Supplementary materials to

Optimization of a GCaMP calcium indicator for neural activity imaging

by

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Supplemental Figure 1. **Signal change ($\Delta F/F_0$) versus $F_{apo}$ for linker 1 mutants of GCaMP3.** Mutants with linker Leu-Glu replaced by Gln-Pro and His-Pro are depicted by green and blue squares, respectively. Note the low $F_{apo}$ for both Gln-Pro and His-Pro linker variants (see inset, X and Y axes linear scale), and the resulting large $\Delta F/F_0$. Log-log plot; inset shows linear plot. Typical GCaMP3 values are shown with the red square.
Supplemental Figure 2. **Effect of linker 2 mutagenesis on GCaMP3.** (A) Fluorescence difference between apo and sat state (ΔF/F₀) plotted versus apo brightness (F_apo). Mutants with linker Thr-Arg replaced by X-Pro are depicted as blue triangles, mutants with linker Pro-X are depicted as orange squares, variants with the original linker Thr-Arg are depicted as green dots. (B) Selection of variants with proline-enriched linkers from (A). Proteins were purified by nickel-NTA purification, and extensively dialyzed. Maximum ΔF/F₀ is plotted for each linker variant. GCaMP3-LP (GCaMP5D) showed the greatest fluorescence increase. (C) SDS-PAGE of the purified GCaMP cpEGFP-CaM linker variants. All variants appeared pure and migrated identically to GCaMP3 on gel electrophoresis.
Supplementary Figure 3. **Rosetta stone.** GCaMP5G sequence with the different protein domains colored (light grey: RSET tag, magenta: M13 peptide, green: cpEGFP, cyan: calmodulin, dark grey: linkers). Above the primary sequence of GCaMP5G the numbering of the different domains is shown, below of GCaMP5G itself. Stars indicate sites of engineering resulting in the different GCaMP5s.
Supplementary Table 1. **Crystallization conditions.** Concentration, precipitant solution and cryo-protectants are listed for all GCaMP proteins crystallized.

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<th>Protein</th>
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Supplementary Figure 4. **Electron density maps.** A portion of the 2Fo-Fc electron density map contoured at 1.5 $\sigma$ for each structure reported here is shown in blue mesh. The region of each structure corresponding to the GFP chromophore and surrounding amino acids is displayed as sticks, while the rest of the cpGFP domain is shown as a semi-transparent cartoon.
Supplementary Figure 5. Structural effects of the **D381Y** mutation. Chromophore environment at the cpGFP/CaM interface in GCaMP2 (top, PDB 3EVR), GCaMP2-T116V, D381Y (middle, PDB 3SG2) and GCaMP5A (bottom, PDB 3SG3) structures reported here. Structures are shown as cartoon and sticks colored by domain (cpGFP: green, linker: white, CaM: cyan). Selected portions of the model around the GFP chromophore (CRO) are represented as sticks with ordered water molecules represented as red spheres.
Supplementary Figure 6. Flexibility of cpEGFP-CaM linker (linker 2). Putty representation of the GCaMP5A and GCaMP5G structures where both the tube thickness and color are proportional to the crystallographic B-factor of that residue (thin, blue: low B-factor → thick, red: high B-factor). Note that the cpEGFP-CaM linker has high B-factors relative to much of the model.
Supplemental Figure 7. **Electron density of the cpEGFP-CaM linker.** A portion of the 2Fo-Fc electron density map contoured at 1σ is shown in blue mesh for the cpEGFP-CaM linker for each of the structures. The linker residues (302 and 303) are labeled.
Supplemental Figure 8. **Conformation of cpEGFP-CaM linker and M13-cpEGFP “KF” linker.** (A) The linker region GCaMP5A and GCaMP5G are shown as cartoon and sticks colored by domain (cpGFP: green, linker: white, CaM: cyan). Selected portions of the model around linker 2 are represented as sticks with ordered water molecules represented as red spheres. Important residues are labeled (CRO: GFP chromophore). *(B next page)* Superposition of the cpGFP domains of the GCaMP3-D380Y (grey) and GCaMP3-KF(1) (red) structures illustrating the conformational change of the CaM domain relative to the cpGFP domain. *(C)* Superposition of the N-terminal lobes (EF-hands 1&2) of GCaMP3-D380Y (grey) and GCaMP3-KF(1) (multi-color) illustrating the conformational change of within the CaM domain. The N-terminal CaM lobe of GCaMP3-KF(1) is red, the C-terminal lobe is green, and the M13 peptide is blue. *(D)* Close-up of the linker 1 region of the GCaMP3-D380Y (left) and GCaMP3-KF(1) (right) structures showing the role of linker 1 in the conformational change.
Supplementary Figure 9

(A) Superposition of the domain-swapped dimeric structures of GCaMP2 (grey), GCaMP2-LIA(1) (blue), and GCaMP5H (red). (B) Close-up of the linker 1 region of the three structures from (A).

Supplementary Figure 9. Crystal structures of mutants at Linker 1 of domain-swapped dimers. (A) Superposition of the domain-swapped dimeric structures of GCaMP2 (grey), GCaMP2-LIA(1) (blue), and GCaMP5H (red). (B) Close-up of the linker 1 region of the three structures from (A).
Supplementary Figure 10

Supplementary Figure 10. Size exclusion chromatography of GCaMP2 (A) and GCaMP2-LIA (B). Proteins were injected at 250 µM in the presence of 5 mM EGTA (-Ca²⁺, red traces) or 2 mM CaCl₂ (+Ca²⁺, blue traces). The calculated mass of the GCaMP proteins is ~50 kD. Note the larger dimer/monomer ratio in the sample of GCaMP2-LIA + Ca²⁺. GCaMP2-LIA is significantly more dimeric than GCaMP2.
Supplementary Figure 11. SDS-PAGE and Gel Filtration. (A) SDS-PAGE analysis of 1: GCaMP2, 2: GCaMP3, 3: 5A, 4: 5D, 5: 5G. (B) Size exclusion chromatogram of GCaMP2, GCaMP3, 5A, 5D and 5G, normalized to the monomer peak. The dimer peak is slightly visible at 13.5ml, GCaMP5 variants show a modest decrease in dimerization compared to GCaMP3.
Supplemental Figure 12. **Brightness of GCaMP5s compared to GCaMP3 in vitro.** (A) $\Delta F/F_0$ of each purified GCaMP5 variant compared to GCaMP3. (B) Brightness of apo- and sat-GCaMP5 compared and normalized to the brightness of apo- and sat-GCaMP3. Note the low apo-brightness of GCaMP5F and GCaMP5H, resulting in a $\Delta F/F$ of over 150 in panel A.
Supplemental Figure 13. Calcium Affinity. (A) Calcium titrations of GCaMP5A, D, G, L and K compared to GCaMP2 and GCaMP3. (B) Hill plots of the same GCaMP5s as in panel A. Note a slight increase in the slope of the GCaMP5 curves compared to the GCaMP3 curve, suggestive of an increase in cooperativity of calcium binding. GCaMP5L could not be fitted with a straight line over the concentration range chosen, and is the only GCaMP5 variant containing the mutation A52V, which increases affinity. Part of the curve fits aligns well with GCaMP3. GCaMP5K shows the highest affinity, and has the largest Hill coefficient. Interestingly, the curve kinks to a slight negative slope for higher calcium concentrations, indicating a potential negative cooperativity.
Supplemental Figure 14. pH titrations of GCaMP2, GCaMP3, GCaMP5A, GCaMP5D and GCaMP5G. Left panels are normalized to the brightness of the saturated state of the indicator, and include the difference in brightness (dotted line) projected on the right Y axis in percent. Right panels depict both curves normalized to 1.
Supplementary Figure 15

Supplementary Figure 15. Excitation and emission scans of GCaMP5 variants. Green dotted line: apo excitation, blue dotted line: apo emission, red line: sat excitation, yellow line: sat emission. Curves normalized to the saturated maximum.
Supplementary Figure 16. (A) $\Delta F/F_o$ vs. 940-nm laser power at the focus, for 500 nM solutions of GCaMPs in pH 7.2 buffer. (B) Fluorescence emission spectra from 1-$\mu$M solutions of GCaMPs in pH 7.2 buffer with free Ca$^{2+}$ (red curves) or with EGTA (blue curves), excited with 4 mW of 940 nm laser light.
Supplementary Figure 17. (A) Absorption, emission and Two-photon action spectra. One-photon absorption (left panel of plots), emission excited by 485nm (center plots) and two-photon-excitation spectrum (right panel) of EGFP and GCaMPs all at 1 µM concentration in MOPS.KCl buffer pH 7.2, in the presence of 10mM Ca-EGTA (sat, red curve) or 10mM EGTA (apo, blue curve). The calcium-dependent signal contrast ΔF/F (green line) is plotted on the right axis. All spectra were taken at constant laser power at the focus (1 mW) across the spectrum.
Supplementary Figure 17 continued. (B) Fluorescence Lifetime. Single-exponential fit to fluorescence decay curves of EGFP and GCaMPs obtaining using time-correlated single-photon counting (TCSPC), at pH 7.2 (left) or pH 9.5 (right), in the presence of Ca$^{2+}$ (sat) or EGTA (apo).
(C) Specific brightness (brightness per fluorescent molecule) excited at 940 nm, as determined by FCS, in kilocounts-sec-1-molecule-1 (kcpsm), in pH 9.5 buffer containing 1 mM CaCl₂.
two-exponential fit  

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Supplemental Table 1. **Two-photon characterization of EGFP and GCaMPs.** Fit to fluorescence decay curves of EGFP and GCaMPs to either a two-exponent decay function (time constants $\tau_1$ and $\tau_2$ with fractional amplitudes in parenthesis), or a single-exponential decay (time constant $\tau$), for pH 7.2 and pH 9.5.
Supplementary Figure 18: Stopped-flow fluorescence characterization of GCaMP3 rise times after Ca\(^{2+}\) steps. Raw traces of individual calcium steps for GCaMP3. Each red trace represents the mean of ~16 trials and the black dashed line is the single-exponential fit.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** On-response overlay, 1 s scale.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** On-response overlay, 500 ms scale.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** On-response overlay, 250 ms scale.
Additional Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** On-response overlay, raw traces, 4 s scale.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** On-response overlay, raw traces, 500 ms scale.
Additional Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3.** Tau (seconds) plotted relative to [Ca$^{2+}$] jump. Calcium concentrations were measured and calibrated with Fura-2, which indicates the purity of Invitrogen tetrapotassium BAPTA to be ~97%.
Additional Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3, GCaMP5G, and GCaMP5D (“GC3-LP”).** Comparison of $k_1$ between several variants for $[\text{Ca}^{2+}]$ jump from 150 nM to 1 µM.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of GCaMP3, GCaMP5G, and GCaMP5D ("GC3-LP").** Tau (seconds) plotted relative to [Ca²⁺] jump. Calcium concentrations were measured and calibrated with Fura-2, which indicates the purity of Invitrogen tetrapotassium BAPTA to be ~97%.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of OGB-1.** [Ca^{2+}] jumps from 0 to 150 nM.
Supplemental Figure 18 continued. **Stopped-flow fluorescence characterization of OGB-1.** [Ca$^{2+}$] jumps from 0 to 150 nM. Overlay of all repeat traces.
Supplementary Figure 18 continued. On-response vs. [Ca]\textsubscript{step} comparison between GCaMP3, GCaMP5D and GCaMP5G.
Supplemental Figure 19. HEK cell acetylcholine titrations. 

(A) Ratio of maximum fluorescence increase over baseline fluorescence for each GCaMP variant plotted against acetylcholine concentration. Insets are fluorescence traces of single-trial responses versus time. (B) Brightness of baseline (light green, no pattern-fill) and maximum response (dark green, diagonal lines). (C) Ratio ($F_{\text{max}}/F_{\text{base}}$) of each GCaMP variant’s maximum response.
Supplemental Figure 20. **Baseline brightness in neuronal culture.** Baseline brightness, normalized to mCherry brightness and GCaMP3, for GCaMP5 variants tested in neuronal culture.
Supplementary Figure 21.

Fluorescence response of GCaMP variants to field stimuli in primary rat hippocampal neurons compared to Oregon Green BAPTA-1 (OGB1) and fluo4. Error bars are SD. Bottom three panels show the G-GECO responses. Note for G-GECO1.2 the Y-axis has been changed due to the large SD.
Supplementary Figure 21 continued. Fluorescence response of GCaMP variants to field stimuli in primary rat hippocampal neurons compared to Oregon Green BAPTA-1 (OGB1) and fluo4. Error bars are SD. Bottom three panels also contain G-GECO variants.
Supplementary figure 22. Average traces of SyGCaMP, LckGCaMP, mGCaMP (GAP43 fusion), and cytoplasmic variants. Trial average + s.e.m. plotted.
Supplemental Figure 23. **GCaMP5s in Astrocytes.** (A) Comparison of maximum projection images of 600 frame movies for astrocytes expressing Lck-GCaMP3 and Lck-GCaMP5G. Still frames of ROI 6 and ROI11 over 50s (1 frame per sec.) (B) Measuring spatial dimensions of spotty Ca\(^{2+}\) signals. Right figures show sample spotty Ca\(^{2+}\) signals. Left figures show average size of spotty Ca\(^{2+}\) signals. There were no significant differences (Lck-GCaMP3 \(n=4\), Lck-GCaMP5G \(n=6\)). (C) Intensity vs. time profiles of 10 ROIs for Lck-GCaMP3 and Lck-GCaMP5G. Note that signal-to-noise ratio is improved in Lck-GCaMP5G. (D) Graphs of number of spotty Ca\(^{2+}\) signals per cell and proportion of cells exhibiting spotty Ca\(^{2+}\) signals over 10min of imaging. Note, Lck-GCaMP5G seems to increase spotty Ca\(^{2+}\) signals per cell. (E) Graphs of signal-to-noise ratio and kinetics. Note, Lck-GCaMP5G significantly increases signal-to-noise ratio. *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\)
Supplemental Figure 23, continued. **GCaMP5s in Astrocytes, comparison of basal fluorescence.** (F) Sample of basal F for Lck-GCaMP3 and Lck-GCaMP5G in astrocytes. (G) Graph of basal F. There are no significant differences.
Supplemental Figure 23 continued. GCaMP5 in Astrocytes, comparison ATP-evoked Ca^{2+} signals. (H) Response of LckGCaMPs to ATP addition. (I), GCaMP5G shows the strongest response of the indicators tested. (J) All GCaMP5 variants are significantly improved relative to GCaMP3.
Supplementary Figure 24. **Mouse Retina expressing GCaMP5D.** Retinas expressing GCaMP5D looked healthy and cells showed normal physiological properties.
Supplementary Figure 25. **Single trial data of GCaMP3, GCaMP5A, GCaMP5G and GCaMP5I.** Response to odor addition depicted in panels A, odor removal in panels B. Average traces are shown.
Supplemental Figure 26. **Response of GCaMP variants in the *Drosophila* larva NMJ assay.**
Supplementary Figure 27. **Sensory-evoked Ca\(^{2+}\) transients from in vivo two-photon imaging in the adult *Drosophila* antennal lobe.** A. Fluorescence changes (\(\Delta F/F\)) from fly antennal lobe DC1 glomerulus projection neurons (PNs) from GCAMP3 and GCAMP5G were obtained by delivering a 1 second odor pulse (grey shading) of 1.0% octanol. The mean of 5 replicate stimulations from 6 antennal lobes (5 animals) is shown along with the s.d. (between antennal lobe means). B. Mean octanol response from the DC1 glomerulus to increasing octanol concentration across 6 antennal lobes (5 animals). Shading represents the s.d. of the mean.
Supplementary Figure 28. **Performance of GCaMP5A in the zebrafish tectal neurites assay.** GCaMP5A performs significantly better than GCaMP3, better even than GCaMP5G.
Supplementary Figure 29. **Spatial activity maps with GCaMP5G (top) and GCaMP2 (bottom) from the pooled sets of tectal neuron somata.** Each square is a neuron color coded for response to left to right stimulus (black 0, red 0.5 ΔF/F) and right to left (black 0, green 0.5 ΔF/F. GCaMP2: 4481 cells from 11 fish, GCaMP5G 4944 cells from 9 fish.
Supplementary Figure 30. Twenty randomly selected fields of view of AAV-syn-GCaMP3 (left) and GCaMP5G (right) *in vivo* in mouse V1. Levels of nuclear filling are comparable between the two.
Supplementary figure 31.

**Supplementary Figure 31.** Spike detection efficiency of GCaMP5K for 1, 2 and 3 action potentials.
Supplementary Figure 32. **Response variability of GCaMP3, GCaMP5G, 5K and OGB1 compared.** The coefficient of variation of GCaMP5G is significantly lower compared to GCaMP3; 5K is similar to GCaMP3.