Supplementary Fig. 1. Selection of sites in GltI for insertion of cpGFP.
Supplementary Fig. 1. Selection of sites in GltI for insertion of cpGFP. Top row, conformational change in MBP upon binding maltose, and homology between MBP and GltI. In MBP, there is movement in the middle of the loop near the hinge (residue 175), but that loop is non-existent in GltI (top center, top right). However, there is also movement at the end of the helix leading into that loop (residue 165 in MBP, middle center, end of yellow helix) and we identified residue 152 of GltI as the homologous insertion point (middle right, end of yellow helix). We also observed that the beginning of the helix traversing the hinge in MBP (bottom center, red helix, residue 311 in MBP) was a point of significant local conformational change, and good insertion position for making a sensor. By homology, we thus chose to insert cpGFP at the beginning of the coil traversing the hinge in GltI (bottom right, red coil, residue 249 in GltI). Expecting the region at the other end of the coil to also undergo local conformational changes (due to its location near the inter-domain interface), we also inserted cpGFP after residue 264 of GltI (bottom right), even though the homologous position in MBP was not tested.

Since the ~120 µM glutamate present in E. coli extracts would saturate GltI (Kd for glutamate ~ 1 µM) and make measurement of the apo-state fluorescence technically challenging, we began sensor development with a hinge region mutation (middle left, green sticks, Ala184Val; numbered 207, if including the 23 amino acid N-terminal periplasmic leader sequence) that decreases glutamate-binding affinity to ~100 µM without compromising the stereochemical integrity of the binding site itself.
Supplementary Fig. 2. EcGltI152-cpGFP amino acid sequence. Insertion of cpGFP after residue 152 of GltI did not yield any expressed protein (data not shown).
**Supplementary Fig. 3.** High throughput screening of linker variants. A small number of linker variants (see ref. 3 for a detailed discussion of linker engineering in this context, and Supplementary Fig. 4 for primer details) were made. Variants of EcGlitI264-cpGFP (Supplementary Fig. 5) showed small changes in fluorescence upon addition of saturating glutamate in the initial assay, but not in a reliably concentration-dependent manner (data not shown). b) Insertion of cpGFP after residue 249 of Glit (Supplementary Fig. 6.) followed by linker screening resulted in a number of variants with small changes in fluorescence that were concentration dependent, but extensive screening did not increase $(\Delta F/F)_{\text{max}}$ beyond 0.5. c) Shifting the insertion point by four residues (after residue 253, Supplementary Fig. 7.) to place cpGFP closer to the inter-domain hinge region, followed by screening of mutations to linker 1 (Glit-cpGFP) yielded a variant (“Glit253.L1LV”) with $(\Delta F/F)_{\text{max}}$ of 1.0. Subsequent randomization of linker 2 (cpGFP-GlI) yielded a variant (“Glit253.L1LV/L2NP”) with $(\Delta F/F)_{\text{max}}$ of 4.5 ± 0.1. Screening details: clarified lysates from individual variants were tested in 96-well format. Fluorescence was measured in the absence and presence of 10 mM glutamate. Positive signals from variants that showed a significant increase in fluorescence in the high throughput screen were confirmed by glutamate titrations. In panel c, the three separate curves represent different groups of primers used and the subsequent improvement on L1LV to yield L1LV/L2NP.
Supplementary Fig. 4. EcGltI Mutagenenic primers. DNA primers encoding the following amino acids were used to generate libraries of variants for screening. All 20 amino acids (“x”) was encoded by NNS.
Supplementary Fig. 5. EcGltl264-cpGFP amino acid sequence.
Supplementary Fig. 6. EcGltl249-cpGFP amino acid sequence.
Supplementary Fig. 7. EcGltl253-cpGFP amino acid sequence.
Supplementary Fig. 8. Gltl253.L1LV/L2NP has a $(\Delta F/F)_{\text{max}}$ of 4.5. Emission spectra for 1 µM eGFP, cpGFP, Gltl253.L1LV/L2NP, and Gltl253.L1LV/L2NP.T203V in PBS (dashed) and PBS + 10 mM glutamate (solid). $\lambda_{\text{ex}} = 485$ nm, 5 nm Ex/Em bandpass.
**Supplementary Fig. 9.** The chromophore of Glu253.L1LV/L2NP has a pKₐ of 6.5 in the glutamate-bound and 7.0 in the ligand-free state. Top, fluorescence of the glutamate saturated (filled circles) and ligand free (open circles) normalized to peak fluorescence. Bottom, measurement of ΔF/F as a function of pH. 1 µM protein was diluted in a triple-buffer containing 10 mM glycine, 10 mM sodium citrate, and 10 mM TRIS.HCl. λₑₓ = 485 nm, 5 nm bandpass; λₑₘ = 515 nm, 5 nm bandpass. The pKₐ of the glutamate-bound form is 6.5, and that of the ligand-free form is 7.0.
Supplementary Fig. 10.
Supplementary Fig. 10. GltI253-cpGFP.L1LV/L2NP has no detectible affinity for a panel of decoy L-amino acids (glutamine, asparagine, cysteine, arginine, histidine, serine, proline, tryptophan, β-alanine, taurine), neurotransmitters (glycine, GABA, acetylcholine, serotonin, D-serine, dopamine and its metabolic precursor L-DOPA), pharmacological glutamate receptor agonists (AMPA, NMDA, kainate) or antagonists (philanthotoxin PhTx-74, D-AP5, NBQX, CNQX, DNQX, CPP), or a glutamate transporter inhibitor (TBOA). Direct titrations (left) show no fluorescent response to any of the compounds. Titrations of glutamate with competing analytes (right), were performed in the presence of 10 mM L-amino acids (or 1 mM tryptophan, due to limiting solubility), 10 mM neurotransmitters, 1 mM agonists, or 50 µM inhibitors. The compounds have no apparent effect on the affinity or response of GltI253-cpGFP.L1LV/L2NP for glutamate.
Supplementary Fig. 11. Stopped-flow characterization of L1LV/L2NP. 0.1 µM GltI253.L1LV/L2NP was mixed rapidly in a Cary Eclipse fluorimeter with stopped-flow attachment (Varian/Agilent Technologies) with varying concentrations of glutamate in HBSS. Rise time is faster than detectible by the instrument, which has a dead-time of 6 msec. The saturation levels reached by kinetics indicate an affinity that is in agreement with the values
determined by standard equilibrium measurements. \( \lambda_{\text{ex}} = 485 \text{ nm}, 5 \text{ nm bandpass}; \lambda_{\text{em}} = 515 \text{ nm}, 10 \text{ nm bandpass}. \)

**Supplementary Fig. 12.** 2-photon photostability. The emission intensity of 1 \( \mu \text{M} \) GltI-cpGFP.L1LV/L2NP in PBS + 10 mM glutamate was measured over one minute of excitation at 940nm (50 mW laser power at the focus). Mutation of Thr203 in GFP to Val has been shown to increase \( (\Delta F/F)_{\text{max}} \) and photostability of the GCaMP calcium sensor \(^4\), so we tested the effect of that mutation in Glt253.L1LV/L2NP. It decreases photostability under 2-photon excitation and also decreases \( (\Delta F/F)_{\text{max}} \) (Supplementary Fig. S8); therefore, it was not characterized further.
Supplementary Fig. 13. (top) 2-photon excitation spectra of GltI253.L1LV/L2NP. Open circles, ligand free. Filled circles, 10 mM glutamate. 1 µM protein in PBS was excited at 930 nm at 1 mW power at the focus point. Emission was read at 550 nm with a 88 nm bandpass filter. (bottom) 2-photon lifetime measurement of GltI253.L1LV/L2NP. Grey dots, ligand free. Black dots, 1 mM glutamate. Single exponential curve fits show a lifetime of 2.51 nsec. for the saturated form, and 2.60 nsec for the ligand-free form. 1.8 µM protein in PBS was excited at 960 nm at 1 mW power at the focus point. Emission was read at 550 nm with a 88 nm bandpass filter.
**Supplementary Fig. 14.** Schematic of expressed proteins: L1LV/L2NP (represented by blue/orange for the GltI component and green for the cpGFP component, matching Fig. 1b.) with an N-terminal histidine tag for purification (grey) from the pRSET-A vector was used for in vitro screening; the pDisplay vector (Invitrogen) includes a secretion sequence (pink), a hemagglutinin (HA) tag (magenta), a myc tag (turquoise), and a PDGFR transmembrane domain (brown); iGluSnFR is L1LV/L2NP in a modified version of pDisplay, lacking the hemagglutinin tag.
Supplementary Fig. 15. Glutamate titrations of bacterially expressed L1LV/L2NP including the pRSET-A tag (black), myc tag (blue), hemagglutinin tag (magenta), and both the myc and hemagglutinin tags (purple). Protein was characterized in clarified lysate. In situ titration of iGluSnFR on HEK cells in yellow (reproduced from one ROI in Fig. 1b) is shown for reference.
Supplementary Fig. 16. Expression of iGluSnFR on surface of neurons and astrocytes is stable for 4 weeks. Confocal image of iGluSnFR on the surface of neurons (left) and astrocytes (right) 4 weeks after infection with AAV2/1.*hSynap*.iGluSnFR (left) or AAV2/1.*GFAP*.iGluSnFR (right). Insets show intensity profiles across the line segments shown in the panels; expression is uniform across the membrane. Scale bars, 10 μm.
Supplementary Fig. 17. Response of iGluSnFR on neurons (hSynap) and astrocytes (GFAP) — co-cultured with neurons — to increasing numbers of field stimulations. Field stimulations were delivered at 40V, 30 Hz, 1 millisecond pulses for the following trains: 1, 2, 3, 5, 10, 20, 40 field stimulations.
Supplementary Fig. 18. pH titration of MBP165.PPYF. (“iMaltSnFR” is a membrane-tethered version of the intensity-based maltose sensor MBP165.PPYF$^3$) Top, fluorescence of the maltose saturated (10 mM maltose, filled circles) and ligand free (open circles) normalized to peak fluorescence. Bottom, measurement of $\Delta F/F$ as a function of pH. 1 µM protein was diluted in a triple-buffer containing 10 mM glycine, 10 mM sodium citrate, and 10 mM TRIS.HCl. $\lambda_{ex} = 485$ nm, 5 nm bandpass; $\lambda_{em} = 515$ nm, 5 nm bandpass.
**Supplementary Fig. 19.** Determination of glutamate concentration in media. ΔF/F was calculated by measuring the fluorescence before and after addition of 10 µL test solution (dilutions of a glutamate stock or media) to 100 µL 0.1 µM GltI-cpGFP253.L1LV/L2NP solution in PBS. The concentration of glutamate in media as determined by both curve fitting and interception with the standard curve is the same. DMEM + 10%FBS contains ~200 µM glutamate; neuronal culture growth medium contains ~100 µM glutamate.
Supplementary Fig. 20. Single trial iGluSnFR signals in response to uncaging at spines. a) Example single trial iGluSnFR traces at 8 spines from 5 neurons during single pulse two-photon glutamate uncaging. b, Relationship between somatically-recorded EPSP amplitude and iGluSnFR signals for the traces shown in (a). Note that the uncaging laser intensities used for this analysis resulted in EPSPs similar in amplitude to those observed under physiological conditions⁵.
**Supplementary Fig. 21.** Confocal fluorescence image of iGluSnFR-expressing ganglion cells in the mouse retina. Image was taken 16 days after in vivo transduction with AAV2/1-hSynapsin-iGluSnFR. iGluSnFR expression is readily visible in the ganglion cell somata (examples marked with asterisks) and their axons (arrowheads) without immunohistochemical amplification (green: iGluSnFR, blue: nuclear stain DAPI).
Supplementary Fig. 22. Fitting of leaky integrator model for predicted RCaMP signal from glutamate input (iGluSnFR signal). Root mean squared error (RMSE) between observed and predicted RCaMP signals can be minimized by adjusting tau, the leak time constant; this is data from trial #7, which is presented in Fig. 5c., shows a time constant of 11.0 ± 5.2 sec. ($n = 8$ trials on a single animal; mean ± s.d.; range: 6-20 sec.)
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**Supplementary Fig. 23.** RMSE and tau values for 8 separate trials.
**Supplementary Fig. 24.** iGluSnFR fluorescence signals in AVA neurons are eliminated in *eat-4* vGluT mutants. iGluSnFR fluorescence is shown in green and RCaMP fluorescence in red, in eight animals expressing both transgenes. RCaMP calcium transients are reduced in frequency, but normal in amplitude, in *eat-4* mutants. iGluSnFR signals are absent.
Supplementary Fig. 25. Glutamate imaging in the zebrafish optic tectum. We tested the ability of iGluSnFR to detect glutamate release evoked by visual stimulation in the optic tectum of larval zebrafish. A neuropil region, containing tectal dendrites and axon terminals of retinal ganglion cells, mosaically labeled with iGluSnFR or GCaMP5G6, was imaged under two-photon excitation, while thin dark bars swept in four different directions (forward or backward along two orthogonal axes) across the fish’s lower visual field. These stimuli elicit calcium transients reliably in tectal neurons, and responses are observed throughout the dendritic tree, as measured using genetically encoded calcium indicators6. In contrast, iGluSnFR responses are much more localized. Visually evoked iGluSnFR fluorescence changes were as high as 100%, with SNR and kinetics sufficient to reveal that glutamate release is sensitive to the stimulus direction (a,b) and that different sites respond at different times (a,c). The spatial organization of these response dynamics can be seen in maps of response magnitude (b) and time to peak (c), and in the frame-by-frame evolution of the fluorescence changes (Supplementary Fig. S26). a) Time course of iGluSnFR fluorescence change in 4 handpicked regions of interest in the left optic tectum of a 6 days post fertilization larval zebrafish. A schematic indicating the imaged region is shown at the lower left. Arrows represent direction of the visual stimulus, with up being tail to head motion. Vertical lines indicate the start and end of stimulus motion. b,c) Spatial maps of peak response magnitude and time to peak.
amplitude (b) and time (c) for the same image region as in a). Response amplitude is \( (\Delta F/F)_{\text{max}} \) during visual stimulus presentation minus the maximum during baseline. Relative peak time is coded from blue (earlier) through white to red (later). Since the overall timing of the response to each stimulus is different, the 267-millisecond window was chosen manually for each direction.
Supplementary Fig. 26. Time course of fluorescence level above peak baseline value ($\Delta F/F$) in zebrafish optic tectum. The spatial organization of response dynamics can be seen in the frame-by-frame evolution of the fluorescence changes. Frame scans collected at 22.47 Hz.
**Supplementary Fig. 27.** Comparison of kinetics of GCaMP5G and iGluSnFR. Local patches of iGluSnFR activity that rise and fall at different times can be clearly distinguished. Visualizing release of glutamate on a sub-100ms timescale is possible because of the rapid kinetics of the indicator. Under the same stimulation conditions, iGluSnFR response decay is $\sim$3 times faster than the state-of-the-art genetically encoded indicator, GCaMP5G$^6$. The normalized average response in the tectal neuropil for GCaMP5G and iGluSnFR is shown, under identical labeling and visual stimulation conditions.
Supplementary Fig. 28. Confocal z-stack image (max-intensity projection) of layer V two weeks after virus injection (800-1,000 μm below pial surface) reveals strong expression of iGluSnFR. An image (right panel) taken from the z-stack (red box), which bisects multiple somata (3 cells marked by red asterisks), shows that iGluSnFR is excluded from the nucleus. Scale bar, 10 μm.
Supplementary Fig. 29. Ketamine/xylazine anesthesia induces repetitive activity of single synapses that lasts for hours. Average ΔF/F at 0 hr was 20 ± 5.7 S.D. (16 events in total) and 19 ± 3.9 S.D. (15 events in total) at 1 hr.
Supplementary Fig. 30. Imaging of apical dendrites during resting-awake state. In vivo two-photon imaging in layer I of an awake animal revealed individual dendrites containing multiple glutamate events (3 ROIs circled). Example traces of fluorescence changes of 3 ROIs during head-fixed treadmill running. 2-second recordings showed repetitive glutamate transients at individual synapses that lasted typically milliseconds with consistent changes in $\Delta F/F$. Scale bar, 10 µm.
Supplementary Fig. 31. Two-photon images of a dendritic branch containing numerous dendritic spines that report glutamate transients during forward running. Six trials of forward running are shown and red arrows indicate a glutamate event. Note that spines sitting adjacent to each other (< 2 μm) report robust glutamate transients.
Supplementary Fig 32. Application of tetrodotoxin (TTX, 1 nM in ACSF) via craniotomy.
Supplementary Fig. 33. Pilocarpine (a non-selective muscarinic receptor agonist, at 300 mg/kg) doubles the amplitude of observed glutamate events.
References