Chapter 16

Imaging Synaptic Glutamate Release with Two-Photon Microscopy in Organotypic Slice Cultures

Céline D. Dürst and Thomas G. Oertner

Abstract

The strength of an excitatory synapse relies on the amount of glutamate it releases and on the amount of postsynaptic receptors responding to the released glutamate. Here we describe a strategy to investigate presynaptic release independently of postsynaptic receptors, using a genetically encoded glutamate indicator (GEGI) such as iGluSnFR to measure synaptic transmission in rodent organotypic slice cultures. We express the iGluSnFR in CA3 pyramidal cells and perform two-photon glutamate imaging on individual Schaffer collateral boutons in CA1. Sparse labeling is achieved via transfection of pyramidal cells in organotypic hippocampal cultures, and imaging of evoked glutamate transients with two-photon laser scanning microscopy. A spiral scan path over an individual presynaptic bouton allows to sample at high temporal resolution the local release site in order to capture the peak of iGluSnFR transients.

Key words Genetically encoded glutamate indicators (GEGIs), iGluSnFR, Synaptic transmission, Glutamate, Organotypic slice cultures, Two-photon imaging

1 Introduction

In the mammalian cortex, glutamatergic excitatory synapses convert presynaptic action potentials into chemical signals that are sensed by postsynaptic glutamate receptors. The smallest information transmission unit at chemical synapses is the release of a single synaptic vesicle filled with neurotransmitters. Its release occurs in a probabilistic manner due to the stochastic nature of the release machinery. Release probability is typically assessed from electrophysiological recordings at the soma of a postsynaptic neuron. However, a pair of connected neurons is usually connected by more than one synapse, precluding the isolation of single-synapse responses. Furthermore, postsynaptic changes such as phosphorylation, desensitization, saturation, lateral diffusion, and internalization of postsynaptic receptors further contribute to the variability of responses measured in the postsynaptic cell. Optical measurements

Jana Dahlmanns and Marc Dahlmanns (eds.), *Synaptic Vesicles: Methods and Protocols*,
Methods in Molecular Biology, vol. 2417, https://doi.org/10.1007/978-1-0716-1916-2_16,

based on fluorescence microscopy are therefore ideal to assess synaptic physiology, as responses from individual synapses can be isolated despite other synapses on the same neuron being active simultaneously. Previous imaging studies used postsynaptic calcium transients as a read-out of synaptic efficacy. However, more recently, thanks to the development of genetically encoded glutamate indicators (GEGIs) with suitable affinity and kinetics to detect cleft glutamate, it is now possible to monitor glutamate transients at the synaptic cleft and thereby isolating presynaptic release of glutamate from postsynaptic parameters. iGluSnFR [1] is an intensity-based glutamate sensing fluorescent reporter constructed from *E. coli* periplasmic glutamate binding protein Gltl and a circularly permuted (cp) EGFP and is the first GEGI that gained great popularity among neuroscientists for investigating glutamate dynamics. In particular, it is well suitable for monitoring cleft glutamate transients thanks to its high fluorescence dynamic range and affinity ($\Delta F/F_{max}$ of 4.5 and K_d of ~4 μM). Further variants with varied biophysical properties displaying different kinetics, affinities, and emission profiles [2–4] enable researchers to select the most appropriate sensor depending on the biological question (bulk tissue vs. single synapse) and imaging system (camera, galvanometric laser scanner, or resonant scanner). Here, we introduce a methodology to investigate presynaptic release, using the GEGI iGluSnFR, to measure glutamate release in the synaptic cleft (Fig. 1). To conduct a single-synapse study, sparse and cell-specific labeling are a prerequisite, making single-cell electroporation the method of choice for GEGI expression. We express the iGluSnFR in CA3 pyramidal cells of rat hippocampal organotypic slice cultures. We elicit individual action potentials in iGluSnFR-expressing CA3 cells and simultaneously monitor released glutamate from individual Schaffer collateral boutons in CA1. To accurately determine the amplitude of individual glutamate transients, we use spiral scans on individual boutons, which provide an increased temporal resolution and a better signal-to-noise ratio.

2 Materials

Experiments involving organ explant must conform to relevant Institutional and National regulations. Follow all waste disposal regulations for disposing waste materials. All solutions have to be prepared using ultrapure water.

2.1 Organotypic Slice Cultures

1. Slice cultures from rodent hippocampus.
2. Slice culture medium: 394 mL MEM, 20% (v/v) Heat-inactivated horse serum, 1 mM L-glutamine, 0.01 mg/mL Insulin, 14.5 mM NaCl, 2 mM $MgSO_4$, 1.44 mM $CaCl_2$,

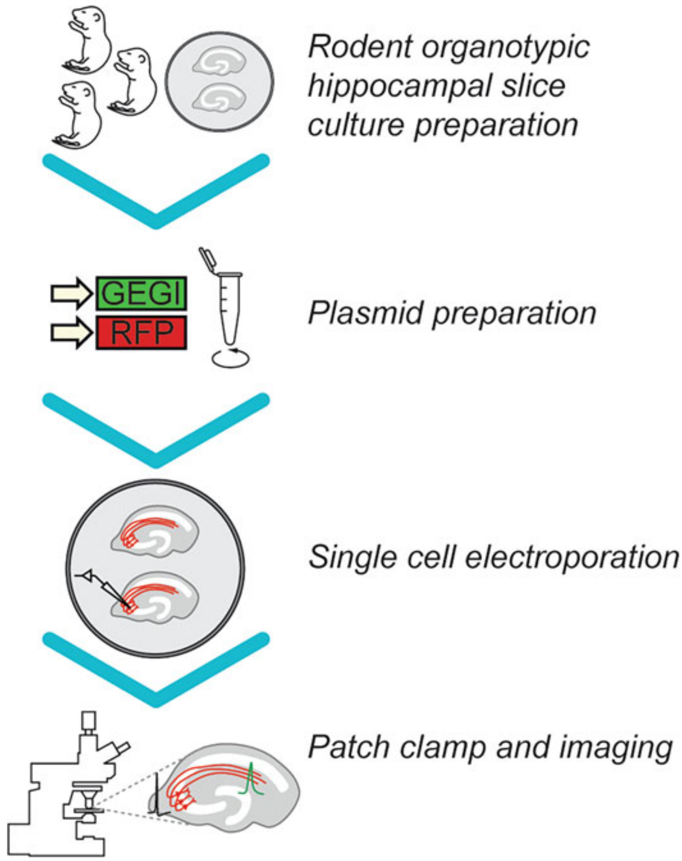

Fig. 1 Experimental workflow of cleft glutamate transient measurements with GEGIs in organotypic hippocampal slice cultures

0.00125% Ascorbic acid, 13 mM D-glucose. Medium has to be sterile filtered (0.2 μm pore size) and stored at 4 °C for up to 4 weeks.

2.2 Single-Cell Electroporation

2.2.1 Electroporation Equipment

1. Upright microscope with motorized stage, CCD camera and IR-DIC (infrared differential interference contrast) or Dodt contrast.
2. 20× or 40× water immersion objective.
3. 4× zoom lens system (0.5–2.0× magnification range).
4. Vibration isolation table.
5. Axoporator 800A with HL-U pipette holder.
6. Motorized micromanipulators.
7. Microscope chamber made of a glass microscope slide (70 × 100 × 1 mm) onto which a Teflon ring (inner diameter ~ 35 mm, 2 mm high) is fixed with silicone aquarium sealant.

8. Plastic syringe body (1 mL) as disposable mouthpiece, connected through a Luer 1-way stopcock and thin silicone tubing to the electrode holder.
9. Headphones.
10. Silver wire (~0.25 mm diameter).
11. Forceps.
12. Hot bead sterilizer.
13. Incubator (37 °C; 5% CO_2) with rapid humidity recovery and copper chamber.
14. Vertical micropipette puller.
15. Thin-walled borosilicate glass capillaries.
16. Tissue culture dishes (60 mm, sterile).
17. Ultrafree centrifugal filter units.
18. Slice culture transduction solution: 10 mM HEPES, 145 mM NaCl, 25 mM D-glucose, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$. Measure the pH using a pH-meter and adjust to pH 7.4 by adding NaOH or HCl. Measure the osmolality with a micro-osmometer and ensure that the osmolality is between 310 and 320 mOsm/kg. Solution must be sterile filtered (0.2 μm pore size), stored at 4 °C for up to 6 months and pre-warmed to 37 °C before use.

2.3 Functional Imaging of Synaptic Transmission

1. Perfusion chamber with a quartz glass bottom.
2. In-line heating of the perfusion solution.
3. Vertical micropipette puller.
4. Micropipettes for whole-cell recording (borosilicate glass with filament, 1.5 mm O.D.).
5. Recording solution, artificial cerebrospinal fluid (ACSF): 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 127 mM NaCl, 25 mM D-glucose, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH adjusted to 7.4, ACSF has to be saturated with 95% O_2 and 5% CO_2. Osmolality should be between 310 and 320 mOsm/kg. Store for max. 1 week at 4 °C. Bubble with carbogen (95% O_2, 5% CO_2) during warm-up to prevent Ca^{2+} precipitation. Maintain perfusion reservoir at 34 °C to prevent bubble formation in recording chamber.
6. K-gluconate-based intracellular solution: 10 mM HEPES, 135 mM K-gluconate, 0.2 mM EGTA, 4 mM $MgCl_2$, 4 mM Na_2-ATP, 0.4 mM Na-GTP, 10 mM Na_2-phosphocreatine, 3 mM L-ascorbic acid, pH adjusted to 7.2 with KOH. Osmolality should be between 290 and 300 mOsm/kg. Solution has to be sterile filtered (0.2 μm pore size), stored at −20 °C. Aliquot in Eppendorf tubes can be stored at −80 °C for max. 6 months. Store on ice during the experiment to slow down ATP hydrolysis.

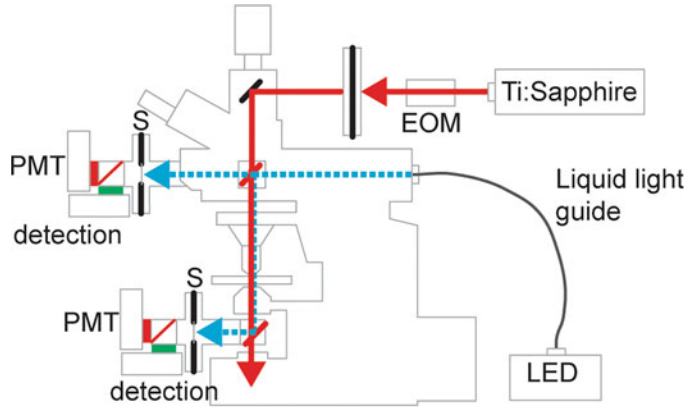

Fig. 2 Setup for simultaneous imaging and somatic induction of individual action potentials. A Ti:Sapphire laser system with dispersion compensation is coupled in through an electro-optical modulator (EOM). Red and green fluorescence is detected through the objective and through the oil immersion condenser (NA 1.4) using two pairs of photomultiplier tubes. 560 DXCR dichroic mirrors and 525/50 and 607/70 emission filters are used to separate green and red fluorescence. Excitation light is blocked by short-pass filters. A high-power blue LED coupled in through the epifluorescence port excites the red morphological marker allowing visualization of the transfected cells. During epifluorescence illumination, PMTs are protected by a mechanical shutter (S)

2.3.1 *Imaging Setup*

1. Two-photon microscope based on an Olympus BX51WI microscope (Fig. 2).
2. Ti:Sapphire laser system with dispersion compensation.
3. Electro-optical modulator.
4. pE-4000 LED light source for epifluorescence.
5. 3× telescope, 5 mm scan mirrors.
6. Compound scan lens ($f = 50$ mm) [5].
7. Dual camera port with IR mirror.
8. Water immersion objective (60×, NA 1.0).
9. Two pairs of photomultiplier tubes.
10. Oil immersion condenser (NA 1.4) (*see* **Note 1**).
11. 560 DXCR dichroic mirrors.
12. 525/50 and 607/70 emission filters.
13. Short-pass filters.
14. NS45B shutter.
15. MP-285 micromanipulator.
16. Motorized stage.
17. Peltier-heating of the oil immersion condenser.

18. Data acquisition boards.
19. Image acquisition software ScanImage [6].

2.3.2 Electrophysiology Setup

1. pE-4000 LED light source for epifluorescence.
2. Infrared Dodt contrast.
3. Patch-clamp amplifier MultiClamp 700B.
4. Electrophysiology software Ephus [7] or Wavesurfer written in MATLAB.
5. Microelectrode manipulator.

3 Methods

Single-cell electroporation is the method of choice to achieve very sparse expression of glutamate sensors in organotypic culture of brain tissue (Figs. 3 and 4b). Sparse expression is necessary to follow the axon of a patch-clamped sensor-expressing neuron to a distal projection area such as CA1. Two-photon microscopy is typically used to detect weak functional signals deep in intact tissue. For expression in neurons, we clone the GEGIs behind the human synapsin 1 promoter and electroporate single neurons in organotypic slice cultures of rat hippocampus. GEGIs are relatively dim in

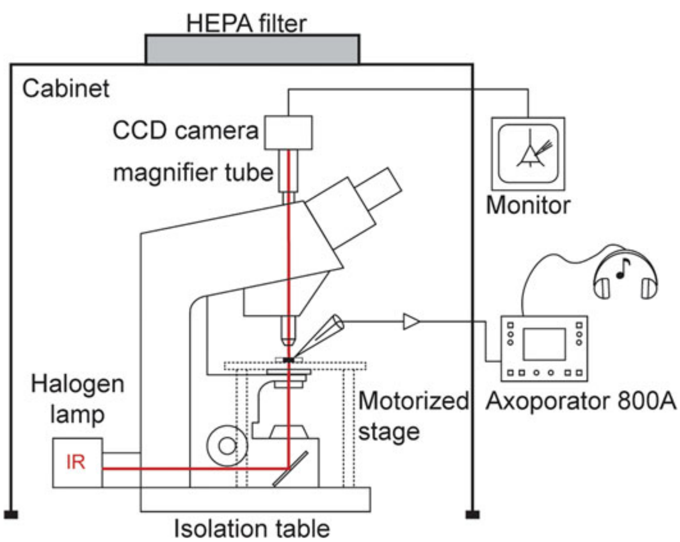

Fig. 3 Electroporation set up. An upright microscope with DIC (Differential interference contrast) and a low magnification objective (20× with a variable magnifier tube before the camera) is used for the electroporation procedure. The electroporation setup is under a laminar flow cabinet with HEPA (high-efficiency particulate arrestance) filter blowing clean air downwards to minimize contaminations. The microscope is on an active anti-vibration table, mechanically isolated from the vibrations generated by the fan in the filter

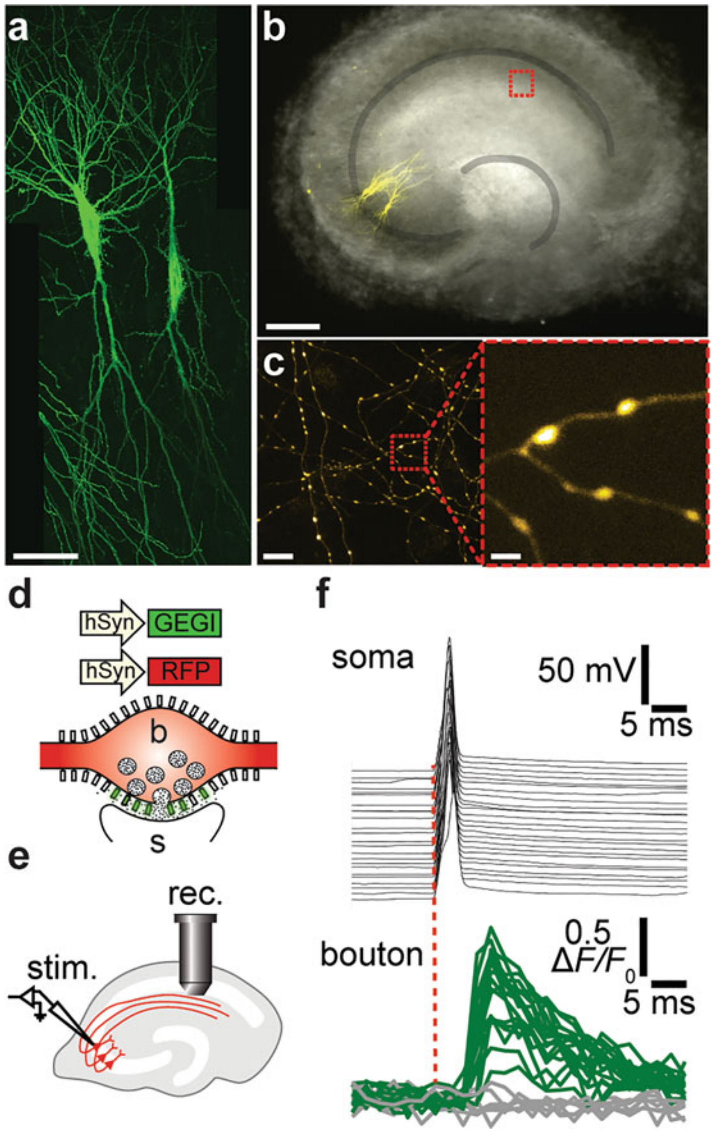

Fig. 4 iGluSnFR expression in CA3 pyramidal cells in organotypic slice cultures of rat hippocampus. (**a**) Maximum intensity projection of two-photon images of CA3 pyramidal neurons expressing a GECI (here, iGlu$_u$ [9]) 4 days after electroporation. The GEGI has its fluorescence mainly localized to the plasma membrane over the entire cell. The scale bar represents 50 μm. (**b**) Transmitted light image (dark field) of a transfected organotypic culture merged with a wide-field fluorescence image showing three transfected CA3 neurons co-expressing tdimer2 and iGluSnFR. The area for synaptic imaging is indicated (red dotted box). Scale bar represents 500 μm. (**c**) Maximum intensity projection of two-photon images of CA3 axons co-expressing tdimer2 and iGluSnFR in CA1 *stratum radiatum* (cells not identical to panel **b**). Scale bar represents 10 μm (*left panel*) and 1 μm (*right panel*). (**d**) Cartoon of a typical Schaffer collateral synapse showing a transfected CA3 bouton (b) synapsing onto a dendritic spine (s). The red fluorescent protein tdimer2 labels the axoplasm while the membrane-anchored iGluSnFR is exposed to the extracellular space. (**e**) Schematic representation of a hippocampal slice with transfected and patch-clamped CA3 pyramidal cell and the optically monitored area in CA1. (**f**) Action potentials are elicited in a transfected neuron by somatic current injections, and glutamate release is simultaneously optically recorded (GEGI fluorescence) from a single Schaffer collateral bouton in

the absence of glutamate, making it difficult to focus on small structures such as axonal boutons. Therefore, we routinely use co-expression of a bright red fluorescent protein (tdimer2 or tdTomato) to label the cytoplasm and follow the axon through the tissue.

3.1 Culture Preparation

For slice cultures preparation from rodent hippocampus, refer to [8] which provides a detailed step-by-step description. Change the medium of the slice culture twice a week (*see* **Note 2**).

3.2 Choice of GEGI Variant

The affinity of the GEGI, expressed as K_d, must be appropriate for the expected glutamate concentration in the cellular or tissue environment. If the goal of the experiment is to distinguish synaptic failures (no glutamate release, no GEGI signal) from successes (stimulation-induced glutamate release), a very high affinity is desirable. If a linear response is important, for instance to estimate the number of vesicles released simultaneously, a slightly lower affinity might be advantageous.

3.3 Neuron Transfection

3.3.1 DNA and Plasmids Preparation

1. Sterile filter an aliquot (0.5 mL) of K-gluconate-based intracellular solution through a Millipore Ultrafree centrifugal unit by centrifugation at 16,000 × g for several seconds in a table-top centrifuge at 4 °C (*see* **Note 3**).
2. Remove the filter insert and add the plasmid DNA to the desired concentration. Use 40–50 ng/μL for pCI.syn.iGluSnFR (a gift from Loren Looger, addgene plasmid #41732) (*see* **Note 4**). For proper visualization of the transfected cells, mix a plasmid encoding for a red fluorescent protein (for example tdimer2; 20 ng/μL) with the GEGI plasmid to achieve co-expression (*see* **Note 5**).

3.3.2 Electroporation

1. Ground the electrodes and coat silver wire tips by bathing them in a saturated Cl_3Fe solution for at least 30 min or overnight prior to first usage.
2. Pull electroporation pipettes using a micropipette puller. Pull thin-walled borosilicate capillaries to obtain a resistance of 10–15 MΩ when filled with the K-gluconate-based intracellular solution (*see* **Note 6**).
3. Back-fill an electroporation pipette with ~2 μL of plasmid mix solution for each slice to electroporate. Store the remaining

Fig. 4 (continued) CA1 showing a broad distribution of amplitudes and occasional failures. Images were acquired at 500 Hz at 34 °C. Individual trials are classified as successes if the peak amplitude of the GEGI transient is $>2\sigma$ (green traces) and as failures when the peak amplitude is $<2\sigma$ (gray traces). Note the propagation delay between presynaptic action potentials and glutamate release events at distal bouton. Figure and legend reproduced from [2] with permission from Nature Protocols electroporation solution containing the plasmid mix at −20 °C for subsequent reuse (*see* **Note 7**).

4. Pipette 1 mL of transduction solution (37 °C) into the microscope chamber.
5. Place the forceps into the hot bead sterilizer for ~10 s before any handling of insert.
6. Transfer one slice culture insert into the chamber by using the sterile forceps and add the transduction solution on top of the slice culture for the water immersion objective.
7. Cover the microscope chamber with a sterile 60-mm dish and transfer the microscope chamber to the microscope to proceed to single-cell electroporation (*see* **Note 8**).
8. Apply positive pressure to the pipette to approach a cell to electroporate.
9. Monitor by audio output of the Axoporator 800A amplifier the tip resistance of the electroporation pipette throughout the entire procedure.
10. Similarly, to patch-clamp recording technique, positive pressure on the electroporation pipette is maintained to keep the tip of the pipette clean while penetrating the tissue. The resistance should be between 10 and 15 MΩ (*see* **Note 9**).
11. Move the tip of the electroporation pipette close to a cell of interest while reducing the positive pressure.
12. Approach the cell without sealing the electrode with membranes from other cells in the tissue (*see* **Note 10**).
13. Touch the plasma membrane, which causes a rise in tip resistance indicated by a rise in pitch.

    Immediately release the pressure and wait for the resistance to increase to 25–40 MΩ (*see* **Note 11**). Do not apply suction and avoid the formation of a GΩ seal as the pulse train might kill the cell.
14. Apply a pulse train (voltage: −12 V, frequency: 50 Hz, pulse width: 500 μs, train duration: 500 ms) (*see* **Note 12**).
15. Slowly retract the pipette and begin applying very light positive pressure once the pipette is retracted 2–4 μm away from the soma (*see* **Note 13**).
16. Increase the positive pressure at more considerable distances from the electroporated cell in order to maintain the pipette tip clean.
17. Using the same electrode, repeat those last eight steps for each cell to be electroporated (*see* **Note 14**).

18. After electroporation of three to four neurons, cover the chamber with a 60-mm dish and transfer back to the tissue culture hood (*see* **Note 15**).
19. Remove all transfection solution and return the insert to the slice culture medium. Typically, 2–4 days are needed for optimal expression levels of GEGIs in hippocampal organotypic slices (*see* **Note 16**).

3.4 Functional Imaging of Synaptic Transmission

1. Ensure that electroporated CA3 neurons are clearly visible under a stereomicroscope (5× objective, DsRed filter set) 2–4 days after electroporation (Fig. 4b).
2. Tune the Ti:Sapphire laser to 980 nm for simultaneous excitation of the red fluorescent protein such as tdimer2 and iGluSnFR (*see* **Note 17**).
3. Start temperature-controlled perfusion system and place the organotypic culture in the recording chamber. Weigh down membrane patch with c-shaped gold wire.
4. Pull patch pipettes (3–5 MΩ) from borosilicate glass capillaries in two stages on vertical puller and back-fill the pipette with internal solution.
5. Approach a transfected CA3 neuron with the patch pipette, switching between red epifluorescence and IR-Dodt contrast (CCD camera) to identify the transfected cell.
6. Establish a GΩ seal and break-in to establish whole-cell configuration (*see* **Note 18**).
7. Move the stage to center the objective in *stratum radiatum*. Search for red fluorescent axons using two-photon excitation (Fig. 4c).
8. Induce a train of 10 action potentials at 100 Hz by injecting current pulses (2–3 ms, 1.5–3.5 nA) 10 times at 100 Hz into the soma and simultaneously acquire rapid frame scans of a single bouton (high zoom, 16 × 16 pixels, 1 ms/line) or line scan (high zoom, 1–2 ms/line).
9. If a bouton shows a train-induced fluorescence increase (green channel), switch to spiral scan mode (Fig. 5a, b; *see* **Note 19**).
10. Acquire single action potentials-induced iGluSnFR transients by injecting somatic current pulses (2–3 ms, 1.5–3.5 nA) at regular intervals (10 s) for ~80 ms, at a sampling rate of 500 Hz (Fig. 4e). A temporal resolution of 500 Hz provides enough temporal resolution for iGluSnFR imaging (Fig. 4f). However, for ultrafast GEGIs [9] a sampling rate of at least 1 kHz might be necessary, whereas for some newer iGluSnFR variants such as SF-iGluSnFR.A184S [3] a slower scanning frequency is sufficient to detect the peak of GEGIs transient (*see* **Note 20**).
11. If necessary, re-adjust for drift between the trials (*see* **Note 21**).

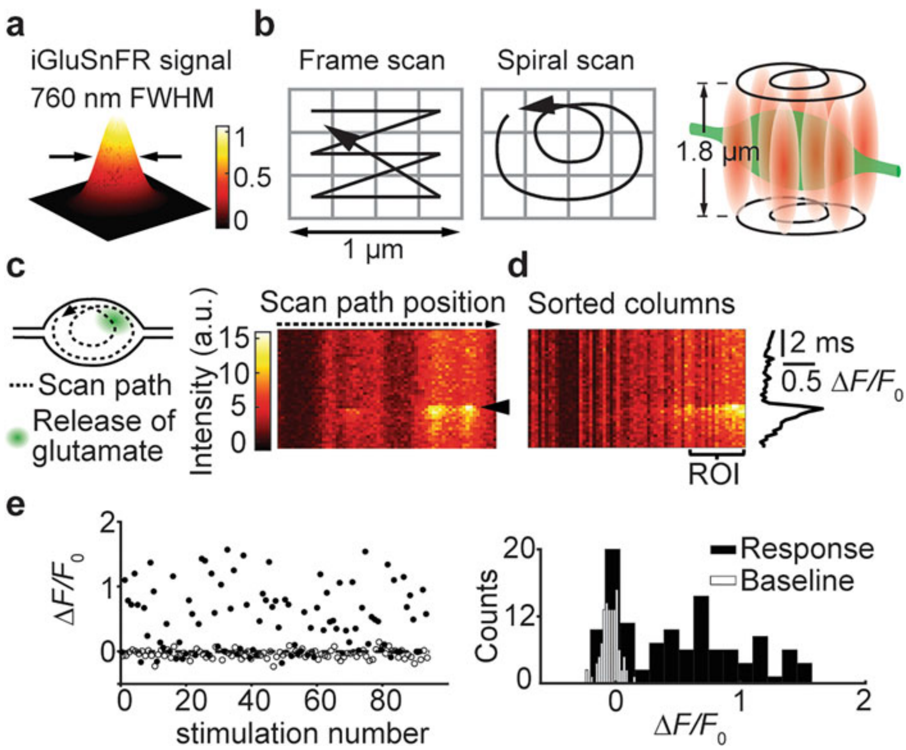

Fig. 5 Signal extraction of GEGI transients from a single Schaffer collateral bouton in CA1. (**a**) The spatial extent of iGluSnFR fluorescence transients is 760 nm, on average (FWHM, short axis of Gaussian fits). No deconvolution was applied. (**b**) Sampling the surface of the bouton by traditional raster scanning requires extreme acceleration of the scan mirrors at the turning points, leading to large positional errors. Spiral scans avoid sharp direction changes (no flyback) and can, therefore, sample the entire bouton surface in 1 or 2 ms. Due to the elongated PSF (1.8 μm in the axial direction), upper and lower surface of a bouton are sampled simultaneously. (**c**) Plotting the unfolded spiral scan lines vs. time (single trial). Raw fluorescence intensity is coded in pseudocolors. A glutamate release event (black arrowhead) from an individual presynaptic terminal occurred and was sampled twice during every spiral scan. (**d**) Only columns with $\Delta F > 1/2$ max (ΔF) are analyzed (ROI, region of interest) resulting in the extracted fluorescence transient (before bleach correction). (**e**) iGluSnFR response amplitude (black markers) of an individual bouton stimulated with single action potentials every 10 s. Note that response amplitudes were constant over time. A short time window before stimulation is analyzed to estimate imaging noise (white markers). The histogram of response amplitudes shows separation between failures of glutamate release (overlap with the baseline histogram) and successes. Figure and legend reproduced from [2] with permission from Nature Protocols

3.5 GEGI Signal Extraction

1. For amplitude extraction, linearize the spiral scans and display them as xt-plots (Fig. 5c).
2. To distinguish successful glutamate release events from failures, perform a statistical comparison of fluorescence fluctuations before stimulation (ΔF baseline, $n = 64$ columns/locations) and response amplitude (ΔF response, $n = 64$ columns/locations). A significant difference suggests a success, whereas lack of significance a failure trial. This classification is preliminary; the final failure analysis is performed after amplitude extraction (**steps 8–11**).

3. Assign a new region of interest (ROI) for each success trial to compensate for eventual lateral drift between individual trials.
4. The spiral scan covering the entire bouton may hit the GEGI transient once or several times per line. Sort the pixel columns (i.e., spatial positions) according to the change in fluorescence (ΔF) in each column (Fig. 5d) (*see* **Note 22**).
5. Include columns with the largest signal ($\Delta F > \Delta F_{max}/2$) in the region of interest (*see* **Note 23**).
6. In failure trials, evaluate identical columns/locations than in the last success trial (*see* **Note 24**).
7. For each bouton, extract the characteristic decay time constant (τ) by fitting a mono-exponential function to the average GEGI fluorescence transient.
8. Estimate the glutamate transient amplitude for every trial by fitting an exponential function to the decay of the fluorescence transient (fixed τ, amplitude as the only free parameter).
9. For each trial, determine the imaging noise (σ) from the baseline of the extracted fluorescence time course (*see* **Note 25**).
10. Classify as "success" trials where average $\Delta F/F_0 > 2\sigma$ above baseline imaging noise, otherwise classify as "failure."
11. Calculate the release probability as the ratio of total number of successes/total number of stimulations (Fig. 5e).

4 Notes

1. To minimize bleaching by excessive excitation, the microscope must be designed to detect emitted photons more efficiently. Using only the objective for fluorescence detection is not enough to achieve single-vesicle sensitivity. Condenser detection (oil immersion, 1.4 NA, large field of view) is essential for the success of single synapse experiments with many trials (Fig. 2). The condenser detection is sensitive to the refractive index of the immersion oil and correct (Köhler) alignment. The oil immersion condenser must be permanently heated (day and night) if a recording temperature above room temperature is desired. This can be achieved with flexible heating pads or Peltier elements.
2. Avoid changing the medium prior to an experiment (~16 h) as this might affect cell excitability.
3. It is important that the DNA-containing solution is not passed through the Millipore Ultrafree centrifugal filter unit.
4. Avoid using very strong promoters (for instance CMV) as they might drive the expression of the plasmids to toxic levels.

5. For different cell types and iGluSnFR variants, the final concentration and optimal expression time have to be determined empirically (typical range: 1–100 ng/μL with this protocol).
6. Ensure constant pipette resistance for reproducible expression. A too high pipette resistance leads to low expression of the plasmids, whereas a too low resistance causes extreme expression levels leading to toxicity problems.
7. Ensure that there are no bubbles in the back-filled pipettes. The back-filled pipettes can be kept in an upright position for up to 2 h before use.
8. Work on an electrophysiology microscope setup in a laminar flow box to prevent contamination (Fig. 3).
9. For reproducible expression level of the plasmids between different electroporation sessions, ensure a constant pipette resistance.
10. If the tip of the pipette seems dirty, withdraw the tip from the tissue and apply strong positive pressure using a syringe. Only once the tip is clean, penetrate again into the tissue while keeping a positive pressure.
11. For more reproducible expression levels of the plasmids between different cells, try to wait for the resistance to increase to a similar value before applying the pulse train.
12. The optimal settings may differ depending on the cell type to electroporate and the desired expression level. Ensure that the pulse is negative otherwise the negatively charged DNA will not be electroporated.
13. To ensure that the constructs are incorporated into the cells, add a fluorescent dye such as Alexa Fluor 594 to the DNA mix. After applying the pulse train to the target neuron, take a fluorescence image to ensure that the DNA solution and fluorescent dye were successfully electroporated.
14. If positive pressure is applied and the tissue surrounding the tip of the electroporation pipette does not appear to be gently blown away, the pipette might be clogged. Clean the tip (away from the tissue) by applying a strong positive pressure with a syringe or use a fresh pipette to pursue the electroporation.
15. To facilitate the search of a bouton that belongs to the stimulated cell, restrict the number of electroporated neurons to three or four in order to make the experiment more effective.
16. The optimal time for a cell to express a given plasmid before starting the experiment has to be determined empirically.
17. As new GEGIs variants (blue, yellow) [3, 4], different combination of GECIs and morphological marker colors can be used according to the available equipment (i.e., laser, filter sets).

18. Eliciting individual action potential in whole-cell configuration of an individual transfected cell as opposed to field stimulation ensures that the GEGI transients originate from the imaged terminal and are not a consequence of glutamate spillover from neighboring terminals.
19. If a galvanometric scanning system is used, the microscope software has to support arbitrary line scans. The code for arbitrary line scans that we developed for our original study is now incorporated in the ScanImage software (Version 2016 and later).
20. Image only for the duration of the GEGI transients to minimize laser exposition. However, the number of trials that can be obtained from a single bouton is limited by the unavoidable bleaching of the indicator molecules and eventual destruction of the release machinery by toxic photoproducts (e.g., oxygen radicals). By using lower laser power, this number can be extended to 300 trials at the cost of a slightly lower SNR. Longer intervals between trials allow replenishing indicator molecules by lateral diffusion, but this strategy is limited by the need for stable whole-cell access during the entire experiment for reliable action potential generation. Furthermore, manual refocusing between trials can lead to substantial bleaching of the indicator. Automated refocusing between trials can minimize this.
21. If the slice is strongly drifting, lower the perfusion rate and ensure that the temperatures of the perfusion solution and of the imaging chamber of the microscope are stable to avoid thermal expansion during the experiment.
22. As opposed to a static ROI, this analysis procedure is robust against minute lateral drift of the tissue between trials and does not require a priori knowledge of the fusion location. As the point spread function (PSF) of a two-photon microscope is typically around 1.8–2 μm along the optical axis, the upper and lower surface of the bouton gets sampled simultaneously and therefore small drifts along the optical axis are unlikely to affect the amplitude of the iGluSnFR transients.
23. The threshold to select pixel columns to be kept in the region of interest is once adjusted according to the noise of the imaging system but should be kept constant for amplitude comparisons between different experiments.
24. If necessary, correct traces for GEGI bleaching prior to amplitude extraction. To this end, fit a mono-exponential or double exponential decay time constant to non-stimulated trials and subtract to the signal of individual trials.

25. In some trials, the baseline fluorescence may show large fluctuations caused by green fluorescent vesicles passing through the axon. Remove these trials from further analysis.

Acknowledgments

The authors thank Iris Ohmert and Sabine Graf for the preparation of organotypic cultures and excellent technical assistance and Mauro Pulin for critical comments on the manuscript. This study was supported by the German Research Foundation through Research Unit FOR 2419 P4, Priority Programs SPP 1665, and Collaborative Research Center SFB 936 B7.

References

1. Marvin JS, Borghuis BG, Tian L et al (2013) An optimized fluorescent probe for visualizing glutamate neurotransmission. Nat Methods 10(2):162–170. https://doi.org/10.1038/nmeth.2333
2. Durst CD, Wiegert JS, Helassa N et al (2019) High-speed imaging of glutamate release with genetically encoded sensors. Nat Protoc 14(5):1401–1424. https://doi.org/10.1038/s41596-019-0143-9
3. Marvin JS, Scholl B, Wilson DE et al (2018) Stability, affinity, and chromatic variants of the glutamate sensor iGluSnFR. Nat Methods 15(11):936–939. https://doi.org/10.1038/s41592-018-0171-3
4. Wu J, Abdelfattah AS, Zhou H et al (2018) Genetically encoded glutamate indicators with altered color and topology. ACS Chem Biol 13(7):1832–1837. https://doi.org/10.1021/acschembio.7b01085
5. Negrean A, Mansvelder HD (2014) Optimal lens design and use in laser-scanning microscopy. Biomed Opt Express 5(5):1588–1609. https://doi.org/10.1364/BOE.5.001588
6. Pologruto TA, Sabatini BL, Svoboda K (2003) ScanImage: flexible software for operating laser scanning microscopes. Biomed Eng Online 2:13. https://doi.org/10.1186/1475-925X-2-13
7. Suter BA, O'Connor T, Iyer V et al (2010) Ephus: multipurpose data acquisition software for neuroscience experiments. Front Neural Circuits 4:100. https://doi.org/10.3389/fncir.2010.00100
8. Gee CE, Ohmert I, Wiegert JS et al (2017) Preparation of slice cultures from rodent hippocampus. Cold Spring Harb Protoc 2017(2). https://doi.org/10.1101/pdb.prot094888
9. Helassa N, Durst CD, Coates C et al (2018) Ultrafast glutamate sensors resolve high-frequency release at Schaffer collateral synapses. Proc Natl Acad Sci U S A 115(21):5594–5599. https://doi.org/10.1073/pnas.1720648115