Genetically encoded calcium indicators (GECIs) are powerful tools for systems neuroscience. Recent efforts in protein engineering have significantly increased the performance of GECIs. The state-of-the-art single-wavelength GECI, GCaMP3, has been deployed in a number of model organisms and can reliably detect three or more action potentials in short bursts in several systems in vivo. Through protein structure determination, targeted mutagenesis, high-throughput screening, and a battery of in vitro assays, we have increased the dynamic range of GCaMP3 by severalfold, creating a family of “GCaMP5” sensors. We tested GCaMP5s in several systems: cultured neurons and astrocytes, mouse retina, and in vivo Caenorhabditis chemosensory neurons, Drosophila larval neuromuscular junction and adult antennal lobe, zebrafish retina and tectum, and mouse visual cortex. Signal-to-noise ratio was improved by at least 2- to 3-fold. In the visual cortex, two GCaMP5 variants detected twice as many visual stimulus-responsive cells as GCaMP3. By combining in vivo imaging with electrophysiology we show that GCaMP fluorescence provides a more reliable measure of neuronal activity than its predecessor GCaMP3. GCaMP5 allows more sensitive detection of neural activity in vivo and may find widespread applications for cellular imaging in general.
molecule calcium-sensitive dyes (Cobbold and Rink, 1987) are both used to report \([Ca^{2+}]\) changes; but GEClIs have the advantage that they enable chronic, noninvasive imaging of defined cells and compartments (Mao et al., 2008). State-of-the-art GEClIs include the Förster resonance energy transfer (FRET) indicators D3cpVenus (D3cpV) (Palmer et al., 2006), TN-XXL (Mank et al., 2008), and YC3.60 (Nagai et al., 2004), and the single-wavelength sensor GCaMP3 (Tian et al., 2009). GCaMP3 is based on circularly permuted green fluorescent protein (cpGFP), calmodulin (CaM), and the \(Ca^{2+}/CaM\)-binding “M13” peptide (M13 pep). Several versions of the original GCaMP sensor (Nakai et al., 2001) have been published (Ohkura et al., 2005; Tallini et al., 2006; Akerboom et al., 2009). Recent versions include “GCaMP4.1” (Shindo et al., 2010), which was used to image Xenopus gastrulation, but no sequence information or comparison with other GEClIs is published. “GCaMP-HS” consists of GCaMP2 with a subset of the “superfolder GFP” mutations (Pédelaçq et al., 2006) and was used for imaging zebrafish motor neurons (Muto et al., 2011), but was also not compared with other sensors. The “G-GECO” sensors (Zhao et al., 2011) were created from GCaMP3 by random mutagenesis; they show \(-2\times\) greater fluorescence increase in purified protein (\(Ca^{2+}\)-saturated vs \(Ca^{2+}\)-free) but were not tested in neurons. However, the sensors are dimmer than GCaMP3 in both the \(Ca^{2+}\)-free and \(Ca^{2+}\)-bound states, which can complicate imaging.

GCaMP3 has been used to detect activity in large neuronal populations in the motor cortex (Tian et al., 2009), barrel cortex (O’Connor et al., 2010), and hippocampus (Dombek et al., 2010) of behaving mice. Long-term imaging of GCaMP3 has revealed learning-related circuit changes in vivo (Huber et al., 2012). GCaMP3 imaging has also been used to probe dendritic excitation in layer 5 dendrites in vivo (Xu et al., 2010; Mittmann et al., 2011), light-evoked responses in populations of neurons in mouse retina (Borghuis et al., 2011), zebrafish tectum (Del Bene et al., 2010), and walking Drosophila (Chiappe et al., 2010; Seelig et al., 2010), among others. However, GCaMP3 remains an imperfect GECl. Single APs are not reliably detected in vivo, and detection of active cells lags synthetic indicators (Tian et al., 2009). GCaMP5 was engineered from GCaMP3 using a combination of structure-guided design (Akerboom et al., 2009) and seminatural library screening. We have enhanced sensitivity by increasing the dynamic range of the fluorescence response \((\Delta F/F = (F - F_a)/F_a)\), the \(Ca^{2+}\)-saturated brightness, and the \(Ca^{2+}\) affinity. We characterized 12 new GCaMP5s in vitro and in vivo under a wide variety of conditions. Each sensor has improved properties relative to the parent sensor GCaMP3. The ideal GCaMP5 indicator for a given study can be selected from this set according to particular experimental requirements.

Materials and Methods

Mutagenesis. Site-directed mutagenesis of GCaMP3 was carried out using the method of Kunkel (1991) or the QuikChange methodology (Agilent Technologies). Single-stranded uracil-containing DNA template of pRSET-GCaMP3 for Kunkel mutagenesis was produced according to established protocols. Mutants were confirmed by DNA sequencing.

Animal use: All experiments were conducted according to protocols approved by the Institutional Animal Care & Use and Institutional Biosafety Committees of the Howard Hughes Medical Institute, Janelia Farm Research Campus, and of the corresponding committees at the other institutions.

Escherichia coli ly sate screen. Libraries were generated using primers containing degenerate codons (NNS) following the site-directed mutagenesis protocols described above and transformed into E. coli XL1-Blue (Stratagene/Agilent). The following day, colonies were scraped off plates, combined, and the plasmid library was isolated with Qiagen Mini-prep kits, following the procedures provided, eluting in 100 µl water. One microfilter of the library was subsequently transformed into E. coli BL21 (DE3) (EMD Biosciences), plated on 244 × 244 mm square LB-agar plates containing 100 µg/ml ampicillin, and grown for 20 h at 30°C. Colonies were selected using a colony picker (QPicX2™; Genetix) and grown in 800 µl ZYM-5052 medium (Studier, 2005) containing 100 µg/ml ampicillin in 96 well-deep blocks for 48 h at 30°C, shaking vigorously at 700 rpm. Two microfilters were taken from each well and mixed into a fresh deep-well block containing 800 µl LB medium + 100 µg/ml ampicillin, grown overnight at 37°C, pelleted, and stored at 4°C for sequence analysis. The E. coli BL21 (DE3) cultures in the deep-well blocks containing the overexpressed mutants were pelleted by centrifugation (4000 × g, 20 min, 4°C), frozen, thawed, resuspended in lysis buffer (20 µl TRIS, pH 8.0, 100 µM NaCl, 1 mg/ml lysozyme, 1.5 Kunitz units/ml DNAsel from Qiagen) and subsequently incubated at 30°C for 2–4 h, shaking. Lysates were clarified by centrifugation (4000 × g, 30 min, 4°C), and 100 µl was taken from each well into Greiner Bio-One black 96-well fluorescence plates (Greiner) in duplicate. To one plate 1 µl 100 µM CaCl2 was added (final Ca2+ concentration ≈1 mM), to the other 1 µl 100 µM EGTA, pH 7.4. Fluorescence was measured in a Tecan Saphire® Spectrophotometer (Tecan), at 485 nm excitation and 510 nm emission, 5 nm slits, gain = 90 V.

Protein expression and purification. Cloning, expression in E. coli, and purification was performed essentially as before (Akerboom et al., 2009). Briefly, for expression in BL21 (DE3), pRSETa-GCaMP variants were transformed to BL21 (DE3), and single colonies were grown for 3 d in ZYM-5052 media (Studier, 2005) at 25°C after which cells were pelleted by centrifugation. Cells were lysed by resuspending them in 4 × w/v lysis buffer 2 (20 µM TRIS.HCl, pH 8.0, 100 µM NaCl), followed by a freeze-thaw cycle and subsequent cell rupture by passing the cell suspension three times through a precooled (4°C) Avestin Emulsiflex-C5 (Avestin) and finally a 15 s sonication step at 30 mW amplitude on ice (Fisher Dismembrator Model 100 equipped with 3 mm tip). Lysate was clarified by centrifugation (30,000 × g, 4°C, 45 min.), and cell-free extract was incubated on a rotary incubator with 5% (v/v) Profinity IMAC Ni-NTA resin (Bio-Rad) at 4°C for 4–16 h. Resin was allowed to settle in 20 ml disposable columns (Bio-Rad), and was washed with 20 ml lysis buffer 2, followed by 10 ml wash buffer (20 µM TRIS.HCl, pH 8.0, 100 µM NaCl, 10 mM imidazole). Proteins were eluted into elution buffer (20 µM TRIS.HCl, pH 8.0, 100 µM NaCl, 300 mM imidazole) and subsequently dialyzed extensively into lysis buffer 2 using Spectra/Por membrane tubing (Spectrum Laboratories), with a molecular weight cutoff (MWCO) of 10,000 Da, at 4°C. Concentration and purity was determined using Agilent Protein 230 chips on an Agilent 2100 Bioanalyzer (Agilent Technologies) as well as NuPAGE Novex BIS-TRIS SDS-PAGE gels (Invitrogen) with 2000 Da MWCO (Fierce). The absorbance of 100 µl protein with 2 µl of either 100 µM CaCl2 or 100 µM EGTA added was measured from 240 to 700 nm, blanked against the chemically identical dialysis buffer.
containing 2 μl of 100 mM CaCl₂ or 2 μl 100 mM EGTA. Spectra were normalized using the absorption at 280 nm.

**Calcium titrations.** Calcium affinity assays were performed by mixing different volumes of the zero-free calcium buffer containing the following (in mM): 10 EGTA, 100 KCl, and 30 MOPS, pH 7.2 and 39 μM free-calcium buffer containing the following (in mM): 10 CaEGTA in 100 KCl, and 30 MOPS, pH 7.2 from the calcium calibration buffer kit (Invitrogen) according to the manufacturer’s instructions. Measurements were performed by mixing 3 μl purified GCaMP (~100 μM) with 100 μl of different ratios of zero-free calcium buffer and 39 μM free-calcium buffer (Invitrogen) in 96-well transparent Greiner Bio-One plates and measuring the fluorescence at 485 nm excitation and 510 nm emission, 5 nm slits, gain = 90 V in duplicate as described earlier.

**GCaMP photophysics.** Photophysical properties for GCaMP2, GCaMP3, and the GCaMP5 variants A, D, and G were investigated in buffer solutions in the presence or absence of free Ca²⁺. Absorption and emission properties, including quantum yield, were determined using a UV/VIS spectrometer (Lambda 35; PerkinElmer) and a fluorescence spectrometer (LS-55; PerkinElmer). Two-photon-excited properties, including fluorescence excitation spectra, two-photon cross section, fluorescence decay time, and emission spectroscopy, were conducted with laser pulses from an 80 MHz Ti:Sapphire laser (Chameleon Ultra II; Coherent). In all cases, near-IR laser pulses were focused into a solution containing the GCaMP proteins via a 60×, 1.2 NA water-immersion objective of an inverted epifluorescence microscope (IX81; Olympus), slightly overfilling the objective back aperture. Fluorescence generated by two-photon excitation was collected by the same 60× objective used to excite the probe, and after passing through a shortpass filter (FF01–720SP; Semrock) and a bandpass filter (FF01–550/88; Semrock), was directed to the input face of a fiber-coupled avalanche photodiode (APD). Two different APDs were used, one optimized for speed for lifetime measurements (model PDF CCTB; Micro Photon Devices), and one optimized for low noise for fluorescence correlation spectroscopy (FCS) and spectral measurements (SPCM-AQRH-14-FC; PerkinElmer) coupled to a 100 μm core multimode fiber (AFS105/125Y; Thorlabs). Output pulses generated by the fast-timing APD were fed to a TCSPC board (TimeHarp200; Picoquant). Output pulses from the low-noise APD were fed to an external autocorrelator (Flex03LQ-01; www.correlator.com) for spectra and FCS measurements. Emission spectra were recorded from an additional microscope port using a fiber-coupled 0.3 m spectograph/CCD (model SP2358 with Pixis 256 CCD camera; Princeton Instruments).

Control of the amount of laser power delivered to the sample in the focal plane of the microscope was accomplished by rotating the linear polarization of the laser output using an achromatic ½-wave plate (AHWP05M-980; Thorlabs), mounted in a computer-controlled rotation stage. The laser beam was focused into the objective by 5× and 10× objectives (GL10-B; Thorlabs). We calibrated the system before measurements, where for each laser wavelength (10 nm increments), the laser power at the focus of the microscope objective was measured while the ½-wave plate was angle adjusted, calibrating at each wavelength a specific power at the focus with a specific orientation of the wave plate. Laser wavelength and power adjustments, as well as data acquisition, were run under computer control. Data analysis was performed using Origin 8.0 software (OriginLab), and FCS fits were performed using a custom software package (V. Iyer, unpublished).

Samples were prepared by dilution from stock solutions of purified proteins into buffers, at either pH 7.25 or pH 9.5, to characterize the fluorescence properties under physiological pH and at elevated pH where most of the protein chromophores are in the deprotonated form in the presence of Ca²⁺. The pH 7.25 buffers (30 mM MOPS, 100 mM KCl) were either purchased as part of a Ca²⁺-calibration kit (C-3008MP; Invitrogen), that contains either 10 mM K₂EGTA or 10 mM CaEGTA, or prepared from MOPS and KCl stock solutions (Meditech) and contain either 1 mM CaCl₂ or 0.5 mM EGTA or 1 mM BAPTA. The pH 9.5 buffer (30 mM CHES, 100 mM KCl) was prepared from stock chemicals and was supplemented with varying concentrations of CaCl₂ or EGTA. Measurements using very low amounts of protein (below 200 nM) such as FCS measurements and one-photon fluorescence measurements, we added 0.1 mg/ml bovine serum albumin (BSA) as a blocking agent to the buffer solutions to prevent loss of proteins from solution to the nearby glass or coverslip surfaces via adsorption over the course of the measurements; this eliminated any decrease in fluorescence signal over the course of the measurements.

**Protein concentration determination for biophysical analysis.** The concentration of chromophore-forming proteins was determined by two methods: alkali-denaturation (Ward, 2005), and two-photon-excited FCS. For both methods, absorption spectra of either enhanced GFP (EGFP) or GCaMP protein solutions were taken in the UV/VIS spectrometer at either pH 7.25 or pH 9.5. In the second method using FCS, GCaMP stock solutions were diluted several thousand fold into pH 9.5 buffer (30 mM CHES, 100 mM KCl) containing 1 mM CaCl₂, and excited at 960 nm in a fluorescence microscope over a range of laser powers. At each laser power, the mean fluorescence rate <N> and its fluctuations were recorded for 50–200 s, and the autocorrelation G(τ) of the fluorescence signal was computed. A fit to the autocorrelation function G(τ), based on a diffusion model, determines the diffusion coefficient of the proteins, and the number of fluorophores in the excitation volume, given by <N> = 1/G(0) (Schwille et al., 1999). This measurement is repeated for a sample containing EGFP at known concentration (determined by alkali denaturation) and dilution in pH 9.5 buffer, providing a reference between a known concentration and a number of fluorophores in the excitation volume.

**Quantum yield.** Quantum yield (QY) was determined for the fluorescent proteins in both pH 7.25 and 9.5 buffer in the presence of 1 mM CaCl₂ using standard methods that measure the optical absorption and total fluorescence yield of samples at a fixed wavelength for both excitation and a standard fluorescence with a QY of 0.93 in aqueous 0.1 N NaOH (Magde et al., 2002) with approximately the same emission spectrum and emission peak.

**Fluorescence decay and lifetime.** The fluorescence lifetime was measured by time-correlated single-photon counting (TCSPC) using two-photon excitation at 960 nm in a fluorescence microscope setup, where detector pulses from the fast-timing APD and trigger signals from a PIN diode monitoring the laser pulse train were fed to the TCSPC board. To achieve improved performance, a pulse-picker (Model 350–160; Conoptics) was inserted in the beam to reduce the laser pulse frequency from 80 to 20 MHz. The fluorescence lifetime of GCaMP samples was determined in either pH 9.5 buffer supplemented with CaCl₂ or EGTA as described earlier, or pH 7.25 buffer (Invitrogen buffer with either 10 mM CaEGTA or 10 mM EGTA). The lifetime reference for the system was fluorescein (τ ≈ 4.1 ± 0.1 ns); in our setup the fluorescein decay was well fit to a single-exponential decay (4.0 ns, χ² = 1.08). Measured fluorescent decays were fit to a single-exponential decay curve, or to a two-exponential decay, when necessary.

**Two-photon excitation spectra.** Two-photon excitation spectra and ΔF/F were measured for the GCaMPs in the presence and absence of free calcium at pH 7.25 and 9.5, respectively, as described above, at 1 μM protein concentration. Two-photon spectra are taken with constant laser power delivered to the sample, although due to a wavelength-dependent pulse width of the femtosecond pulses, and changes in focal spot size (focused beam diameter scales as the excitation wavelength), the laser intensity varies gradually across the spectrum. We do not correct for this variation in intensity. Together with each run of GCaMP samples, a reference two-photon excitation spectrum of fluorescein was recorded, allowing us to determine the absolute two-photon cross section of the GCaMPs being measured. These cross sections were used to calculate the fluorescence quantum yield of the molecules decreases with higher intensity, due to photobleaching of the fluorophores in the volume of the focused laser beam. While the peak brightness will strongly depend on...
the molecular environment (in vitro vs intracellular/in vivo), this value can be used as a quantitative measure in comparing the photostability of different fluorophores.

To determine the peak brightness, GCaMPs were diluted to nominally 50 nM in pH 9.5 buffer containing either 1 mM CaCl₂ or 0.5 mM EGTA. Solutions also contained 0.1 mg/ml BSA to prevent adsorption of fluorophores to the nearby coverslip surface. As a control, EGFP at 50 nM was prepared and measured in the same buffer, without CaCl₂ or EGTA. Measurements were taken for a series of laser powers (with power measured in the focal plane) at 940 nm, where fluorescence time course data was acquired for 50–200 s at each laser intensity. For FCS, we used the low-noise APD. The output of the APD was fed to an autocorrelator and associated software to generate two quantities: the time-average fluorescence rate $<F>$ and the measured autocorrelation $G(t)$ of the fluorescence data. FCS theory equates the quantity $1/G(0)$ to $<N^2>$, the average number of emitting molecules in the excitation volume. By acquiring $<F>$ and $G(t)$ simultaneously for each probe over a range of laser intensities, and fitting $G(t)$ to determine $<N^2>$, we can define the two-photon brightness at each intensity as $<F>/<N^2>$, the effective detected fluorescence rate per emitting molecule at each intensity. This quantity has a maximum value or peak brightness, since as the intensity is raised, saturation and photobleaching begin to diminish the fluorescence rate.

**Intensity dependence of ΔF/ΔF** under two-photon excitation. Without using FCS, GCaMPs can be characterized by the power dependence of their $(\Delta F/\Delta F)_{max}$. For these measurements, GCaMPs were diluted to 0.5 μM in pH 7.25 buffer $\pm$ 2.5 mM and recorded for laser excitation at 940 nm under increasing power over the range of 0.5 mW–70 mW.

**Human embryonic kidney cell assay.** For expression in human embryonic kidney (HEK) 293 cells, DNA was PCR amplified from the pRSCE-GCaMP construct, purified, and digested with BglII and NotI (NEB), and ligated into digested pEGFP-N1 (Takara-Bio), which had been digested with BglII and NotI to remove EGFP, resulting in pCMV-GCaMPs. Successful clones, with the EGFP gene replaced with the gene coding for GCaMP, were confirmed by sequence analysis, and plasmids were prepared using the endo-free plasmid Maxi kit (Qiagen). Equal amounts of plasmid for each GCaMP variant were transfected into HEK293 cells using the 96-well Nucleofector plates (Lonza), with each variant in 16 wells for eight duplicate measurements. Cells were grown and incubated at 37°C for 2 d, after which growth medium was aspirated and replaced with 100 μl of lysis buffer 2, after which EGTA was added to 1 mM final concentration. This mixture was allowed to equilibrate at room temperature for 30 min, after which CaCl₂ was added to 2.5 mM final concentration. Aliquots (100 μl) of each GCaMP were then injected onto a Superdex 200 10/300 GL column (GE Healthcare) with 20 mM Tris, pH 8.0, 100 mM NaCl, and 2 mM CaCl₂ as the running buffer, and protein was eluted at a flow rate of 0.5 ml/min.

**Hippocampal neuronal culture imaging.** Primary cultures of hippocampal neurons were obtained from P0 rat pups by dissection, papain-based disso- lution, and plating onto Matrigel-coated (BD Biosciences), 24-well glass-bottom plates (MatTek) and then cultured in DMEM/B27 medium (Invitrogen). SIV-based lentiviral vectors containing hsyn1-GCaMP variant-ires-ns-mCherry-WPRE-SV40 polyadenylation signal (hsyn1: human synapsin-1 promoter) constructs were produced through quadruple transfection of HEK293T cells in 10 cm plates. Harvested lentiviral particles were used to infect hippocampal neuronal cultures on the third day in vitro for 16 h, and medium was replaced with DMEM/B27/4 mM AraC (Invitrogen) and Sigma on day 16–18 in vitro. Infected neurons were stimulated using a custom-built, 24-well multiplexed field stimulator with platinum wires and imaged using an Olympus IX81 motorized, inverted microscope (10× objective, 0.4 NA, Chroma ET-EGFP or ET-TxRed filter sets) Prior Scientific H117 ProScanII motorized stage; Cairn Research optical feedback OptoLED illumination system; and an EMCCD camera (Andor iXon+ 897, 34.8 frames per second). Field stimuli were delivered at 40 V, 83 Hz, 1 ms pulses for the following trains: 1, 3, 5, 10, 20, 40, 80, and 160 field stimuli. The whole system was automated using MetaMorph (MM: Molecular Devices) and MATLAB (MathWorks) software. Imaging buffer including the following (in mM): 145 NaCl, 2.5 KCl, 10 glucose, 10 HEPES, pH 7.4, 2 CaCl₂, 1 MgCl₂, 0.013 (2-carboxyxyperazine-4-yl)-propyl-1-phosphonic acid (Tocris Biosciences), 0.01 6-cyano-7-nitroquinoline-2,3-dione (Tocris Biosciences), 0.01 gabazine (Tocris Biosciences), and 1 γ-methyl-4-carboxyphenylglycine (Tocris Biosciences). Images were processed and analyzed using custom software.

**Astrocye imaging.** Methods were identical to those described previously (Shigetomi et al., 2010b). Briefly, we used an Olympus IX71 microscope equipped with an IXON DVS875DCS EMCCD camera (Andor), 5×-dramatic optical correction condenser, 0.8×-dramatic objective. Simultaneous collection was achieved using the TILLVision software. We used an Olympus 60×1.45 NA objective lens. Images were typically taken every 1 s. Exposure time and pixel binning were optimized to visualize fluorescence signals for each experiment (maximum binning was 4 × 4). Cultures were perfused with recording buffer containing the following (in mM): 110 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 v-glucose, and 10 HEPES at pH 7.4 (adj usted with NaOH).

For imaging astrocyte activity in neuron/astrocyte cocultures, Lck-GCaMP3 and Lck-GCaMP5G were subcloned into an AAV vector driven by the astrocyte-specific GFAP promoter (Brenner et al., 1994). Neuron-astrocyte coculture was infected at day 3 in vitro and cells were imaged 10 d postinfection.

**Mouse retina in vitro imaging.** Methods were identical to Borchius et al. (2011).

**Caenorhabditis elegans imaging.** The same experimental protocol was used as in previous GCaMP experiments (Tian et al., 2009). All recordings are from the AWC nervous system. After 10 s of observation, odor ([IAA] was 10−9 M) was added and animals imaged for 30 s. Odor was delivered for 5 min, during the last 10 s of odor application, and Polychrome V monochro mator (TILL Photonics). The control of excitation and image acquisition was achieved using TILL Vision software. We used an Olympus 60×1.45 NA objective lens. Images were typically taken every 1 s. Exposure time and pixel binning were optimized to visualize fluorescence signals for each experiment (maximum binning was 4 × 4). Cultures were perfused with recording buffer containing the following (in mM): 110 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 v-glucose, and 10 HEPES at pH 7.4 (adjusted with NaOH).
Expression levels of *C. elegans* transgenes from extrachromosomal arrays can show considerable animal-to-animal variation, complicating analysis of imaging results. To linearize the imaging measurements and improve comparisons across different expression levels, we first selected AWC pixels whose intensity exceeded nonfluorescent background, then calculated average pixel intensity.

**Drosophila larval neuromuscular junction preparation.** To allow imaging access to transgenic presynaptic neuromuscular junction (NMJ) boutons that expressed variants of GCaMP3, third instar Drosophila larvae were dissected using methods similar to those described previously (Jan and Jan, 1976). Genetic constructs were prepared in the pFRC7–20XUAS-IVS vector (Pfeiffer et al., 2010) and inserted in the VK00005 site (Venken et al., 2006) to allow expression in motor neurons using the OK6-Gal4 driver (Aberle et al., 2002). The combination of OK6-Gal4, VK00005, and pFRC7–20XUAS-IVS provided good labeling of Type 1b and 1s, but not type II boutons in heterozygous animals (+/yellow1 white1; +/OK6-Gal4; +/UAS-GCaMPx3). Actively wandering larvae were dissected in ice-cold Schneider’s insect medium (Sigma), pH shifted to 7.2 using NaOH. A 35 mm Petri dish previously one-third filled with Sygard (Dow Corning), was used to pin down the cuticle and body wall muscles. Umost care was taken during dissection to reduce the potential for muscle movement during imaging (ensuring central longitudinal muscles were not contacted with instruments at any time, cuticle stretching was sufficient to keep the preparation in place but not so much to cause spontaneous calcium release, choosing pin insertion locations that minimized potential damage to imaging region). Imaging commenced within 15 min of the segmental nerves being cut close to the ventral nerve cord. Before dissecting the Schneider’s insect medium was replaced with HL6 (Macleod et al., 2002) supplemented with 2 mM CaCl2, adjusted to 275 mOsm, pH 7.4–7.6 (start to end) while temperature stayed relatively constant (±2 ± 0.5°C).

**Drosophila larval NMJ stimulation parameters.** Segmental nerves were drawn by suction into a heat-polished glass pipette, ~12 μm internal diameter (Macleod et al., 2002), which was connected to an Iso-Flex stimulator (A.M.P.I.) to deliver suprathreshold electrical pulses (~2.9 V, ~5 mA). After 3 s of baseline, each stimulus was 2 s in duration with individual 300 μs impulses delivered at a frequency of 1, 5, 10, 20, 40, 80, and 160 Hz. The interstimulus interval was ~20 s and each stimulus frequency was repeated five times, with a pause of ~35 s, before moving to a higher stimulus frequency. The stimulus order was kept constant as higher frequency stimuli were potentially deleterious and outside the normal range of firing frequencies (Chouhan et al., 2010). We only included data from each FOV if the ΔF/F0 continuously increased with increasing stimulation and peaked at 80 Hz, as previously observed from intracellular recordings (Chouhan et al., 2010). When this was not observed, presumably due to muscle damage or overstretching during the dissection, the data from the entire FOV was discarded.

For each GCaMP construct, 10 FOV that met the quality control parameters described above were used. These FOV were collected from seven animals (i.e., more than one FOV collected from one animal). To describe the performance of an indicator, boutons within an FOV were averaged, replicate trials of a stimulus frequency were averaged, and the 10 FOV were averaged.

**Preparation and odor delivery for adult Drosophila experiments.** Flies were reared on standard cornmeal agar medium. We used the Gal4/UAS system (Brand et al., 1994) to direct the expression of the calcium sensors to projection neurons (PNs). *GAL4-H6-Gal4* flies were a gift from L. Luo (Oxford University). *GAL4-UAS-GCaMPx3* (Mall et al., 2011) adult females, 3–5 d after eclosion, Adult flies were dissected using previously described methods (Jayaraman and Laurent, 2007). Flies were anesthetized in a vial on ice until movement stopped (~15 s) and then gently inserted into a hole in a piece of aluminum foil. Small drops of wax (55°C) were used to suspend the fly in the hole, with the edge of foil defining a horizontal plane around the head and thorax, from the first antennal segment anterior to the scutellum posteriorly. The dorsal side of the foil was bathed in saline, while the ventral side (including antennae and maxillary palp) remained dry and accessible to odors. A window was cut in the dorsal head cuticle between the eyes, extending from the ocelli to the first antennal segment. Fat and air sac dors and anterior to the brain were removed, but the perineural sheath was left intact. The proboscis was affixed with a small drop of wax to a strand of human hair to limit brain movement. Spontaneous leg movements were typically observed in this preparation for the duration of the recording (~2–3 h). The saline composition used in all olfactory experiments contained the following (in mM): 103 NaCl, 3 KCl, 5 N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, 10 trehalose, 10 glucose, 26 NaHCO3, 1 NaH2PO4, 2.0 CaCl2, and 4 MgCl2, adjusted to 275 mOsm, pH 7.4. Different concentrations were delivered using a custom-made odor-delivery system designed by Dmitry Rinberg, and a Teflon nozzle (entry diameter 1/8") directed toward the antennae. Odors...
were delivered at different concentrations diluted in paraffin oil (paraffin oil alone, 0.001, 0.01, 0.1, 1.0, and 10%) in a constant stream of air (1 L/min) with an additional 10% dilution in air. For each concentration, five replicate deliveries were performed and the data averaged. Odor delivery times were measured using a mini-PID (Aurora Scientific). Odors were presented for 1 s. All comparisons of sensor performance were made using experiments with identical odor presentation times. The results reported are based on data obtained from five GCaMP3-expressing flies (six antennal lobes; ALs) and five GCaMP5-expressing flies (six ALs).

Figure 1. Design of GCaMP5s. A, Schematic of the GCaMP3 structure with sites of engineering shown. B, Structural effects of the D381Y mutation (D380Y in GCaMP3 numbering). Chromophore environment at the cpGFP/CaM interface in GCaMP2 (top, PDB 3EVR) (Akerboom et al., 2009) and GCaMP5G (bottom, PDB 3SG4) structure reported here. Structures are shown as a diagram 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. C, (ΔF/F)max versus F_apo for both linker 1 variants of GCaMP3 (left) and linker 2 variants of GCaMP3 (right) in bacterial lysate. Left, The green square denotes L1-Gln-Pro, the blue square denotes L1-His-Pro. Right, Linker variants L2-Pro-X are depicted as red squares, L2-X-Pro as blue triangles, and original GCaMP3 linker variants (L2-Thr-Arg) as green dots. D, One-photon absorption (left), one-photon emission (middle), and two-photon excitation (right) spectra of both GCaMP3 (top) and GCaMP5G (bottom). Calcium-free spectra are depicted by dashed blue lines and calcium-saturated spectra by solid red lines. Dashed green lines depict (ΔF/F)_max plotted on the right axis.
GCaMP5D T302L, R303P 22.0
GCaMP5J L59H, E60P, D380Y ND ND ND 42.2

Data collection
Oligomeric state Monomer Monomer Monomer Dimer Dimer Monomer
PDB ID 3SG2 3SG3 3SG4 3SG5 3SG6 3SG7

Structures of GCaMP variants have been deposited in the Protein Data Bank (http://www.pdb.org/).

GCaMP5A D380Y 17.4
GCaMP5L A52V, T302L, R303P 17.7
GCaMP5S also contain the mutations R2 deletion, M65K, T115V, and N362D listed for GCaMP3.

First values are measured at a calcium concentration of 670 nM, second at a calcium concentration of 8.52 nM.

Unique reflections 45514 40352 26965 29184 40438 32056
Resolution (Å) 2.00 2.10 2.40 1.90 1.70 1.90
Redundancy 13825 13825 13825 13825 13825 13825
Completeness (%) 99.9 (100) 99.9 (100) 99.6 (100) 99.3 (73.9) 95.5 (95.6) 95.5 (95.6)
Refooion
Resolution (Å) 2.00 2.10 2.40 1.90 1.70 1.90
Unique reflections 45514 40352 26965 29184 40438 32056
Rmerge (Rint) 0.173/0.207 0.165/0.199 0.172/0.213 0.180/0.225 0.203/0.249 0.184/0.230
No. atoms (B-factors (Å²))
Protein 3465 (33.0) 3470 (33.6) 3396 (36.5) 3228 (19.8) 3358 (19.6) 3356 (19.2)
Ligand/ion 3182 (32.6) 3187 (33.4) 3167 (36.5) 3089 (18.9) 3082 (18.3) 3206 (20.1)
Water 279 (36.7) 279 (35.9) 225 (37.0) 169 (34.5) 272 (35.2) 186 (39.6)
RMSD values
Bond lengths (Å) 0.027 0.028 0.024 0.022 0.025 0.024
Bond angles (°) 2.02 2.09 1.91 1.90 2.02 1.98
Ramachandran plot
Favored/outliers (%) 97.7/0.3 95.9/0.3 95.6/0.5 99.2/0.0 98.7/0.0 97.7/0.3

Structures of GCaMP variants have been deposited in the Protein Data Bank (http://www.pdb.org/).
Calcium imaging in adult Drosophila. We imaged on a two-photon microscope using PrairieView software and an Olympus 40×, 0.8 NA LUMPlanFL/IR objective. A mode-locked Ti:Sapphire Chamele Ultra II laser (Coherent) tuned to 920 nm was used for excitation. Fluorescence was collected using multimode optical fibers (Hamamatsu) after bandpass filtering. Images were acquired in frame scan mode (20 Hz) for a single plane of one AL. Fluorescence time series were then obtained by averaging across the spatial extent of the glomerulus in the frame. In all cases fluorescence changes were calculated relative to baseline fluorescence levels as determined by averaging >2 s just before odor presentation. In vivo imaging of visually evoked calcium transients in larval zebrafish tectal neuropil. *mitf(+/−) (nacre) zebrafish larvae (Lister et al., 1999) expressing elav3 under the control of the pan-neuronal elav3/Huc promoter (elav3:Gal4) were injected at 1–8 cell embryonic stage with a solution containing 0.04% phenol red, 50 ng/μl transposable mRNA, and 25 ng/μl plasmid DNA with the coding sequence of GCaMP5A or GCaMP5G downstream of 14 upstream activation sequences (UASs) and flanked by two Tol2 sequences for stable genomic integration. An elav3:Gal4, UAS:GCaMP3 stable line (Del Bene et al., 2010) was used for experiments with GCaMP3.

Six days postfertilization (dpf) larvae were embedded in 2% low melting point agarose and imaged at 7 dpf at 28°C. A custom-built two-photon microscope equipped with a mode-locked Ti:Sapphire Chameleon Ultra II laser (Coherent) tuned to 920 nm and controlled by ScanImage v3.6 software (Pologruto et al., 2003) was used for acquiring image time series at 17 Hz. Two-photon imaging of visually evoked calcium transients in larval zebrafish retinal bipolar cell terminals. All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the UK Home Office. Fish were maintained on a 14:10 h light/dark cycle at a temperature of 28°C and bred naturally. Transient expression of GCaMP2 or GCaMP5G was driven by constructs containing the Birbach-A promoter (Dreosti et al., 2009), which targets ribbon synapses of sensory neurons. Constructs at a concentration of 0.084 μg/μl were injected into embryos at the 1–4 cell stage. To inhibit melanin formation, larvae were kept in 200 μM 1-phenyl-2-thiourea (Sigma) from 28 h postfertilization. Imaging was carried out as described previously (Dreosti et al., 2009). Briefly, whole zebrafish larvae (9–11 dpf) were immobilized in 2.5% low melting point agarose on a glass coverslip. The temperature of the room was 23–24°C. Bipolar cell terminals were imaged in vivo using a custom-built two-photon microscope equipped with a mode-locked Chameleon titanium–sapphire laser tuned to 915 nm (Coherent) with an Olympus LUMPlanFL 40× water-immersion objective (NA 0.8). Emitted fluorescence was captured through both the objective and a substage oil condenser, filtered through an HQ 500/60 nm 2P GFP emission filter (Chroma Technology), and detected by a set of GaAsP photodiodes (Hamamatsu). Imaging and image acquisition were controlled under ScanImage v.3.6 software (Pologruto et al., 2003). Movies were processed using the SARIAS suite of analysis routines (Dorostkar et al., 2010) running in Igor Pro 7 (WaveMetrics). These routines begin with the automated extraction of the fluorescence change in each terminal by defining ROIs using a filtering algorithm based on a Laplacian transform.
close to eye of the fish. The stimulus time course was controlled in Igor Pro v. 4.01 (WaveMetrics) and time locked to image acquisition through ScanImage. The mean intensity of light stimuli was $2 \times 10^5$ photons/μm$^2$/s, which corresponds to a low photopic intensity.

Mouse preparation for in vivo imaging. Mice were anesthetized using isoﬂurane (3% for induction, 1.5–2% during surgery). A circular craniotomy (2–3 mm diameter) was placed above V1 (centered 2.7 mm lateral from lambda suture). Oregon Green BAPTA-1 (OGB-1)-AM (Invitrogen) was injected as previously described (Stosiek et al., 2003; Komiyama et al., 2010; Zariwala et al., 2011). A custom titanium head post was fixed to the skull using black dental cement (Contemporary Ortho-Jet). The craniotomy was covered with agarose (1.2–1.5%) and a round glass cover slip (Warner Instrument, 5 mm diameter, #1 thickness) was cemented to the skull using black dental cement (Contemporary Ortho-Jet). The moving grating stimulus was generated using the Psychophysics Toolbox (Brainard, 1997; Pelli, 1997) in MATLAB. Each stimulus trial consisted of a 4 s blank period (uniform gray at mean luminance) followed by a 4 s drifting sinusoidal grating (0.05 cycles per degree, 1 Hz temporal frequency). The visual stimuli were synchronized to individual image frames using frame-start pulses provided by ScanImage 3.7. The gratings were presented through an LCD monitor (30 × 40 cm), placed 25 cm in front of the center of the right eye of the mouse. The monitor subtended an angle of ±38° horizontally and ±20° to ±38° vertically around the eye of the mouse. For cell-attached recording, we used a smaller LCD monitor (12 × 16 cm) placed 10 cm in front of the right eye. Each stimulus trial consisted of a 4 s blank period followed by a 2 s drifting square wave grating (0.05 cycles per degree, 2 Hz temporal frequency).

Mouse data analysis. Slow drifts in brain position in the X and Y directions were corrected using the TurboReg plug-in in ImageJ (The´venaz et al., 1998). All remaining analyses were performed in MATLAB. ROIs corresponding to visually identifiable cell bodies were selected using a semi-automated algorithm. For GCaMP3, GCaMP3G, and GCaMP5K, ring-shaped ROIs were placed at the cytosolic regions of the cells (excluding the nucleus; GCaMP expression is typically restricted to the cytoplasm; Tian et al., 2009). For OGB-1, circular ROIs covering the whole soma were used. The fluorescence time course of each cell was measured by averaging all pixels within the ROI. The neuropil contamination was corrected using a semi-automated algorithm. For GCaMPs and 800 nm for OGB-1. The objective was a 40× dipping lens (Olympus, 40×, 0.8 NA). Image acquisition was performed using ScanImage 3.7 (http://www.scanimage.org) (Pologruto et al., 2003). Images (512 × 250 pixels, 250 × 250 μm) were collected at 4 Hz.

In vivo cell-attached recording was performed using glass pipettes (−5−7 ΜΩ) filled with solution containing the following (in μM): 125 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 CaCl$_2$, 2 MgSO$_4$, and 0.1 Alexa Fluor 594). Signals were amplified using an AxoPatch 200B amplifier (Molecular Devices), filtered at 5 kHz, and digitized at 10 kHz. Images (32 × 20 pixels, 20 × 20 μm) were acquired at 50 Hz.

Figure 2. Neuronal testing of GCaMPs. A, GCaMP3 and 5G responses in neurons. DIC (left) and false-colored image of fluorescence response to 40 field stimuli (right). B, Trial-averaged responses of GCaMP3 and 5G, and OGB-1 and Fluo-4, to 1 and 10 field stimuli. C, Peak ΔF/ΔF$_{0}$ versus stimuli. Error bars indicate SEM. Right, blow-up of 1–5 stimuli. D, SNR including SEM. SNR was computed as the ratio between the peak fluorescence response amplitude (ΔF) and the SD of the fluorescence trace before stimulus onset. Fluo-4 was omitted from the blow-ups.
the grating that produced the strongest response. The orientation-tuning curve was constructed by measuring the mean $\Delta F/F$ over the 4 s stimulus period for each orientation. We then fitted the tuning curve as the sum of two Gaussian functions centered on $\theta_{\text{pref}}$ and $\theta_{\text{pref}} + \pi$ with equal width $\sigma$, different amplitudes $A_1$ and $A_2$, and a constant baseline $B$ (Niel and Stryker, 2008). The value of $\sigma$ was required to be larger than 15° to reflect the limit of our stimulus set (45° separation) in resolving sharper tuning. The OSI was defined as follows:

$$R_{\text{pref}} - R_{\text{ortho}}$$

where $R_{\text{pref}}$ and $R_{\text{ortho}}$ are the response amplitude at the preferred ($\theta_{\text{pref}}$) and the orthogonal orientation ($\theta_{\text{pref}} + \pi$). Tuning width was defined as the half-width at half-maximum of the fitted Gaussian ($\sigma \cdot \sqrt{2 \cdot \ln 2}$). Finally, DSI was calculated as follows:

$$R_{\text{pref}} - R_{\text{opposite}}$$

where $R_{\text{opposite}}$ is the response in the opposite direction ($\theta_{\text{pref}} + \pi$).

For simultaneous imaging and cell-attached recording, ring-shaped ROIs were placed at the cytosolic regions of the cells. Neuronal signals were measured from the image region with the cell excluded. Neuronal compensation was performed as above ($r = 0.7$) before calculating $\Delta F/F$. For visual-evoked response (see Fig. 9A–E), the baseline fluorescence ($F_0$) was measured over a 1 s period immediately before the start of grating stimulation. For responses to a few isolated AP (Fig. 9F, $E$), we searched the spike trace for events with 1, 2, and 3 APs within a 200 ms window, and no other APs during a 1 s period before and a 0.5 s period after the first AP. The baseline fluorescence ($F_0$) was measured over a 0.2 s period before the first AP. AP detection was quantified using template matching with the average trace of 1, 2, and 3 AP events as templates for detecting 1, 2, and 3 APs, respectively. The 0 AP traces (478 traces) were taken from the same fluorescence recordings during periods where no AP was detected for at least 2 s. The decision variable was the projection of the fluorescence traces along the direction of the template vector. Detection efficiency was defined as the fraction of correctly detected events given a 5% false positive rate.

The single exponential model fit of the GCaMP5K signal (Fig. 9H) was calculated by linear convolution of the detected spike point process with a single exponential kernel: $h(t) = A \cdot e^{-\tau t}$. The amplitude $A$ and the decay time constant $\tau$ were adjusted to minimize the mean square error of the fit. For the nonlinear model, the output of the linear mode $\Delta f(x(t))$ was passed to a nonlinear stage to generate the final output as follows:

$$\Delta f_{\text{non-linear}}(t) = a \cdot b \cdot x(t)^2 + c \cdot x(t)^3.$$

The four parameters $A$, $\tau$, $b$, and $c$ were adjusted independently to minimize the mean square error.

Statistical methods. Unless specified otherwise, all statistical methods were implemented in MATLAB or Microsoft Excel, using standard packages.

Results

Structure-guided engineering of GCaMP5s and biophysical characterization

A small family of GCaMP5 variants was produced from the GCaMP3 scaffold by combining improvements generated from

Figure 3. Targeted GCaMPs and astrocyte testing. A, Heat map showing peak $\Delta F/F$ of hippocampal neurons transfected with SyGCaMP5s following 10 field stimuli (20 Hz). B, Fluorescence response versus field stimuli for SyGCaMPs. C–E, Performance of LckGCaMPs in astrocytes. C, Top, Baseline fluorescence of LckGCaMP3 and 5G. Middle, Spotty calcium signals. Bottom, Quantified baseline fluorescence: red, GCaMP3; blue, GCaMP5G. D, Spotty calcium transients for LckGCaMP3 and Lck-GCaMP5G, respectively. E, Peak $\Delta F/F$ for spontaneous (left) and ATP-evoked (right) responses in astrocytes. F, Peak $\Delta F/F$ for neuronal AP-evoked astrocyte responses. Error bars indicate SEM. G, Fluorescence micrograph of GFAP-GCaMP5G-transfected astrocytes and fluorescence response of GFAP-GCaMP5G to field stimulation (30 Hz) of cocultured neurons; 1–120 field stimulations. Raw traces in gray, trial-average in blue.
site-directed mutagenesis at the cpGFP/CaM proto-interface and targeted library screening at the M13pep-cpGFP and cpGFP-CaM linkers. We also tested the effect of mutations to the M13 peptide and near the third Ca\(^{2+}\)-binding site of CaM. We found that Asp380Tyr increases the Ca\(^{2+}\) accessibility of the cpGFP chromophore; indeed this mutation reorganizes nearby side chains and the water network in close proximity to the cpGFP chromophore, resulting in an apparent decrease in solvent accessibility of the cpGFP barrel (Fig. 1B). Consequently, the Asp380Tyr mutation raises the brightness of the calcium-bound state of GCaMP3 for both GCaMP3 and GCaMP5G; in addition, calcium affinity and cooperativity (Hill coefficient) are increased by approximately 25% for GCaMP5A (Table 1).

The structure of the M13pep-to-cpGFP linker (“linker 1”) is known to be critical to sensor function (Nakai et al., 2001). Crystal structure analysis of Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free GCaMP2 (Wang et al., 2008; Akerboom et al., 2009) revealed that both amino acids of linker 1 (Leu60 and Glu61) make significant contacts to cpGFP and CaM and stabilize both the apo and Ca\(^{2+}\)-bound conformations. After screening mutagenic libraries at the linker 1 positions (59–60), we found a number of variants that substantially increased the dynamic range of GCaMP3 in E. coli lysates (Table 1; Fig. 1C). Mutation of linker 1 from Leu-Glu (GCaMP3) to His-Pro (GCaMP5B) or Glu-Pro (GCaMP5C) approximately doubles or triples the \(\Delta F/F\) max of GCaMP3, respectively (Table 1; Fig. 1C).

Mutation of the cpGFP-to-CaM linker (“linker 2”) has also been shown to affect sensor function (Souslova et al., 2007). Accordingly, we also screened targeted libraries at the linker 2 positions (302–303) and identified variants with increased dynamic range in E. coli lysates (Table 1; Fig. 1C). Most prominent was Leu-Pro (GCaMP5D), which has a \(\Delta F/F\) max approximately twice that of GCaMP3, but with decreased affinity (Table 1).

Presuming that the improvements to sensor function resulting from the individual mutation of the cpGFP/CaM interface and linker 1 (L1) and linker 2 (L2) might be additive, different combinations of these (in the GCaMP3 scaffold, Fig. 1A) named GCaMP5E-J, were characterized extensively in vitro (Table 1). Compared with GCaMP3, all these GCaMP5 variants have significantly higher \(\Delta F/F\) (Table 1). The Arg380Tyr mutation not only increases the brightness of both the saturated (sat) and apo states in the GCaMP3 background (GCaMP5A), it also increases the brightness of both states for several of the other sensors with modified linkers, e.g., 5G/D and 5H/F. Linker mutations L1-QP (GCaMP5C), L1-HP (GCaMP5B), and L2-LP (GCaMP5D) decrease the fluorescence of the apo state, and their combination has a cumulative effect: GCaMP5E, GCaMP5F, GCaMP5H, and GCaMP5I are all exceptionally dim in the apo state. This effect is strongest for GCaMP5F and GCaMP5H, both containing combinations of L1-QP and L2-LP, leading to an ~160-fold fluorescence increase upon calcium binding in vitro (Table 1).

A GCaMP5 sensor incorporating the CaM mutation Arg90Gly (Sorensen and Shea, 1996) was constructed in the background of GCaMP5A, to further increase Ca\(^{2+}\) affinity (GCaMP numbering Arg92Gly, GCaMP5K). While GCaMP5K, as intended, has a higher affinity (and Hill coefficient) for Ca\(^{2+}\) than GCaMP3 \(K_a = 190 \text{ nM}, n = 3.8\), it comes at a cost of \(\Delta F/F\) max (Table 1). Another gain in affinity was achieved by mutation of the M13 peptide, Ala52Val, in a “hydrophobic anchor” position (Hultschig et al., 2004) and similar to the “D2”

Figure 4. GCaMP5D in mouse retina. A, Top, Fluorescence micrograph of retinal ganglion cells (RGCs) expressing GCaMP5D. Bottom, Two-photon fluorescence image of RGCs expressing GCaMP5D. B, Fluorescence response of six indicated RGCs to infrared LED stimulus and to full-field blue LED flash. Black dashed lines show responses of a representative population of GCaMP3-labeled RGCs recorded under identical stimulus conditions (data replotted from Borghuis et al., 2011). C, Peak \(\Delta F/F\) distribution for 55 imaged RGCs. Corresponding data for GCaMP3 and OGB-1 labeled neuron populations are shown in red and black, respectively (data reproduced from Borghuis et al., 2011). D, SNR distribution of GCaMP3 and GCaMP5D.
M13 variant (Palmer et al., 2006). This mutation, acquired serendipitously in GCaMP5D (resulting in GCaMP5L), increases calcium affinity ~2-fold while preserving ($\Delta F/F_{\text{max}}$) (Table 1).

We used an in-depth biophysical characterization of this panel of variants to reduce the number taken forward for in vivo testing, and identified some with particularly useful characteristics. GCaMP5G has a significantly higher apo $pK_a$ (Table 1), indicating that the Ca$^{2+}$/H$^{+}$ free state is more likely to have a prototated, dim chromophore at physiological pH. Apparent rates of fluorescence conversion were determined by stopped-flow mixing, beginning at 0 [Ca$^{2+}$] and stepping to 250 nM–10 $\mu$M. Initial rates ($k_i$) of fluorescence after steps to 10 $\mu$M [Ca$^{2+}$] were significantly faster for 5D and 5G than for GCaMP3 (Table 1), although rates after steps to 500 nM [Ca$^{2+}$] were comparable. Peak brightness and QY of GCaMP5s tested were comparable to GCaMP3, and GCaMP5G revealed that a larger fraction of molecules is in the fluorescent, deprotonated state for GCaMP5G.

Characterization in HEK293 cells and cultured neurons

We next characterized the GCaMP5 variants in HEK293 cells in response to acetylcholine-induced Ca$^{2+}$ mobilization (Tian et al., 2009). The ratio of peak-to-baseline fluorescence was increased for most variants, by up to ~17 fold for GCaMP5H (Table 1). This is the direct result of lower baseline fluorescence for all variants except GCaMP5A, in agreement with in vitro data (Table 1). GCaMP5s containing L1-QP (GCaMP5C, GCaMP5F, and GCaMP5H) were not selected for further analysis in cultured neurons, due to the low baseline brightness of variants containing this linker. GCaMP5K was directly tested in primary rat hippocampal neurons due to its high affinity; the HEK293 cell testing was omitted for this variant.

A selection of eight GCaMP5 variants was expressed in primary rat hippocampal neurons by lentivirus-mediated gene transfer. After 16–18 d, infected neurons appeared healthy (Fig. 2A); most GCaMP5s were dimmer than GCaMP3 at baseline, except GCaMP5K (“5K”), which was approximately twice as bright before stimulation (Table 1). APs were elicited at 83 Hz via a custom-built extracellular field stimulator (1 AP per stimulus, data not shown) and imaged at 34.8 Hz with an EMCCD camera. In parallel experiments we incubated neurons with cell-permeable versions (acetoxymethyl ether, “AM” derivatives) of the small molecule calcium dyes OGB-1 or Fluo-4. Robust fluorescence responses were seen from 1 to 160 field stimuli (Fig. 2B–D). We extracted fluorescence from individual neurons and computed background-subtracted fluorescence transients. Single field stimuli, corresponding to one AP, evoked fluorescence transients that were detectable in single trials (Fig. 2B). Peak $\Delta F/F$ (Fig. 2C) and signal-to-noise ratio (SNR; Fig. 2D) were significantly improved for most GCaMP5 variants compared with GCaMP3, over at least part of the stimulus regime (Fig. 2C,D; $\Delta F/F$ and SNR for each variant including GCaMP3, OGB-1, Fluo-4, and G-GECO1.2).

Fusion-directed localization in neurons and astrocytes

GCaMP5G (“5G”), which showed the highest response at maximum stimulation in the cultured neuron screen, was selected for testing in the context of specific protein fusions. Variant 5G was fused to synaptophysin (Dreosti et al., 2009) creating “SyGCaMP5G” (targeted to the outside of synaptic vesicles), and to the Lck domain (Shigetomi et al., 2010b) (“Lck-GCaMP5G”; inside of the plasma membrane). SyGCaMP constructs were transfected into hippocampal neurons, and boutons were imaged (Fig. 3A); the response of SyGCaMP5G (“Sy5G”) to acetylcholine-induced Ca$^{2+}$ mobilization in the context of specific protein fusions is shown in Figure 3B. This demonstrates the ability of GCaMP5 to be used in the context of protein fusions and highlights the potential for additional applications of GCaMP5 in neuroscience.
small field stimuli was significantly higher compared with SyG-CaMP2 and SyGCaMP3 (Fig. 3B).

5G also proved superior to GCaMP3 for detection of Ca$^{2+}$ hot-spots in astrocytes. The membrane-targeted Lck-GCaMP5G (“Lck-5G”) detected spontaneous Ca$^{2+}$ transients (“spotty calcium” signals) (Shigetomi et al., 2010a) and ATP-induced responses with ~2-fold greater response magnitude than Lck-GCaMP3 in astrocytes; the 5G-determined responses also had more apparent local

Figure 6. GCaMPs in Drosophila. A, Schematic of larval NMJ preparation, and close-up of Type 1b boutons from muscle 13 (segments A3–A5) used for wide-field imaging. Scale bar, 30 μm. B, Single trials of electrically evoked Ca$^{2+}$ transients from wide-field imaging in the Drosophila larval NMJ. Top: Fluorescence changes (ΔF/F) traces from presynaptic terminals obtained by delivering 2 s of electrical stimulus at different frequencies. Bottom: SNR of the same data. Left, GCaMP3. Right, GCaMP5G. C, Two-photon imaging frame scan of PNs innervating the DC1 glomerulus in the adult fly AL (dorsal view) Scale bar, 20 μm. D, The mean of five replicate stimulations from six ALs (5 animals) is shown along with the SD (between AL means). Response to a 0.1% octanol, 1 s odor pulse from DC1 PNs. E, Mean octanol response from PNs from DC1 glomerulus (averaged over 5 flies) to increasing concentration. All panels show mean ± SD.
Figure 7. In vivo imaging in zebrafish. A, Schematic representation of area imaged (red square; retinal bipolar cell terminals) including fluorescence micrograph of bipolar cell. B, Two-photon imaging of calcium spikes in axon terminals of retinal bipolar cells in Tg(Ribeye-A:GCaMP2) (green line) and Tg(Ribeye-A:GCaMP5G) (blue line) fish. Mean (± SEM) of 20 spontaneous calcium spikes plotted. C, Schematic of tectal neuropil imaged in zebrafish (red square). Micrograph with dashed yellow lines marking the borders of the tectal neuropil. D, Imaging Ca$^{2+}$ transients in RGC axons and tectal neuron dendrites in GCaMP3, 5A, and 5G fish. Single-trial (gray) and trial-average (GCaMP3, red; 5A, cyan; 5G, blue) $\Delta F/F$ traces recorded during 2 s visual stimulation to contralateral eye (black bars below traces); stimulus bar moves through the receptive field of the imaged neurons, and is unlikely to be visible to the imaged neuron for the entire 2 s. E, Histograms depicting average ($\Delta F/F)_{av}$ values (left), maximum (middle) ($\Delta F/F)_{max}$ values, and SNR (right), over the neurons analyzed. Error bars indicate SEM, **p < 0.01, ***p < 0.001, (Figure legend continues.)
structure, significantly increasing the resolution of spotty cal-
cium signal detection (Fig. 3C–E). Furthermore, the threshold of
detection was lowered from ~10 field stimuli for Lck-GCaMP3
to ~2 field stimuli for Lck-5G for transfected astrocytes co-
cultured with neurons, indicating that both dynamic range and
sensitivity have been substantially improved for membrane-
targeted 5G compared with GCaMP3 (Fig. 3F). SNR was slightly
improved for most of the stimulus range, although variability was
higher due to lower baseline fluorescence (Fig. 3F).

In parallel, membrane-targeted 5G and GCaMP3 were expressed
in astrocytes (under control of the glial fibrillary acidic protein
(GFAP) promoter) cocultured with neurons. Fluorescence changes
in astrocytes following neural activity evoked by electrical field stim-
ulation were easily recorded using GFAP-5G (Fig. 3G). These results
demonstrate that the GCaMP5 sensors will be useful to study Ca2+
dynamics in a range of cell types, and that these indicators are suited
for membrane targeting and protein fusions.

Characterization in mouse retina
GCaMP5D ("5D") displayed large (∆F/∆F)max to 1–3 field stim-
uli in cultured neurons (Fig. 2D), and was selected for further testing in in vitro mouse retina, as before (Borghuis et al., 2011). We introduced 5D into AAV under the neuron-specific
hsyn1 promoter and infected mouse retinas in vivo (see Mate-
rials and Methods)(Borghuis et al., 2011). Retinal ganglion
cells (RGCs) were bright and appeared healthy, with nuclear-
excluded fluorescence (Fig. 4A, top). We recorded light-
evoked responses from 5D-expressing RGCs in dissociated
retinas (Fig. 4A, bottom). RGCs showed fluorescence changes both to the onset of the infrared scan laser (910 nm; 20 mW after
the objective)(Borghuis et al., 2011) and to a flash of visible light (420 nm) delivered with an LED 2 s after scan onset (Fig. 4B).
Time course, amplitude, and polarity of the responses varied
across the labeled population, consistent with the known diver-
Sibility of RGC types (e.g., ON and OFF, brisk and sustained).
The peak amplitude (∆F/∆F)max of the evoked fluorescence response
was 1.6 ± 1.3 (n = 55; mean ± SEM; range 0.0–4.5) (Fig. 4C). Of
all 5D-expressing cells, 65% responded with (∆F/∆F)max > 0.6
(mean 2.3 ± 1.0). For comparison, GCaMP3 and OGB-1 pro-
duced distributions of (∆F/∆F)max with peaks around 0.2, with
only ~30% of GCaMP3- and OGB-labeled RGCs showing (∆F/
F)max > 0.15 (Borghuis et al., 2011) (Fig. 4C). SNR for
GCaMP5D was significantly improved compared with GCaMP3
(GCaMP3: SNR average = 8.97 ± 1.08, n = 187; GCaMP5D: SNR average 13.43 ± 1.16; n = 64; p = 0.025) (Fig. 4D).

Imaging sensory-evoked Ca2+ transients in worms
To test the in vivo performance of GCaMP5 in worms, we selected
GCaMP3, 5A, and 5G for analysis. We monitored the activity of the
C. elegans AWC (\textsuperscript{11005}A) chemo sensory neuron, which responds to odor
presentation with graded calcium decreases and to odor removal

(Figure legend continued.) n.s., not significant; two-tailed t test. ∆F/∆F, Cumulative distributions of amplitudes of visually evoked calcium transients: p = 0.03 for 5G, p < 0.001 for 5A (compared with GCaMP3); two-sample Kolmogorov–Smirnov test. For all experiments n = 35 calcium transients recorded in ≥3 larvae. G, Schematic of two-photon imaged location (optic tectum somata of Tg(eve:3;GCaMP2, 3, and 5G) fish) including micrograph. H, Left, fraction of cells with detectable response (for 5G vs GCaMP3: paired t test, one-tailed p < 0.01). Right, Cumu-
lative histogram of peak ∆F/ΔF values. I, Trial-averaged (n = 13, 9, and 5 larvae) for GCaMP3, GCaMP5D, and GCaMP5G, respectively responses to visual stimuli of top 50% of responding cells, ranked by peak ∆F/ΔF. J, SNR of trial-averaged responses. Cell traces were divided by the SD of 10 s preceding visual response. The average of this SNR trace for each indicator is plotted.

with graded calcium increases (Tian et al., 2009) (Fig. 5A). All
GCaMPs were expressed from the same promoter; in all cases, no
defect was observed in AWG-dependent spontaneous Ω turning fre-
quency was unaffected by the transgene (Tian et al., 2009) (Fig. 5B).
Individual worms were imaged in a microfluidic chamber during an
odor addition–removal sequence with the odor isoamyl alcohol
(IAA) (Fig. 5C,D). All GCaMPs detected the known decrease in
AWG calcium upon odor addition and the calcium increase upon
odor removal (Tian et al., 2009). GCaMP5G performed comparably
to GCaMP3 for odor presentation and for odor removal, based on
total fluorescence change and SNR. GCaMP5A showed a strong sup-
pression in baseline fluorescence upon odor addition, and a large
increase after odor removal. In addition, the higher baseline fluores-
cence of 5A resulted in a threefold improvement in SNR for odor
presentation, and a twofold increase in SNR for odor removal, over
GCaMP3. However, the 5A fluorescence plateaued near peak, sug-
gestig a truncation of the response to the highest calcium levels,
unlike GCaMP3. These results are consistent with the higher affinity
and cooperativity of 5A Ca2+ binding compared to GCaMP3 (Table
1). Many neurons in C. elegans exhibit graded responses to stimuli,
and 5A extends the observable range to calcium fluctuations too low
for GCaMP3 to detect reliably. These results indicate that 5A may be
an improved indicator for C. elegans neurons, with the choice be-
tween 5A and GCaMP3 dictated by the specific application.

Characterization of GCaMPs in larval Drosophila
Both GCaMP3 and 5G were expressed in Drosophila melanogaster
larvae using a motor-neuron promoter (OK6-Gal4). Type 1b
NMJs boutons of third instar larvae were imaged following activity
evoked from electrically stimulating motor neuron axons using
a suction electrode (Macleod et al., 2002) (Fig. 6A). Larval
NMJs showed robust fluorescence changes to evoked APs across
a range of stimulation frequencies. GCaMP5G showed a three-
fold increase in (∆F/∆F)max over GCaMP3, saturating at ~8. Sin-
gle APs were clearly detectible in trial-averaged 5G responses but
only rarely in single trial responses (Fig. 6B). The shapes of the
max (∆F/∆F) and SNR values for both indicators, but
responses with 5G were significantly greater than those with
GCaMP3 across all stimuli frequencies (n = 6, 10 NMJs/GCaMP,
7 larvae, for electrical stimuli at 1, 5, 10, 20, 40, 80, and 160 Hz, p
values at ∆F/∆Fmax were 0.0006, 0.01, 0.002, 4.8e-06, 6.6e-10, 1.5e-
11, and 1.3e-12, respectively, and p values at SNRmax were 6.6e-
06, 0.006, 0.001, 4.7e-06, 1.2e-09, 9.7e-10, and 1.2e-06, re-
spectively).

Imaging sensory-evoked Ca2+ transients in adult Drosophila
In parallel we crossed UAS:GCaMP Drosophila flies with a Gal4
fly line (GH146-Gal4) expressing broadly in the olfactory projec-
tion neurons, and imaged calcium changes in the AL (Jayaraman
and Laurent, 2007), in the DCI glomerulus, in response to pre-
sentations of octanol (Fig. 6C). For 1% octanol (the highest in-
tensity stimulus we presented) we observed: 5G, 8.96 ± 3.02
(∆F/∆F)max (range 6.11–14.08, n = 6 ALs); GCaMP3, 3.18 ± 0.90
(∆F/∆F)max (range 2.02–4.72, n = 6 ALs), a threefold improve-
mment (Fig. 6D) in dynamic range. We presented a range of concen-
trations of octanol to obtain a tuning curve with GCaMP3 and
5G (Fig. 6E). The shapes of the ∆F/∆Fmax and SNRmax curves were
similar for both indicators, but responses with 5G were sig-
ificantly greater than those with GCaMP3 across all concentra-
tions except 0.01% octanol (n = 6 ALs/GCaMP, five flies, for concen-
trations of 0.0001, 0.001, 0.01, 0.1, and 1% octanol, p values for
∆F/∆Fmax were 0.007, 0.005, 0.07, 0.0003, 0.0002, and 0.0003, re-
Optimization of a GCaMP Calcium Indicator

**Figure 8.** Comparing 5G and 5K with GCaMP3 and OGB-1 in mouse visual cortex. **A,** Left, Schematic showing experimental setup. Right, GCaMP5G expression in layer 2/3 neurons of V1 3 weeks following AAV injection. **B,** Normalized fluorescence intensity along a line through the center of a cell (red line in A, right). Mean in red and standard deviation in gray. **C,** Responses of three cells to eight oriented moving grating stimuli; gray, single trials, blue, trial-average. **D,** Visual responses (\(\Delta F/F\)\textsubscript{max}) of 438 responsive cells, rank ordered by signal level, to eight orientations aligned in columns starting with the preferred orientation. **E,** Fraction of visually responsive neurons (GCaMP3, 10.2%; 5G, 21.5%; 5K, 20.6%; OGB-1, 36.5%; neuropil compensation factor \(r = 0.7\)). **F,** Fraction of responsive neurons as a function of the strength of neuropil compensation applied. **G,** Averaged visually evoked calcium transients of the 10% most responsive cells at their preferred orientations, normalized to the end of the stimulus period. **H,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **I,** Fluorescence half-decay time after stimulus offset (quantified for the 10% most responsive cells at preferred orientation). **J,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **K,** Fraction of visually responsive neurons (GCaMP3, 10.2%; 5G, 21.5%; 5K, 20.6%; OGB-1, 36.5%; neuropil compensation factor \(r = 0.7\)) as a function of the strength of neuropil compensation applied. **L,** Averaged visually evoked calcium transients of the 10% most responsive cells at their preferred orientations, normalized to the end of the stimulus period. **M,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **N,** Fluorescence half-decay time after stimulus offset (quantified for the 10% most responsive cells at preferred orientation). **O,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **P,** Fraction of visually responsive neurons (GCaMP3, 10.2%; 5G, 21.5%; 5K, 20.6%; OGB-1, 36.5%; neuropil compensation factor \(r = 0.7\)) as a function of the strength of neuropil compensation applied. **Q,** Averaged visually evoked calcium transients of the 10% most responsive cells at their preferred orientations, normalized to the end of the stimulus period. **R,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **S,** Fluorescence half-decay time after stimulus offset (quantified for the 10% most responsive cells at preferred orientation). **T,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **U,** Fraction of visually responsive neurons (GCaMP3, 10.2%; 5G, 21.5%; 5K, 20.6%; OGB-1, 36.5%; neuropil compensation factor \(r = 0.7\)) as a function of the strength of neuropil compensation applied. **V,** Averaged visually evoked calcium transients of the 10% most responsive cells at their preferred orientations, normalized to the end of the stimulus period. **W,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **X,** Fluorescence half-decay time after stimulus offset (quantified for the 10% most responsive cells at preferred orientation). **Y,** Average \(\Delta F/F\) at the preferred orientation for low responder (50 – 80th percentile), mid responder (80 – 97th percentile), and high responder (> 97th percentile) cells. **Z,** Fraction of visually responsive neurons (GCaMP3, 10.2%; 5G, 21.5%; 5K, 20.6%; OGB-1, 36.5%; neuropil compensation factor \(r = 0.7\)) as a function of the strength of neuropil compensation applied.

**In vivo imaging of evoked and spontaneous APs in zebrafish**

We tested GCaMP2, GCaMP3, 5A, and 5G *in vivo* in zebrafish (*Danio rerio*), at three different locations in the visual pathway (Nevin et al., 2010). An overview of the fish visual system for each of the three imaging locations is shown (Fig. 7A, C, G). GCaMP2 and 5G were first expressed in sensory neuron ribbon synapses, under control of the Ribeye-A promoter. In Ribeye-A GCaMP fish (Fig. 7A), 5G showed a ~2.5 fold larger (\(\Delta F/F\)\textsubscript{max}) compared with GCaMP2 in bipolar cell terminals in the inner plexiform...
Figure 9. Relationship between spiking and GCaMP5K signal in vivo. A, Visually evoked GCaMP5K response (top) and simultaneously recorded spikes (bottom) in a layer 2/3 pyramidal cell in V1. Arrow, Putative single spike-induced signal. B, GCaMP5K responses (top: gray, individual trials; purple, average of 5 trials) and corresponding spike raster (middle) and peristimulus time histogram (bottom) during the presentation of eight oriented grating stimuli. C, Peak GCaMP5K response during 2 s visual stimulation as a function of spike rate. D, Peak GCaMP5K (Figure legend continues.)
layer following a visual stimulus (Dreosti et al., 2009) (Fig. 7B), which might be related to Ca\(^{2+}\) spiking in some of these terminals (Dreosti et al., 2011). GCaMP3, 5A, and 5G were next expressed pan-neuronally, using the elavl3 (HuC) promoter. In RGC axons and tectal dendrites (Fig. 7C), 5A and 5G responses to visual stimuli were greater compared with GCaMP3 (Fig. 7D) in both average and maximum peak response (Fig. 7E). SNR was improved for both 5A and 5G. Interestingly, as for the worm odor addition/removal imaging, 5A outperformed GCaMP3 and 5G (Fig. 7D–F). In tectal neuron somata (Fig. 7G), (ΔF/F)\(_{\text{max}}\) for 5G was over threefold improved compared with GCaMP3 (Fig. 7H, I). More importantly, the number of cells with detectable visual responses increased by sixfold (35% of the total number of cells) for 5G compared with GCaMP3 (Fig. 7H). SNR was fourfold improved for 5G compared with GCaMP3 (Fig. 7J).

**In vivo imaging of visual stimulus-evoked activity in mouse cortex**

L2/3 neurons in the mouse primary visual cortex (V1) show a broad distribution of spike rates in response to visual stimulation (0–20 Hz; median 4 Hz) (Niell and Stryker, 2008). In contrast to other sensory areas in the rodent (O’Connor et al., 2010), the majority of V1 neurons can be driven to spike. L2/3 in V1 thus provides an ideal system to assay the sensitivity of different calcium indicators in vivo. A larger fraction of responding neurons in V1 indicates higher sensitivity for detecting APs.

We thus tested GCaMPs in V1 under similar experimental conditions used in published studies (Niell and Stryker, 2008; Kerlin et al., 2010; Zariwala et al., 2012). Both 5G and GCaMP5K (“5K”) were tested in vivo because of their superb SNR in cultured neurons (Figs. 2D, 4). Both GCaMP5s and GCaMP3 were delivered by AAV-

**Figure legend continued.** response plotted against spike rate for nine cells. **E.** Average GCaMP3 response to 1, 2, and 3 APs within 200 ms search windows. Gray traces are mean ± SEM (n = 225, 81, and 21 for 1, 2, and 3 APs, respectively). **F.** Spike detection efficiency. **G.** Peak ΔF/F response to 1, 2, and 3 APs. **H.** Single exponential model (yellow trace) and nonlinear model (green trace) fit of the GCaMP5 signal (gray) from the simultaneously recorded spike response (black trace, bottom). Arrows, Underestimation of large events. Arrowheads, Overestimation of small events by the single exponential model. **I.** Trial-to-trial variability of GCaMP5 (coefficient of variation) during repeated presentation of preferred stimuli calculated using different measures. (**p = 0.0012; n.s., nonsignificant; n = 7 cells). **J.** Trial-to-trial variability of fluorescent responses at preferred orientation quantified for all visually responsive neurons, for all four calcium indicators.
iciency of 5K under our imaging conditions. The detection efficiency was 29.3% for 1 AP, 63.0% for 2 APs, and 95.2% for 3 APs, at a 5% false positive rate (Fig. 9F).

The supralinear relationship between spiking and GCaMP5K fluorescence has to be taken into account in the interpretation of GCaMP-based calcium imaging. This supralinearity provides superb SNR for imaging highly active neurons; however, the supralinear relationship also emphasizes differences in spike rate across trials, leading to a larger trial-to-trial variability. Indeed, trial-to-trial variability of GCaMP5K responses was larger compared with spikes (Fig. 9I) and OGB-1 responses (Fig. 9F) during repeated presentation of preferred stimuli.

This difference in variability is completely explained by a quantitative model relating 5K signals and spiking activity. For synthetic calcium indicators convolving spikes with a single exponential kernel accounts well for the signal of several synthetic calcium indicators (Yaksi and Friedrich, 2006; Greenberg and Kerr, 2009; Kerlin et al., 2010; Komiyama et al., 2010; Sato and Svoboda, 2010). When applied to GCaMP5K, however, a single exponential model underestimates the response during strong activity (Fig. 9H, arrows), and overestimates the response during weak activity (Fig. 9G, arrowheads). Adding a simple supralinearity after the linear convolution (see Materials and Methods) greatly improves the fit (Fig. 9G, green curve). The nonlinear model reproduces the higher trial-to-trial variation of the GCaMP5K response compared with spikes (Fig. 9I). Furthermore, removing the nonlinear fit from the data, and replac-}

Discussion

GCaMP3 has been widely used in diverse model organisms, facilitating a large number of new neuroscience applications. However, GCaMP3 has remained lacking in detection of sparse spiking activity. All of the GCaMP5s described showed improved dynamic range compared with GCaMP3. Several GCaMP5 indicators (5A, 5D, 5G, and 5K) were further characterized in a wide variety of neuronal and astrocyte activity imaging preparations, and in several model organisms in vivo. Depending on the application, the user will have the option to pick the ideal GECI.

GCaMP5s were produced from the GCaMP3 scaffold by targeted library screening at the cpGFP/CaM proto-interface and the two interdomain linkers. Functional analysis of GCaMP variants has largely validated the conjecture that crystal structures provide snapshots of conformational states that are directly relevant to the sensing mechanism. The primary utility of GCaMP crystal structures has been to delineate the protein domain proto-interfaces and the GFP chromophore environment, which guided targeted library mutagenesis, while also supporting some atomic-level predictions. The critical importance of linker length and composition to GCaMP function is consistent with recent results in other sensor classes (Horikawa et al., 2010; Alicea et al., 2011; Marvin et al., 2011). In GCaMP and other sensors based on circularly permuted fluorescent proteins, the linkers are in close proximity to the FP chromophore, and may both directly modulate fluorescence and contribute to the efficiency of larger scale ligand-dependent domain rearrangements. We have consistently found proline to be enriched in the interdomain linkers of high-SNR variants of cpGFP-based indicators (Alicea et al., 2011; Marvin et al., 2011); the increased rigidity of the proline polypeptide backbone may limit interdomain conformational sampling to states that differ more widely in fluorescence.

Some GCaMP5 variants show a >150-fold increase in fluorescence upon calcium binding in vitro; this is approximately equivalent to the best small-molecule probes (but both have low baseline fluorescence). This 10-fold improvement did not translate into increased performance for these specific GCaMPs in more demanding preparations because of the low baseline fluorescence, underlining the importance of these medium-throughput cell-based assays in biosensor development. Performance in various in vivo and reduced preparations in a variety of cell types shows that GCaMP5A, GCaMP5G, and GCaMP5K variants consistently outperform GCaMP3. This level of improvement was also consistent for GCaMP5G fused to synaptic vesicles and the plasma membrane. These results suggest that the improvements are “intrinsic” to the sensor, rather than due to “extrinsic” factors such as [Ca2+] levels in specific cells or subcellular locations, temperature, expression level, or vagaries of the particular system tested. Biophysical characterization showed that several factors contribute to this improvement: lower Ca2+-free fluorescence, higher Ca2+-bound fluorescence, and higher Ca2+ affinity.

GCaMP5G showed the largest responses to maximal stimulation when expressed in cultured neurons, and was therefore tested in all model organisms described. Comparative experiments with GCaMP5A and GCaMP5G in zebrafish and worm, and GCaMP5G and GCaMP5K in mouse, showed that all these GCaMP5s outperformed GCaMP3 in ΔF/ΔF and/or SNR. In worm and zebrafish, GCaMP5A showed the largest (ΔF/ΔF)max and SNR, indicating that for some preparations GCaMP5A might be the preferred version over GCaMP5G and GCaMP3. In mouse, GCaMP5G and GCaMP5K performed similarly well.

A recent report describes variants of GCaMP3, termed “G-GECOs” (Zhao et al., 2011), optimized by selecting for maximum ΔF/ΔF in E. coli colonies expressing random mutagenic libraries. Each of the G-GECO variants described is significantly dimmer than GCaMP3 in both the Ca2+-free and Ca2+-bound states, with a greater decrease in the former leading to a higher ΔF/ΔF. Although G-GECO1 displayed improved KCl-evoked signal change in dissociated rat hippocampal neurons at maximum stimulation, an intrinsically dimmer sensor may complicate imaging in more complex preparations. Indeed, SNR of G-GECO1.2 was lower than GCaMP3 over the complete range of field stimuli (Fig. 2). In vitro (ΔF/ΔF)max for the best G-GECOs are ~2-fold higher than GCaMP3, whereas some GCaMP5 variants show a 14-fold increase in the (ΔF/ΔF)max of GCaMP3 in vitro. GCaMP5G, the most consistently high-performing variant across in vivo assays, outperforms G-GECOs in terms of (ΔF/ΔF)max in vitro. The improved performance of the GCaMP5s versus the G-GECOs supports the strategy of structure-guided engineering as an efficient way to improve sensors, rather than random mutagenesis.

We compared the in vivo responses of mouse visual cortical neurons labeled with two of the best GCaMP5s (GCaMP5G and GCaMP5K), GCaMP3, and OGB-1, upon presentation of visual stimuli to the mouse. The GCaMP5 variants and OGB-1 showed similar fluorescence responses for most cells at the optimal stimulus orientation; GCaMP3 fluorescence responses were significantly lower. For all GCaMPs, after stimulation, fluorescence intensity returned to baseline level significantly faster compared with OGB-1. Annotation of the total fraction of visually responsive cells by GCaMP5 was twofold improved over GCaMP3, although still trailing OGB-1. This is in agreement with the zebrafish imaging, where GCaMP5G resulted in a larger fraction of visually responsive cells as well. These results are consistent with simultaneous imaging/cell-attached recordings; GCaMP5K
detects single APs and bursts of 2–3 APs much better than GCaMP3 (Tian et al., 2009), although less well than OGB-1. Detection of larger bursts of activity is also much improved for GCaMP5s compared with GCaMP3 and OGB-1, increasing the effective dynamic range of imaging. A nonlinear model of fluorescence dependence on spike rate accurately fits the in vivo data, and reduces trial-to-trial variability. Together these results show that GECIs are approaching small molecule indicators in terms of detection of sparse activity and neural activity quantification.

With its improved performance, GCaMP5 will directly enable more experiments in neuroscience and other fields of biology. Improvements in GECI transgene delivery will also contribute to increased utility. We have recently published a Cre-dependent GCaMP3 reporter mouse, allowing stable long-term expression in genetically defined neurons (Zariwala et al., 2012). Trans-synaptic delivery of calcium indicators is possible using rabies virus (Osakada et al., 2011). Zinc finger nucleases have facilitated chromosomal knock-ins in a variety of organisms, including mouse, allowing stable long-term expression in vivo. Improvements in GECI transgene delivery will also contribute to more experiments in neuroscience and other fields of biology.

Although the GCaMP5s are currently the best GECIs for single-wavelength calcium monitoring, FRET-based sensors offer the advantage of easy donor/acceptor ratioing, primarily for motion artifact control. Several versions of the FRET-based GECI Yellow Cameleon, including YC2.6, YC3.6 (Nagai et al., 2004), and the high-affinity YC-Nano (Horikawa et al., 2010), have been peer reviewed. Several versions of the FRET-based GECI GCaMP5 harboring similar mutations, fusion to a second FP, or stoichiometric expression using viral 2A peptides may offer increased performance levels with a ratiometric output.

Although we have shown that the GCaMP5 variants constitute a significant improvement over G-GECO and GCaMP3, further GECI engineering remains. Detection of sparse spiking activity should be brought in line with the best small molecule indicators. Improvements in rise and decay kinetics are required to precisely monitor spike number and time. Long-term overexpression artifacts, such as the cytromboid nuclear-filling phenotype, must be understood and eliminated, either through protein engineering or fine-tuned control of expression by promoter and enhancer adaptation. Improved GECIs, in combination with recent advances in light delivery and collection, fast scanning, image analysis, and behavioral paradigms, are setting the stage for chronic neural activity imaging to address fundamental questions in learning and memory, development, and the neural basis of behavior.

Notes
Supplemental material consisting of 32 indexed supporting figures is available at http://www.janelia.org/lab/looger-lab. This material has not been peer reviewed.

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