High-speed imaging of glutamate release with genetically encoded sensors

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EDITORIAL SUMMARY This Protocol describes the design, *in vitro* characterisation and imaging applications of iGluSnFR-based genetically-encoded glutamate indicators (GEGIs) in tissue culture of rat hippocampus

TWEET A new protocol for the design, characterisation and high-speed imaging applications of genetically-encoded glutamate indicators (GEGIs).

COVER TEASER High-speed imaging of glutamate release

Up to three primary research articles where the protocol has been used and/or developed.

1. Helassa, N. et al. Ultrafast glutamate sensors resolve high-frequency release at Schaffer collateral synapses. Proc. Natl. Acad. Sci. U. S. A. 115, 5594–5599 (2018).

2.

3.

Abstract

The strength of an excitatory synapse depends on its ability to release glutamate and on the density of postsynaptic receptors. Genetically-encoded glutamate indicators (GEGIs) allow eavesdropping on synaptic transmission at the level of cleft glutamate to investigate properties of the release machinery in detail. Based on the sensor iGluSnFR, we recently developed accelerated versions that allow investigating synaptic release during 100 Hz trains. Here we describe the detailed procedures for design and characterization of fast iGluSnFR variants *in vitro*, transfection of pyramidal cells in organotypic hippocampal cultures, and imaging of evoked glutamate transients with two-photon laser scanning microscopy. As the released glutamate spreads from a point source - the fusing vesicle - it is possible to localize the vesicle fusion site with a precision exceeding the optical resolution of the microscope. By using a spiral scan path, the temporal resolution can be increased to 1 kHz to capture the peak of fast iGluSnFR transients. The typical time frame for these experiments is 30 min per synapse.

Introduction

One of the fundamental parameters setting synaptic strength is the release probability of the presynaptic bouton. Release probability is classically assessed in electrophysiological recordings from the postsynaptic neuron. In neocortex and hippocampus, however, two neurons are frequently connected by more than one synapse, which makes it very difficult to achieve a situation where responses from a single synapse can be electrophysiologically isolated. Furthermore, both presynaptic changes (vesicle depletion, changes in release probability) and changes on the level of postsynaptic receptors (e.g., phosphorylation, desensitization, saturation, lateral diffusion, and internalization) contribute to the variability of postsynaptic responses [1,2]. These effects become even more difficult to disentangle during high-frequency stimulation, when all physiological parameters change simultaneously as the synapse struggles to maintain transmission. Due to these complications, it is an attractive proposition to assess synaptic physiology with functional imaging methods, as they convincingly isolate responses from a single synapse even if other synapses on the same neuron are active at the same time. While a large number of imaging studies used postsynaptic Ca^{2+} transients as a read-out of synaptic efficacy, it is now possible to intercept synaptic transmission at the level of cleft glutamate, effectively isolating presynaptic dynamics from postsynaptic changes.

Overview of optical glutamate sensors

In the last decade, two types of optical glutamate sensors have been developed: chemically-labeled and genetically-encoded glutamate indicators (GEGIs). Both types of sensors utilize a glutamate-binding protein which is either labeled with a synthetic fluorophore or fused to a fluorescent protein. Sensors based on the ligand-binding domain of AMPAR subunit conjugated with a small fluorescent dye molecule near the glutamate-binding pocket have been used to image bulk extrasynaptic glutamate dynamics in the brain [3,4]. A chemical FRET-based approach, combining a donor and acceptor fluorophore with the glutamate binding protein iGluR5-S1S2 (Snifit-iGluR5) [5] showed an improved fluorescence change in cultured cells but has not been applied to brain tissue yet. Sparse and cell-specific labeling without background fluorescence, a precondition for single-synapse studies, seems to be difficult to achieve with chemically labeled sensors.

The first fully genetically-encoded glutamate indicator (GEGI) called FLIPE [6] was FRET-based and contained a glutamate binding protein (GltI from *E.coli*) located between an N-terminal enhanced cyan fluorescent protein (ECFP) and a C-terminal yellow fluorescent protein called Venus. Later improved to reach a maximum CFP/YFP ratio change of 44% and a K_d of 2.5 µM (Hires et al., 2008), the sensor named SuperGluSnFR allowed measurements of the time course of synaptic glutamate release and spillover in hippocampal cultures. However, the signal-to-noise ratio (SNR) of SuperGluSnFR was still low, and ~30 traces had to be averaged to measure glutamate release in response to single action potentials. A significant improvement was the development of iGluSnFR [9]. iGluSnFR is an intensity-based glutamate sensor constructed from *E. coli* GltI and circularly permuted (cp) EGFP. Its high fluorescence dynamic range ($\Delta F/F_{max}$ of 4.5) and K_d of ~4 µM make it a very suitable tool for investigating cleft glutamate dynamics. iGluSnFR has been used to measure glutamate in a variety of tissues such as the retina [10], visual cortex [11] and olfactory bulb [12]. We and others developed variants displaying different kinetics, affinities and emission profiles [13–15]. Those new GEGIs with varied biophysical properties 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).

Comparison with other methods to image presynaptic function

To image presynaptic function, fluorescent glutamate sensors are not the only possibility. The change in vesicular pH during vesicle exocytosis and recycling/reacidification has been successfully exploited to measure the activity of individual presynaptic terminals. Synapto-pHluorin, the first genetically-encoded pH indicator, was based on a pH-sensitive GFP variant fused to the C-terminus of synaptobrevin/VAMP2 (vesicular associated membrane protein-2) to target the sensor to the inner surface of synaptic vesicles [16]. Other vesicular targeting strategies used fusion to synaptophysin [17], synaptotagmin [18] and the vesicular glutamate transporter VGLUT [19]. Spectrally red-shifted sensors with a red fluorescent pH-sensitive protein like VGLUT-mOrange2 [20] and sypHTomato [21] were developed, and the ratiometric sensor Ratio-sypHy [22] was instrumental in revealing the arrested development of synapses in dissociated neuronal culture. In addition, in primary cultures, pHluorins are sufficiently sensitive to detect single vesicle release events [23–25]. It is even possible to localize individual fusion events with a precision exceeding the resolution limit of the microscope [26]. Analysis of release during high-frequency activity, however, is difficult with pH-based methods: reuptake and reacidification are slow processes, leading to rapid accumulation of green fluorescence inside active synaptic terminals. Furthermore, pH-based indicators provide no information about the glutamate content (filling state) of individual vesicles. Another technique to study presynaptic function is to image the loading and unloading of amphiphilic styryl dyes (FM dyes), initially developed to study vesicle recycling at the neuromuscular junction (NMJ) [27]. The lack of cellular selectivity prevents the use of FM dyes at individual synapses in the densely packed neuropil. In addition, the relatively long partitioning time of FM dyes in- and out of the membrane (seconds) renders the relation between staining/destaining events and sub-millisecond glutamate release rather obscure.

Overview of the Procedure

The Procedure can be divided into two sections (see Fig. 1); sensor development (Steps 1-42) and functional imaging of synaptic activity in hippocampal slice cultures (Steps 43-62).

Sensor development (Steps 1-42): While iGluSnFR is an excellent general-purpose GEGI, it may be necessary to further optimize specific properties such as affinity for glutamate (K_d), brightness, or kinetics for specific experiments. Optimization starts with structure-guided mutations of residues close to the glutamate binding pocket (Fig. 1a) (Step 1). Newly generated variants are expressed in *E. coli*, purified and tested *in vitro* for glutamate-induced changes in fluorescence (Steps 2-20). If the dynamic range is deemed sufficient, affinity and kinetics are determined by stopped-flow fluorimetry (Steps 21-37). The most promising candidates are expressed and characterized in HEK cells (Steps 38-42) and finally, in neurons (Steps 43-62). We found considerable differences in the absolute affinity and kinetics of sensor molecules in solution compared to the same molecules tethered to the plasma membrane of cells [13]. Relative differences between GEGIs, however, were conserved, validating the use of *in vitro* calibrations for sensor optimization.

Imaging synaptic function (Steps 43-62): Single-cell electroporation is the method of choice to achieve very sparse expression of glutamate sensors in organotypic culture of brain tissue (Fig. 1b)

(Steps 46-56). The sparse expression makes it easy to follow the axon of a patch-clamped sensor-expressing neuron to a distal projection area, e.g., CA1. While camera-based systems are ideal for functional imaging in dissociated neuronal culture, two-photon microscopy is typically used to detect weak functional signals deep in scattering tissue (Steps 57-62). The optimal strategy for functional imaging depends on the goal of the experiment: to obtain spatial information about the fusion sites of vesicles on individual presynaptic boutons, we use fast frame scans and slower GEGIs (iGluSnFR) (Step 62 Option A). To accurately determine the amplitude of individual glutamate transients, a prerequisite for optical quantal analysis, spiral scans on individual boutons provide increased temporal resolution and better signal-to-noise ratio (SNR) (Step 62 Option B). While 500 Hz provide sufficient temporal resolution for iGluSnFR imaging, we increase the spiral scan frequency to 1 kHz for ultrafast GEGIs.

Limitations of the method

Glutamate diffuses out of the synaptic cleft in less than 1 ms. Even the fastest GEGIs cannot monitor the true kinetics of free glutamate diffusion as the sensor needs time to rearrange its conformation to become fluorescent. In addition, scanning microscopy has limited temporal resolution. For capturing sub-millisecond fluorescence changes, it would be necessary to park the excitation beam on the synaptic cleft. This is not a technical problem, but in practice, point-scan experiments are extremely sensitive to small lateral movements of the active bouton in the tissue. At the moment, galvanometric scanning can still adequately sample the fastest GEGIs.

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). Therefore, the laser exposition per single AP should be reduced to a minimum. To measure GEGI transients in response to individual APs, we image in spiral mode for ~ 80 ms. We routinely acquire ~100 trials from single boutons without any decay in amplitude or release probability (see Experimental design and Supplemental Fig. 1c). By using lower laser power, this number can be extended to 200 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.

Experimental design

Development and characterization of fast glutamate probes: Site-directed mutagenesis and protein expression/purification are done following standard procedures and should lead to high yields of the GEGIs with a purity > 90% in a single-step purification process (determined by SDS-PAGE). One of the most important parameters is the fluorescence dynamic range (F_{+glu} - F_{-glu} / F_{-glu}) which is a measure of the fluorescence change upon glutamate binding. If the dynamic range of the new GEGI variant is < 2, the probe's response to glutamate is not high enough to be suitable for cellular experiments.

The affinity of the GEGI, expressed as K_d, has to 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, e.g., to estimate the number of vesicles released simultaneously, a slightly lower affinity might be advantageous. For a GEGI to be a useful probe for *in vivo* imaging, it also needs to be specific for glutamate. Therefore, binding to other ligands has to be assessed (Fig. 2a). As iGluSnFR is based on the glutamate/aspartate ABC transporter protein (GltI), it is expected that the sensor retains a significant affinity for aspartate. However, it should be unresponsive to serine or glutamine. Most of the GEGIs indeed show a fluorescence response to aspartate binding, sometimes even with higher fluorescence dynamic range than for glutamate. However, the affinity is often lower, and fortunately, aspartate does not act as a neurotransmitter. Nevertheless, aspartate sensitivity needs to be considered when monitoring glutamate in non-neuronal tissues or cellular compartments.

To observe fast events like neurotransmission in synapses, the kinetics of the formation and decay of the fluorescence state are critical. Thus, the association and dissociation of the purified GEGIs are determined *in vitro* by stopped-flow fluorimetry (Fig. 2b). While performing association measurements, it is essential to record baselines for the buffer to obtain the zero level of the PMT and for the glutamate-free GEGI to obtain the starting point of the fluorescence increase. This recording is essential to detect rapid phases (>1000 s^{-1}) that are faster than the resolution of the stopped-flow device (about 1 ms mixing time) and thus appear as jumps. Recording the dissociation of glutamate from the GEGIs is especially challenging, as the glutamate needs to be removed from the sensor, which is difficult due to lack of chemical traps. We circumvent this obstacle by mixing the glutamate-bound GEGI with the high affinity GluBP 600n (K_d about 600 nM) [6]. However, for low affinity variants, these measurements are limited by the concentration of GluBP 600n available and by the very small decrease in fluorescence amplitude. Low-affinity GEGIs (K_d > 1 mM) have to be saturated with glutamate concentrations in the mM range, however, GluBP 600n is at best concentrated to be ~ 1 mM in the optical cell. As only a small fraction of the GEGI is dispossessed of its glutamate, only a very small decrease in fluorescence occurs and, thus a small signal is observed.

For *in vivo* use of the GEGIs, the sensors need to be attached to the outer membrane of a cell. Thus, the sensors are cloned in mammalian expression vectors, which add a mouse Ig κ-chain for secretion and a PDGFR transmembrane helix for membrane attachment. In order to confirm correct localization, the sensor is expressed in cell lines (HEK293T cells) and titrated with glutamate to determine the cellular K_d. We found that the attachment to the outer membrane of the cell increases the variants' affinity for glutamate by a factor of up to 20-fold. Relative differences between the variants, however, are conserved [13]. This affinity increase needs to be considered when choosing a suitable sensor for *in vivo* applications.

Imaging synaptic glutamate release with two-photon microscopy: 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 the absence of glutamate, making it difficult to focus on small structures such as axonal boutons. 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; the newly developed CyRFP1 [28] is also an excellent choice for this purpose. The red fluorescence also

provides additional information about the volume of individual boutons. Electroporated CA3 neurons are clearly visible under a stereomicroscope (5x objective, DsRed filter set) 2-4 days after electroporation (Fig. 3a-c). Two-photon excitation at 980 nm reveals axons and boutons of the expressing neurons in CA1 *stratum radiatum*, far away from the somata in CA3 (Fig. 3c). Targeted patch-clamp recording from a transfected neuron allows triggering single action potentials (APs) by brief depolarizing current injections (Fig. 3e). Simultaneous imaging of a single bouton (spiral scan path at high zoom (Fig. 5a-c)) reveals green fluorescence transients time-locked to the action potentials, indicating glutamate release from the stimulated bouton. In spite of reliable action potential generation, synapses frequently failed to release glutamate (Fig. 3e, gray traces), indicating a stochastic vesicle fusion process.

Fusion site localization: While spiral scans are optimal to determine the peak amplitude of the glutamate signal, we use fast frame scans (16 x 16 pixels, frame rate 62.5 Hz) to localize the likely location of the fusing vesicle in individual trials. The spatial peak of the averaged signal does not necessarily occur in the center of the bouton, but often close to the edge (Fig. 4b), reflecting the random orientation of the synaptic cleft on the surface of the bouton. To localize individual fusion events in noisy images, we fit a two-dimensional Gaussian kernel (Fig. 4c) to the first frame after stimulation. Plotting the center positions of Gaussian fits (Fig. 4c) relative to the morphological outline of the bouton (red channel) revealed a small region of release, the active zone (Fig. 4d). Increasing the extracellular Ca^{2+} concentration from 1 mM to 4 mM did increase the amplitude of single-trial responses, but not the size of the apparent active zone (Fig. 4e). The higher cleft glutamate concentrations caused by single action potentials in 4 mM Ca^{2+} suggest the simultaneous release of multiple vesicles or a switch from partial to full release. No clustering was found when we fitted green fluorescence before stimulation or in trials classified as failures (Fig. 4f and g).

Spiral scans and amplitude extraction: As we do not know a priori where on the bouton the highly localized and short-lived GEGI signals will appear (Fig. 5a), we need to sample the entire surface of the bouton as fast as possible. Traditional raster scanning (Fig. 5b) requires extreme acceleration of the scanning mirror at the end of every scan line, limiting the maximum frame rate to ~120 Hz. By scanning a spiral pattern, we are able to sample the same area at frequencies up to 1 kHz. The point spread function (PSF) of our two-photon microscope is 0.5 µm in the imaging plane and 1.7 µm along the optical axis (FWHM, measured with 170 nm fluorescent beads). As the PSF is elongates in the axial direction, we sample the upper and lower surface of the bouton simultaneously. Our goal is to extract the amplitude of fluorescent transients from spiral scans independent of the exact position of the fusion site on the bouton. To do so, the unfolded spiral scans are plotted as straight lines underneath each other, resulting in a space-time plot (Fig. 5c). Typically, the spiral scan intersects the diffusing cloud of glutamate two or three times per scan, resulting in multiple 'hot spots' that all contained information about the same release event. To extract amplitude information, columns (corresponding to positions on the bouton) are sorted according to the change in fluorescence (Fig. 5d). The columns with the largest signal ($\Delta F > \Delta F_{max}/2$) are averaged (region of interest, ROI). In trials where no fluorescence change is detected (failures), the same columns as in the last 'success' trial are analyzed. As opposed to a static region-of-interest (ROI), this analysis procedure is robust against minute drift of the tissue between trials and does not require a priori knowledge of the fusion location. We extract the amplitude from the resulting fluorescence trace by fitting a single exponential function to the decay of the fluorescence transient. To

estimate the noise level (photon shot noise) in every experiment, we perform the same fitting procedure to a section of the baseline (before stimulation). As expected, the baseline amplitudes are close to zero (Fig. 5f, gray dots and columns). Typically, the histogram of all responses (green) also show one cluster around zero (failures of release), the remaining responses (successes) form an asymmetric, broad peak between 40 and 160% $\Delta F/F_0$. A close inspection of the spatial distribution of the signal (Fig. 5e, average of 10 successes) shows a rapid decay of the peak, but no lateral spread as might be expected from a diffusion process. It is important to note that the diffusion of free glutamate out of the synaptic cleft happens in less than 1 ms and cannot be resolved by iGluSnFR or another GEGI. Instead, what we observe is the relatively slow unbinding of glutamate from quasi-stationary iGluSnFR molecules, explaining the lack of lateral spread of the signal.

Materials

Reagents

- Plasmids
  - pCMV iGluSnFR (Addgene plasmid #41732) [9], iGluSnFR in mammalian expression vector, can be used for expression in HEK293T cells and as starting point for SDM to induce new mutations
  - pET41a(+) (Novagen, Merck cat. no. 70556-3), bacterial expression vector used to express GEGI variants in *E. coli*
  - pET30b (Merck cat. no. 69909), bacterial expression vector used to express GEGI variants in *E. coli*
  - pCI syn iGluSnFR (Addgene plasmid #106123), mammalian expression vector to express iGluSnFR in neurons (hippocampal slices)
  - pDisplay FLIPE-600n (Addgene plasmid # 13545) [6], vector encoding for GluBP 600n for expression of glutamate-binding protein GluBP 600n in *E. coli*. Purified GluBP is used for kinetic analysis *in vitro*.
  - pCI syn $iGlu_f$ (Addgene plasmid #106121)[13], mammalian expression vector for expression of $iGlu_f$ in neurons
  - pCI syn $iGlu_u$ (Addgene plasmid #106122)[13], mammalian expression vector for expression of $iGlu_u$ in neurons
  - pCI syn tdimer2, a gift of Roger Y. Tsien [29], mammalian expression vector for expression of dimeric red fluorescent protein in neurons
- Cloning and molecular biology
  - Restriction enzymes: BglII, NotI (NEB cat. no. R0144 and R0189)
  - T4 DNA ligase (NEB cat. no. M0202)
  - QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies cat. no. 200516)
  - NucleoSpin® Plasmid kit (Machery and Nagel cat. no. 740588)
  - HiSpeed Plasmid Midi Kit (Qiagen cat. no. 12643)
  - LB Broth (Powder) - Lennox (Fisher BioReagents cat no. BP1427)
  - LB Agar, Lennox (Granulated) (Fisher BioReagents cat no. BP9745)
  - Kanamycin sulfate (Fisher BioReagents cat no. BP906)

CAUTION: toxic. Wear protective gloves, clothing, and eye protection. Wash hands thoroughly after handling.

- PureLink HiPure Plasmid Maxiprep (Life Technologies cat. no. K210006)

- General reagents
  - HEPES (Fine White Crystals/Molecular Biology) (Fisher Scientific cat. no. BP310-1)
  - Sodium chloride, BioXtra, ≥99.5% (AT) (Merck cat. no. S7653)
  - 1 M $MgCl_2$ solution (Invitrogen AM9530G)
  - Sodium hydroxide (Merck cat no. S8045)

    CAUTION: Danger. Corrosive to metals and causes severe skin burns and eye damage. Wear protective gloves, clothing, and eye protection. Wash hands thoroughly after handling.
  - D(+)-Glucose (Merck cat no. D9434)
  - Potassium chloride, BioXtra, ≥99.0% (Merck cat no. P9333)
  - NaH_2PO_4 (Merck cat no. S0751)
- Protein expression and purification
  - Pierce™ Protease Inhibitor Tablets, EDTA-free (Thermo Fisher Scientific cat. no. A32965)

    CAUTION: Danger, causes severe skin burn and eye damage. Wear protective gloves, clothing and eye protection. Wash hands thoroughly after handling.

    CRITICAL: EDTA-free inhibitor is critical to ensure binding of the His tagged protein to the HisTrap High Performance column
  - HisTrap™ High Performance (GE Healthcare cat. no. 17524801)
  - SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 22 mm (Thermo Fisher Scientific cat. no. 68035)
- Glutamate, aspartate, and serine titration
  - L-Glutamic acid (Merck cat. no. G1251)
  - L-Aspartic acid (Merck cat. no. A8949)
  - L-Serine (Merck cat. no. S4500)
- Testing in cell lines
  - HEK293T cells (Merck cat. no. 85120602)

    CAUTION: The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
  - Sensorplate, 24 well, PS, F-bottom, glass bottom, black, lid, sterile, single packed (Greiner bio one cat. no. 662892)
  - DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Gibco, Thermo Fisher Scientific cat. no. 31966047)
  - MEM Non-Essential Amino Acids Solution (100X) (Gibco, Thermo Fisher Scientific cat. no. 11140035)
  - Fetal Bovine Serum (FBS), qualified, heat inactivated, E.U.-approved, South America Origin (Gibco, Thermo Fisher Scientific cat. no. 10500056)
  - Penicillin-Streptomycin (10,000 U/mL) (Gibco, Thermo Fisher Scientific cat. no. 15140122)

CAUTION: Causes skin irritation, eye irritation, may cause an allergic skin reaction and respiratory irritation. Wear protective gloves, clothing, and eye protection. Wash hands thoroughly after handling.

- Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific cat. no. 11668027)

- Slice culture and recording
  - Slice cultures from rodent hippocampus [30]

    CAUTION: Any experiments involving live rats must conform to relevant Institutional and National regulations. In our case, organ explant procedures were approved by the veterinary of the University Medical Center Hamburg-Eppendorf, Germany
  - MEM (Sigma-Aldrich cat. no. M7278)
  - Heat-inactivated horse serum (Gibco cat. no. 16050-122)
  - L-glutamine (200 mM; Gibco cat. no. 25030-024)
  - L-ascorbic acid (Sigma-Aldrich, cat. no. A5960)
  - Insulin (1 mg/mL; Sigma-Aldrich cat. no. I1882)
  - HEPES (Sigma-Aldrich cat. no. H4034)
  - K-gluconate (Sigma-Aldrich, cat. no. G4500)
  - EGTA (Sigma-Aldrich, cat. no. E0396)
  - Na_2-ATP (Sigma-Aldrich, cat. no. A3377)
  - Na-GTP (Sigma-Aldrich, cat. no. G8877)
  - Na_2-phosphocreatine (Sigma-Aldrich, cat. no. P7936)
  - $NaHCO_3$ (Sigma-Aldrich, cat. no. S5761)
  - NaH_2PO_4 (Sigma-Aldrich, cat. no. S5011)
  - Potassium chloride (Sigma-Aldrich, cat. no. S5886)
  - 1 M KCl (Fluka cat. no. 60121)
  - 1 M $MgSO_4$ (Fluka cat. no. 63126)
  - 1 M $MgCl_2$ (Fluka, cat. no. 63020)
  - 1 M $CaCl_2$ (Fluka cat. no. 21114)
  - D-glucose (Fluka cat. no. 49152)
  - Cl_3Fe (Fluka cat. no. 10695862)

Reagent Setup

Resuspension buffer for expression: 50 mM HEPES-Na^+, 200 mM NaCl, pH 7.5, filtered (0.2 µm pore size) and stored at 4°C for 2 weeks

Elution buffer for expression: 50 mM HEPES-Na^+, 200 mM NaCl, 500 mM imidazole, pH 7.5, filtered (0.2 µm pore size) and stored at 4°C for 2 weeks

Storage buffer for expression: 50 mM HEPES-Na^+, 100 mM NaCl, pH 7.5, stored at 4°C for 2 weeks

Assay buffer for biophysical characterization: 50 mM HEPES-Na^+, 100 mM NaCl, 2 mM $MgCl_2$, pH 7.5, stored at 4°C for 2 weeks

Association buffers for biophysical characterization:

- 1 µM GEGI in assay buffer, stored at 4°C for one day.
- 0.1x K_d to 10x K_d glutamate in assay buffer, stored at 4°C for one day

CRITICAL: In order to measure the full range of response in dependence of the glutamate concentration, the glutamate concentration mixed with the GEGI has to be distributed around the K_d

Dissociation buffers for biophysical characterization:

- 2 mM GluBP 600n in assay buffer, stored at 4°C for one day.
- 1 µM GEGI in assay buffer, stored at 4°C for one day.
- 1 µM GEGI in assay buffer with saturating glutamate (10x K_d), stored at 4°C for one day

Complete DMEM: DMEM, 1x NEAA, 10% (v/v) FBS, 100 U/ml penicillin-streptomycin, stored at 4°C for 2 month.

HEK293T cell imaging buffer: 20 mM HEPES-Na^+, 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, pH 7.4 stored at 4°C for up to 6 month.

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 $CaCl_2$, 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.

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 310-320 mOsm/kg. If the osmolality is out of range, a mistake was made during solution preparation. Solution has to 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.

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 310-320 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.

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 290-300 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.

Equipment

- Equipment for protein expression/purification

- Sonicator for lysing *E. coli* (Sonics & Materials Inc., VibraCell)
- ÄKTA Purifier or Explorer (GE healthcare)

- Equipment for biophysical characterization
  - Fluorescence spectrometer with magnetic stirring function (Fluorolog3, Horiba Scientific)
  - Hellma® fluorescence cuvettes, ultra Micro (Merck, cat. no. Z802336-1EA)
  - Hellma fluorescence cuvette QS 3500 µL (Merck, cat. no. Z600172-1EA)
  - ALADDIN syringe pump (World Precision Instruments, cat. no. AL-1000)
  - SGE syringe 250 µL, barrel inner diameter 2.30 mm (Trajan Scientific and Medical, cat. no. P/N 006230)
  - 'KinetAsyst' Dual-mixing Stopped-Flow System (TgK Scientific, cat. no. SF-61DX2) equipped with two circulating water baths for temperature control and a long-pass filter >530 nm. The equipment should be set up in a dark lab with red light illumination and temperature control (20°C)
- Equipment for imaging in cell lines
  - Inverted spinning-disk confocal fluorescence microscope (3i Marianas)
- Electrophysiology equipment
  - pE-4000 LED light source (CoolLED) for epifluorescence
  - infrared Dodt contrast (Luigs & Neumann)
  - Patch-clamp amplifier (Axon Instruments, model no. MultiClamp 700B)
  - Microelectrode manipulator (Sutter Instrument, MP-285).
  - Micropipettes for whole-cell recording (Borosilicate glass with filament, 1.5 mm O.D.)
- Electroporation equipment
  - Upright microscope with a motorized stage, CCD camera and IR-DIC (infrared differential interference contrast) or Dodt contrast
  - 20x water immersion objective (Zeiss Achroplan)
  - 4x zoom lens system (0.5 – 2.0x magnification range)
  - Vibration isolation table (Table Stable LTD, TS-150)
  - Axoporator 800A with HL-U pipette holder (Molecular Devices)
  - Plastic syringe body (1 ml) as disposable mouthpiece, connected through a Luer 1-way stopcock and thin silicone tubing to the electrode holder
  - Headphones and speakers
  - Microscope chamber made of a glass microscope slide (70 x 100 x 1 mm) onto which a Teflon ring (inner diameter: ~ 35 mm, height: 2 mm) is fixed with silicone aquarium sealant
  - Motorized micromanipulators (Luigs & Neumann)
  - Silver wire (diameter: ~ 0.25 mm)
  - Forceps (Fine Science Tools, cat. no. 11002-16)
  - Hot bead sterilizer (Fine Science Tools, cat. no. 18000-45)
  - Incubator (37°C; 5% CO_2) with rapid humidity recovery and copper chamber (Heracell 150i/160i, Thermo Scientific)
  - Micropipette puller (PC-10, Narishige)
  - Thin-walled borosilicate glass capillaries (WPI, cat. no. TW150F-3)

- Tissue culture dishes (60 mm, sterile; Sarstedt, cat. no. 83.1801)
- Ultrafree centrifugal filter units (Millipore, cat. no.UFC30GV0S)
- Micro-osmometer (Fiske Model 210)

  CRITICAL: The electroporation microscope should be situated close to the tissue culture hood (in the same room) to prevent contamination. We built a laminar flow cabinet with HEPA filter unit around the electroporation setup. The microscope has to be mechanically isolated from the vibrations generated by the fan in the filter box; we use an active anti-vibration table.

- Software
  - ImageJ2 (https://imagej.net/ImageJ2)
  - GraphPad Prism 7 (https://www.graphpad.com/)
  - ScanImage 3.8 [31] (https://vidriotechnologies.com/)
  - Ephus [32] (https://www.janelia.org/open-science/ephus)

Equipment Setup

- **Equipment for functional imaging in tissue:** We built a two-photon microscope based on an Olympus BX51WI microscope with pE-4000 LED light source (CoolLED) for epifluorescence and infrared Dodt contrast (Luigs & Neumann). A Ti:Sapphire laser system with dispersion compensation (MaiTai DeepSee, Spectra Physics) was coupled in through an electro-optical modulator (EOM, Conoptics), a 3x telescope (Thorlabs), 5 mm scan mirrors (Cambridge), a compound scan lens (f = 50 mm [33]), a dual camera port with IR mirror (Olympus) and a water immersion objective (LUMPLFLN 60XW, 60x, NA 1.0, Olympus). Red and green fluorescence was detected through the objective and the oil immersion condenser (NA 1.4, Olympus) using 2 pairs of photomultiplier tubes (H7422P-40SEL, Hamamatsu). 560 DXCR dichroic mirrors and 525/50 and 607/70 emission filters (Chroma Technology) were used to separate green and red fluorescence. Excitation light was blocked by short-pass filters (ET700SP-2P, Chroma). During epifluorescence illumination, sub-stage PMTs were protected by a NS45B shutter (Uniblitz). For electrical stimulation of individual neurons, we mounted the head-stage of a MultiClamp 700B amplifier (Molecular Devices) on a MP-285 micromanipulator (Sutter Instruments) on a motorized stage (40-40, Danaher Motion) that also moved the perfusion chamber (quartz glass bottom). Temperature was controlled by Peltier-heating of the oil-immersion condenser and in-line heating of the perfusion solution (Warner Instruments). The setup was controlled by Matlab software (ScanImage [31] and Ephus [32]) via data acquisition boards (National Instruments). At the start of a trial, electrophysiology and image acquisition were synchronized by a hardware trigger (TTL pulse). During a trial (2 s, typically), laser power was regulated via EOM and restricted to the periods of expected glutamate release (20 - 80 ms window, depending on GEGI kinetics) to minimize bleaching.

  CRITICAL: To minimize bleaching by excessive excitation, the microscope has to be designed to detect emitted photons very efficiently. Using only the objective for

fluorescence detection is not sufficient 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. Replace aging PMTs with excessive dark counts.

CRITICAL: The oil-immersion condenser has to 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. As the thermal mass of the condenser is very large, constant-current heating is sufficient, provided that the temperature of the perfusion solution is additionally regulated by a feedback control circuit (in-line heater, Warner Instruments). A climate chamber would be an attractive solution but is not compatible with direct-mounted PMTs.

CRITICAL: If a galvanometric scanning system is used, the microscope software has to support arbitrary line scans or spiral scans[34]. The code for arbitrary line scans that we developed for our original study is now incorporated in the ScanImage software (Version 2016 and later). ScanImage is developed and supported by Vidrio Technologies, LLC as an open-source research resource. A resonant scanning system may be sufficiently fast in frame mode if extreme zoom-in (few scan lines) can be realized.

Procedure

Generation of GEGIs Timing 1 week (5 h hands-on time)

CRITICAL: We have made a number of GEGI-encoding plasmids available via Addgene (see Reagents). Follow Steps 1-42 in order to design GEGIs with tailored biophysical parameters.

1. Analyze protein 3D structures of the 99% homolog of GltI of *Shigella flexneri* (PDB 2VHA) and literature [35,36] to assign critical residues involved in glutamate binding. Substitute essential residues with amino acids with similar physical properties. Avoid radical changes in amino acid size or charge as this will frequently result in misfolded or otherwise non-functional proteins.
2. Subclone the iGluSnFR gene from a mammalian expression vector into a bacterial expression vector (pET41a) using restriction digestion of BglII and NotI and ligation (T4 DNA ligase) following the manufacturer's protocol.
   CAUTION: Subcloning requires DNA to be analyzed by agarose gels. This requires the use of DNA intercalating fluorescent dyes (e.g. ethidium bromide or Sybr green) which highly toxic as mutagens and should be handled with care. DNA imaging systems are based on UV lamps so appropriate personal protective equipment should be used.
3. Insert point mutations using the QuikChange XL site-directed mutagenesis kit following the manufacturer's instructions and confirm new variants by DNA sequencing.
4. Subclone *glti* gene encoding GluBP 600n from pRSET FLIPE 600n (ECFP-ybeJ-Venus) into pET30b (His-fusion expression vector) at BglII and NotI restriction sites.

Expression and purification of new GEGIs Timing 3 days (10 h hands-on time)

5. Transform 1 µL of iGluSnFR variants or GluBP 600n plasmid DNA into 50 µL of *E. coli* BL21 (DE3) gold chemically competent cells.
6. Pick one colony and grow in 10 mL LB-medium supplemented with 100 µg/mL kanamycin for overnight at 37°C and 180 rpm (pre-culture).
7. Inoculate 1 L LB-medium containing 100 µg/mL kanamycin with whole pre-culture, incubate at 37°C and 180 rpm until OD_{600} reaches (0.6-0.8).
8. Cool the cells down to 20°C.
9. Induce protein expression with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubate at 20°C and 180 rpm overnight.
   CRITICAL STEP: Best protein yields are obtained when inducing expression during exponential phase of growth (OD_{600} 0.6-0.8), overnight at 18-20 °C.
10. Harvest cells by centrifugation at 3000 *g* for 15 min at room temperature, resuspend cells in 40 mL resuspension buffer supplemented with Pierce Protease Inhibitors and lyse via sonication on ice for 2 min (2 sec "on" and 8 sec "off").
    CAUTION: Wear ear protection equipment during sonication.
    CRITICAL STEP: Sonication produces heat and may result in the degradation of your protein of interest. Performing sonication on ice and in the presence of protease inhibitors will dramatically limit this phenomenon.
11. Remove the cell debris by ultracentrifugation at 100,000 *g* for 45 min at 4°C.
    CRITICAL STEP: After cell lysis, all steps are performed at 4°C when possible to avoid protein digestion by cellular proteases (steps 12-14).
12. Load the supernatant on equilibrated HisTrap HP column (nickel affinity resin) mounted on an ÄKTA system (flow rate 4 mL/min) and wash with 40 mL resuspension buffer.
13. Elute the protein with 10 column volumes of a linear gradient of resuspension and elution buffer (0 to 0.5 M imidazole) and collect in 2 mL fractions. Analyze the purified protein by SDS-PAGE and stain the gel with Coomassie blue.
14. Pool fractions of interest and dialyze overnight at 4°C in a snakeskin dialysis tubing (3.5 kDa) against 4 L storage buffer.
    CRITICAL STEP: It is essential to perform dialysis to remove the imidazole from your buffer. Otherwise, protein precipitation will occur upon defrosting (from step 15). The dialysis molecular weight cut-off used can be higher than 3.5 kDa as long as it is below 15 kDa.
15. Store purified protein in fractions of 1 mL in the -80°C freezer.
    PAUSE POINT: The purified protein can be stored at -80°C for up to 3 years.

Determining the dynamic range Timing 1 h

16. Prepare 50-100 nM iGluSnFR proteins in assay buffer, add to a Hellma micro cuvette (50 µL) and place the cuvette into a fluorescence spectrometer pre-equilibrated to 20 °C.
17. Record the fluorescence emission spectrum (λ_{ex} = 492 nm and λ_{em} = 497-550 nm) ($\boldsymbol{F_{-glu}}$).
18. Add 10 mM glutamate solution to the micro cuvette, mix well and record the fluorescence emission spectrum (λ_{ex} = 492 nm and λ_{em} = 497-550 nm) ($\boldsymbol{F_{+glu}}$).
19. To analyze the data, take the maximal emission (around 514 nm) of each measurement and calculate the fluorescence dynamic range $((F_{+glu}- F_{-glu})/F_{-glu})$.

20. Repeat Steps 16-19 twice in order to generate 3 independent replicates.
    CRITICAL STEP: If the fluorescence dynamic range is < 2 the fluorescence change upon glutamate binding is too low for imaging in hippocampal slices. In that case, try using higher concentrations of glutamate. If the fluorescence dynamic range has not improved, return to the "Selection of residues close to binding pocket step" and select another mutation.

Determining K_d and specificity **Timing 2 h for each ligand**

21. Prepare 50-100 nM GEGI in 3 mL assay buffer in a 3500 µL Hellma Quartz cuvette (QS).
22. Add a magnetic stir bar and place the cuvette into spectrofluorometer.
    CRITICAL STEP: Make sure stir bar is moving rigorously.
23. Fill a 250 µL Hamilton syringe with assay buffer with appropriate ligand: L-glutamate (10x K_d 10-50 mM), L-aspartate (10x K_d 10-50 mM) or L-serine (10x K_d 10-50 mM).
    CRITICAL STEP: Make sure there are no air bubbles in the syringe nor in the tubing.
24. Install the syringe in an Aladdin syringe pump, set the flow rate to 10 µL/min and place the tubing outlet carefully into the micro cuvette.
25. Simultaneously start the recording of the fluorescence emission over time (λ_{ex} = 492 nm and λ_{em} = 514 nm) and the syringe pump.
26. To analyze the data, use time information to calculate the ligand concentration in the cuvette at each given time point. Correct the fluorescence emission for dilution/photobleaching and normalize it. Plot the corrected and normalized fluorescence against the ligand concentration and fit with a Hill equation for specific binding (GraphPad Prism 7) to obtain affinity for glutamate (K_d) and cooperativity of binding (n).
27. Repeat Steps 21-26 twice in order to generate at least 3 independent experiments.

Measuring kinetics **Timing 5 h (4h for association, 1h for dissociation)**

CRITICAL: Temperature control is essential for all kinetic measurements. Furthermore, washing the instrument firmly after changing the ligand concentration is critical. To correctly analyze the data baselines and maximum fluorescence intensity lines should be recorded as described below. Always average at least five records (“shots”) for each measurement to obtain a representative trace.

28. ***Association (Steps 28-32):*** Mix 1 µM GEGI in assay buffer with the maximal glutamate concentration in assay buffer (10x K_d, as determined in Step 27). Set the fluorescence level after mixing to reach 80% detector saturation by adjusting the gain on the PMT (reference of 1 for normalization). This step will prevent detector overload in future experiments.
29. Mix assay buffer with assay buffer and record the baseline (reference of 0 for normalization).
30. Mix 1 µM GEGI in assay buffer with assay buffer. This measurement should result in a straight line and shows the basal fluorescence without ligand bound.
    CRITICAL STEP: Steps 28-30 need to be performed before starting any association kinetic measurement of a GEGI. It ensures that the instrument is calibrated for maximum/minimum fluorescence detection levels and prevents detector damage.
31. Glutamate dependent association kinetics: Mix 1 µM iGluSnFR variant in assay buffer with increasing glutamate concentrations in assay buffer (0.1x to 10x K_d). Record and average five measurements for each glutamate concentration.

CRITICAL STEP: You may have to perform measurements of different time scales to have enough data points for an accurate exponential fitting. Make sure you use about 10 different glutamate concentrations to measure the fluorescence increase with increasing glutamate and also the saturation as shown by reaching a maximum in fluorescence and on-rate.

32. To analyze the data, normalize the recorded time traces to the PMT baseline and the maximal fluorescence level. Fit the time traces with mono- or biexponential decays (Kinetic studio or GraphPad Prism 7). Plot the obtained observed rate constants against the glutamate concentration.

33. ***Dissociation (Steps 33-37):*** Mix 1 µM GEGI in glutamate saturating assay buffer (10x K_d, as determined in Step 27) with the same buffer. Set the fluorescence level after mixing to reach 80% detector saturation by adjusting the gain on the PMT (reference of 1 for normalization). This step will prevent detector overload in future experiments.
34. Perform step 29 to obtain the reference for 0.
35. Perform step 30 to obtain the basal fluorescence level.
    CRITICAL STEP: Steps 34-35 need to be performed before starting any dissociation kinetic measurement of a GEGI. It ensures that the instrument is calibrated for maximum/minimum fluorescence detection levels and prevents detector damage.
36. Dissociation kinetics: Mix 1 µM GEGI in assay buffer with saturating glutamate concentration (10x K_d) with 2 mM GluBP 600n in assay buffer. Higher concentrations of GluBP 600n are not advisable as precipitation might occur. Record and average five measurements.
    CRITICAL STEP: You may have to perform measurements of different time scale to have enough data points for an accurate exponential fitting.
37. To analyze the data, normalize the recorded time traces to the PMT baseline and the maximum fluorescence level. Then fit the time trace with mono- or biexponential decays (Kinetic studio or GraphPad Prism 7). Plot the obtained observed rate constants against the glutamate concentration.

Determining fluorescence dynamic range and K_d in HEK293T cells **Timing 2 days (5 h hands-on time)**

38. Seed ~200,000 cells onto 24-well glass bottom fluorescence plates in complete DMEM and let them attach for 24 h in the incubator (37°C, 5% (v/v) CO_2).
39. Transfect the cells with GEGI plasmids generated by SDM of promising mutations into the mammalian expression vector pCMV iGluSnFR using Lipofectamine 2000 following the manufacturer's protocol.
40. Examine the cells 24 h post-transfection at 37 °C with a confocal microscope (light source 488 nm, using GFP settings). Check for localization at the plasma membrane.
41. Glutamate titration: add stepwise glutamate to final concentrations between 0-10x K_d (as determined in Step 27) and take an image of the cells before and after each addition step.
    CRITICAL STEP: Focus drift may happen when you add glutamate. If it is the case, discard the last data-point and move to a different well.
42. Define elliptical regions of interest (ROI) along cell membrane and determine the fluorescence intensity (ImageJ). Normalize intensity for each individual cell and average over a total number

of >20 cells. Plot relative intensity versus glutamate concentration and fit data with Hill equation (GraphPad Prism 7).

Cloning in neuronal expression vector **Timing 7 days (5h hands-on time)**

43. Use QuikChange XL site-directed mutagenesis kit to insert mutation of promising GEGI variants into pCI syn iGluSnFR vector and confirm by DNA sequencing.
44. Prepare plasmid for electroporation using a plasmid DNA preparation kit (e.g. PureLink HiPure Maxiprep kit from Life Technologies)

Culture preparation **Timing 15 min/brain**

45. Prepare organotypic slice cultures (rat hippocampus)as described in [30].

Single-cell electroporation **Timing 10 min plus 10-20 min per slice, depending on slice quality and number of cells to transfect**

46. ***Preparation of plasmids and DNA (Steps 46-47):*** 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.
47. Add the GEGI plasmids to the desired concentration after removal of the filter insert. Use 40 to 50 ng/µL for pCI syn $iGlu_f$ (Addgene plasmid #106121) or pCI syn $iGlu_u$ (Addgene plasmid #106122) [13]. For different cell types and GEGIs, the final concentration may have to be determined empirically (range: 1-100 ng/µL).
    CRITICAL STEP: It is important that the DNA-containing solution is not passed through the Millipore Ultrafree centrifugal filter unit.
    CRITICAL STEP: To aid visualization of axons and boutons of transfected neurons, mix plasmid encoding for red fluorescent protein (e.g. tdimer2; 20 ng/µL) with GEGI plasmid to achieve co-expression.

    PAUSE POINT: The electroporation solution containing the plasmid can be stored between electroporation sessions at -20°C for up to 1 year.

48. ***Electroporation (Steps 48-56):*** Coat silver wires tips and ground electrodes with AgCl by bathing them in a saturated Cl_3Fe solution for at least 30 min or overnight prior to first use.
49. Pull electroporation pipettes using a micropipette puller (e.g. PC-10, Narishige). Pull thin-walled borosilicate capillaries to obtain a resistance of 10-15 MΩ when filled with the intracellular solution.
    CRITICAL STEP: Ensure constant pipette resistance for reproducible expression. A too high pipette resistance leads to low expression, whereas a too low resistance causes extreme expression levels and toxicity.
50. Back-fill an electroporation pipette with ~1.2 µL of plasmid mix solution (from Step 47) for each slice to electroporate. Back-filled pipettes can be kept (in an upright position) for up to 2 hours before use. Pipette 1 mL of transduction solution (37˚C) into the microscope chamber. Transfer one slice culture insert into the chamber (sterile forceps) and add transduction solution on top of

the slice culture for the water immersion objective. Use a sterile 60-mm dish to cover the microscope chamber to transfer to the microscope to proceed to single cell electroporation.
CRITICAL STEP: To avoid any contamination place forceps into the hot bead sterilizer for ~ 10 sec before any handling of insert.
CRITICAL STEP: Work on an electrophysiology microscope setup in a laminar flow box (see Equipment section) to prevent contamination.

51. Apply positive pressure to the pipette to approach a cell to electroporate. Monitor by audio output of the Axoporator 800A amplifier the tip resistance of the electroporation pipette throughout the entire procedure. The resistance should be between 10–15 MΩ.
CRITICAL STEP: 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.
CRITICAL STEP: For reproducible expression level of the plasmids between different electroporation sessions, ensure that the pipette resistance is constant.
52. Move the tip of the electroporation electrode close to a cell of interest while reducing the positive pressure.
53. Approach the cell without sealing the electrode with membranes from other cells in the tissue. 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. Do not apply suction and avoid the formation of a GΩ seal.
54. Apply a pulse train (e.g., voltage: −12 V, frequency: 50 Hz, pulse width: 500 µs, train duration: 500 ms). The optimal settings may differ depending on the cell type to electroporate.
CRITICAL STEP: 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.
55. Slowly retract the pipette and begin applying very light positive pressure once the pipette is retracted 2–4 µm away from the soma. Increase the positive pressure at more considerable distances from the electroporated cell in order to maintain the pipette tip clean. Using the same electrode, repeat steps 51-55 for each cell to be electroporated.
56. Cover the chamber with a 60-mm dish and transfer back to the tissue culture hood. Remove all transfection medium 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. However, the optimal time for a cell to express a given plasmid before starting the experiment has to be determined empirically [37].

Stimulating transfected neurons **Timing 30-90 min per recording depending on slice quality, the number of cells expressing the electroporated plasmids and the length of the recording**

57. Start temperature-controlled perfusion system and place the organotypic culture in the recording chamber. Weigh down membrane patch with c-shaped gold wire.
? TROUBLESHOOTING
58. Tune the Ti:Sapph laser to 980 nm for simultaneous excitation of tdimer2 and GEGI.
? TROUBLESHOOTING

59. Approach a transfected CA3 neuron with the patch pipette, switching between red epifluorescence and IR-Dodt contrast (CCD camera).
? TROUBLESHOOTING

60. Establish a GΩ-seal and break in to establish whole-cell configuration.
CRITICAL STEP: Stimulate an individual transfected cell to avoid stimulation of presynaptic terminals close to the terminal under scrutiny. This will ensure that the GEGI transients originate from the imaged terminal and are not a consequence of glutamate spillover.

61. Move the stage to center the objective on CA1. Search for red fluorescent axons using two-photon excitation.
? TROUBLESHOOTING

Imaging synaptic glutamate release

62. Scan modality and signal analysis depend on the synaptic parameter under scrutiny. To localize the fusion site, acquire fast frame scans followed by fitting with a Gaussian kernel (Option A). To analyze the amplitude of glutamate transients, acquire spiral scans (Option B). To extract the amplitude, define a region of interest in every trial and fit an exponential decay function to the extracted time course.

(A) **Fusion site localization** **Timing 1 hour per recording**

i. Inject current pulses (2-3 ms, 1.5-3.5 nA) into the soma and acquire rapid frame scans of a single bouton (high zoom, 16 x 16 pixels, 1 ms/line).

ii. Treat the raw images by a wavelet method to reduce photon shot noise [38] and improve SNR.
? TROUBLESHOOTING

iii. Upsample the images to 128 x 128 pixels (Lanczos kernel).

iv. Align the images using a Fast Fourier transform (FFT) performed on the red fluorescence signal (tdimer2).

v. Define a morphology mask to define a continuous area encompassing bouton and axon (pixel intensity ≥ 10% to 30% maximal intensity).

vi. Calculate the relative change in GEGI fluorescence ($\Delta F/F_0$) pixel by pixel using the mean of 5 baseline frames as F_0. Calculate the relative change and average the top 3% pixel values (of the GEGI signal) within the bouton mask to obtain the peak amplitude.

vii. Construct a template (2-D anisotropic Gaussian kernel) from the average of 5 trials classified as success. A trial is classified as success when the peak amplitude is above 2σ of the baseline frames.

viii. Perform a first round of analysis where fitting the template to every single frame by adapting only the amplitude and keeping the location and shape of the kernel fixed at the template values to obtain a preliminary classification of 'successes' ($\Delta F/F_0 > 2\sigma$ of baseline noise) and 'failures' ($\Delta F/F_0 < 2\sigma$ of baseline noise).

ix. Repeat the fitting procedure on all trials classified as successes (step viii) allowing for variable location in order to localize the fusion site
? TROUBLESHOOTING

x. As a control, apply the same localization procedure to the failure trials and to the frame before stimulation.

? TROUBLESHOOTING

(B) **Amplitude extraction and failure analysis** **Timing 1 hour per recording**

i. Inject current pulses (2-3 ms, 1.5-3.5 nA) into soma and acquire rapid frame scans of a single bouton (high zoom, 16 x 16 pixels, 1 ms/line).
ii. If a bouton shows AP-induced fluorescence increase (green channel), switch to spiral scan mode.
? TROUBLESHOOTING
iii. Acquire AP-induced GEGI transients at regular intervals (10 s), using 500 or 1000 Hz sampling. Image only for the duration of the GEGI transients (~20 to 80 ms) to minimize laser exposition.
iv. For amplitude extraction, linearize the spiral scans and display them as xt-plots (Fig. 5c).
v. 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, lack of significance a failure trial. This classification is preliminary; the final failure analysis is performed after amplitude extraction (step xi).
vi. As there may be lateral drift between individual trials, it is necessary to assign a new region of interest (ROI) for each success trial. 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). In a given trial, only the columns which display a clear change in fluorescence ($\Delta F > ½$ max ΔF) are analyzed (ROI). The threshold is once adjusted according to the noise of the imaging system but should be kept constant for amplitude comparisons between different experiments.
vii. In failure trials, evaluate identical columns/locations than in the last success trial.
viii. If necessary, correct traces for GEGI bleaching (see Box 1).
ix. For each bouton, extract the characteristic decay time constant (τ) by fitting a mono-exponential function to the average GEGI fluorescence transient.
x. 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).
? TROUBLESHOOTING
xi. For each trial, determine the imaging noise (σ) from the baseline of the extracted fluorescence time course. Classify as 'success' trials where average $\Delta F/F_0 > 2\sigma$ above baseline imaging noise, otherwise classify as 'failure'.

Box 1: Bleaching of GEGI

During imaging, some GEGI molecules bleach, leading to a decrease in baseline fluorescence during each trial (Supplementary Figure 1). This may cause problems when fitting an exponential function to the decay of the glutamate response, since at least two time constants have to be taken into account. To correct individual trials from one bouton for bleaching, fit an exponential decay function to the average of several 'failure' trials. Subtract this function from each trial (failures and successes; step 63B viii). Between trials, fluorescence partially recovers, indicating lateral diffusion of GEGI molecules in the axonal membrane. Some loss of GEGI fluorescence (20-40%) during the course of the experiment can be

tolerated since it does not affect the glutamate-induced relative change in fluorescence ($\Delta F/F_0$, Supplementary Figure 1). We found that manual refocusing between trials can lead to substantial bleaching of the indicator. This can be minimized by automated refocusing between trials.

Troubleshooting

Step 57: Slices are contaminated. See [30] for proper slice culture handling.

Step 58: No cells express the construct. Ensure that the constructs are incorporated into the target cells by adding a fluorescent dye such as Alexa Fluor 594 to the DNA mix (Step 47). After applying the pulse train to the target neuron (Step 54), take a fluorescence image (e.g., Leica Z6 APO) to ensure that the DNA solution and fluorescent dye were successfully electroporated. For more details for the electroporation procedure refer to [37].

Step 59: Cells are dying after transfection with the constructs. Lower the expression of the GEGI, ensure that the pipette resistance (Step 49) is not lower than 10-15 MΩ in the bath before electroporation, and/or reduce expression time. A large pipette tip diameter (low resistance) can lead to overexpression of the GEGI and cell toxicity. Cells should be imaged 2-4 days after electroporation, as longer expression of GEGIs can affect cell health.

CAUTION: Very strong promoters (CMV) should not be used for physiological experiments in neurons.

Step 61: Slice is drifting, focus is not stable. Lower the perfusion rate. Check that the temperatures of the perfusion solution and of the imaging chamber of the microscope are stable to avoid thermal expansion during the experiment.

Step 62A (ii): The responses are very weak and barely above noise. Wait longer after electroporation for a higher expression level. If the expression levels are too low, the GEGI signal from a single vesicle may be below the detection limit. The detection limit is determined by the noise level of the optical recording setup. Minimize background fluorescence, which can be caused by leaking room light, stray pump laser photons (green), or excessive dark counts in aging PMTs. Condenser detection is sensitive to the refractive index of the immersion oil and correct (Köhler) position.

Step 62A (ix): The localization seems inaccurate. Calibrate the optical and mechanical performance of your system using fluorescent microbeads. Imaging of microbeads (0.17 µm diameter) positioned next to a fluorescent presynaptic terminal allows quantifying the accuracy of the response localization procedure.

Step 62A (x): In cases where the positions of apparent 'failures' clusters in a second area of the bouton, exclude the bouton from further analysis as it might be a multi-synapse bouton.

Step 62B (ii): The success rate in finding a bouton releasing glutamate is very low. This can be due to low release probability. Check $[Ca^{2+}]$ of the ACSF.

Step 62B (x): 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.

Timing

Steps 1-4	Generation of GEGIs	7 days (5 h hands-on-time)
Steps 5-15	Expression and purification of new GEGIs	3 days (10 h hands-on-time)
Steps 16-20	Dynamic range determination	1 h
Steps 21-27	K_d determination	2h/ligand
Steps 28-32	Association kinetics	4 h/variant
Steps 33-37	Dissociation kinetics	1 h/variant
Steps 38-42	Determination of dynamic range and K_d in cells	2 days (5 h hands-on time)
Steps 43-44	Subcloning into neuronal expression vector	7 days (5 h hands-on time)
Step 45	Culture preparation	15 min/brain
Steps 46-47	Preparation of plasmids and DNA	10 min
Steps 48-56	Single-cell electroporation	10-20 min/slice
Steps 57-61	Stimulating transfected neurons	30-90 min/recording
Steps 62A (i-x)	Fusion site localization	1 h/recording
Steps 62B (i-xi)	Amplitude extraction and failure analysis	1h/recording

Anticipated results

Assessing the properties of neighboring boutons

Once a responding bouton is identified, several neighboring boutons along the same axon can be imaged sequentially. Neighboring boutons frequently have similar release probabilities and response amplitudes (Fig. 6a). In rare cases, however, we found dramatic differences in response amplitude between neighboring boutons (Fig. 6b). To test whether boutons on the same axon are functionally similar, we generated random pairs by drawing from our entire set of characterized boutons. The differences between randomly selected boutons are normally distributed (black bars in Fig. 6d). The actual difference between neighboring boutons (red line in Fig. 6d) is at the low end of the distribution, indicating that neighboring boutons tended to have similar release probabilities. A similar result, however non-significant, was found when response amplitudes were analyzed (Fig. 6e and f).

Application of fast GEGIs

While iGluSnFR has an excellent SNR, it is too slow to resolve vesicle fusion events during high-frequency transmission [39]. Recently developed ultrafast GEGIs, $iGlu_u$ and $iGlu_f$ [13] resolve individual responses during 100 Hz trains, albeit with slightly lower SNR as shorter transients correspond to fewer photons collected (Fig. 7a). For these experiments, scan speed was increased to 1 kHz, and a high Ca^{2+} solution was used to increase release probability. Under these conditions, individual boutons typically had a release probability of 1 on the first AP, which rapidly dropped to ~0.2 towards the end of the 100 Hz train. After a brief recovery period (0.5 s), most boutons could restore their initial high release probability (Fig. 7b and c). Interestingly, the depression also affected the amplitude of individual successes, suggesting a

switch from multivesicular release (MVR) to univesicular release during high-frequency activity [1,40]. Alternatively, a switch from full fusion to partial fusion of synaptic vesicles[41] could explain this observation. Responses from an iGluSnFR-expressing bouton during high-frequency stimulation are shown for comparison (blue traces, Fig. 7a). Summation of 100 Hz release events drives this slow GEGI towards saturation, making it impossible to disentangle single-pulse responses by deconvolution. As saline with high calcium concentration (4 mM) was used in these experiments, the prevalence of MVR under more physiological conditions remains to be investigated. In this context, an important advantage of GEGI measurements compared to GECIs[42] is their independence from extracellular [Ca^{2+}], allowing to investigate the impact of changes in $[Ca^{2+}]_e$ on presynaptic function[43]. In summary, ultrafast GEGIs allow direct visualization of short-term plasticity at individual synapses and may help unraveling the underlying biophysical mechanisms.

Data availability

The data that support the findings of this study are available from the corresponding author (Email: thomas.oertner@zmnh.uni-hamburg.de) on request.

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Author contributions

C.D.D., J.S.W, K.T., and T.G.O. designed the experiments and prepared the manuscript. C.D.D. performed synaptic imaging experiments. N.H., S.K., C.C. and M.G. created and characterized novel iGluSnFR variants, C.S. wrote software to acquire and analyze GEGI data.

Competing interest statement

The authors declare no competing interests.

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Figure legends

Fig 1: Overview of the protocol workflow for the development of glutamate sensors and 2-photon imaging of glutamate transients in individual synapses.

Fig 2: Characterization of GEGIs.

(**a**) Left: Setup for affinity and selectivity determination. GEGI in assay buffer is placed into a fluorescence cuvette with a magnetic stirrer and placed inside the sample chamber of the fluorescence spectrometer (Fluorolog3, Horiba Scientific). With an Aladdin pump, the ligand (Glu, Asp, Ser) is continuously added to the cuvette while the fluorescence is recorded (λ_{ex} = 492 nm, λ_{ex} = 514 nm). Right: Examples of affinity curves of a GEGI for glutamate (black squares) and aspartate (black circles). The fluorescence emission is corrected for dilution and bleaching and plotted against the glutamate concentration in the chamber. The data is then fitted with a Hill equation (green and orange traces for glutamate and aspartate, respectively). (**b**) Left: Setup for stopped-flow kinetic measurement. The solutions are rapidly mixed in the mixing chamber and then pushed into the optical cell where the fluorescence is excited at 492 nm and emission is detected by a PMT with a cut-off filter (>530 nm). For association the GEGI in assay buffer without glutamate is loaded into the drive syringe B and mixed with assay buffer containing increasing concentrations of glutamate filled in drive syringe A. For dissociation the GEGI in assay buffer with saturating glutamate concentration is loaded into the drive syringe B and mixed with GluBP 600n in assay buffer filled into drive syringe A. For both measurements the PMT zero level is determined by mixing assay buffer with assay buffer and the intrinsic fluorescence of the GEGI is recorded by mixing the GEGI in assay buffer with assay buffer (both without Glu). Right: Examples for recorded time traces. Top: Fluorescence increase observed when GEGIs are mixed with increasing glutamate concentration. Bottom: Decrease in fluorescence when glutamate is retained from GEGI by GluBP 600n. The raw data are fitted with monoexponential decays (dark green line).

Fig 3: iGluSnFR expression in CA3 pyramidal cells in organotypic slice culture of rat hippocampus.

(**a**) Co-expression of two plasmids in individual CA3 pyramidal cells in organotypic slice culture. The red fluorescent protein tdimer2 labels the axoplasm while the membrane-anchored iGluSnFR is exposed to the extracellular space. (**b**) Transmitted light image (dark field) of a transfected organotypic culture merged with a wide-field fluorescence image showing three transfected CA3 neurons. The area for synaptic imaging is indicated (red dotted box). Scale bar represents 500 µm**.** (**c**) Two-photon image stack (maximum intensity projection) of CA3 axons in CA1 *stratum radiatum* (cells not identical to panel b). Scale bar represents 10 µm. Image from [13]. (**d**) Maximum intensity projection of two-photon images of CA3 pyramidal neuron expressing $iGlu_u$ 4 days after electroporation (fluorescence intensity is shown as inverted gray values). $iGlu_u$ shown here and other GEGIs had their fluorescence mainly localized to the plasma membrane over the entire cell. The scale bar represents 50 µm (left image) and 5 µm (right image). (**e**) 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 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 >2σ (green traces) and as failures when the peak amplitude is <2σ (gray traces). Note propagation delay between presynaptic APs and glutamate release events at distal bouton.

Fig 4: Localization of fluorescence transients in low and high $[Ca^{2+}]_o$.

(**a**) Morphology of individual boutons. Red fluorescence was upsampled (16 x 16 pixels to 128 x 128 pixels)**,** aligned and averaged over all trials. Scale bars represent 0.5 µm. (**b**) Average response of iGluSnFR superimposed with bouton outline (black line) from red channel (morphology). The bouton outline was generated by thresholding the red channel followed by smoothing. (**c**) Two-dimensional Gaussian fit to average response. On average, the full width at half maximum (FWHM) was 763 ± 29 nm (n = 12; 5 boutons shown here) (**d**) Plotting the center position of 2D Gaussian fits to individual trials. Fusion appears to be localized to a small region on the bouton (active zone). Amplitude ($\Delta F/F_0$) of individual trials is color-coded. Scale bars represent 0.5 µm. (**e**) Increasing the extracellular Ca^{2+} concentration increased the amplitude of individual responses, but did not lead to release events outside the active zone. (**f**) Fitting responses classified as failures (< 2σ of baseline noise) did not reveal any clustering, indicating that there was indeed no localized signal in these trials (true negatives). (**g**) Fitting frames before stimulation (green baseline fluorescence) did also not result in clustering.

Fig. 5: Signal extraction of GEGI transients from a single Schaffer collateral bouton in CA1.

(**a**) The spatial extent of iGluSnFR fluorescence transients was 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. At t = 58 ms, a glutamate release event from an individual presynaptic terminal occurred and was sampled twice during every spiral scan. (**d**) Only columns with ΔF > ½ max (ΔF) were analyzed (ROI, region of interest). Green trace: Extracted

fluorescence transient (before bleach correction). (**e**) *Upper panel:* Average of 10 trials (single APs) to analyze lateral spread of signal from t=0 to t=18 ms. *Lower panel:* Decay of fluorescence transient (5 scan lines plotted = 18 ms). Note the lack of lateral spread of the signal due to slow diffusion of membrane-anchored GEGI. (**f**) iGluSnFR response amplitude (green markers) of a single bouton stimulated with single APs every 10 s. Note that response amplitudes were constant over time. A time window before stimulation was analyzed to estimate imaging noise (gray markers). The histogram of response amplitudes shows separation between failures of glutamate release (overlap with the baseline histogram) and successes.

Fig. 6: Release statistics of neighboring boutons on the same axon.

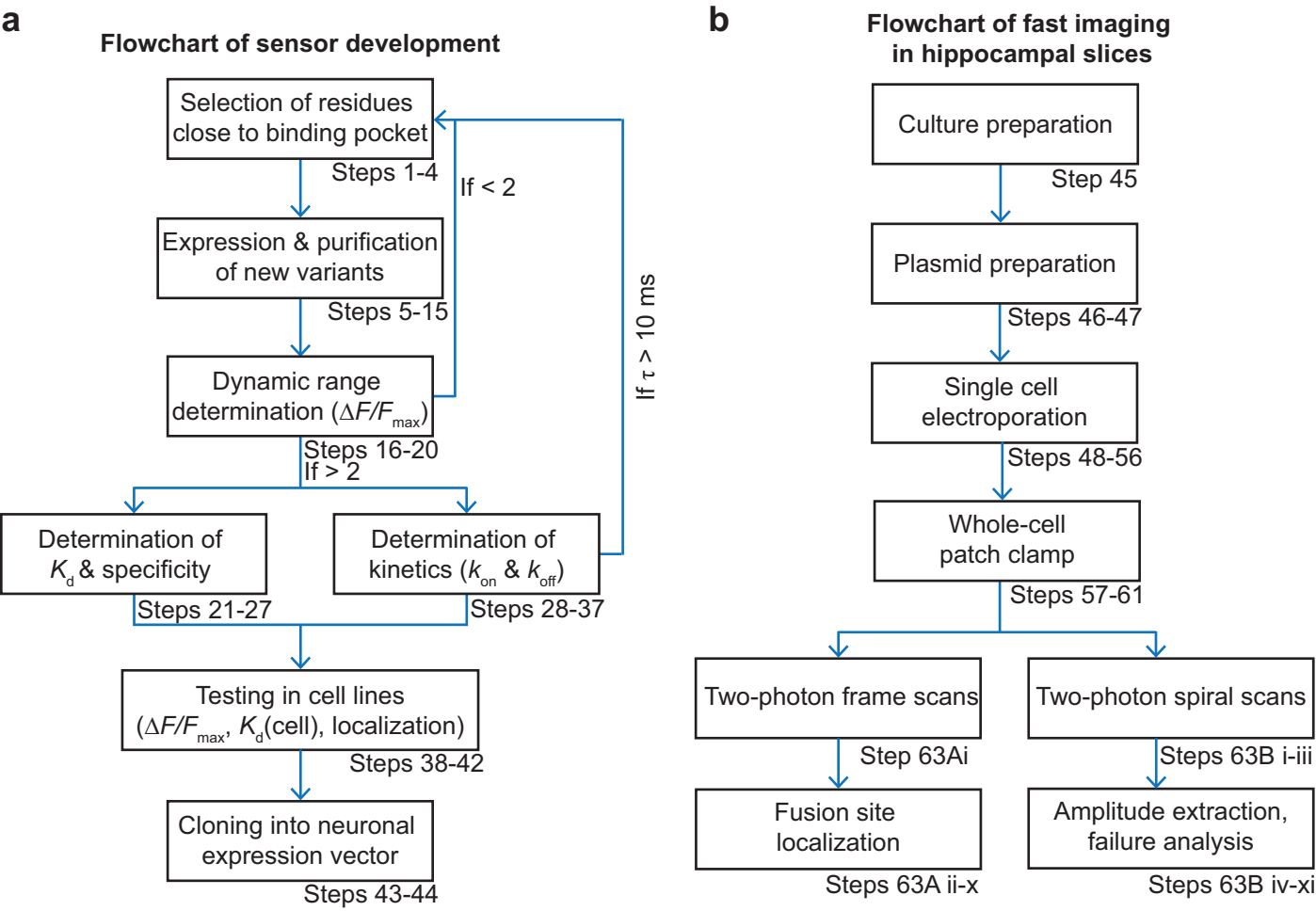
(**a**) Glutamate transients (green dots) and baseline fluorescence (grey dots) of two neighboring boutons measured in ACSF containing 2 mM Ca^{2+} and 1 mM Mg^{2+} located on the same axon (left panels) and their corresponding histogram counts (right panels). (**b**) Glutamate transients (blue dots) and baseline fluorescence (grey dots) of two neighboring boutons located on the same axon (left panels) and their corresponding histograms (right panels) measured in ACSF containing 2 mM Ca^{2+} and 1 mM Mg^{2+} and their corresponding histogram counts (right panels). (**c**) Synaptic release probability (p_r) (calculated out of ~ 100 trials) of individual boutons (B1) and their neighboring bouton on the same axon (B2); n=10. The pair of neighboring boutons from (a) and (b) are shown in green and blue, respectively. (**d**) Histogram of $\Delta\ p_r = |p_r\text{BX}-p_r\text{BY}|$. BX and BY are randomly paired from the dataset in (c). $|\Delta p_r\text{B2}-\ \Delta p_r\text{B1}|$ (red vertical line) is significantly more similar than mean $\Delta\ p_r$ of two boutons paired randomly from the same dataset; (p-value: 0.0148). (**e**) Amplitude of the iGluSnFR signal given a success of a bouton B1 and its neighbor on the same axon (B2); n=10. The pair of neighboring boutons from (a) and (b) are shown in green and blue, respectively. (**f**) Histogram count of the difference between the average $\Delta F/F_0$ of successes only of two random neighboring boutons. The difference of the average $\Delta F/F_0$ of successes from two neighboring boutons (red vertical line) is not significantly more similar than the randomly connected pairs of boutons.

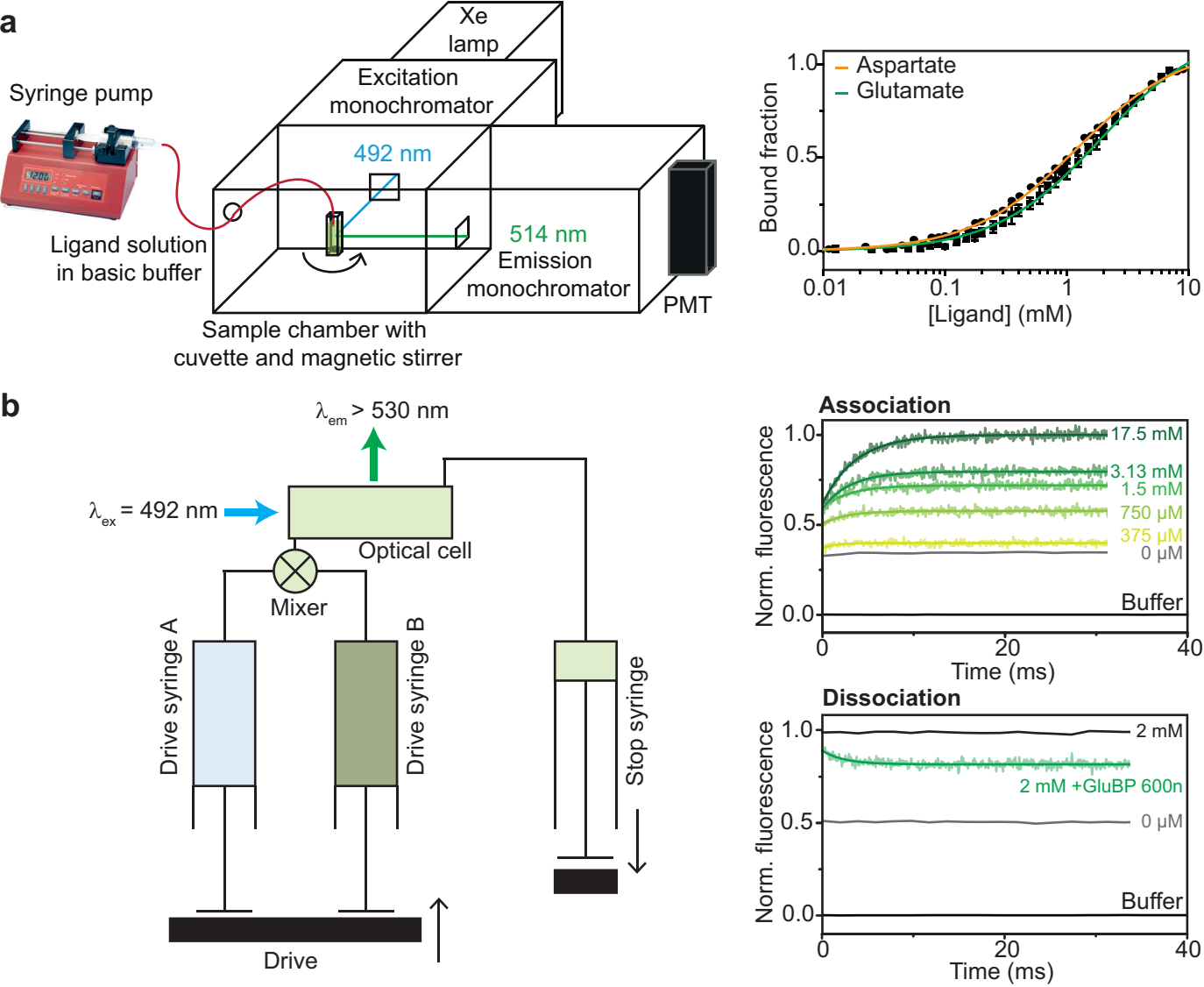
Fig. 7: Resolving high-frequency transmission with ultrafast GEGI, $iGlu_u$.

(**a**) The presynaptic neuron was driven to spike at 100 Hz (10 APs). After a pause of 0.5 s, one more AP was triggered to quantify recovery from depression. iGluSnFR signals (blue) or $iGlu_u$ signals (green) were recorded at single Schaffer collateral boutons (only during the 100 Hz train) in *stratum radiatum*. Recordings were performed in 4 mM Ca^{2+} and 1 mM Mg^{2+} to ensure very high release probability. Note summation and saturation of iGluSnFR (but not $iGlu_u$) during the high-frequency train. (**b**) $iGlu_u$ responses to the 1^{st} AP of the 100 Hz train, to the 9^{th} AP of the train, and to the recovery pulse. To minimize bleaching, the bouton was only imaged (spiral scans) during pulses 1, 9 and 11. Note frequent failures in response to pulse 9. (**c**) Extracted single-trial amplitudes reveal strong depression and full recovery of this bouton. Failures of glutamate release can be seen in response to pulse 9. Note the large amplitude of initial responses compared to depressed responses. Plots were generated with violinplot.m (GitHub, ©Bastian Bechtold).

SUPPLEMENTARY MATERIAL

- **Supplementary Figure 1**


a
Flowchart of sensor development
Selection of residues close to binding pocket
Steps 1-4
Expression & purification of new variants
Steps 5-15
Dynamic range determination (ΔF/F_max)
Steps 16-20
If < 2
If > 2
If τ > 10 ms
Determination of K_d & specificity
Steps 21-27
Determination of kinetics (k_on & k_off)
Steps 28-37
Testing in cell lines (ΔF/F_max, K_d(cell), localization)
Steps 38-42
Cloning into neuronal expression vector
Steps 43-44
b
Flowchart of fast imaging in hippocampal slices
Culture preparation
Step 45
Plasmid preparation
Steps 46-47
Single cell electroporation
Steps 48-56
Whole-cell patch clamp
Steps 57-61
Two-photon frame scans
Step 63Ai
Fusion site localization
Steps 63A ii-x
Two-photon spiral scans
Steps 63B i-iii
Amplitude extraction, failure analysis
Steps 63B iv-xi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514 nm
Emission monochromator
PMT
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Glutamate
Bound fraction
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0.5
0.0
0.01
0.1
1
10
[Ligand] (mM)
b
λem > 530 nm
λex = 492 nm
Optical cell
Mixer
Drive syringe A
Drive syringe B
Stop syringe
Drive
Association
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3.13 mM
1.5 mM
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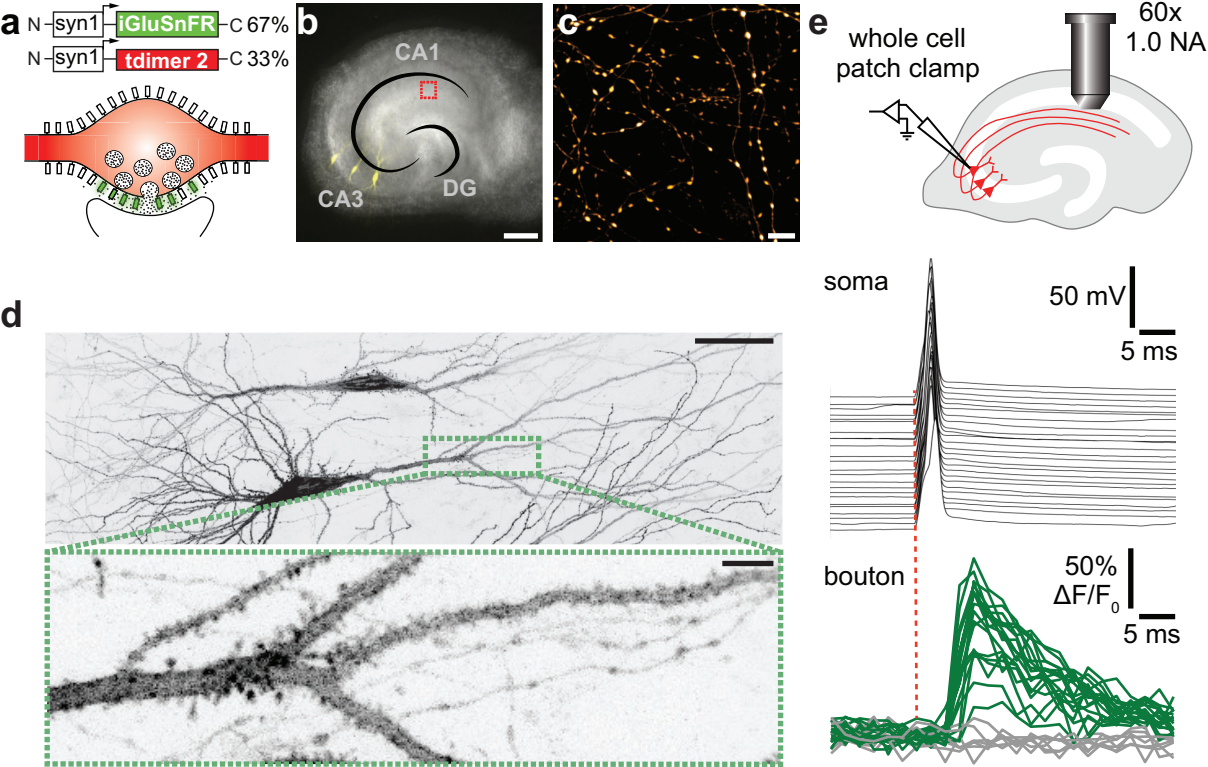
Buffer
Time (ms)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CA3
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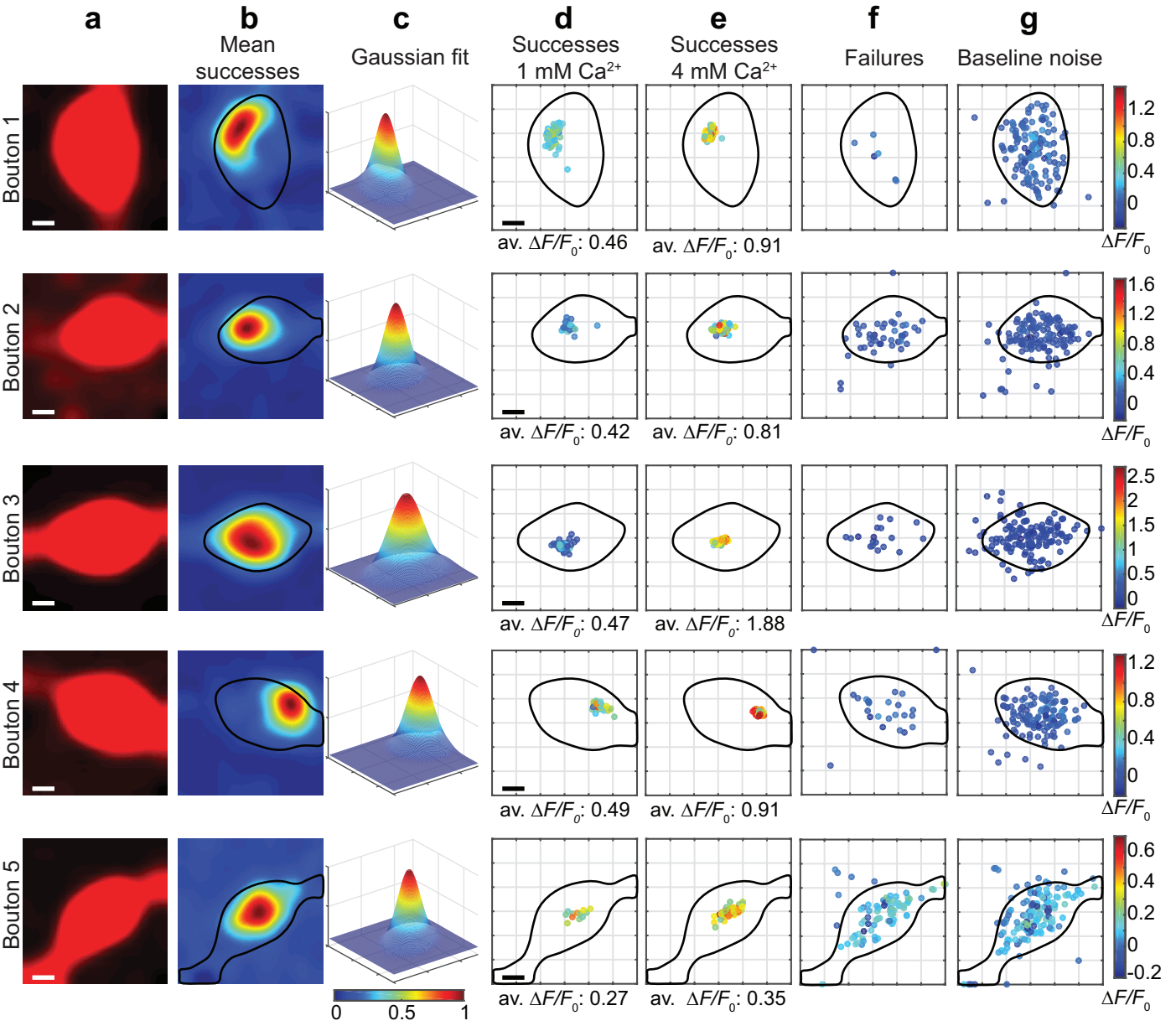
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b
Mean successes
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Gaussian fit
d
Successes 1 mM Ca2+
e
Successes 4 mM Ca2+
f
Failures
g
Baseline noise
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Bouton 2
Bouton 3
Bouton 4
Bouton 5
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0.5
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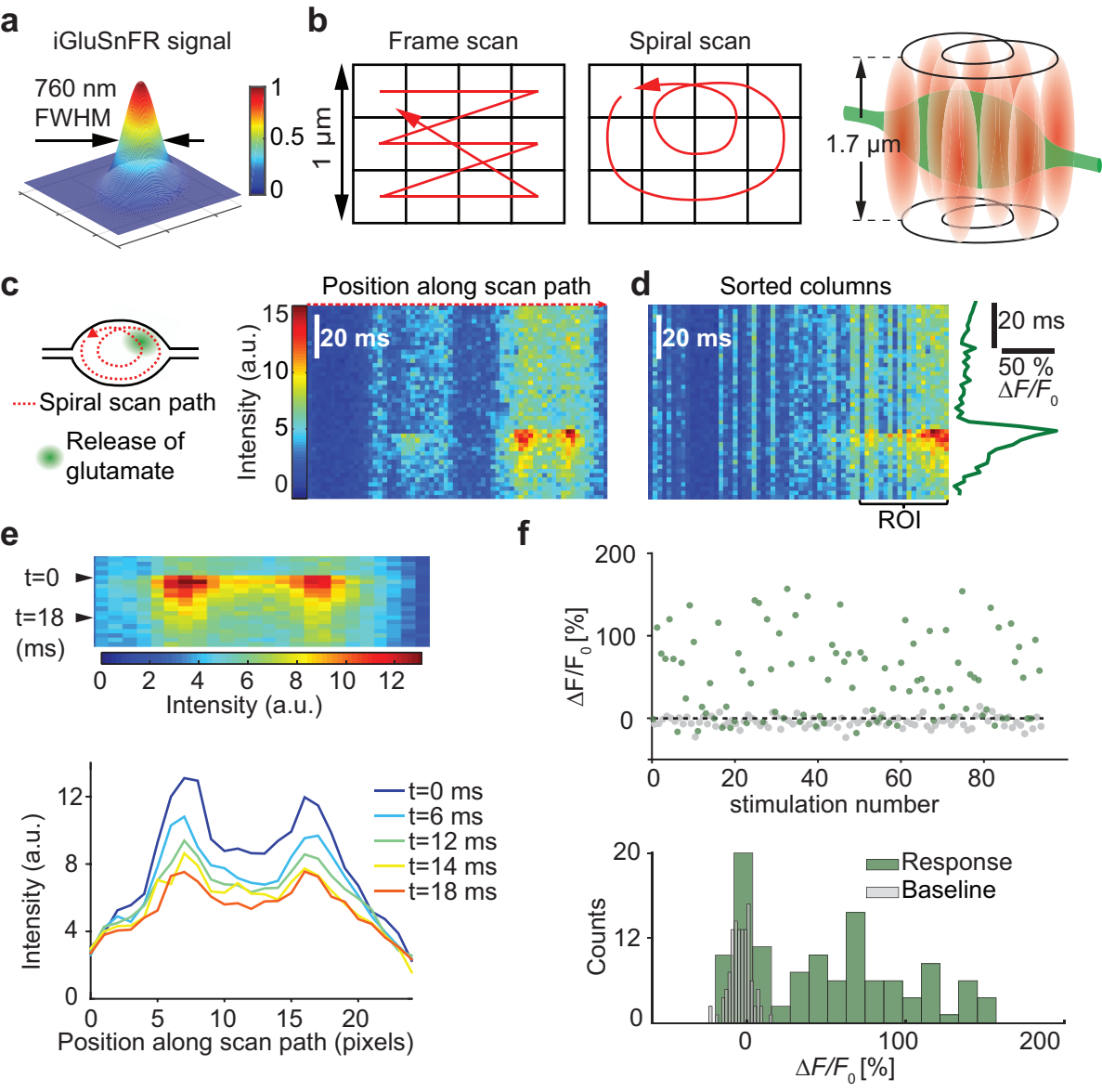
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iGluSnFR signal
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FWHM
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0.5
0
b
Frame scan
Spiral scan
1 µm
1.7 µm
c
Spiral scan path
Release of
glutamate
Position along scan path
Intensity (a.u.)
20 ms
d
Sorted columns
20 ms
20 ms
50 %
ΔF/F0
ROI
e
t=0
t=18
(ms)
Intensity (a.u.)
t=0 ms
t=6 ms
t=12 ms
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Counts
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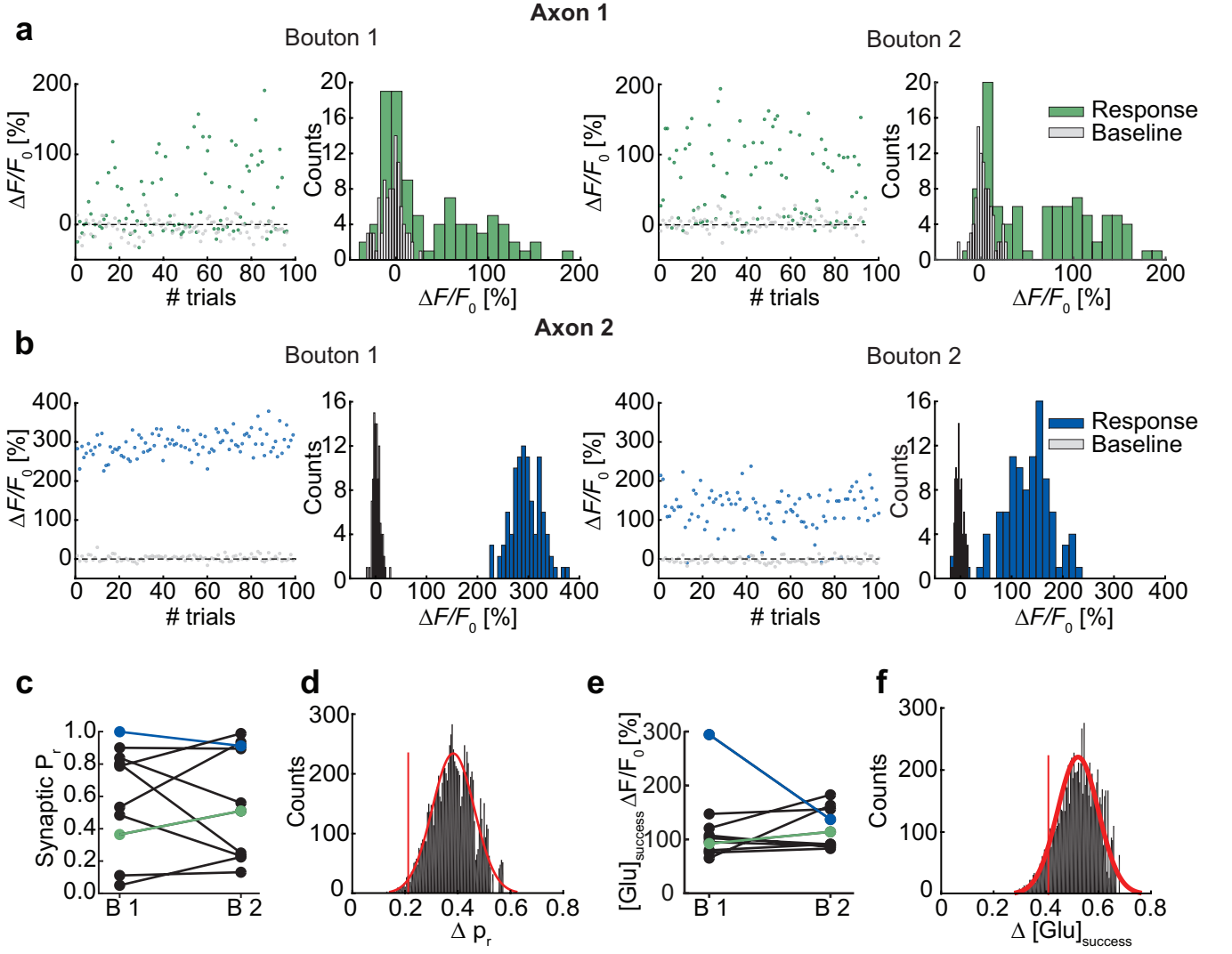
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Bouton 1
Bouton 2
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trials
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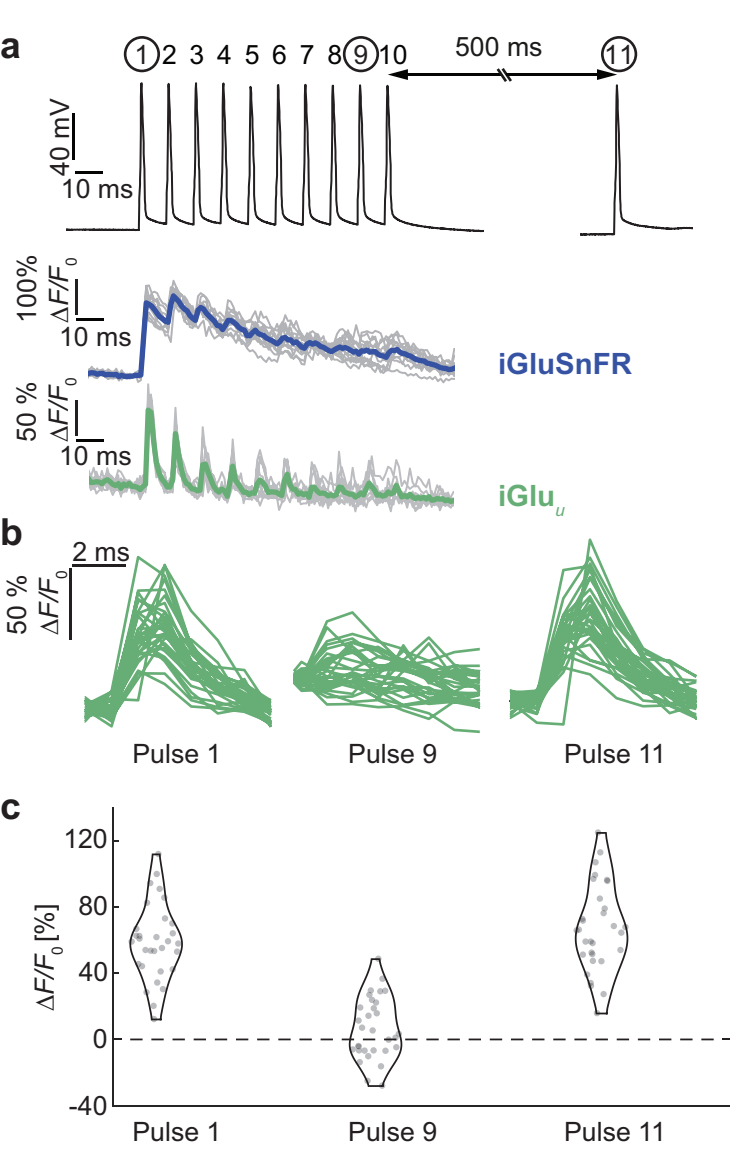
Δ [Glu]success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100%
ΔF/F0
10 ms
iGluSnFR
50 %
ΔF/F0
10 ms
iGluu
b
2 ms
50 %
ΔF/F0
Pulse 1
Pulse 9
Pulse 11
c
120
80
40
0
-40
ΔF/F0 [%]
Pulse 1
Pulse 9
Pulse 11