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Multiplexed and spatiotemporal measurement of glutamate secreted by neurons and bacteria

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Materials and Methods

Materials

The bacterial iGluSnFR expression vector pRSET.GltI253-cpGFP.L1LV/LVNP was a gift from Loren Looger (Addgene plasmid No. 41733). Carbenicillin (Carb) was from Gold Biotechnology (St. Louis, MO). Streptavidin, bovine serum albumin (BSA) (A3059), and adenosine 5'-triphosphate (ATP) were from Sigma-Aldrich (St. Louis, MO). QIAprep Spin Miniprep, QIAquick Gel Extraction, Phusion DNA polymerase, and Ni–NTA resin were obtained from Qiagen (Seoul, Korea). Restriction enzymes and T4 ligase were obtained from New England Biolabs (Ipswich, MA). Alexa-labeled secondary antibodies, bacterial cell lines (DH10B and BL21 (DE3)), neuronal cell culture media, streptavidin conjugated with horseradish peroxidase (SAV-HRP), Biocytin-Alexa 594, D-biotin, Dynabeads® M-280 Streptavidin, Dulbecco's phosphate buffered saline (DPBS), Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit, and Pierce BCA Protein Assay Kit were from Thermo Fisher Scientific Inc. (Waltham, MA).

Sensor engineering

The GltI.253-cpGFP gene was amplified from pRSET.GltI253-cpGFP.L1LV/L2NP plasmid [1] by PCR using the primer pairs, biGSFR-F (5′- cga tgg GGA TCC gcc gca gg -3′, *BamH*I) and biGSFR-R (5′- ggc agc GAA TTC GCG GCC GCt gcg gcc CTG CAG ttt cag tgc ctt gtc att cg -3′, *EcoR*I, *Not*I, and *Pst*I). The PCR product of GltI.253-cpGFP was digested with BamHI/EcoRI and ligated with plasmid pRSET-A (Invitrogen) restricted with the same enzyme. To incorporate the N-terminal biotinylation motif sequence, the sequence for AP-tag was amplified from the pNICE-HA-H6-NL1-H8-RFP-AP vector (submitted) by PCR using the primer pairs, GS.Avi-F (5′- att gca CTG CAG gga ggc agc ga -3′, *Pst*I) and Avi4-R (5′- ggc agc GCG GCC GCt tac tcg tgc cac tcg atc ttc tgg gcc tcg aag atg tcg ttc -3′, *Not*I). The PCR product of AP-tag was digested with *Pst*I/*Not*I and sequentially inserted into the GltI.253-cpGFP carrying plasmid restricted with the same enzymes. The resulting plasmid (pRSET-biGSFR) was used to transform *E. coli* BL21 (DE3) by electroporation.

Protein expression and purification

The recombinant cells were grown overnight in 2 mL of liquid lysogeny broth (LB) medium with 100 μg/L Carb at 37 ºC and 150 rpm. The inoculum was refreshed with 200 mL of LB medium containing 100 μg/L Carb. The cells were grown until mid-log phase (OD_{600} = 0.5~0.6) when isopropyl β-D-1-thiogalactopyranoside (IPTG) was

added to a final concentration of 0.2 mM. The cells were cultivated for an additional 20 h at 18 ºC and 150 rpm, and harvested by centrifugation (13,000 rpm) for 10 min at 4 ºC. The cells were broken by sonication and the sensor protein was purified by Ni-NTA affinity chromatography. The purity was analysed by SDS-PAGE and the concentration of the purified protein was determined by BCA assay.

Conjugation of sensor proteins with magnetic SAV microbeads

The purified biGSFR (540 µL, 1.5 mg/mL) was incubated with Dynabeads Streptavidin M280 suspension (60 µL, 6×10^5 beads/µL) at 25 ºC until saturation. The biGSFR microbeads were magnetically separated from the solution, washed briefly, and resuspended in 0.6 mL DPBS before use.

Animals and ethical approval

All animal studies and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University.

Primary culture of hippocampal neurons

Primary hippocampal neurons were obtained from Sprague-Dawley rat embryos at day 18 of gestation (E18). Briefly, hippocampus dissected from E18 rat embryos were rinsed with HBSS, and then incubated with papain and DNase with 60 rpm for 30 min at 37 °C. After sequential rinsing with solution of 10% and 5% FBS in HBSS, individual single cells were mechanically isolated by trituration 10 times in 2 mL HBSS containing DNase with silanized Pasteur pipette (the pipette tip was barely polished with fire). The cell suspension was diluted to density of 2×10^5 cells/mL with plating media containing MEM supplemented with 0.6% (w/v) glucose, 10 mM sodium pyruvate, 1 mg/mL FBS, and 1% penicillin-streptomycin. Then, the cell-media solution plated on the PDK-coated glasses placed in Petri dish. After 3 h the cell culture media was exchanged with B27-supplemented neurobasal media containing 2 mM glutamax. Cultures were maintained in an incubator at 37 °C and 5% CO_2 atmosphere.

Culture of *Corynebacterium glutamicum*

The culture medium (glucose 40 g/L, urea 8.0 g/L, biotin 0.003%, K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 2.5 g/L, and $MnSO_4 \cdot 7H_2O$ 0.1 g/L, $CaCO_3$ 1.6 g/L, pH 7.0) was inoculated with *C. glutamicum* ATCC 13032 (American Type Culture Collection) and cultured at 30 ºC for 25 and 54 h when 10 mL of the supernatant was harvested by centrifugation, autoclaving, and filtering for the measurement of glutamate in the culture medium using biGSFR microsensors, Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit, and HPLC. The culture medium before inoculation was used as a control.

Determination of glutamate concentration

For the measurement of glutamate spillover, hippocampal neurons were cultured for 15 DIV. The culture medium was exchanged with DPBS containing $CaCl_2$ before the application of biGSFR microsensors (3×10^5). After incubation for 10 min fluorescence images were captured by a fluorescence microscope (Eclipse Ti-E, Nikon, Japan) with excitation/emission at 485/515 nm at 4.8 frame per second (fps), during which the neurons were stimulated by applying KCl (200 mM final in DPBS). The standard titration curve was also obtained in DPBS with 209 ms exposure time corresponding to 4.8 fps. The fluorescence images were analysed by NIS-Element software (Nikon) where at least ten region of interests (ROIs) were selected for mean fluorescence intensity (MFI)

calculation. After the fluorescence measurements, changes in fluorescence intensity of the ROIs were calculated as $\Delta F/F$, where F is the average baseline fluorescence and ΔF is the fluorescence intensity at glutamate input minus the baseline fluorescence ($\Delta F/F = (F_{glutamate} - F_0)/F_0$).

For the measurement of glutamate production by *C. glutamicum*, we used a 96-well microplate reader (Synergy Mx, BioTek) with excitation/emission at 480/510 nm and 2.4×10^5 microsensors in 4 μL culture medium per 100 μL sample per well in triplicate. The ligand-binding data were fit to a single-site binding isotherm: $S = (F - F_0)/(F_{sat} - F_0) = [L]/(K_d + [L])$, where S is saturation, [L] is ligand concentration, F is fluorescence intensity, F_0 is fluorescence intensity in the absence of ligand, and F_{sat} is fluorescence intensity at saturation with ligand.[2]

For the HPLC analysis of glutamate in cell medium, we used Dionex Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific Inc., USA) equipped with a VDSpher 100 C_{18}-E column (VDS Optilab, Germany) and a UV/Vis detector (338 nm).

The Amplex Red assay was done by the manufacturer's protocol with additional non-linear fitting for higher Glu concentrations. The Glu standard solution was prepared using the *C. glutamicum* culture media instead of the provided buffer solution to fix the reagent ratio during serial dilution of the cultured sample.

Examination of the effects of metal ions

For the investigation of the effect of biological metal ions, 2 mM stocks of $CaCl_2$, $FeSO_4$, $MgCl_2$, $ZnSO_4$, and $MnSO_4$ were prepared with the cell culture media for *C. glutamicum*. The stocks were then serially diluted for the incubation with biGSFR in the presence of 1 and 2 mM Glu.

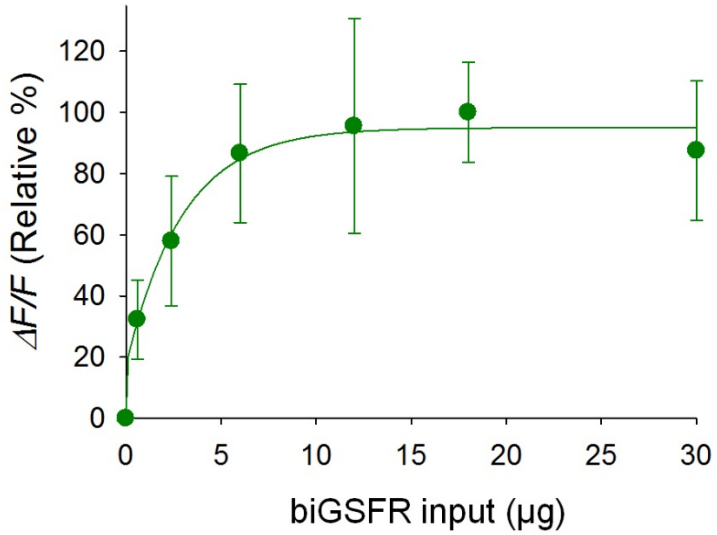

Fig. S1. Titration of biGSFR immobilization on SAV microbeads. About 6 μg of biGSFR (~5.5×10^{13} molecules) is enough to saturate 6×10^5 of the microbeads, giving ~9×10^7 proteins/bead.

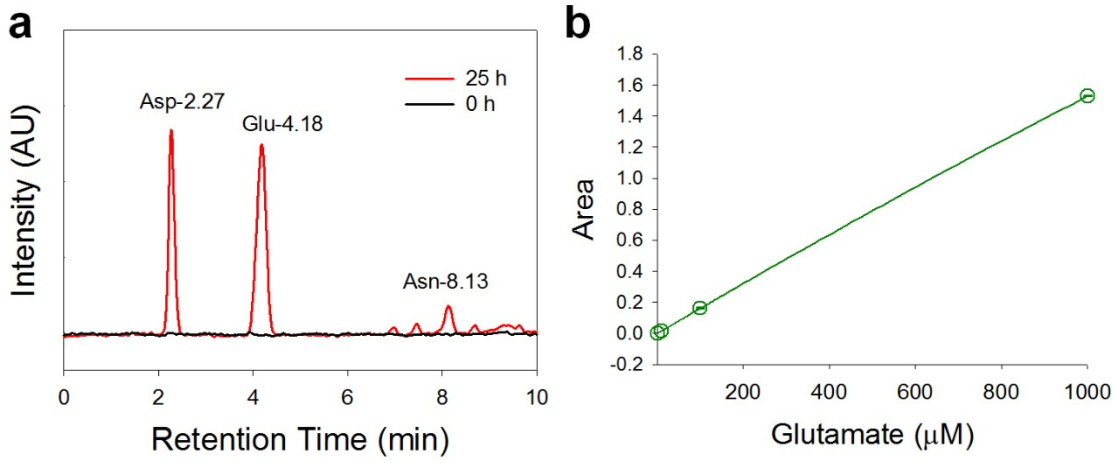

Fig. S2. Quantification of glutamate produced by *C. glutamicum* using HPLC. (a) Chromatogram profile (part) of the supernatant of the cell culture media cultured for 0 and 25 h. (b) Standard curve obtained using the solutions with known amount of glutamate. The cell culture media found to contain 20.6 μM Glu. The Glu concentration increased up to 58.2 μM if the cells were grown for 54 h.

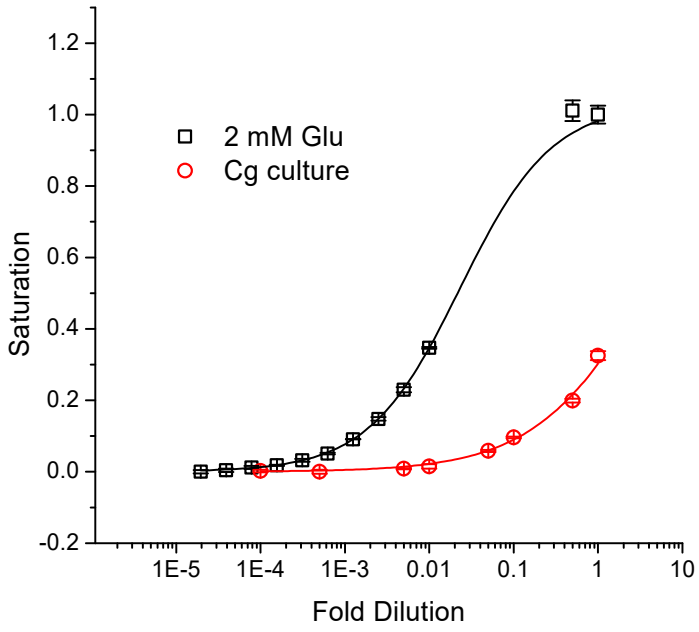

Fig. S3. Quantification of glutamate produced by *C. glutamicum* using Amplex Red Glu assay kit. Standard curve was generated using the culture media containing 2 mM Glu (open square). The concentration of Glu generated by the cells grown for 54 h was determined to be 12.6 μM, a 158.3 fold lower than the 2 mM Glu standard solution.

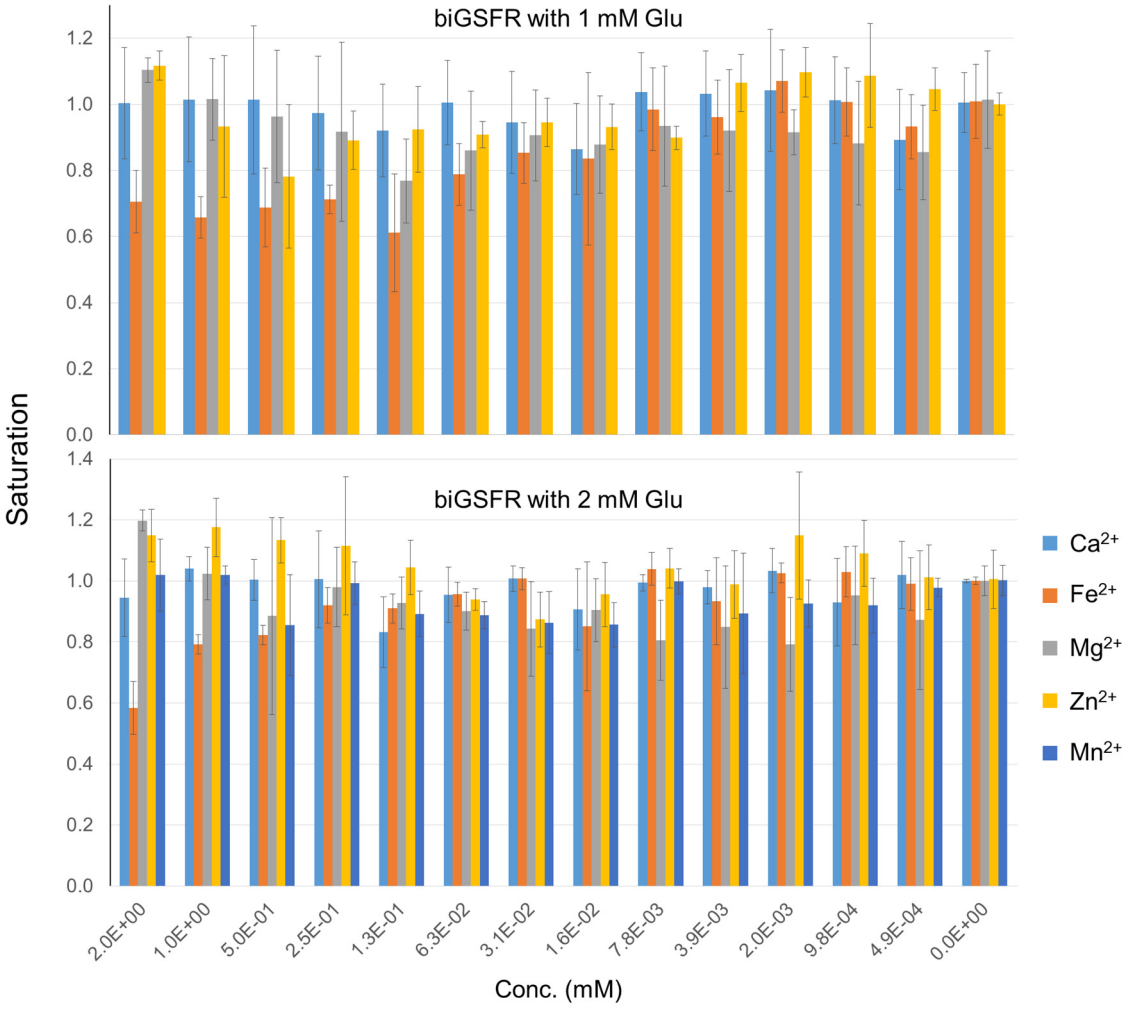

Fig. S4. The effect of biological metal ions on biGSFR activity determined in the presence of 1 mM (upper panel) and 2 mM (lower panel) Glu.

1. J. S. Marvin, B. G. Borghuis, L. Tian, J. Cichon, M. T. Harnett, J. Akerboom, A. Gordus, S. L. Renninger, T.-W. Chen, C. I. Bargmann, M. B. Orger, E. R. Schreiter, J. B. Demb, W.-B. Gan, S. A. Hires and L. L. Looger, *Nat. Methods*, 2013, **10**, 162-170.
2. S. Okumoto, L. L. Looger, K. D. Micheva, R. J. Reimer, S. J. Smith and W. B. Frommer, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 8740-8745.