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9 **Single-Color, Ratiometric Biosensors for Detecting Signaling Activities in Live Cells**
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Abstract

Genetically encoded fluorescent biosensors have revolutionized the study of signal transduction by enabling the real-time tracking of signaling activities in live cells. Investigating the interaction between signaling networks has become increasingly important to understanding complex cellular phenomena, necessitating an update of the biosensor toolkit to allow monitoring and perturbing multiple activities simultaneously in the same cell. We therefore developed a new class of fluorescent biosensors based on homo-FRET, deemed FLuorescence Anisotropy REporters (FLAREs), which combine the multiplexing ability of single-color sensors with a quantitative, ratiometric readout. Using an array of color variants, we were able to demonstrate multiplexed imaging of three activity reporters simultaneously in the same cell. We further demonstrate the compatibility of FLAREs for use with optogenetic tools as well as intravital two-photon imaging.

Introduction

Genetically encoded biosensors have revolutionized the study of cell signaling by allowing the real-time monitoring of signaling activities, such as enzymatic activity or the release of second messengers, in live cells. They are therefore critical tools for uncovering the precise spatial and temporal regulation of signal transduction cascades. These biosensors can be divided into two broad classes: single-color and ratiometric. Single-color sensors, with an intensimetric activity readout, only occupy a single color channel, allowing for more flexibility in multiplexed imaging experiments. However, they are sensitive to variations in probe concentration caused by changing expression levels or cell shape, as well as differences in imaging conditions, such as

illumination intensity and focus. On the other hand, sensors with a ratiometric readout, such as those based on Förster Resonance Energy Transfer (FRET), cancel out many of these variations, enabling quantitative measurements of second messenger concentrations and better comparisons between experiments. However, the requirement for two distinct color channels limits their application in multiplexed imaging.

We therefore aimed to develop sensors that only occupy a single channel while still cancelling out the effects of varying imaging conditions and probe concentrations. Hence, rather than use the emission ratio between the FRET and donor channels, we instead used the loss of polarization of emitted light as a readout for FRET between two fluorescent proteins (FPs). Because this approach does not require the donor and acceptor to have distinct emission wavelengths, it can be used for either hetero-FRET (e.g., between different chromophores) or homo-FRET (e.g., between identical chromophores). Homo-FRET measurements have been useful for detecting protein clustering and protein oligomerization in live cells^{1,2,3}, but only recently has the possibility of using homo-FRET in biosensor designs been explored^{4,5}. Here we describe the development of a panel of single-color, genetically encodable biosensors based on homo-FRET for detecting kinase activity and second messenger dynamics. We call these sensors FLuorescence Anisotropy REporters, or FLAREs.

Results and Discussion

To create our FLARE probes, we adapted existing FRET-based biosensors for homo-FRET measurements by replacing the traditional FRET pair with two FPs of the same color. The resulting biosensors include a molecular switch, which changes conformation in the presence of a particular biochemical activity, flanked by two spectrally similar FPs at the N- and C-termini.

Changes in the conformation of the molecular switch, and thus the biochemical activities under study, are then monitored by observing the fluorescence anisotropy of the sensor using fluorescence polarization microscopy, with increased anisotropy corresponding to a lower-FRET state of the sensor, similar to the effect of increasing the intramolecular distance between the FRET pair (Figure 1 Supplement 1).

To develop a Protein Kinase A (PKA) activity FLARE, the molecular switch from A Kinase Activity Reporter 4 (AKAR4)^{6,7}, composed of an FHA1 domain and PKA substrate (Figure 1a), was flanked between two FPs of the same color. The FHA1 domain binds to the PKA substrate when the latter is phosphorylated, altering the conformation of the molecular switch and leading to a change in FRET between the flanking homo-FRET pair. To test the effect of FP circular permutation on these biosensors, we developed two FLARE-AKAR variants based on the yellow FP mVenus: one in which the C-terminal FP was circularly permuted at position 172 (cp172Venus), consistent with the hetero-FRET AKAR4 sensor, and one without circular permutation. We expressed mVenus-cp172Venus FLARE-AKAR in HEK293T cells and captured a time-course using fluorescence polarization microscopy. Following PKA activation using a cocktail of 50 μ M forskolin (Fsk), an adenylyl cyclase activator, and 100 μ M 3-isobutyl-1-methylxanthine (IBMX), a general phosphodiesterase inhibitor, the anisotropy decreased from 0.29 ± 0.003 to 0.26 ± 0.003 , a decrease of 0.028 ± 0.001 (N=44, biological replicates, unpaired, two-tailed t-test, $p < 0.0001$), with the kinetics of the decrease matching those observed with AKAR4 (Figure 1b, Figure 1 Supplement 2a). The yellow variant without the circular-permutation in the C-terminal FP showed slightly reduced changes in anisotropy upon stimulation with Fsk/IBMX (Figure 1c, Figure 1 Supplement 2c), consistent with previous

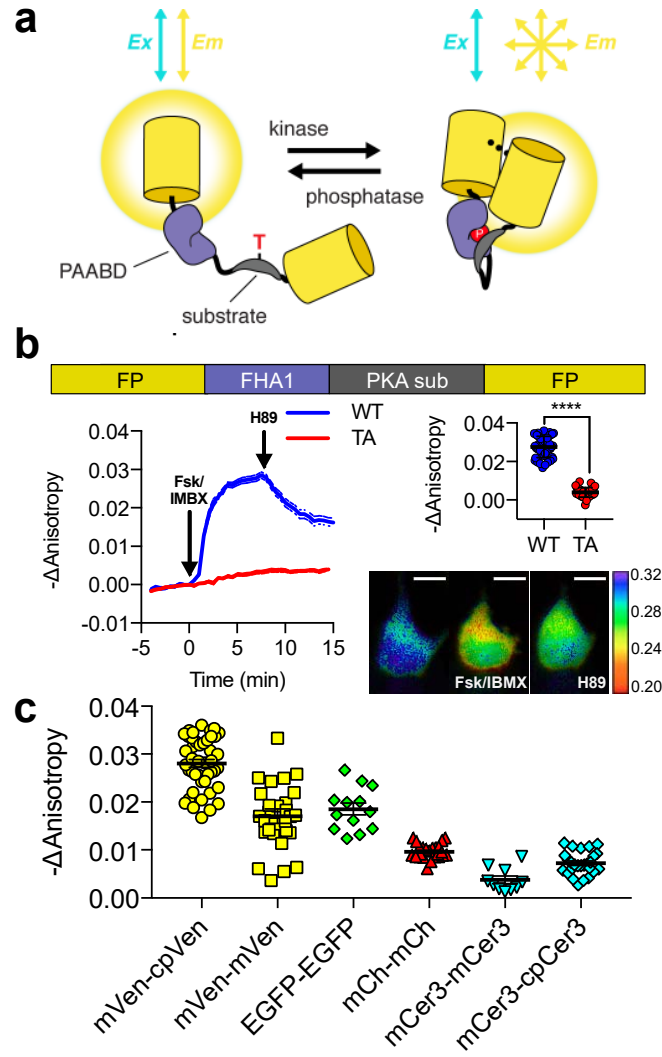


Figure 1 Design and Characterization of FLARE AKAR a). Schematic of a kinase activity FLARE b). Diagram illustrating domain structure of FLARE-AKAR (top). Time-course of mean fluorescence anisotropy of Venus-cp172Venus FLARE-AKAR wild type (blue, N=44) and kinase insensitive T-to-A mutant (red, N=38) expressed in HEK293T cell, stimulated with 50 μ M forskolin and 100 μ M IBMX at t=0 min, and 20 μ M H-89 at t=24 min (left). Dashed lines above and below represent standard error of the mean. Changes in anisotropy upon Fsk/IBMX stimulation for both FLARE-AKAR WT and T-to-A mutant (upper right, two-tailed t-test, $p < 0.0001$), calculated as the difference between the mean anisotropy from t=5 min to t=7.5 min and the mean anisotropy of the baseline before drug addition. The mean for each is shown, with the error reflecting the standard error of the mean. Representative anisotropy pseudocolor image before Fsk/IBMX stimulation (t=0 min), after Fsk/IBMX stimulation (t=7.5 min), and after inhibition of PKA with H-89 (t=24 min) (lower right) c). Comparison of the magnitude of the anisotropy change for different color variants of FLARE-AKAR upon stimulation with Fsk/IBMX including Venus-cp172Venus AKAR (N=44), Venus-Venus FLARE AKAR (N=32), EGFP-EGFP FLARE AKAR (N=13), mCh-mCh FLARE AKAR (N=22), mCerulean3 FLARE-AKAR (N=10), and mCerulean3-cp173Cerulean FLARE-AKAR (N=26). The mean for each is shown, with the error reflecting the standard error of the mean.

observations in hetero-FRET-based biosensors^{8,9,10}. We observed a slight positive

correlation between intensity and anisotropy change for Venus-cp172Venus FLARE AKAR;

however the expression level does not significantly impact the reporting ability of these sensors in general (Figure 1 Supplement 3a). The signal-to-noise ratio (SNR) of Venus-cp172Venus FLARE-AKAR was calculated to be 32 by dividing the magnitude of the anisotropy change upon maximal PKA stimulation by the standard deviation of the baseline before stimulation.

Subsequent control experiments confirmed that this change in anisotropy is caused by a change in the FRET state due to the conformational change of the sensor upon stimulation of PKA activity. PKA inhibition using 20 μ M H-89 led to an immediate slope change and increase in anisotropy (Figure 1b). On the other hand, a mutant version of the biosensor with a threonine-to-alanine (T-to-A) mutation at the phosphorylation site showed no change in anisotropy upon PKA stimulation with Fsk/IBMX or inhibition with H-89 (Figure 1b, Figure 1 Supplement 2b), suggesting that the observed changes in anisotropy were due to phosphorylation of the PKA substrate. Furthermore, isoproterenol, a β -adrenergic agonist, induced FLARE-AKAR responses in a dose-dependent manner (Figure 1 Supplement 4). To further demonstrate that the change in anisotropy upon PKA stimulation was due to a change in FRET, we mutated the chromophore of the C-terminal cp172Venus in Venus-cp172Venus FLARE AKAR from GYG to GGG. We observed that the magnitude of the response to Fsk/IBMX decreased to approximately one-third of that of the wild-type sensor (Figure 1 Supplement 5). The remaining response was likely due to intermolecular FRET that occurs when the FHA1 domain of one molecule binds to the phosphorylated PKA substrate in an adjacent molecule.

In addition to the yellow sensors, we developed a panel of color variants of FLARE-AKAR, including EGFP-EGFP, mCherry-mCherry, mCerulean3-mCerulean3¹¹, and mCerulean3-cp173Cerulean3 versions. All of these variants exhibited a decrease in anisotropy in cells treated with Fsk/IBMX; however, the magnitude of the anisotropy decrease depended on

the choice of FP, with the mVenus-cp172Venus variant having the largest response (Figure 1c, Figure 1 Supplement 2). As with the Venus variants, the Cerulean3-based FLARE-AKAR showed an increased dynamic range with a circularly-permuted fluorescent protein at the C-terminal position. mCherry-mCherry FLARE-AKAR, being spectrally shifted from the AKAR4 heteroFRET sensor allowed for direct comparison of FLARE and heteroFRET sensors within the same cell. As shown in Figure 1 Supplement 6, changes in anisotropy in mCherry-mCherry FLARE-AKAR corresponded with the changes in normalized emission ratio in AKAR4, with similar kinetics.

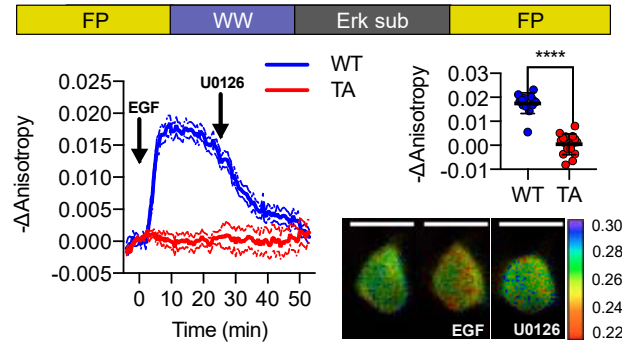
We furthermore demonstrated the ability of FLARE-AKAR sensors to monitor kinase activity at particular subcellular compartments. By fusing Venus-cp172Venus FLARE-AKAR to targeting motifs from Lyn kinase and DAKAP1, we were able to detect PKA activity at the plasma membrane and outer mitochondrial membrane, respectively (Figure 1 Supplement 7). Moreover, we used untargeted FLARE sensors to detect differential PKA activity dynamics in different compartments; diffusable Venus-cp172Venus FLARE AKAR in HeLa showed that PKA activity has slower kinetics and a lower magnitude in the nucleus than the cytosol (Figure 1 Supplement 8).

To demonstrate the generalizability of FLAREs, we developed a family of single-color kinase activity or activation reporters in various colors (Figure 2). To construct a single-color Erk activity biosensor, we replaced the PKA sensor domain from Venus-cp172Venus FLARE-AKAR with the sensor domain from EKAR-EV, composed of a WW domain (PAABD), a flexible EV linker, and an Erk substrate peptide (Figure 2a)^{12,13}. When expressed in HEK293T cells, Venus-cp172Venus FLARE-EKAR-EV exhibited a decrease in anisotropy of 0.02 ± 0.001 (N=13) after treatment with 100 ng/mL epidermal growth factor (EGF) to activate the MAPK

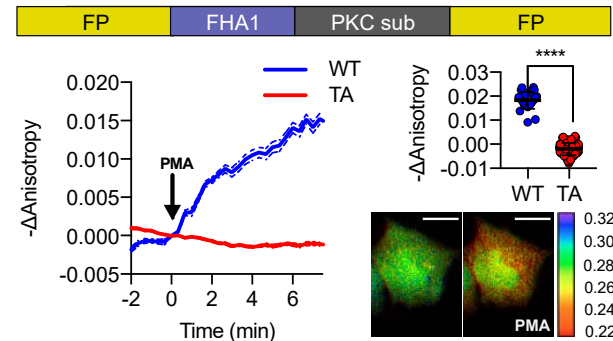
pathway (Figure 2a, Figure 2 Supplement 1). This response was reversed upon MEK inhibition using 20 μ M U0126, and no change in anisotropy was observed with a T-to-A mutant sensor. Likewise, we developed a panel of PKC activity reporters, called FLARE-CKARs, by flanking a PKC sensor domain composed of an FHA1 domain and a PKC substrate¹⁴ from a CKAR2 construct (Figure 2 Supplement 2) with mVenus-cp172Venus. We observed an anisotropy decrease of 0.02 ± 0.001 (N=26) upon activation of PKC with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Figure 2b, Figure 2 Supplement 3). For both FLARE-EKAR (Figure 2 Supplement 1) and FLARE CKAR (Figure 2 Supplement 3), we likewise repeated this process for various color variants, and like the FLARE-AKARs, the sensors based on mVenus exhibited the largest responses. Furthermore, a myosin light chain kinase (MLCK) sensor was converted to a FLARE by exchanging the cyan FP for mVenus¹⁵. Calmodulin (CaM) association with the MLCK-CaM binding domain in between the FPs decreases FRET, leading to an increase in fluorescence anisotropy upon forced calcium (Ca^{2+}) entry with 30 mM KCl (N=13) (Figure 2c, Figure 2 supplement 4).

In addition to biosensors for monitoring enzymes, we also developed FLAREs for monitoring second messenger dynamics (Figure 3a). We developed a Ca^{2+} FLARE by utilizing the sensor domain from the Cameleon family of biosensors, composed of CaM and the Ca^{2+} /CaM-binding peptide M13^{8,16}. When expressed in HEK293T cells, Venus-cp172Venus FLARE-Cameleon exhibited a decrease in anisotropy of 0.03 ± 0.002 (N=10) upon addition of 1 μ M ionomycin and 5 mM CaCl_2 , with the Venus-Venus, mCerulean3-mCerulean3, and mCherry-mCherry versions also showing detectable responses (Figure 3b, Figure 3 Supplement 1a,b). The mVenus-based FLARE sensors tend to show larger dynamic ranges for a variety of

a mVenus-cpVenus172 FLARE EKAR-EV



b mVenus-cpVenus172 FLARE CKAR



c EYFP-mVenus FLARE MLCK

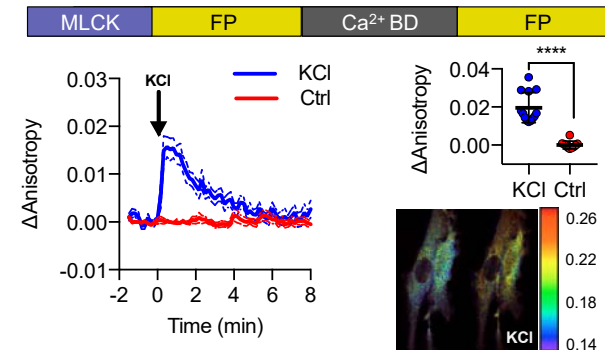


Figure 2 A panel of kinase activity and activation sensors a). Domain structure of FLARE-EKAR-EV (above). Time-course of mean fluorescence anisotropy of Venus-cp172Venus FLARE-EKAR-EV WT (blue, N=13) and kinase-insensitive mutant (red, N=16) expressed in HEK293T cell, with addition of 100 ng/mL EGF at t=0min and 20 μM U0126 at t=25min (left). Summary of anisotropy changes (upper right, two-tailed t-test, p<0.0001), calculated as the difference between the mean anisotropy from t=10 min to t=15 min and the mean anisotropy of the baseline before drug addition. The mean is shown, with the error reflecting the standard error of the mean. Representative anisotropy pseudocolor image before EGF stimulation (t=0 min), after EGF stimulation (t=25 min), and after inhibition of MEK with U0126 (t=47.5 min) (lower right). b). Domain structure of FLARE-CKAR (above). Time-course of fluorescence anisotropy of Venus-cp172Venus FLARE-CKAR WT (blue, N=26) and kinase-insensitive mutant (red, N=119) with addition of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) at t=0min. Summary of response magnitudes (upper right, two-tailed t-test, p<0.0001), calculated as the difference between the mean anisotropy from t = 10 to t = 11.33 min and the mean anisotropy of the baseline before drug addition. The mean is shown, with the error reflecting the standard error of the mean. Representative anisotropy pseudocolor image before PMA addition (t=0 min) and after PMA addition (t=15 min). c). Domain structure of FLARE-MLCK (top). Anisotropy time course of a representative REF52 cell expressing YFP-Venus FLARE-MLCK treated with 30 mM KCl at t=0 (N=13, blue), or vehicle control (N=10). Summary of anisotropy changes, calculated as the mean difference between the anisotropy at t = 0.333 min and the anisotropy of the baseline, before KCl addition (upper right, two-tailed t-test, p<0.0001). Representative pseudocolor anisotropy images before and after KCl treatment (lower right). Dashed lines above and below time course traces reflect the standard error of the mean. FP, fluorescent protein; CaM BD, MLCK calmodulin binding domain

208 FLARE sensors than mCerulean3 or mCherry variants, likely due to the superior extinction
209 coefficient and quantum yield of mVenus, which make it a good FRET donor and acceptor. We
210 further demonstrated the ability of Venus-cp172Venus FLARE Cameleon to detect submaximal
211 responses by monitoring calcium transients in histamine-stimulated HeLa cells (Figure 3
212 Supplement 1c). In order to determine the dissociation constant and Hill coefficients, we purified
213 Venus-cp172Venus Cameleon and measured the fluorescence anisotropy in solutions of known
214 free Ca^{2+} concentration at different temperatures (Figure 3 Supplement 2). The resulting
215 parameters are in good agreement with other Cameleon sensors⁸. Furthermore, we developed
216 another calcium FLARE sensor, based on D1-ER¹⁷, with a sensitivity appropriate for calcium
217 monitoring in the ER. The anisotropy decreases upon increasing calcium concentration and
218 increases when calcium is depleted from the ER upon treatment with thapsigargin (Figure 3
219 Supplement 3). In addition to Ca^{2+} , we developed a FLARE to detect intracellular cAMP based
220 on the ICUE family of sensors, in which a conformational change in the cAMP effector EPAC
221 leads to a decrease in FRET efficiency in the presence of cAMP¹⁸ (Figure 3a). When expressed
222 in HEK293T cells, the fluorescence anisotropy of Venus-cp172Venus FLARE-ICUE increased
223 by 0.02 ± 0.001 (N=40) upon stimulation with Fsk/IBMX (Figure 3c, Figure 3 Supplement 4).

224 The fact that FLAREs only occupy a single color channel and are highly generalizable for
225 different biosensors, as well as color variants, highlights their utility for multiplexed imaging
226 applications. While multiplexing of hetero-FRET sensors is generally limited to two probes^{19,20},
227 we demonstrate that FLAREs can facilitate co-imaging of three biosensors simultaneously. We
228 co-expressed mCherry-mCherry FLARE-AKAR, Venus-cpVenus FLARE-EKAR-EV, and
229 mCerulean3-mCerulean3 FLARE-Cameleon in HEK293T cells and acquired a time-course with
230 sequential treatment using Fsk/IBMX, EGF, and thapsigargin. Clear and distinct decreases in

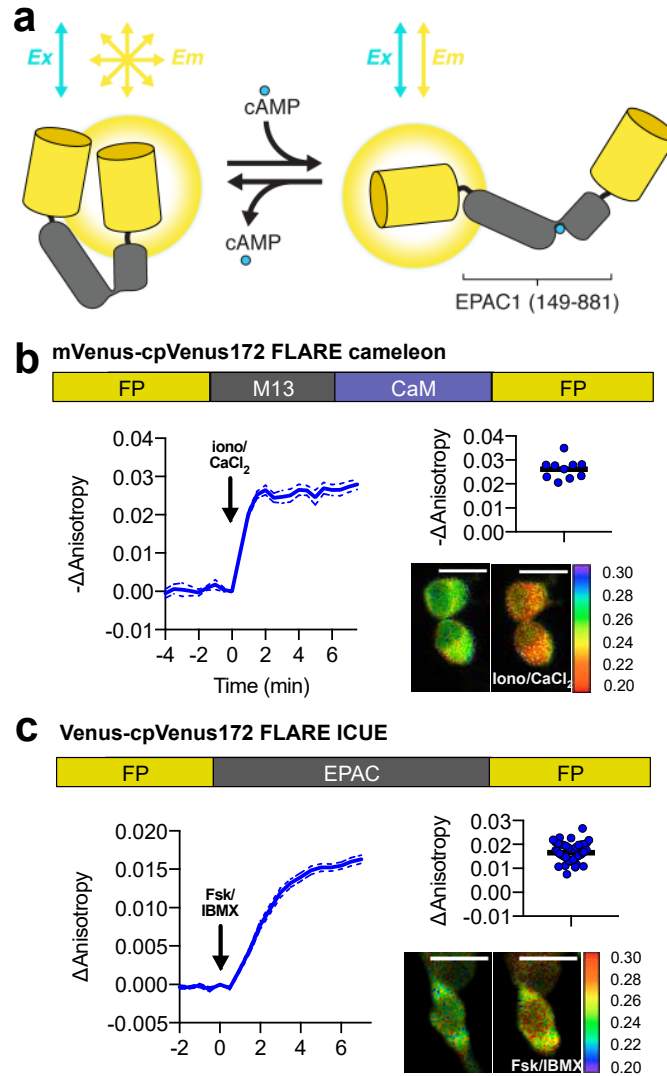


Figure 3 Design and characterization of FLARE second messenger sensors a). Schematic of FLARE ICUE cAMP second messenger sensor b). Domain structure of FLARE-Cameleon (top). Time-course of mean fluorescence anisotropy of Venus-cp172Venus FLARE-Cameleon (N=10) with addition of 1 μ M ionomycin and 5 mM CaCl_2 . Summary of anisotropy changes after 1 μ M ionomycin and 5 mM CaCl_2 (upper right) calculated as the difference between the mean anisotropy from $t=5$ min to $t=7.5$ min and the anisotropy of the baseline before drug addition. Representative anisotropy pseudocolor image before and after 1 μ M ionomycin and 5 mM CaCl_2 addition. c). Domain structure of the cAMP sensor FLARE-ICUE (top). Time-course of mean fluorescence anisotropy of Venus-cp172Venus FLARE-ICUE (N=40) with addition of 50 μ M forskolin and 100 μ M IBMX at $t=0$ (left). Summary of anisotropy changes after 50 μ M forskolin and 100 μ M IBMX with respect to baseline (upper right), calculated as the difference between the mean anisotropy from $t=5$ min to $t=7.5$ min and the anisotropy of the baseline before drug addition. Representative anisotropy pseudocolor image before ($t=0$ min) and after ($t=7.5$) stimulation with Fsk/IBMX (lower right). Dashed lines above and below time course reflect standard error of the mean.

anisotropy were observed in the red channel after Fsk/IBMX treatment, in the yellow channel after EGF stimulation, and in the cyan channel after thapsigargin treatment, corresponding to an increase in PKA activity, Erk activity, and intracellular Ca^{2+} , respectively (N=17) (Figure 4a, Figure 4 Supplement 1).

We further aimed to show that FLARE sensors can be used to monitor multiple signaling activities simultaneously in different cellular contexts. For example, mCerulean3-mCerulean3 FLARE Cameleon, co-imaged with other FLARE sensors in HeLa cells, can detect calcium responses to physiologically relevant stimulation conditions, such as histamine (Figure 4 Supplement 2). Additionally, when mCerulean3-mCerulean3 FLARE AKAR was co-imaged with Venus-cp172Venus cameleon in HEK293-T cells, activation of the β -adrenergic with isoproterenol led to a transient decrease in anisotropy in the cyan-channel (Figure 4 Supplement 3). Furthermore, we used FLAREs to study the cAMP- Ca^{2+} oscillatory circuit in pancreatic β -cells. MIN6 β -cells were transiently transfected with Venus-cp172Venus FLARE-ICUE and mCerulean3-mCerulean3 FLARE-Cameleon to simultaneously monitor cAMP and Ca^{2+} dynamics, respectively (N=19). We observed clear fluorescence anisotropy oscillations in both channels following stimulation with 20 mM tetraethylammonium chloride (TEA) (Figure 4b, Figure 4 Supplement 4). Ca^{2+} and cAMP exhibit synchronized oscillations, with Ca^{2+} increases corresponding to cAMP decreases, consistent with previous findings^{21,22}. These data demonstrate that even the lower-signal mCerulean3-based FLARE sensors can be used under sub-maximal and physiologically relevant stimulation conditions in multiplexed imaging experiments.

In addition to multiplexed biosensor imaging, the fact that FLARE sensors occupy only a single color channel also permits simultaneously perturbing and monitoring biochemical activities using optogenetics and FLAREs, respectively. We coexpressed an mCherry-tagged,

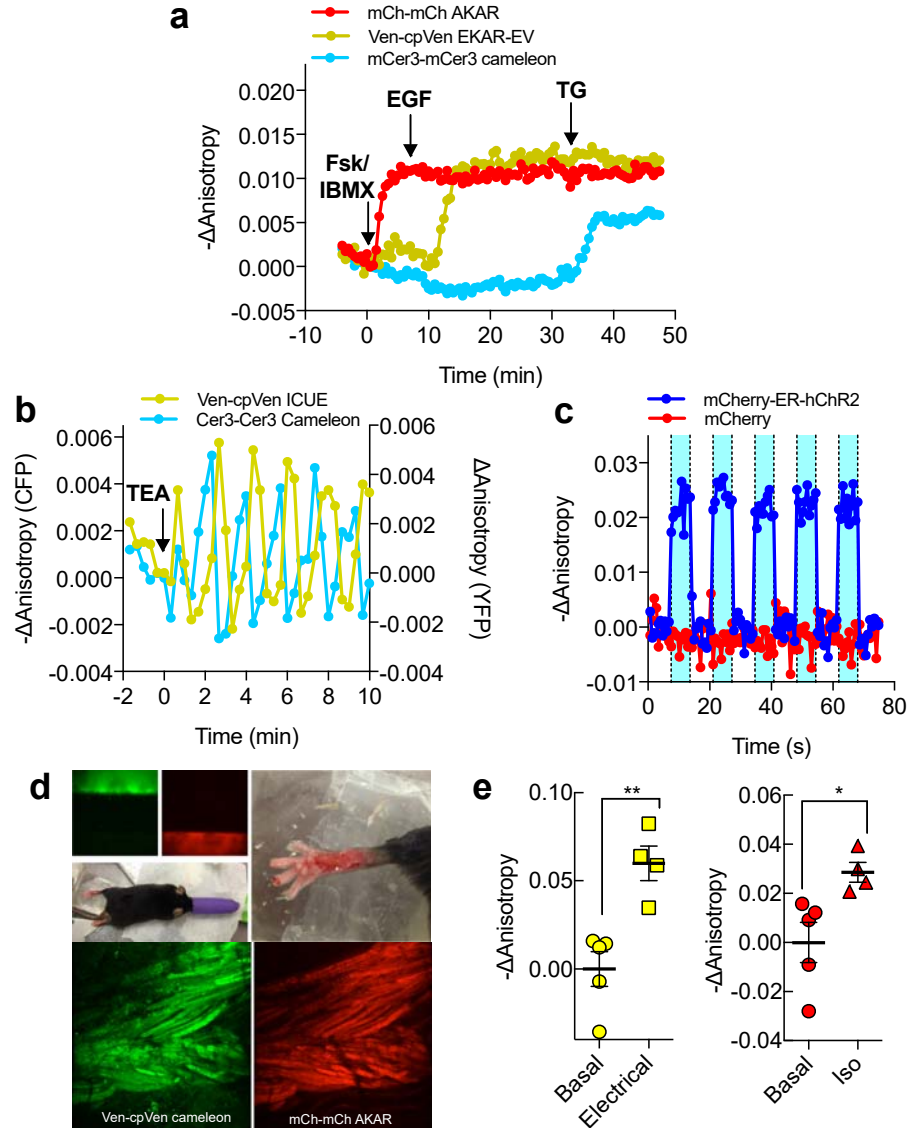


Figure 4 Multiparameter Imaging with FLAREs a). Time-course of a representative HEK293T cell co-expressing mCherry-mCherry FLARE-AKAR, mVenus-cp172Venus FLARE-EKAR-EV, and mCerulean3-mCerulean3 FLARE-Cameleon, with 50 μ M forskolin/100 μ M IBMX added at $t=0$ min, 100 ng/mL EGF at $t=7.5$ min, and 1 μ M thapsigargin added at $t=32.5$ min ($N=17$). b). Anisotropy response of a representative MIN6 cell co-expressing Cerulean3-Cerulean3 FLARE-Cameleon and Venus-cp172Venus FLARE-ICUE, showing robust oscillations after stimulation with TEA at $t=0$ min ($N=19$). c). Venus-cp172Venus FLARE-Cameleon anisotropy time-course in REF52 cells co-expressing the sensor and either mCherry-tagged humanized channel rhodopsin2 (blue-curve) or mCherry alone (red-curve), with periodic illumination with 455nm light (indicated by cyan shading). d). Purified mVenus and mCherry proteins were placed in separate capillary tubes and imaged with 855 nm and 1200 nm two-photon excitation. Plasmids encoding Venus-cp172Venus FLARE-Cameleon and mCherry-mCherry FLARE-AKAR were electroporated into the skeletal muscle of the foot of a live mouse for *in vivo* imaging. Below are Z-stack projections of skeletal muscle co-expressing these two sensors, excited with 855 nm (left) and 1200 nm (right) light. e). Electrical stimulation decreased fluorescence anisotropy in the 855nm channel, consistent with increased cytoplasmic Ca^{2+} (left) (two-tailed T-test, $p=0.0037$). Intraperitoneal injection of isoproterenol activates mCherry-mCherry FLARE AKAR (two-tailed T-test, $p=0.0239$), as indicated by decreased anisotropy in the 1200nm channel (right). The mean for each is shown, with the error reflecting the standard error of the mean.

ER-targeted channelrhodopsin2 (hChR2)^{23,24,25}, a light-gated calcium ion channel, with Venus-cp172 FLARE-Cameleon in REF52 cells, a rat embryonic fibroblast cell line. Illumination with blue light produced an immediate decrease in anisotropy, corresponding to an increase in intracellular Ca²⁺ (Figure 4c, Figure 4 Supplement 5). Control cells lacking hChR2 expression showed no change in anisotropy in the yellow channel.

In vivo, two-photon imaging of FLAREs was tested using a skeletal muscle preparation (Figure 4d,e). Exclusive excitation of mVenus (855 nm) or mCherry (1200 nm) was verified by imaging capillary tubes filled with recombinant proteins (Figure 4d). Plasmids encoding Venus-cp172Venus FLARE-Cameleon and mCherry FLARE-AKAR sensors were electroporated into the flexor digitorum brevis muscle of a live mouse^{26, 27}. Administration of an electrical current stimulated a rise in intracellular Ca²⁺ concentration, as indicated by a decrease in FLARE-Cameleon anisotropy, independent of changes in FLARE-AKAR anisotropy (Figure 4e). Activation of AKAR was then induced by intraperitoneal injection of isoproterenol (0.5 mg/kg). Thus, FLAREs enable *in vivo* multiparametric biosensor measurements.

We have demonstrated that FLAREs are a highly generalizable, accessible platform for creating single-color sensors to detect biochemical activities in individual cells in real time. Their ratiometric readout allows for fluctuations in light intensity and probe concentration to be cancelled out, permitting quantitative measurements of intracellular concentrations. We showed that these sensors have an SNR of 3-32 (Table 1) and a dynamic range comparable with first generation FRET sensors^{28,29}. Future development and optimization should further enhance their performance. We demonstrated that current FLAREs are already useful for multiplexed imaging applications. They can also be used in conjunction with optogenetic tools to enable all-optical interrogation of cellular regulation, and for intravital two-photon imaging to facilitate studies in

tissues and living animals. FLAREs, by allowing researchers to monitor multiple activities within the same cell, as well as to both monitor and optogenetically perturb activities in the same cell, could be used to study the how the spatiotemporal regulation of biochemical activities in highly integrated pathways allows a small number of signals to produce diverse cellular behaviors.

Materials and Methods

Reagent type (species) or resource	Designation	Source or reference	Identifiers
Cell Line (<i>Homo sapiens</i>)	HEK293-T		RRID:CVCL_0063
Cell Line (<i>Homo sapiens</i>)	HeLa		RRID:CVCL_0030
Cell Line (<i>Mus musculus</i>)	Min6		RRID:CVCL_0431
Cell Line (<i>Rattus norvegicus</i>)	REF-52		RRID:CVCL_6848
Chemical Compound, Drug	Forskolin	LC Laboratories	F9929
Chemical Compound, Drug	3-Isobutyl-1-methylxanthine (IBMX)	Sigma	I5879
Chemical Compound, Drug	H-89	LC Laboratories	H5239
Chemical Compound, Drug	Epidermal Growth Factor (EGF)	Sigma	E9644
Chemical Compound, Drug	U0126	Sigma	U120
Chemical Compound, Drug	Phorbol 12-Myristate 13-Acetate (PMA)	LC Laboratories	P-1680
Chemical Compound, Drug	Tetraethylammonium chloride (TEA)	Sigma	T2265
Chemical Compound, Drug	ionomycin	EMD Millipore	407951
Chemical Compound, Drug	thapsigargin	Cayman Chemical	10522
Chemical Compound, Drug	histamine	Sigma	H7250
Commerical Kit	Lipofectamine-2000	Invitrogen	11668019
Commerical Kit	Polyjet	SignaGen	SL100688
Recombinant DNA Reagent	pCDNA3 AKAR4	PMID: 20838685	
Recombinant DNA Reagent	pCDNA3 EKAR-EV	PMID: 21976697	

Recombinant DNA Reagent	pCDNA3 CKAR1	PMID: 12782683	
Recombinant DNA Reagent	FRET MLCK sensor	PMID: 15071183	
Recombinant DNA Reagent	pCDNA3 YC3.6 Cameleon	PMID: 10051607	
Recombinant DNA Reagent	pCDNA3 ICUE3	PMID: 19603118	
Strain (<i>Escherichia coli</i>)	BL-21 Codon Optimized Plus	New England Biolabs	C2527
Software	FIJI		RRID:SCR_014294
Software	MetaFluor		RRID:SCR_002285
Software	Micromanager		RRID:SCR_000415
Software	Zeiss Actiovision		RRID:SCR_002677
Software	MATLAB		RRID:SCR_001622
Software	GraphPad Prism		RRID:SCR_002798

Plasmid and Construct Construction

Cloning was performed using the pRSET-B vector using typical molecular cloning methods using polymerase chain reaction (PCR) with Phusion polymerase (New England Biolabs), restriction enzyme digestion, and ligation with T4 DNA ligase. To clone Venus-cp172Venus FLARE, AKAR4 was sub-cloned from a modified pCDNA3 to pRSET-B between the BamHI and EcoRI restriction enzyme sites. mVenus was then PCR amplified with primers encoding a BamHI site at the 5' end and an SphI site at the 3' end, and the resulting PCR product was digested with BamHI and SphI and ligated to pRSET-B AKAR4 digested with BamHI and SphI, with the mCerulean1 gene removed. Venus-cp172Venus FLARE AKAR was then sub-cloned back into a modified pCDNA3 vector using the BamHI and EcoRI sites. Other color variants were created by replacing the genes for the fluorescent proteins in other FLARE AKAR variants in pRSET-B, either between the BamHI and SphI sites for the N-terminal fluorescent protein, or SacI and EcoRI for C-terminal fluorescent proteins. Finalized constructs intended for mammalian expression were then sub-cloned into a modified pcDNA3 expression vector between the BamHI and EcoRI sites. FLARE variants of other sensors were created by

amplifying the molecular switch from EKAR-EV, CKAR2, Cameleon and ICUE3 with primers encoding the SphI and SacI sites, digesting the PCR product with SphI and SacI enzymes, and ligating them to the relevant pRSET-B FLARE AKAR plasmid digested with SphI and SacI to remove the domains involved in the molecular switch for FLARE AKAR. The final constructs were then subcloned into a modified pCDNA3 expression vector between the BamHI and EcoRI sites. Targeted versions of the sensors were created either by PCR amplifying the sensor with primers containing the targeting sequence and ligating it to the pCDNA3 expression vector between BamHI and EcoRI, or by subcloning the construct into a plasmid already containing the targeting sequence. N-terminal targeting sequences were placed between HindIII and EcoRI, and C-terminal targeting sequences between EcoRI and XbaI. All cloning steps were performed using DH5 α strain of *E. coli*.

The threonine to alanine mutants for Venus-cp172Venus FLARE AKAR and FLARE EKAR were created by performing site-directed mutagenesis using a standard single-primer PCR-based protocol. The threonine to alanine mutant for Venus-cp172Venus FLARE CKAR, as well as the chromophore-dead variant of Venus-cp172Venus FLARE AKAR, was created using Gibson assembly, amplifying the appropriate fragment with a primer containing the desired mutation.

The YFP MLCK FLARE sensor was created by replacing the CFP portion of an existing two color sensor^{15,30} with a PCR amplified mVenus fragment flanked by XhoI and AgeI restriction sites. Moreover, the coding sequence for the CFP D1 ER sequence was manufactured by Genewiz. The sensor consists of two oxmCer3 proteins (<https://www.ncbi.nlm.nih.gov/pubmed/26158227>) flanking the D1 ER calcium sensing domains¹⁷ and a C-terminal KDEL ER retention sequence.

369

370 *Cell Culture and Transfection*

371 HEK293-T and HeLa cells were maintained using Dulbecco's Modified Eagle's Medium
372 (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
373 Cells were seeded onto a 35-mm glass-bottom imaging dish and incubated at 37°C with 5%
374 ambient carbon dioxide. HEK293-T, HeLa, Min6, and REF-52 cell lines were maintained
375 separately from other cells and were screened regularly to confirm the absence of mycoplasma
376 contamination using Hoechst staining. As the origin of the cells was not central to the nature of
377 these experiments, we did not further validate the identity of the cell lines. Cells were transfected
378 using Lipofectamine 2000 (Invitrogen), Polyjet (SignaGen), or calcium phosphate and incubated
379 for 12-48 hours before imaging. The growth media was removed immediately before imaging,
380 and the cells were washed two or more times with Hanks Balanced Salt Solution (HBSS) buffer
381 with glucose at room temperature. The cells were imaged in HBSS buffer with glucose at either
382 room temperature or 37°C.

383

384 *Fluorescence Polarization Microscopy*

385 Widefield images were collected using an Zeiss AxioObserver equipped for fluorescence
386 polarization microscopy, using one of two setups. In the first setup, a wire grid polarizer
387 (Meadowlark Optics) was placed in the excitation pathway between the LED illuminators and
388 reflector turret containing filter cubes specific for CFP (Zeiss), YFP (Zeiss), and mCherry
389 (Semrock). Images were generally collected using a 20× 0.75 NA objective lens. Polarizations
390 parallel and perpendicular to the excitation polarizations were separated using Optical Insights
391 Dual-View using their polarization splitting module. Both images were simultaneously collected

in a single image collected by a water-cooled Orca-R2 (Hamamatsu). In the second setup, a polarizer (Chroma) was placed in the excitation pathway between the xenon arc lamp and the excitation filters. Images were collected using a 20× 0.45 NA objective lens. Polarizations were separated using an Opto-Split II LS image splitter, with two wire grid polarizers (Meadowlark) oriented parallel and perpendicular to the excitation polarizer. Images of both polarizations were collected using a Hamamatsu Flash 4.0 sCMOS camera. Two-photon imaging was performed using a Zeiss 7 MP with GaAsP non-descanned detectors housed at the University of Maryland School of Medicine confocal facility. Coherent Chameleon and OPO lasers were used for excitation. Fluorescence was filtered using an ET680 short pass filter for two-photon microscopy (Chroma) prior to separating polarizations with a 1 inch broadband polarizing beamsplitter cube (Thorlabs) mounted using a custom 3D printed cube. Images were collected using a 10×, 0.3 NA Plan-apochromat objective lens. *In vivo* imaging was performed on C57Bl/6 mice under isoflurane anesthesia.

Image Analysis

Image analysis was performed using Fiji (ImageJ) open-source image processing software. Polarization images were cropped and aligned using either the Zeiss Axiovision software or Fiji's built-in StackReg registration plugin. In Fiji, regions of interest (ROIs) were drawn around each cell, as well as one in the background. ROI intensities were background subtracted in each channel to estimate fluorescence emission intensity, and anisotropy was calculated as described by Lakowicz *et al*³¹. Anisotropies were calculated using the conventional equation³⁰:

$$r = \frac{P - gS}{P + 2gS}$$

Where *g* is the correction factor that accounts for differences in polarization transmission

efficiencies within the instrument. The g-factor was calculated using an isotropic fluorescein solution as described by Piston *et al.*³² Δ anisotropy was calculated by subtracting the anisotropy at each time point by the anisotropy at the time point right before drug addition. The magnitude of the anisotropy changes were calculated by taking the difference between the average anisotropy when the signal peaked or plateaued and the average anisotropy of the baseline time points before drug was added.

Protein Purification

Purification of the FLARE-Cameleon sensors was done using the BL21-RIL Codon Plus strain of *E. coli*, which were transformed with the construct cloned in the pRSET-B vector, with a Poly-His tag in the header sequence to allow for metal ion binding. The cells were grown to an OD of 0.2, when expression was induced with IPTG and allowed to grow overnight. The cells were then pelleted, frozen, resuspended, and lysed by sonication. Protein purification was performed using column chromatography with Ni-NTA resin. Fractions were collected and analyzed using SDS-PAGE; fractions showing sufficiently pure protein product were pooled.

In vitro Calcium Calibration

In vitro calibration of the FLARE-Cameleon sensor was performed using fluorescence anisotropy spectroscopy in solutions with varying concentrations of free calcium in a temperature-controlled environment. These solutions were made by titrating known concentrations of free EGTA and calcium-saturated EGTA at pH 7.1 (Calcium Concentration Kit #1--Thermo Scientific). The fluorescence anisotropy was measured using a Photon Technology International QuantaMaster spectrofluorometer equipped with a Xenon flash lamp, fluorescence

polarizers, and a Peltier cuvette holder for temperature control. Anisotropies were calculated using integrated intensities of S- and P-polarized emission spectra. The correction factor G was measured by measuring the P- and S-polarized emission spectra of fluorescein, which is assumed to be isotropic, and taking the ratio of their measured integrated fluorescence intensities. To determine the dissociation constant (K_d) and Hill coefficient (n), the anisotropy vs. calcium concentration data were fit to the following equation:

$$r = r_{min} + (r_{max} - r_{min}) * \left(\frac{[Ca^{2+}]}{K_d + [Ca^{2+}]} \right)^n$$

Acknowledgements

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Competing Financial Interests

The authors declare no competing financial interests.

Source Data Files Legends

Source Data Figure 1. a). Sheet 1, Figure 1b Time Course. Time course for mVenus-cp17Venus FLARE AKAR, wild type and kinase-inactive threonine-alanine (TA) mutant. b). Sheet 2, Figure 1b Summary: Comparison of magnitude of responses for individual cells for Venus-cp172Venus FLARE AKAR, wild type and kinase-inactive threonine-alanine (TA)

mutant, as well as statistics comparing the two groups. c). Sheet 3, Figure 1c. Summary of magnitude of responses for various color variants of FLARE AKAR after stimulation with forskolin and IBMX.

Source Data Figure 2. a). Sheet 1, Figure 2a. Time course for Venus-cp172Venus FLARE EKAR, both wild type and kinase-inactive (TA) mutant. b) Sheet 2, Figure 2a. Comparison of magnitudes of anisotropy changes for Venus-cp172Venus FLARE EKAR, both wild type and kinase-inactive (TA) mutant, of upon EGF addition, and relevant statistical tests to compare the two variants. c). Sheet 3, Figure 2 Supplement 1c. Changes of magnitudes of anisotropy change for various FLARE EKAR variants upon EGF addition. d). Sheet 4, Figure 2b. Time course for Venus-cp172Venus FLARE CKAR, both wild type and kinase-inactive (TA) mutant. e) Sheet 5, Figure 2b. Comparison of magnitudes of anisotropy changes for Venus-cp172Venus FLARE EKAR, both wild type and kinase-inactive (TA) mutant, of upon EGF addition, and relevant statistical tests to compare the two variants. f). Sheet 6, Figure 2 Supplement 2. Time courses for CKAR1 and CKAR2. g). Sheet 7, Figure 2 Supplement 3c. Changes of magnitudes of anisotropy change for various FLARE CKAR variants upon PMA addition. h). Sheet 8, Figure 2c. Time course for FLARE MLCK, with either addition of KCl and vehicle only control. i). Sheet 9, Figure 2c. Summary for magnitude of responses for FLARE MLCK.

Source data Figure 3. a). Sheet 1, Figure 3b. Time course for Venus-cp172Venus FLARE Cameleon. b). Sheet 2, Figure 3b. Summary of magnitude of responses for Venus-cp172Venus FLARE Cameleon upon addition of calcium chloride and ionomycin. c). Sheet 3,

Figure 3 Supplement 1b. Summary of magnitudes of responses for various FLARE Cameleon variants upon addition of calcium chloride and ionomycin. d). Sheet 4, Figure 3 Supplement 2. *In vitro* calibration of Venus-cp172Venus FLARE Cameleon, both raw data and sigmoidal curve fits. e). Sheet 5, Figure 3 Supplement 3. Summary of magnitude of anisotropy changes for CFP FLARE D1ER upon addition of ionomycin and three different doses of calcium. f). Sheet 6, Figure 3c. Time course for Venus-cp172Venus FLARE ICUE. g). Sheet 7, Figure 3c Summary of magnitudes of changes in anisotropy for Venus-cp172Venus FLARE ICUE upon addition of forskolin and IBMX.

Source Data Figure 4. a). Sheet 1, Figure 4a. Time course for multiplexed imaging of mCherry-mCherry FLARE AKAR, Venus-cp172Venus FLARE EKAR, and mCer3-mCer3 FLARE Cameleon, expressed in HEK293-T cells and treated with forskolin and IBMX, EGF, and thapsigargin at t=0 min, t=7.5 min, and t=32.5 min, respectively. b). Sheet 2, Figure 4b. Time course for Venus-cp172Venus FLARE ICUE and mCer3-mCer3 FLARE Cameleon in Min6 cells, treated with TEA at t = 0 min. c). Sheet 3, Figure 4c. Time course for Venus-cp172Venus FLARE Cameleon co-expressed with mCherry tagged hChR2-ER or mCherry alone. d). Sheet 4, Figure 4 e. Summary data of 2-photon *in vivo* imaging of Venus-cp172Venus FLARE Cameleon and mCherry-mCherry FLARE AKAR, in the muscle cells in the feet of live mice.

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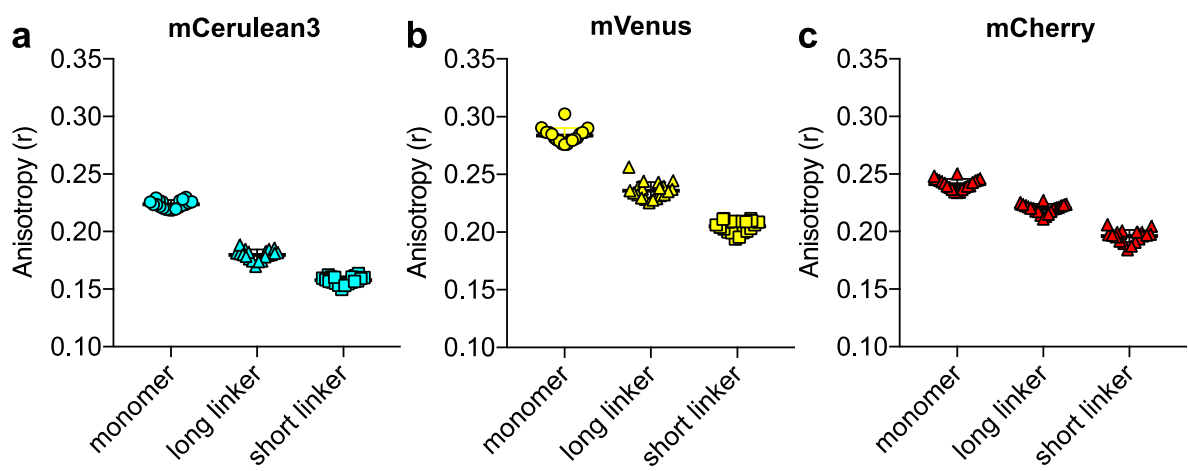
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577 **Figure Supplements**



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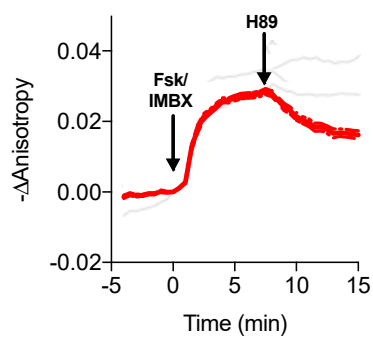
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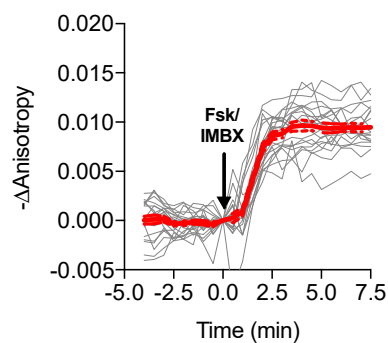
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Figure 1 Supplement 1 Anisotropy vs. Intramolecular Distance. Anisotropy measurements of monomer, as well as tandem dimers separated by either a long (44 amino acid) or a short (10 amino acid) linker, of a). mCerulean3, b). mVenus, and c). mCherry. The mean for each is shown, with the error reflecting the standard error of the mean.

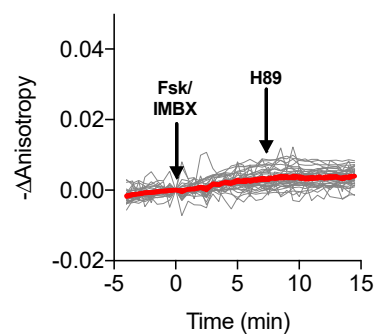
a Venus-cp172Venus FLARE AKAR



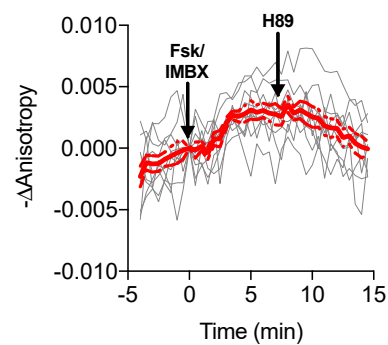
e mCh-mCh FLARE AKAR



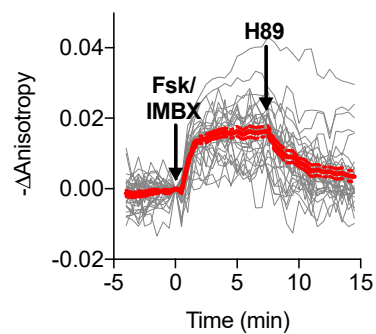
b Venus-cp172Venus FLARE AKAR (T/A mutant)



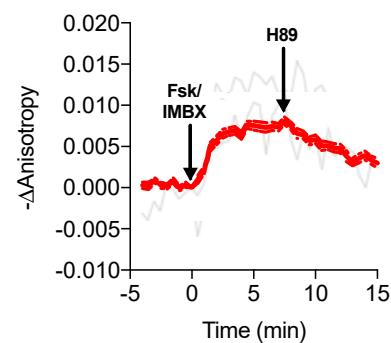
f mCer-mCer FLARE AKAR



c Venus-Venus FLARE AKAR



g mCer-cp173Cer FLARE AKAR



d EGFP-EGFP FLARE AKAR

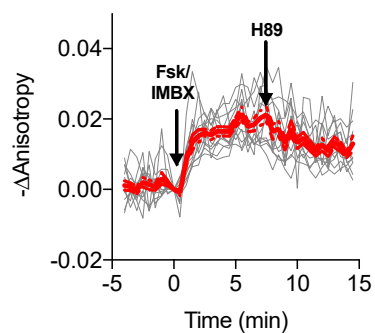


Figure 1 Supplement 2 Individual and average traces for FLARE-AKAR variants, including a). Venus-cp172Venus AKAR (N=44), b). Venus-cp172Venus FLARE-AKAR T/A mutant (N=38), c). Venus-Venus FLARE AKAR (N=32), d). EGFP-EGFP FLARE AKAR (N=13), e). mCh-mCh FLARE AKAR (N=22), f). mCerulean3-mCerulean3 FLARE-AKAR (N=10), and g). mCerulean3-cp173Cerulean FLARE-AKAR (N=26). Gray lines are individual cell traces, while the red solid line is the average curve. Red dotted lines represent the average curve plus or minus the standard error of the mean.

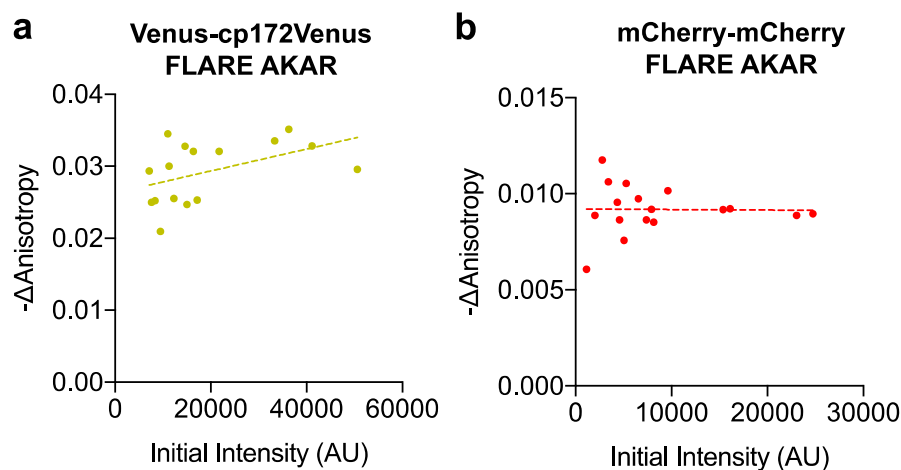


Figure Supplement 3 Scatter plots showing the relationship between the magnitude of the anisotropy change after treatment with 50 μ M forskolin and 100 μ M IBMX and the initial intensity for a). Venus-cp172Venus FLARE AKAR and b). mCherry-mCherry FLARE AKAR, in HEK293-T cells. The magnitude of the signal was calculated by taking the mean anisotropy from t = 5min to t = 7.5 min and subtracting the mean anisotropy of the baseline before drug addition.

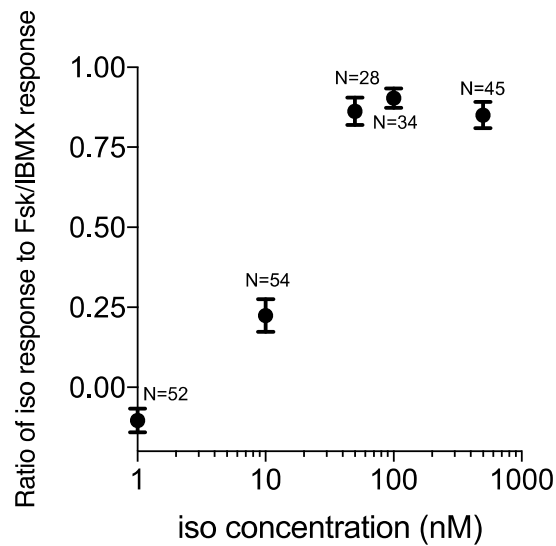


Figure 1 Supplement 4 Venus-cp172Venus FLARE-AKAR isoproterenol dose response. HEK cells were transiently transfected with Venus-cp172Venus FLARE-AKAR, and stimulated with varying doses of isoproterenol, followed by maximal stimulation with 50 μ M forskolin and 100 μ M IBMX. The ratio of the anisotropy decrease following isoproterenol dose to that after Forskolin and IBMX stimulation vs. the isoproterenol dose is plotted.

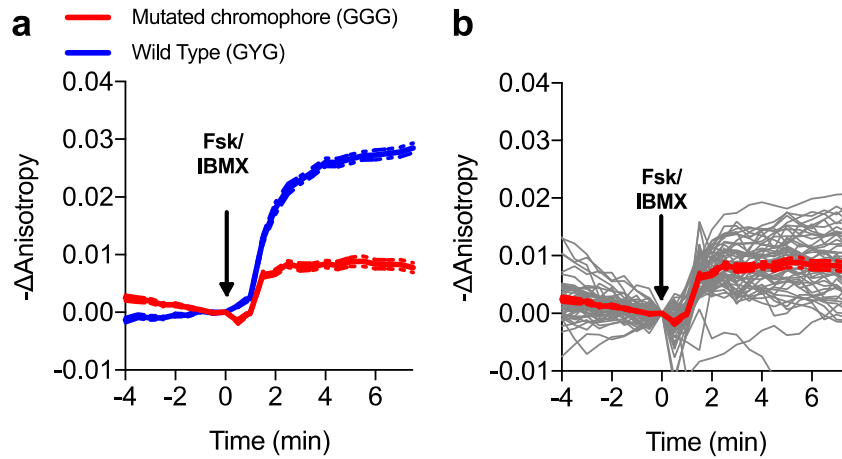
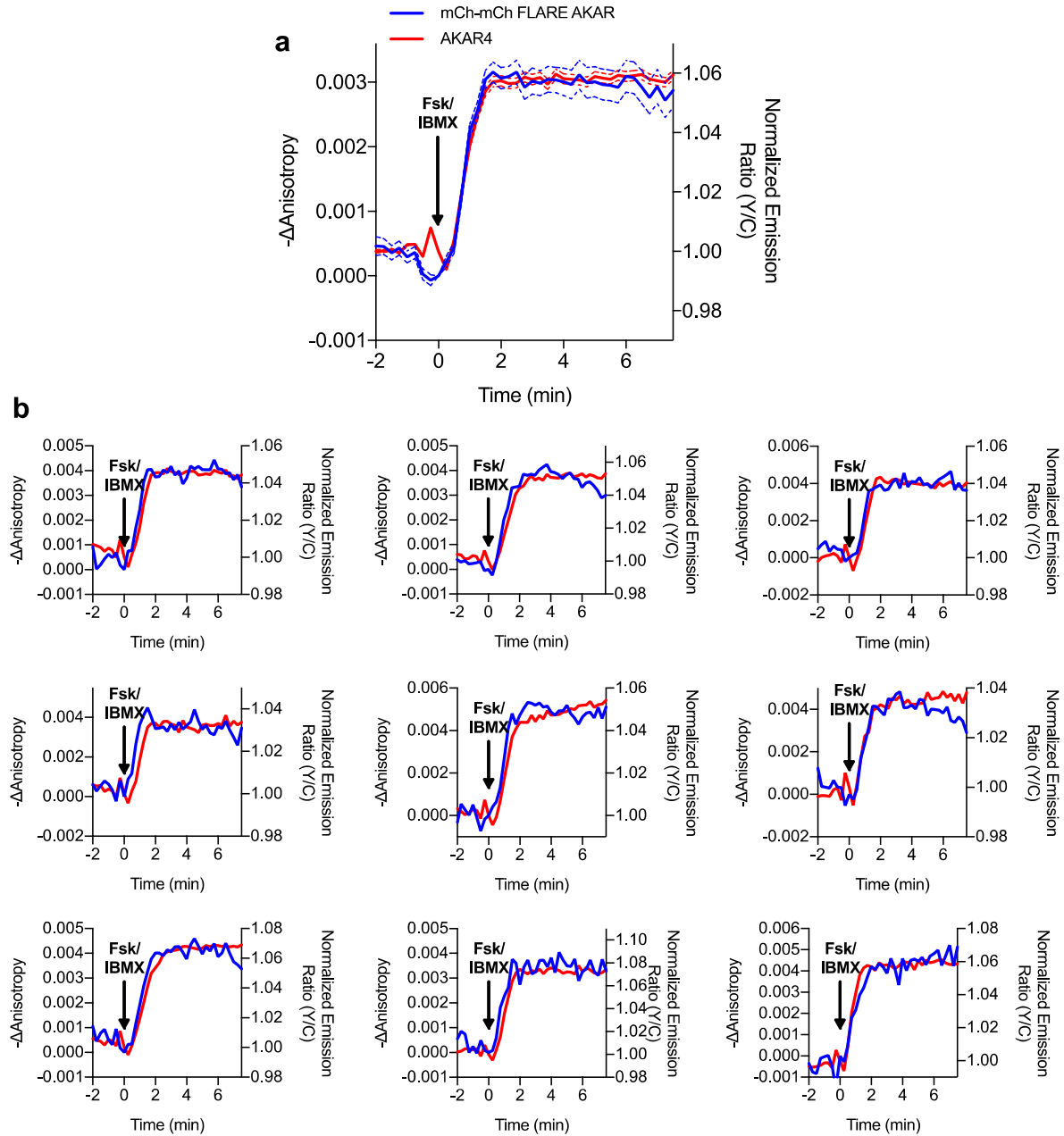


Figure 1 Supplement 5 Characterization of the chromophore-dead FLARE AKAR mutant a).

Comparison of Venus-cp172Venus FLARE AKAR responses of the wild type sensor and a mutant sensor in which the chromophore of the C-terminal cp172Venus was mutated from GYG to GGG (N=53), expressed in HEK293-T cells and treated with 50 μ M forskolin and 100 μ M IBMX. b). Individual cell traces for Venus-cp172Venus FLARE AKAR (GGG mutant). The red curve represents the mean for all the cell traces, with the dotted lines above and below reflecting the standard error of the mean.



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Figure 1 Supplement 6 Direct comparison of mCherry-mCherry FLARE-AKAR and

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heteroFRET AKAR4. mCherry-mCherry FLARE-AKAR and AKAR4 were co-expressed in

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HEK cells, which were stimulated with 50 μ M forskolin and 100 μ M IBMX at t=0 min (N=74).

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a). Average anisotropy traces for mCherry-mCherry FLARE AKAR and average normalized

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emission ratio traces are shown for AKAR4. The dashed lines above and below the curve

represent the standard error of the mean. b). Nine representative cell traces showing both mCherry-mCherry FLARE AKAR and AKAR4 traces for the above experiment.

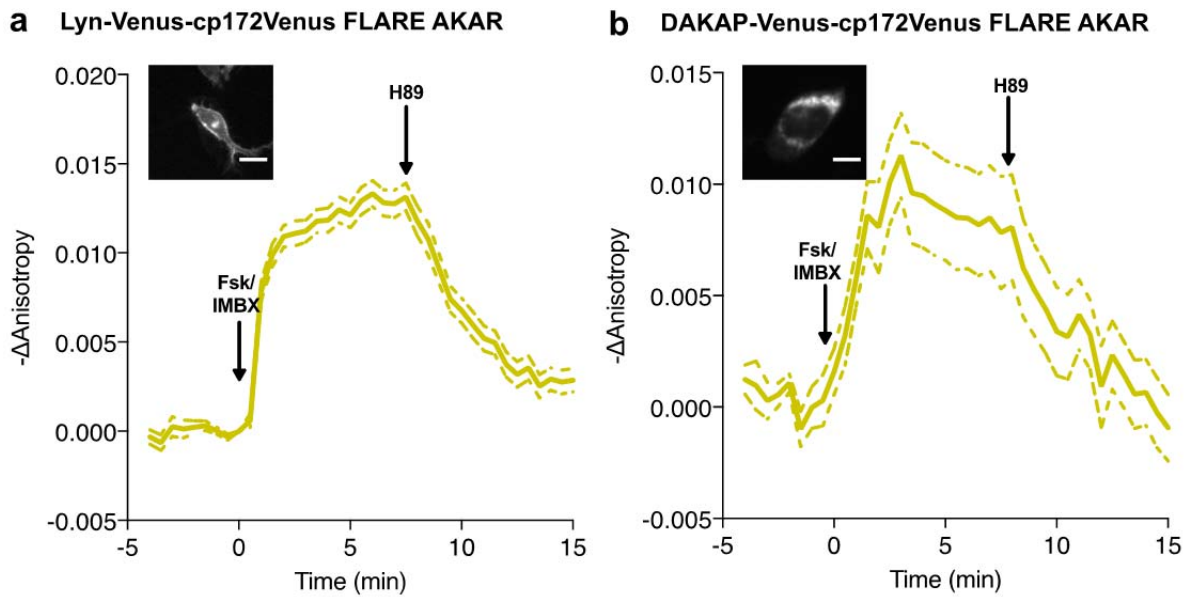


Figure 1 Supplement 7 Subcellular targeted FLARE-AKARs. Average anisotropy traces of Venus-cp172Venus FLARE AKAR subcellularly targeted to a) the plasma membrane with the Lyn-kinase targeting motif (N=77) b) the mitochondrial membrane with the targeting sequence from DAKAP (N=32), expressed in HEK cells stimulated with 50 μM forskolin and 100 μM IBMX at t=0 min, and 20 μM H89 at t=7.5 min. Scale bars are 10 μm . Dashed lines above and below the curves reflect the standard error of the mean.

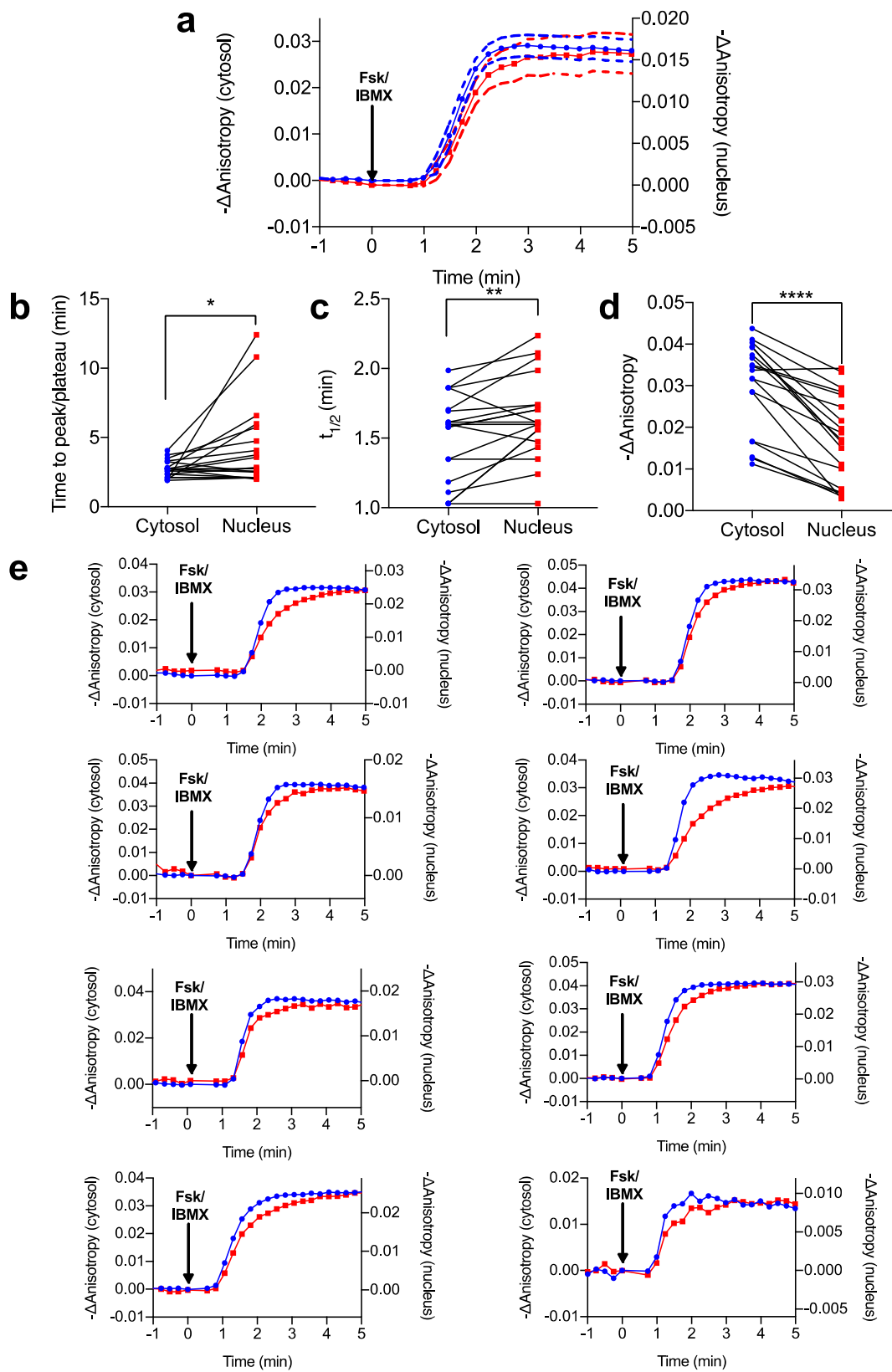


Figure 1 Supplement 8 Differential PKA activity kinetics in the cytosol and the nucleus a). Comparison of Venus-cp172Venus FLARE AKAR anisotropy changes in the cytosol and the nucleus in HeLa cells treated with 50 μ M forskolin and 100 μ M IBMX (N=19). Dashed lines above and below the curves reflect the standard error of the mean. b). Comparison of the time for the anisotropy change to peak or plateau in the cytosol and the nucleus (two-tailed paired T-test, $p=0.0295$). c). Comparison of the time for the anisotropy change to reach half of the peak ($t_{1/2}$) in the cytosol and the nucleus (two-tailed paired T-test, $p=0.0065$). d). Comparison of the magnitude of the anisotropy change in the cytosol and the nucleus (two-tailed, paired T-test, $p<0.0001$). e). Eight representative anisotropy time traces for individual cells, comparing the cytosolic and nuclear anisotropies.

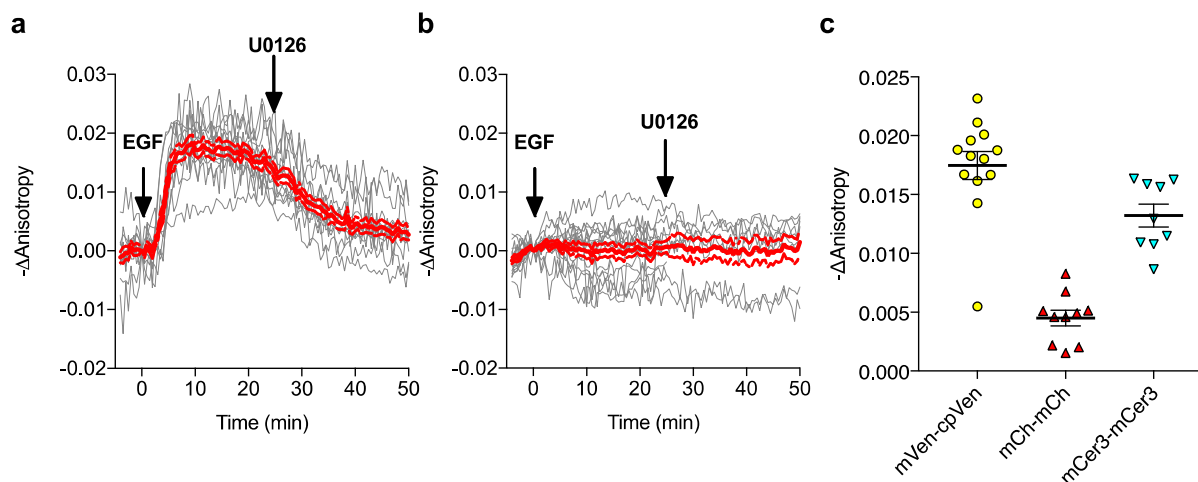


Figure 2 Supplement 1 FLARE-EKAR Characterization. a). Individual (gray) and average (red) cell traces for Venus-cp172Venus FLARE EKAR (N=13), expressed in HEK293T cells and treated with 100 ng/mL epidermal growth factor (EGF) at t=0 min and the MEK inhibitor U0126 at t=25 min. b). Individual (gray) and average (red) cell traces for Venus-cp172Venus FLARE EKAR with a threonine-to-alanine (T-to-A) at the phosphorylation site (N=16), expressed in HEK293T cells and treated with 100 ng/mL epidermal growth factor (EGF) at t=0 min and the MEK inhibitor U0126 at t=25 min. c). A summary of anisotropy changes for various color-variants of FLARE-EKAR, upon stimulation with 100 ng/mL EGF. The magnitude of the signal was calculated by taking the mean anisotropy from t = 10min to t = 15 min and subtracting the mean anisotropy of the baseline before drug addition.

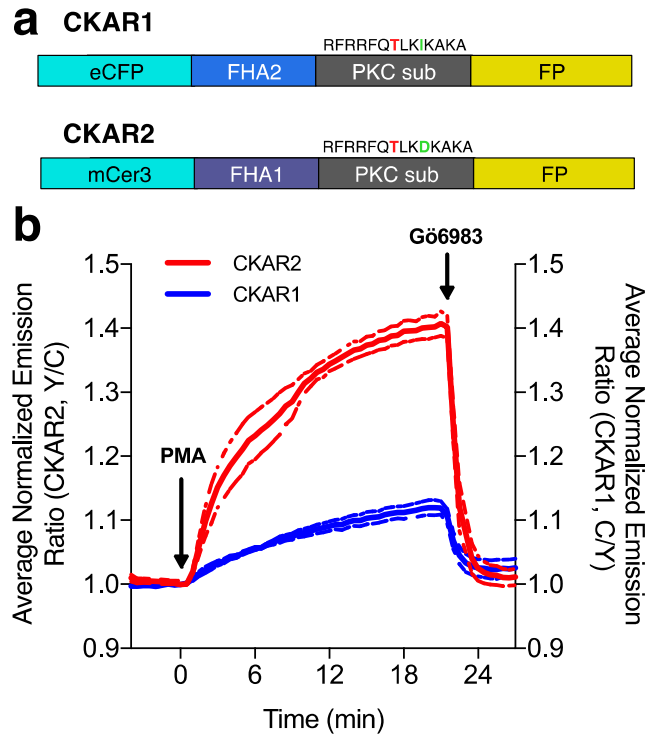
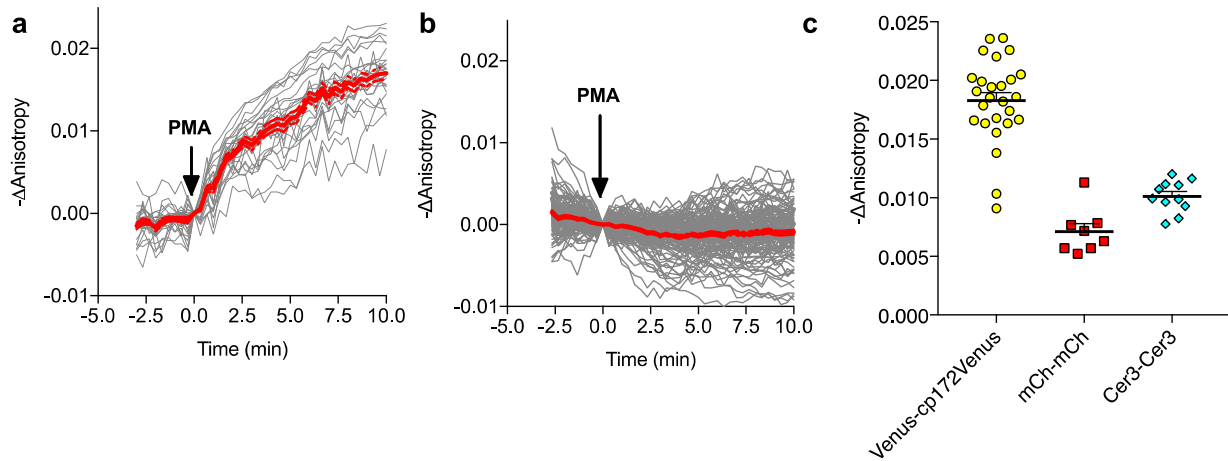


Figure 2 Supplement 2 Characterization of the CKAR2 hetero-FRET sensor. a). Comparison of CKAR1 and CKAR2 domain structures. b). Comparison of FRET responses of CKAR1 and CKAR2 expressed in HeLa cells to Protein Kinase C (PKC) stimulation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) as well as PKC inhibition with 1 μ M Gö6983. Curves here represent mean curves from one representative experiment with biological replicates N=7 for CKAR1 and N=4 for CKAR2; the experiment was repeated three times.

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689 **Figure 2 Supplement 3** FLARE CKAR characterization. a). Individual (gray) and average (red)
 690 cell traces for Venus-cp172Venus FLARE CKAR (N=26), expressed in HEK293-T cells treated
 691 with 50ng/mL PMA at t=0 min. Dotted lines above and below the curves reflect the standard
 692 error of the mean. b). Individual (gray) and average (red) cell traces for the kinase-inactive
 693 mutant of Venus-cp172Venus FLARE CKAR (TA mutant) (N=119), expressed in HEK293-T
 694 cells treated with 50ng/mL PMA at t=0 min. c). A summary of anisotropy changes for various
 695 color-variants of FLARE-ICUE in HEK293T cells upon stimulation with 50ng/mL PMA. The
 696 magnitude of the signal was calculated by taking the mean anisotropy from t = 10 min to t =
 697 11.33 min and subtracting the mean anisotropy of the baseline before drug addition.

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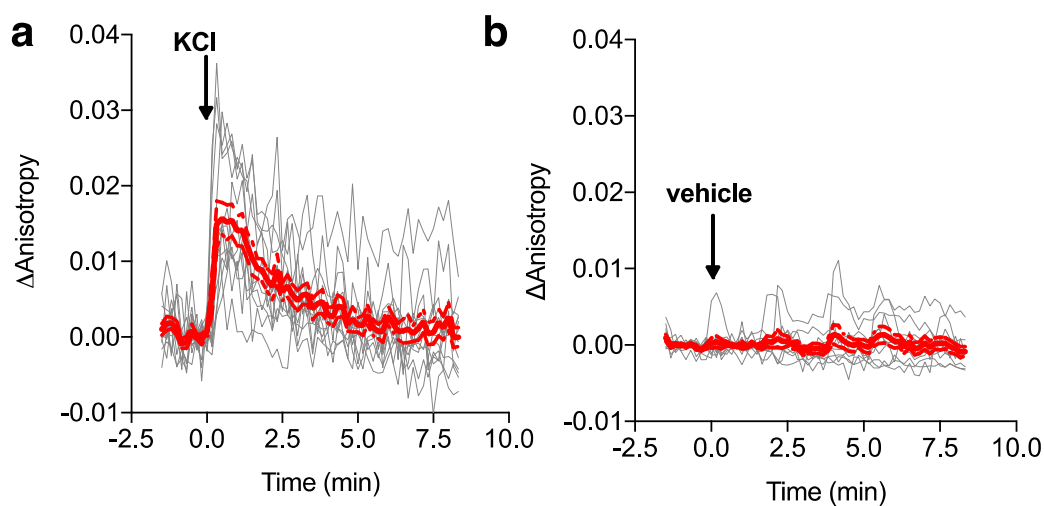


Figure 2 Supplement 4 a). Individual (gray) and average (red) cell traces for FLARE-MLCK (N=13) with KCl added at t=0 min (N=13). b). Individual (gray) and average (red) cell traces for FLARE-MLCK with vehicle only added at t=0min (N=10). The dashed lines above and below the red curve reflect the standard error of the mean. The magnitude of the signal was calculated by taking the Δ anisotropy at t = 0.33 min and subtracting the mean Δ anisotropy of the baseline before drug addition.

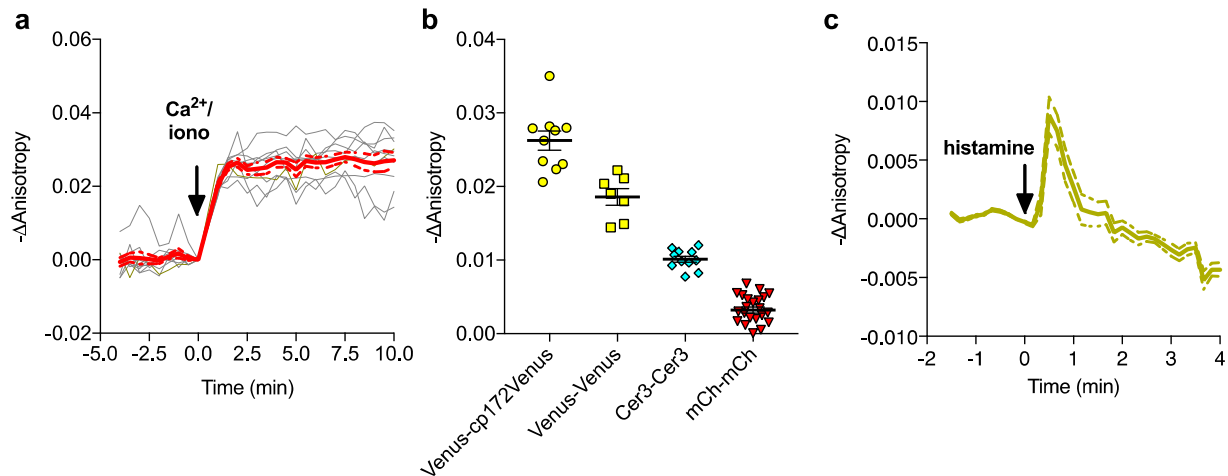


Figure 3 Supplement 1 Characterization of FLARE Cameleon. a). Individual (gray) and average (red) cell traces for Venus-cp172 FLARE Cameleon (N=10). b). A summary of anisotropy changes for various color-variants of FLARE-Cameleon in HEK293T cells upon stimulation with 1 μ M ionomycin and 5mM calcium chloride. The mean for each is shown, with the error reflecting the standard error of the mean. The magnitude of the signal was calculated by taking the mean anisotropy from t = 5 min and t = 7.5 min and subtracting the mean anisotropy of the baseline before drug addition. c). Average anisotropy trace for Venus-cp172Venus FLARE-Cameleon expressed in HeLa cells upon stimulation with histamine (100 μ M). Dotted lines above and below the curves reflect the standard error of the mean.

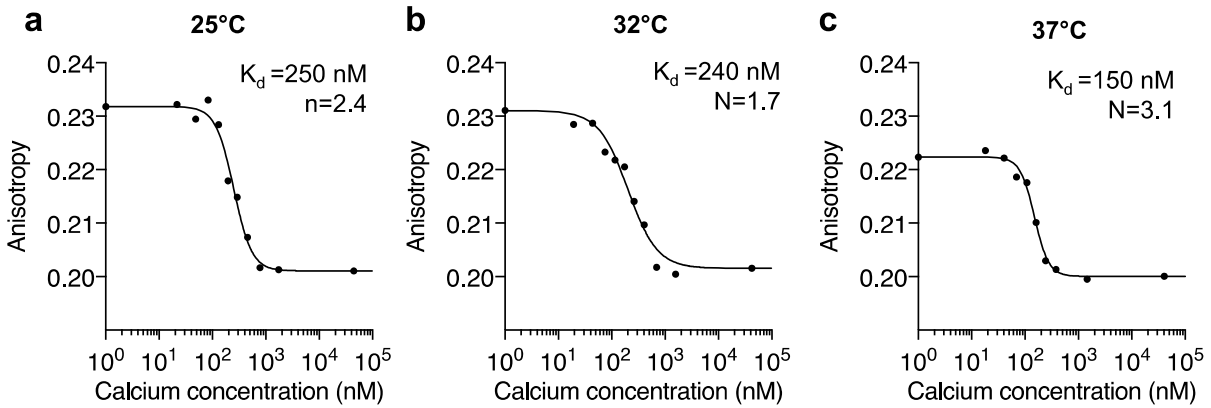


Figure 3 Supplement 2 *In vitro* calibration of purified Venus-cp172 FLARE-Cameleon. Anisotropy vs. calcium concentration calibration curves of purified Venus-cp172 Venus Cameleon for a). 25°C b). 32°C, and c). 37°C.

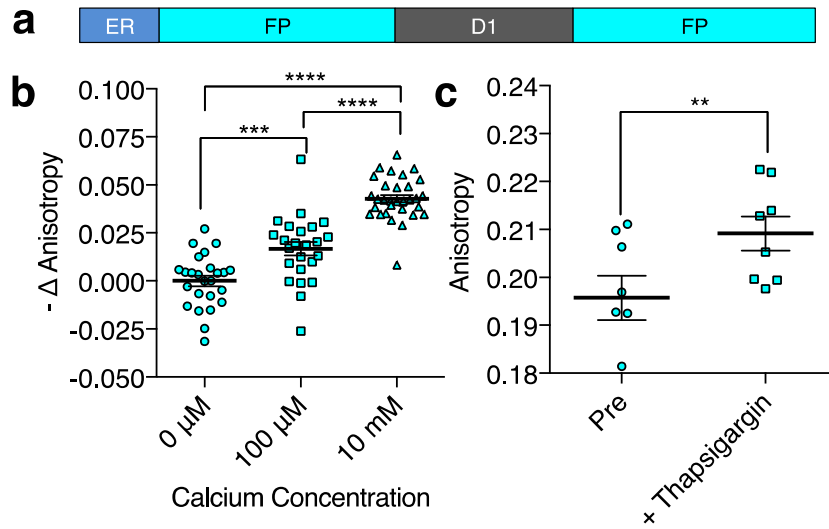


Figure 3 Supplement 3 Characterization of CFP FLARE-D1ER. a). Schematic of the ER calcium sensor. b). REF52 cells expressing CFP FLARE D1ER were permeabilized using 5 μ M ionomycin and equilibrated with 3 mM EGTA to remove Ca^{2+} or the indicated Ca^{2+}

concentration. Fluorescence anisotropy values were normalized to the Ca^{2+} free condition. Statistical significance was assessed using an ANOVA and Tukey Multiple comparison test ($p < 0.001$, $**** p \leq 0.0001$). c) REF52 cells expressing CFP FLARE D1ER were treated with thapsigargin (1 μM , 5 min), to reduce ER Ca^{2+} levels (N=8, $** p < 0.01$ by t-test). The mean for each of the above is shown, with the error reflecting the standard error of the mean

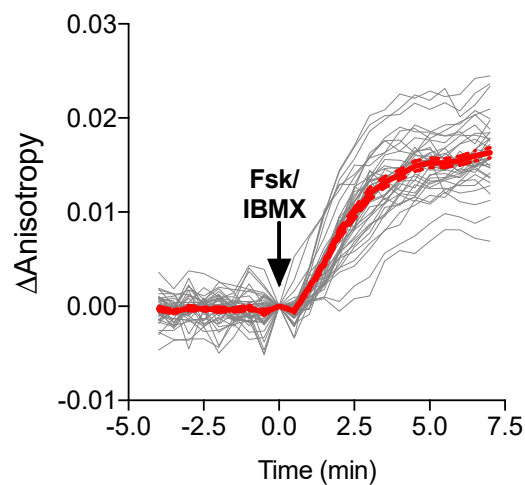


Figure 3 Supplement 4 a). Individual (gray) and average (red) cell traces for Venus-cp172 FLARE-ICUE (N=40). Dashed lines above and below the average curve reflect the standard error of the mean.

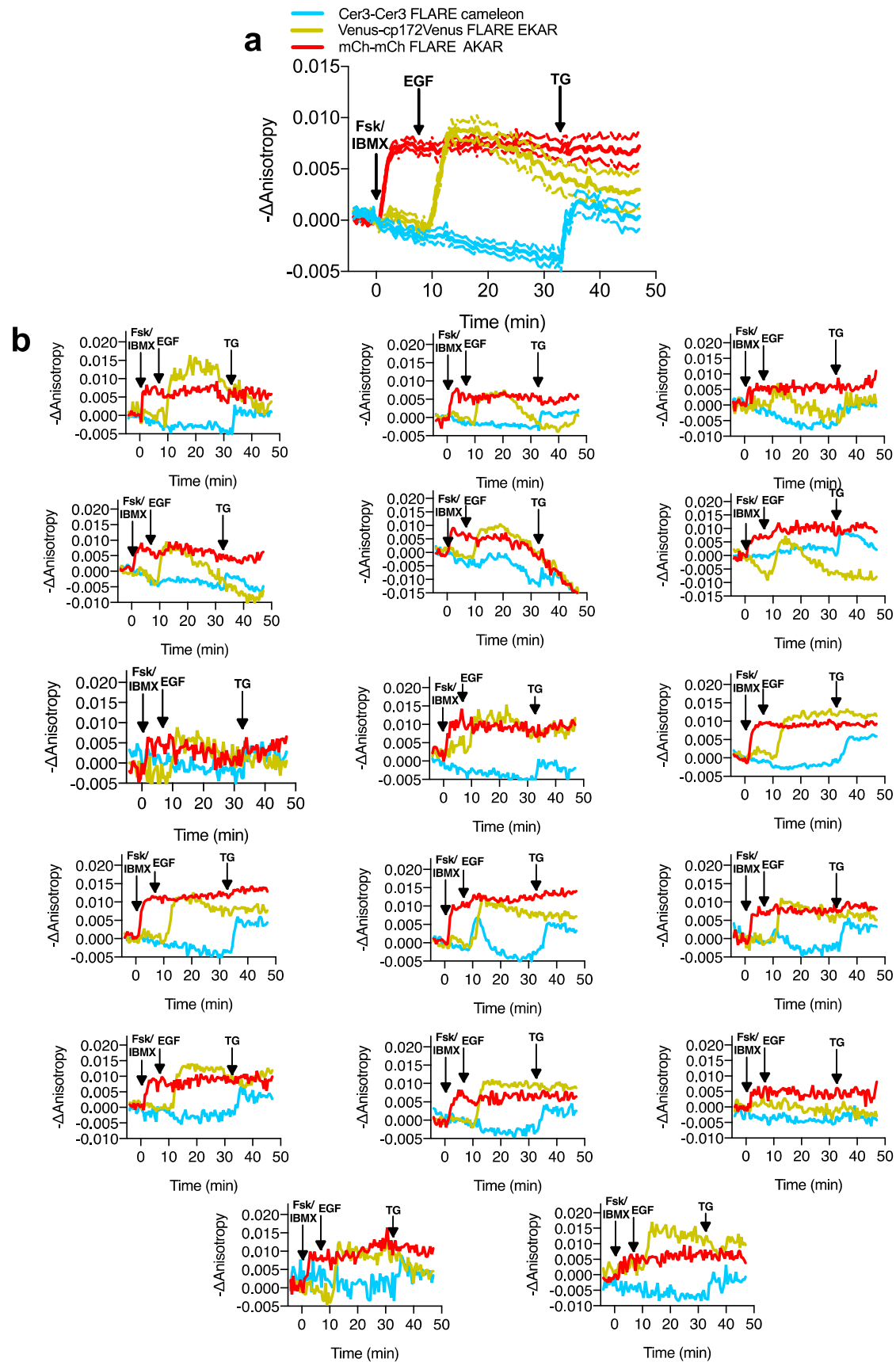
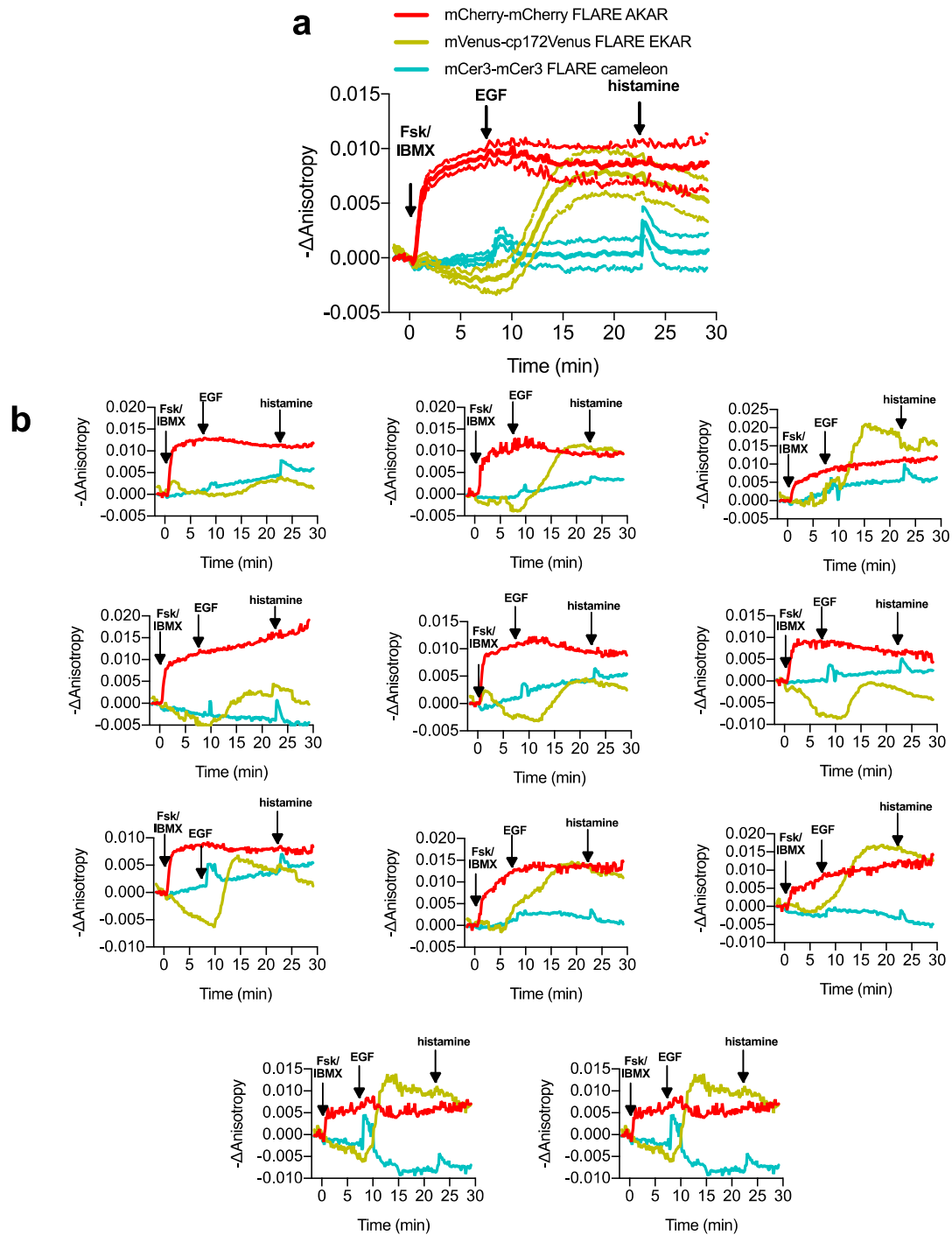


Figure 4 Supplement 1 a). Average cell traces for mCherry-mCherry FLARE-AKAR, Venus-cp172Venus FLARE-EKAR, and mCer3-mCer3 FLARE Cameleon, expressed in HEK293T cells, and stimulated with 50 μ M forskolin and 100 μ M IBMX at t=0min, 100 ng/mL EGF at t=7.5 min, and 1 μ M thapsigargin at t=32.5min. (Dashed lines reflect the standard error of the mean). b). Single cell anisotropy traces for above experiment.



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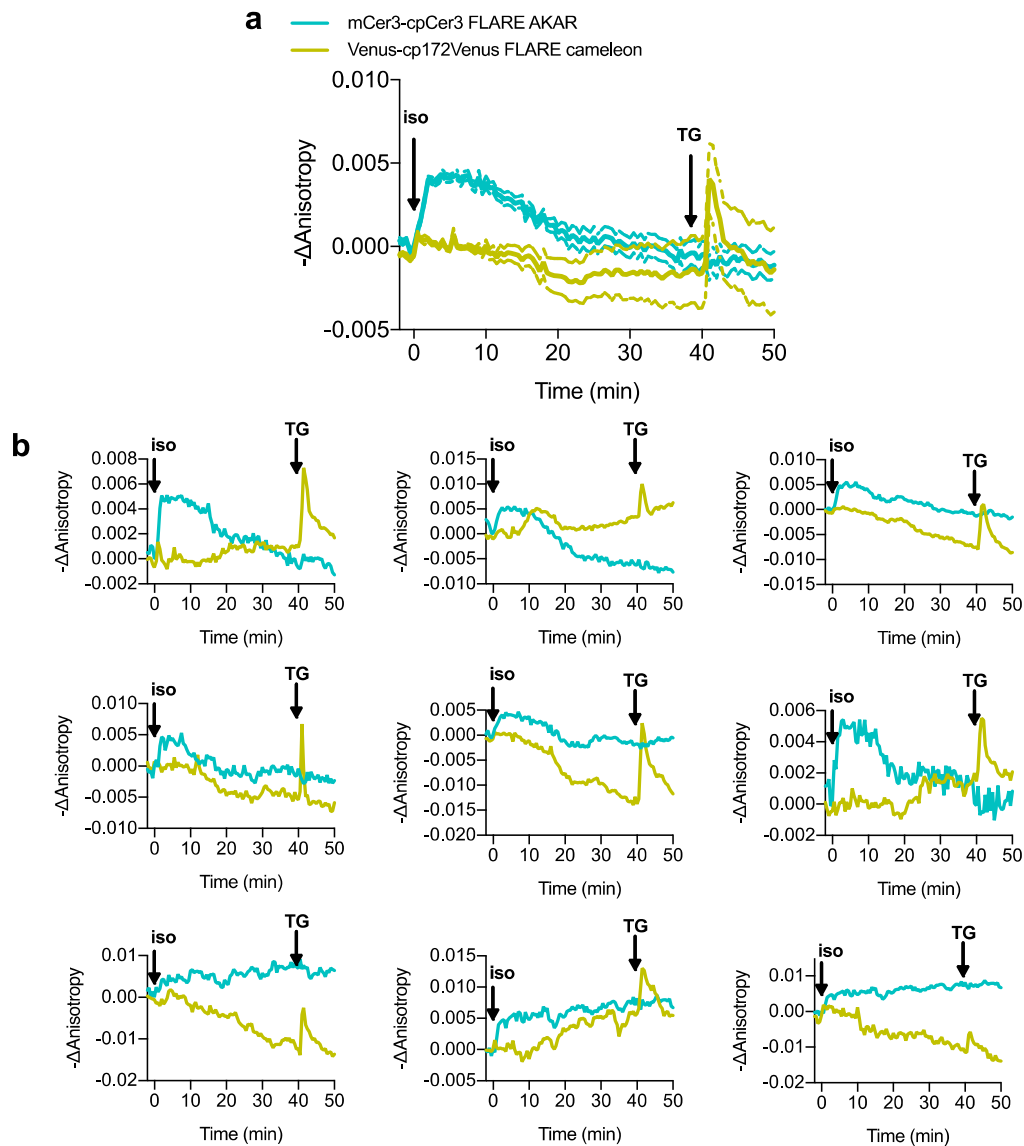
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Figure 4 Supplement 2 a). Average cell traces for mCherry-mCherry FLARE-AKAR, Venus-cp172Venus FLARE-EKAR, and mCer3-mCer3 FLARE Cameleon, expressed in HeLa cells, and stimulated with 50 μ M forskolin and 100 μ M IBMX at t=0min, 100 ng/mL EGF at t=7.5 min, and 100 μ M histamine at t=32.5min (N=11). (Dashed lines reflect the standard error of the mean). In the cyan channel, transient decreases in anisotropy can be observed both after both EGF and histamine stimulation. b). Representative cell anisotropy traces for above experiment.

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798

799 **Figure 4 Supplement 3 a).** Average cell traces for mCerulean3-mCerulean3 FLARE-AKAR,
 800 and mVenus-cp172Venus FLARE Cameleon, expressed in HEK293-T cells, and stimulated with
 801 100 nM isoproterenol at t=0min and 1 μ M thapsigargin at t=40 min (N=28). (Dashed lines reflect
 802 the standard error of the mean). b). Representative cell anisotropy traces for above experiment.

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— mCer3-mCer3 FLARE cameleon
— Venus-cp172Venus FLARE ICUE

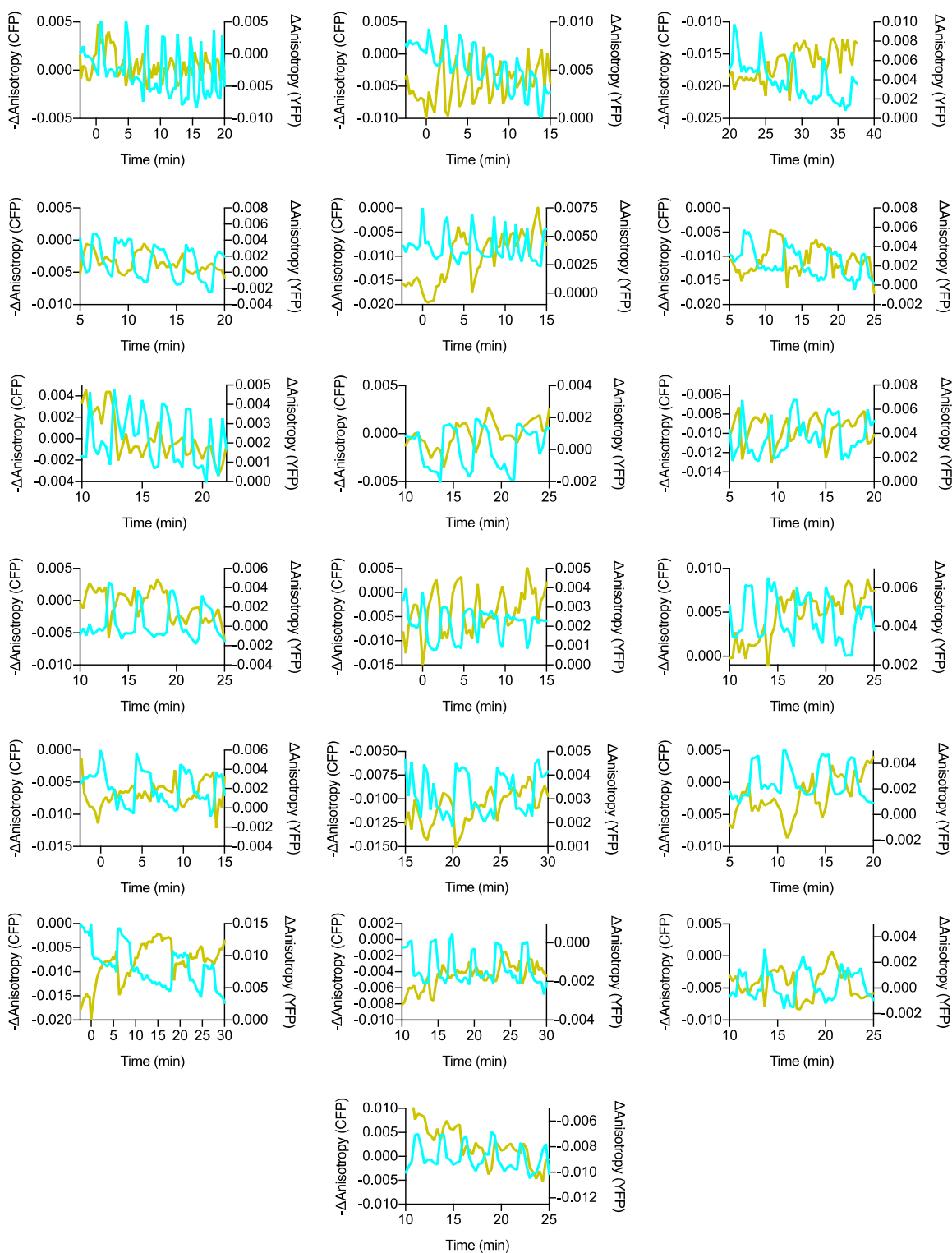


Figure 4 Supplement 4 Monitoring calcium and cAMP oscillations in pancreatic beta cells. Additional cell traces for mCer3-mCer3 FLARE-Cameleon and Venus-cp172Venus ICUE, coexpressed in Min6 cells, showing out of phase oscillations upon stimulation with TEA (N=19).

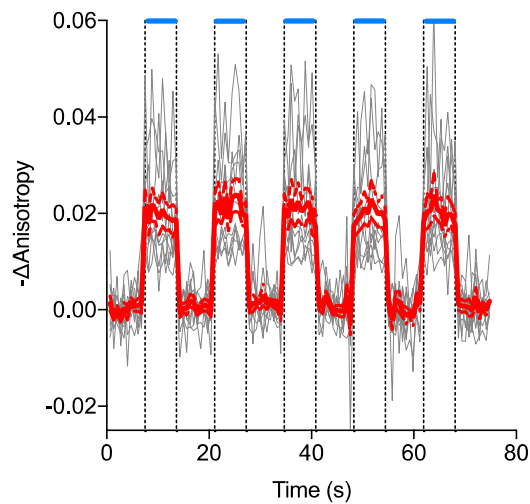


Figure 4 Supplement 5 Individual (gray) and average (red) cell traces for Venus-cp172Venus FLARE AKAR co-expressed with hChR2-ER, with intermittent exposures to 455nm light (N=11). Dashed lines reflect the standard error of the mean.

822 **Table 1**

823

Sensor	Signal to Noise Ratio ($\pm$ SEM)
Venus-cp172Venus FLARE AKAR	32 ± 2.0 (N=32)
mVenus-mVenus FLARE AKAR	10 ± 1.6 (N=32)
EGFP-EGFP FLARE AKAR	6 ± 0.6 (N=32)
mCherry-mCherry FLARE AKAR	14 ± 1.5 (N=22)
mCerulean3-mCerulean3 FLARE AKAR	3 ± 0.5 (N=10)
mCerulean3-cp173 Cerulean3 FLARE AKAR	5 ± 0.3 (N=26)
Venus-cp172Venus FLARE EKAR	17 ± 3.1 (N=8)
mCherry-mCherry FLARE EKAR	4 ± 0.8 (N=10)
mCerulean3-mCerulean3 FLARE EKAR	9 ± 2.3 (N=9)
Venus-cp172Venus FLARE CKAR	27 ± 3.3 (N=26)
mCherry-mCherry FLARE CKAR	14 ± 3.0 (N=8)
mCerulean3-mCerulean3 FLARE CKAR	9 ± 1.7 (N=6)
FLARE-MLCK	8 ± 0.8 (N=13)
Venus-cp172Venus FLARE Cameleon	19 ± 4.8 (N=10)
mCherry-mCherry FLARE Cameleon	5 ± 0.8 (N=23)
mCerulean3-mCerulean3 FLARE Cameleon	9 ± 1.5 (N=11)
Venus-cp172 Venus FLARE ICUE	19 ± 1.2 (N=40)

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