU* in the Ni-S Elements software*)*. We use 1%–2% laser power and have not noticed significant photobleaching.
   g. Adjust the *FITC> gain* to adjust the preferred signal intensity.
   h. Set the scan area to 256×256 pixels in the software and set the Zoom-in index to 8×.
   i. Move the scan area to cover 3–4 terminal boutons of the muscle target (segment A3, muscle 6, choose either motor neuron Ib or Is based on NMJ size (Figure 7A).
   j. Set the average speed of the confocal microscope to 1 and select Resonant scanning as the scanning mode. Set the scanning direction to "bidirectional".

   Note: Using a Nikon A1R confocal, go to *A1plus Pad> Averaging Speed> 1, Channel mode> 1, Scanning> Resonant, Settings> Scanning direction> bidirectional*. Check if the fps is at least 60. If below 60, reduce the scanning size to increase the frame rate. These settings can be changed for different microscope settings accordingly to achieve the preferred frame rate.

   k. Set up time-lapse imaging for a 1-min duration and begin acquisition (Methods video S2).
   l. Save the file.

   Note: For this recording, a fps = 60–100 is preferred. Setting the scan area to a rectangle shape can increase the fps in some cases.

Part 3. Image analysis for either pre- or post-synaptic Ca^{2+} imaging

Timing: 30 min per sample

This section describes the steps necessary to analyze pre- and post-synaptic Ca^{2+} signals.

5. **Area scan:** Analyze the area scan image by importing into ImageJ (troubleshooting 2).
   a. Check the alignment of frames. Only use the frames when they are positionally stable across frames. If there is drifting among the frames, crop out those frames.
   b. Draw a region of interest (ROI) using ImageJ (*freehand selections* tool) surrounding 1–2 terminal boutons (Figure 4A).
   c. Save ROI: (press T) to *ROI manager> More> Multi Measure* (mean intensity of selected ROI)> export the results to an Excel file.

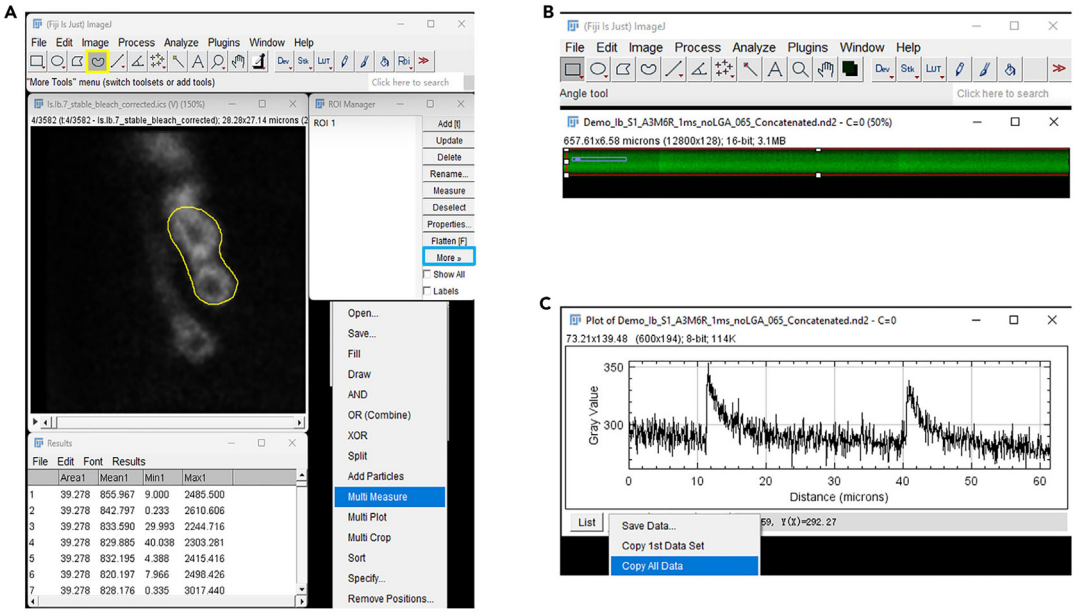

Figure 4. Quantal SynapGCaMP8f analysis using Fiji ImageJ

(A) Fiji software control panel for selection of ROI and measurement of intensity across multiple frames.

(B) Selection of rectangular ROI for 1D concatenated line scan images.

(C) Example result of profile plot results for 1D concatenated line scan images.

6. **Line scan:** Analyze the line scan image by importing into Image J.
   a. Convert the time-lapse line scan data into a 1D concatenated image using Nikon NIS Elements software.

   Note: For alternative confocal systems, there may be other software options available to achieve the same function. Unconcatenated images can also be processed by ImageJ in the form of multiple frames.

   b. Open the concatenated image with ImageJ. Turn the image 90° left to make it horizontal (Figure 4B).
   c. Select the ROI with the "rectangle" tool, then go to *Analyze* > *plot profile* (control + K). The plot gives the mean intensity profile of each line inside the selected rectangular area (Figure 4C).
   d. Export all data to an Excel sheet.
7. Ca^{2+} fluorescence intensity analysis using Excel.
   a. Calculate baseline fluorescence intensity (F) using the function: =PERCENTILE.INC(array,0.1).
   b. Calculate ΔF/F using the function: *=(Data point – F)/F.*
   c. Go to *Insert* > *line* > *plot ΔF/F.* Visualize the Ca^{2+} intensity traces.

   Note: When calculating baseline F in Step 7.a, for each data point, *array* = the continuous 100 data points starting from each data point.

8. Ca^{2+} transient amplitude and kinetic analysis using MiniAnalysis.
   a. Copy the single column of "mean intensity" data into a separate Excel sheet, save it as a Text (Tab delimited) format file (Demo.txt).
   b. Open the Demo.txt file in Clampfit. Set the Units as "Au", Acquisition Mode as "Gap-Free" and input the correct sampling interval per signal (Figure 5A).
   c. In Clampfit, save the Demo.txt file as ABF 1.8 (integer) format (Demo.abf) so it can be processed by MiniAnalysis.

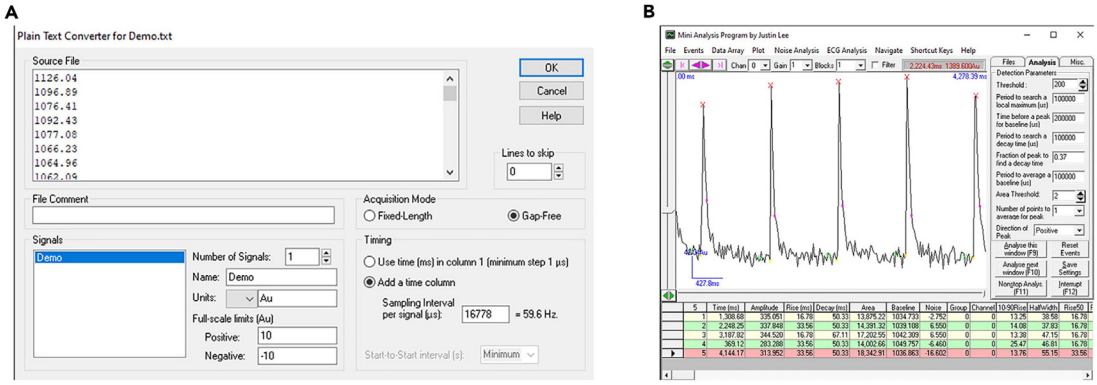

Figure 5. Ca^{2+} intensity peak-detection using MiniAnalysis

(A) Clampfit (11.2) software interface for importing intensity trace data in .txt format. Note the setting of Units, Acquisition Mode, and timing is flexible.

(B) Mini Analysis (6.0.7) interface for detecting peaks of intensity trace data in .abf format. The detection parameter should be adjusted accordingly to best catch the peak of a given trace.

d. Open the Demo.abf with MiniAnalysis, measure the peak by setting the detection parameters at appropriate values so that the baseline and peak can be properly detected.

Note: For example, we set "Threshold" at 50 (a value higher than maximum noise but lower than the minimum signal); "Period to search a local maximum" at 100 ms; "Time before a peak for baseline" at 100 ms; "Period to search a decay time" at 200 ms; "Period to average a baseline" at 100 msec.

⚠ CRITICAL: These values, especially the "Threshold" value, should be adjusted according to the level of noise and dynamics of the empirical trace until the signal spikes can be accurately detected. MiniAnalysis generates the information about the peak amplitude, baseline level and rise & decay time constants (Figure 5B).

Note: Step 3 and Step 4 are interchangeable.

EXPECTED OUTCOMES

For successful recordings of presynaptic mScar8f (Figure 6A) under 1.5 mM extracellular Ca2+ saline, a single action potential is expected to yield ~30% ΔF/F0 in the GCaMP8f signal and no significant change in mScarlet fluorescence. Low frequency resonant area scan generates a smoother signal trace compared to line scanning. This signal represents the response of one or more whole boutons with high signal to noise ratios but low temporal resolution (Figures 6B and 6C; see Methods video S1). High frequency Galvano line scanning generates a signal trace representing a line of pixel-sized area with high temporal resolution but low signal to noise ratio (Figure 6D). For successful postsynaptic quantal Ca2+ imaging using confocal microscopy, the ΔF/F signal should be around 0.3–1.3 in 0.4 mM Ca2+ extracellular saline (Figures 7B and 7C), which corresponds to 0.2–1.5 mV mEPSP amplitudes recorded by sharp electrode current clamp electrophysiology (Figure 7D). The ΔF/F traces should be smooth, with little noise, and each quantal peak should be clear and distinguishable from the baseline signal (Figure 7C; Methods video S2).

LIMITATIONS

Due to the point scanning nature intrinsic to confocal microscopy, the frame rate is limited to area scan rates of ~8 fps when performing postsynaptic Ca^{2+} imaging in the described setup. Hence, line scanning is necessary to achieve higher frame rates. However, if the microscope is equipped with a resonant scanner, the frame rate for area scans can be enhanced to 60–100 fps. In addition, drifting of the ROI over time can occur due to muscle movement and/or stage vibration; software

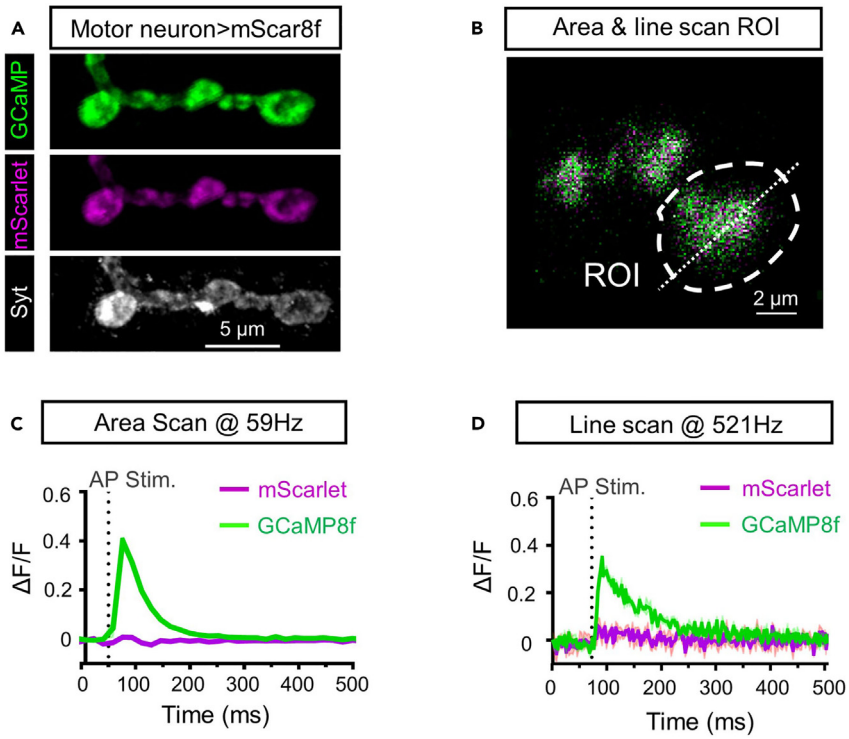

Figure 6. Example images and quantified presynaptic mScar8f signals

(A) Immunostaining of motor neuron expressing mScar8f. Scale bar: 5 µm.

(B) Selection of ROI for area scan and line scan measurements. Scale bar: 2 µm.

(C) Example traces of resonant area scan data at 59 Hz. Traces shown are an average of 5 continuous stimuli at 1 Hz, with shadow representing the +/-SEM. Dash line indicates the timing of electrical stimuli.

(D) Example traces of line scan data at 521 Hz. The average of 5 continuous traces elicited by 1 Hz stimulation is shown, with the shadow representing the +/-SEM.

such as NIS Elements software and Huygens SVI deconvolution software can help to correct for these artifacts. Finally, GCaMP8f might induce toxicity when highly expressed and combined with certain genetic mutations.

TROUBLESHOOTING

Problem 1

Sample exhibits XY drifting over time (as in step 4 above).

Potential solution

- Check the sample preparation, make sure the tissue is secured by pins, and the glass slide is secured by the stage pins.
- Check the air table that is holding the microscope and make sure it is turned on and stable.
- Check the microscope settings such as image area, Z-axis setting, and Piezo settings.
- Wait ~5 min after dissecting the preparation for the sample to stabilize.
- Use software to correct for the drift post-acquisition (e.g., *NIE analysis software > Auto alignment feature).*

Problem 2

GCaMP8f signals are noisy, and/or signals are difficult to distinguish from background (as in step 5 above).

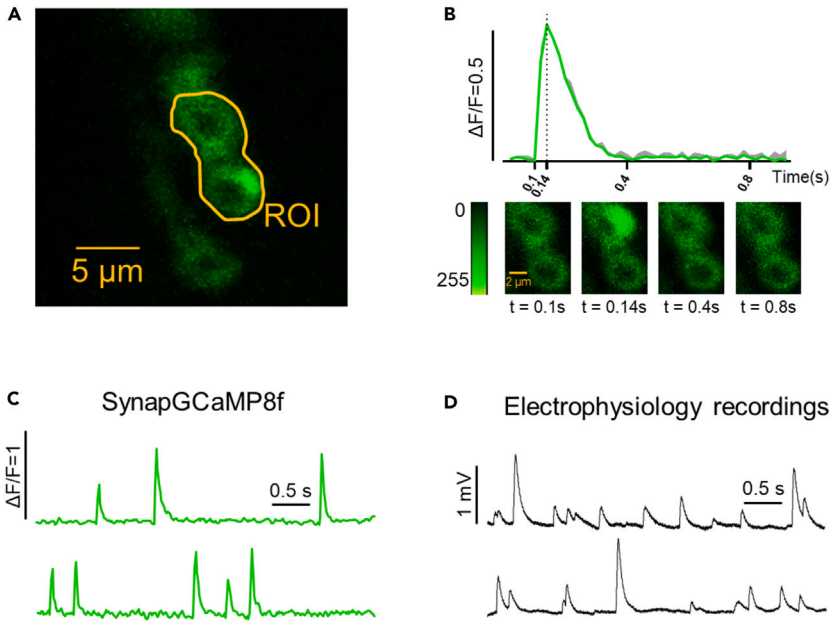

Figure 7. Example images and quantified postsynaptic SynapGCaMP8f signals

(A) Representative images of boutons expressing SynapGCaMP8f in the postsynaptic compartment. Scale bar: 5 μm.

(B) Quantification of fluorescence intensity changes in single spontaneous Ca^{2+} signals reported by SynapGCaMP8f. Data are represented as mean +/- SEM. Scale bar: 2 μm.

(C) Representative traces of spontaneous postsynaptic Ca^{2+} transients. Scale bars: ΔF/F = 1; 0.5 s.

(D) Representative recordings of miniature excitatory postsynaptic potentials (mEPSPs) at the Drosophila NMJ. Scale bars: 1 mV; 0.5 s.

Potential solution

- Check the lighting in the recording room and make sure the environment is dark. Turn off all lights if necessary.
- Check the gain and pinhole and to calibrate responses.
- Check the filter and make sure it is the correct one.
- Check the sample to ensure it is responsive to touch and still maintains a stable resting potential through electrophysiological recording.
- Choose another terminal bouton at a different muscle segment; this might have a better signal response.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dion Dickman (dickman@usc.edu).

Technical contact

Technical support and information will be provided by the technical contacts, Jiawen Chen (jiawenc@usc.edu) and Kaikai He (kaikaihe@usc.edu).

Materials availability

The *Drosophila* transgenic probes relevant to this study (SynapGCaMP8f and mScar8f) are available free of charge to researchers upon request to the lead contact.

Data and code availability

This study did not generate datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102832.

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AUTHOR CONTRIBUTIONS

D.D. conceptualized the study. Y.H. formulated the method. J.C. and K.H. standardized and refined the experimental and data analysis procedures and wrote the manuscript. All authors reviewed, revised, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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