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Simultaneous dual-color fluorescence lifetime imaging with novel red-shifted fluorescent proteins

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Abstract

We describe a red-shifted fluorescence resonance energy transfer (FRET) pair optimized for dual-color fluorescence lifetime imaging (FLIM). This pair utilizes a newly developed fret donor, monomeric cyan-excitable red fluorescent protein (mCyRFP), which has a large stokes shift and a monoexponential fluorescence lifetime decay. When used together with EGFP based biosensors, the new pair enables simultaneous imaging of the activities of two signaling molecules in single dendritic spines undergoing structural plasticity.

FRET imaging of biosensors allows researchers to investigate the spatiotemporal dynamics of signaling cascades in living cells¹. Additionally, it has been demonstrated that two-photon fluorescence lifetime imaging (2pFLIM) is useful for imaging signal transduction in small neuronal compartments in brain slices^{2–4}, revealing principles of biochemical integration and computation in single spines⁵. However, on account of the limited number of suitable FRET–FLIM pairs, it has been difficult to simultaneously image multiple FRET sensors with 2pFLIM. Thus, we engineered a new red FRET pair well suited for simultaneous FLIM alongside EGFP-based biosensors.

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Author contributions

T.L. performed FLIM characterization, FLIM imaging in slices, and *in vivo* imaging. J.C. initially engineered and characterized CyRFP1; B.B.K. engineered mCyRFP1 and characterized new variants. A.J.L. engineered mMaroon1. M.Z.L. and R.Y. provided supervision. T.L., M.Z.L., and R.Y. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

As many FLIM sensors use EGFP or monomeric EGFP (mEGFP) as a FRET donor, we aimed to develop a red or orange FRET donor that could be excited simultaneously with EGFP-based biosensors but would have separable fluorescence emission. We started with cyan-excitable orange fluorescent protein (CyOFP1), as it features high quantum yield, a long monoexponential fluorescence lifetime decay, and excitability at the same wavelength as EGFP⁶. However, CyOFP1 has two less desirable features: its short-wavelength emission tail limits collection of EGFP emission to a narrow band; and, although many fusion proteins localize correctly, CyOFP1, it is dimeric at concentrations $\geq 1 \mu\text{M}$ ⁶.

We set out to further red shift and monomerize CyOFP1. Introducing mutations at chromophore-interacting residues, we found that mutation of His-61 to Met (H61M) caused red shifted fluorescence (Fig. 1a,b) while maintaining high fluorescence quantum yield (ϕ) of 0.77. However, like CyOFP1, this 'CyRFP1' mutant was dimeric at $10 \mu\text{M}$ (Supplementary Fig. 1a). To increase the monomericity of CyRFP1, we examined the crystal structure of Neptune, the closest crystallized predecessor of CyOFP1; and we noticed hydrophobic contacts by Ala-161, a position occupied by a charged Arg residue in the monomeric red fluorescent protein mCherry. Mutating Ala-161 to charged residues Arg or Lys, we found that the CyRFP1 A161K mutant was more monomeric than CyRFP1 (Supplementary Fig. 1b).

However, CyRFP1 A161K matured more poorly than its dimeric predecessor in bacteria (Supplementary Table 1). Rescreening of chromophore-interacting sites yielded an N41A mutant with improved maturation (Supplementary Table 1), which proved to be monomeric even at the relatively high concentration of $100 \mu\text{M}$ (Supplementary Fig. 1a). This mutant, designated mCyRFP1, exhibited broad absorbance and excitation centered near 500 nm (Supplementary Fig. 1c,d); peak and average emissions of 596 and 600 nm, respectively (Fig. 1a,b and Supplementary Fig. 1d); and ϕ of 0.65 (Supplementary Table 1). mCyRFP1 fluorescence demonstrated a pK_a of 5.6 (Supplementary Fig. 1e) and photobleached with a normalized halftime of 45 s (Supplementary Fig. 1f), a halftime similar to that of yellow fluorescent proteins⁷. Finally, both mCyRFP1 and CyRFP1 displayed long, monoexponential fluorescence decays with fluorescence lifetimes of 3.55 ns and 3.71 ns, respectively (Fig. 1c, Supplementary Fig. 2, and Supplementary Table 2).

We next compared the performance of CyOFP1 and its new mutants in mammalian cells. Cells expressing CyRFP1 were as bright as cells expressing CyOFP1. Cells expressing mCyRFP1, while dimmer than those expressing CyRFP1, were three-fold brighter than those expressing CyRFP1 A161K (Supplementary Fig. 3a), consistent with improved maturation of mCyRFP1. Cells expressing mCyRFP1 could be continuously excited under typical widefield imaging conditions for minutes before losing half their initial brightness (Supplementary Fig. 3b). mCyRFP1 fusion to histone H2B did not interfere with mitosis (Supplementary Fig. 4a), and fusions to various subcellular markers were properly localized (Supplementary Fig. 4b). To further characterize the monomericity of mCyRFP1, we quantified performance in an organized smooth endoplasmic reticulum (OSER) assay, an assay reported to be sensitive for FP monomericity⁸. We observed that the percentage of normal cells with mCyRFP1 fused to endoplasmic reticulum (ER) membrane protein (CytERM) was 82.4% (Supplementary Fig. 4c), a value most similar to the published scores

for mVenus and mKate2⁹. We also tested mEGFP and mCardinal as references, obtaining scores of 96.8% and 41.8%, similar to previously published scores⁹ (Supplementary Fig. 4c). Thus, consistent with its monomericity at 100 μ M *in vitro*, mCyRFP1 performs well as a fusion tag in cells.

A major advantage of CyRFP1 and mCyRFP1 is their ability to be coexcited along with EGFP while their emissions are easily separable from those of EGFP (Fig. 1a,b). Thus, we further characterized two-photon (2p) excitation spectra of CyRFP1 and mCyRFP1 compared with those of mEGFP (Supplementary Fig. 5a,b). While both CyRFP1 and mCyRFP1 have a broad 2p excitation peak around 1,000 nm, they show higher brightness than EGFP when excited at 900–940 nm, wavelengths commonly used for EGFP imaging in neurons¹⁰. Since CyRFP1 is brighter and shows less bleedthrough into green channel than mCyRFP1 under 2p excitation (Supplementary Fig. 5c), we tested the usability of CyRFP1 as a structural marker for simultaneous 2p imaging alongside green fluorescent sensors in single dendritic spines *ex vivo* and *in vivo*. By biolistically transfecting neurons with fluorescent calcium indicator GCaMP6s¹¹ and CyRFP1, we were able to simultaneously image Ca²⁺ transients and spine volume changes during spine structural plasticity induced with 2p glutamate uncaging in CA1 pyramidal neurons in organotypic hippocampal slices (Fig. 1d,e). Under these conditions, CyRFP1 and mCyRFP1 did not show any detectable photobleaching during repeated 2p imaging of dendritic spines (Supplementary Fig. 5d). Furthermore, these fluorophores did not exhibit aggregation in the nucleus of neurons following long-term expression (~1 week) in organotypic slices, as opposed to mCherry, which tends to aggregate and accumulate in lysosomes¹² (Supplementary Fig. 6). Lastly, we tested performance of CyRFP1 during *in vivo* imaging in live animals. Following transfection of layer 2/3 pyramidal neurons with GCaMP6s and CyRFP1 using *in utero* electroporation, we simultaneously imaged spontaneous calcium events and spine structure in dendritic spines through a cranial window in the motor cortex of adult mice (Fig. 1f,g and Supplementary Video 1). Bright CyRFP1 fluorescence in spines and dendrites under these conditions indicated that optical properties of CyRFP1 remain stable even after months of expression. Furthermore, these results μ Q15 demonstrate the feasibility of using stable CyRFP1 fluorescence to correct for movement artifacts and to quantify relative intracellular [Ca²⁺] normalized to structural volume.

The long fluorescence lifetime and monoexponential decay of mCyRFP1 (Fig. 1c) suggest that it would be an effective FLIM–FRET donor. We next turned our attention to identifying a far-red fluorescent protein that could function as an effective FLIM–FRET Q34 acceptor for mCyRFP1 in mammalian cells¹³. We measured fluorescence lifetimes in HEK cells of mCyRFP1 fused to candidate acceptors mCardinal¹⁴, mNeptune2 (ref. 14), and mMaroon1 (ref. 15). The mCyRFP1–mMaroon1 fusion demonstrated the highest FRET efficiency (62.3 $\pm$ 0.2%) and fraction of protein undergoing FRET (folding efficiency = 69.2 $\pm$ 0.3%, Supplementary Fig. 7a,b). The calculated Förster radius (r_0) of 6.3 nm for the mCyRFP1–mMaroon1 pair, assuming random interfluorophore orientations, was greater than that of other commonly used FLIM–FRET pairs mEGFP–sREACH and mEGFP–mRFP (Supplementary Table 3). Furthermore, mMaroon1 is minimally excited by wavelengths used to excite EGFP and mCyRFP1 (920 nm in 2p excitation; Fig. 2a and Supplementary Fig. 5a,b), minimizing contamination arising from mMaroon1 emission when measuring

mCyRFP1 fluorescence lifetime (Supplementary Fig. 7c). FLIM analysis of HEK cells coexpressing EGFP and mCyRFP1, either alone or fused to FRET acceptors, confirmed our ability to simultaneously measure lifetimes of bound or unbound states of EGFP and mCyRFP1 with minimal crosstalk between the two channels (Supplementary Fig. 7c,d).

To validate FLIM–FRET sensors based on mCyRFP1 and mMaroon1, we created and tested sensors for the small GTPases Cdc42 and RhoA using these fluorophores. We replaced EGFP and mCherry in previously established intermolecular sensor systems for Cdc42 and RhoA³ with mCyRFP1 and mMaroon1, respectively, creating new sensors named Cdc42–CyRM (mCyRFP1–mMaroon1) and RhoA–CyRM (Fig. 2b–f). In HEK cells, FLIM measurements showed that the binding fractions for constitutively active variants of Cdc42–CyRM and RhoA–CyRM were significantly higher than those of dominant-negative and wild-type variants. The sensitivities of the sensors were similar to those of the previously reported sensors³, establishing the usefulness of mCyRFP1–mMaroon1 as a new FLIM–FRET pair.

Finally, we tested if it is possible to perform simultaneous, dual-color 2pFLIM of two biosensors based on mEGFP and mCyRFP1 donors. Specifically, we attempted to image CaMKII and RhoA activation during structural plasticity of single spines induced by 2p glutamate uncaging in organotypic hippocampal slices¹⁶. To image CaMKII activity, we used Green-Camuia, a FRET sensor that uses mEGFP and the dark YFP variant dimVenus in an intramolecular arrangement⁴. For RhoA imaging, we used RhoA–CyRM (Fig. 3a). We verified lack of contamination from mEGFP–CaMKII–dimVenus to mCyRFP1 lifetime (Supplementary Fig. 7c,d) as well as the lack of FRET between dimVenus and mMaroon (Supplementary Fig. 8a; see Supplementary Table 4 for comparison of fluorescent proteins used). We excited both sensors simultaneously with a 2p laser tuned to 920 nm, and we separated the green emission of GreenCamuia and the red emission of RhoA–CyRM using a dichroic mirror. Previous work using 2pFLIM sensors in separate experiments found that, during induction of structural plasticity, CaMKII activation is spatially confined to stimulated spines and decays within 1 min, whereas RhoA activation spreads substantially along the dendrite near the stimulated spine and lasts for tens of minutes^{3,4}. Using Green-Camuia and RhoA–CyRM, we found that glutamate uncaging at a single spine induces short and confined CaMKII α activation followed by long-lasting and spreading RhoA activation from the same spine (Fig. 3b–d). Through a series of control experiments, we further verified that dual-color FLIM measurements are not influenced by experimental artifacts or crosstalk between different channels (Fig. 3e and Supplementary Fig. 8b–d). These results demonstrate that simultaneous imaging of two biochemical sensors is possible using mEGFP and mCyRFP1 FRET donors. In addition, dual-color FLIM revealed that, during structural plasticity of a single spine, CaMKII activity is more confined and transient than RhoA activity, consistent with the previous studies using single-color FLIM^{3,4}.

The improved properties of CyRFP1—including high brightness, large Stokes shift, and emission separation from EGFP—enabled simultaneous imaging of CyRFP1 and GCaMP6s in brain slices and *in vivo*. Expressing CyRFP1 throughout the neuron should enable better quantification of calcium dynamics in small microcompartments such as dendritic spines¹⁷. Moreover, we have engineered the monomeric variant mCyRFP1, which performs well

when fused to a variety of proteins. The new mCyRFP1–mMaroon1 FLIM–FRET pair enables dual-color FLIM together with mEGFP-based FRET probes. As mCyRFP1 and mEGFP can be coexcited by the same wavelengths of light while their emissions can be separated, truly simultaneous FRET–FLIM is possible, as we demonstrated by simultaneous imaging of CaMKII and RhoA activities. The recent development of acceptors for mEGFP that are even darker than REACh will further simplify multicolor FLIM–FRET imaging¹⁸.

Dual FRET–FLIM would allow more accurate quantification of spatiotemporal relationships between protein activities by enabling imaging in the same cells. Moreover, spatiotemporal correlation of two signaling pathways using multicolor FLIM could help decipher the dynamics of biochemical signaling under less controlled conditions such as those in live animals.

Supplementary Material

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Figure 1. Characterization of CyRFP1 and dual-imaging with GCaMP6

(a) Normalized absorbance spectra of mCyRFP1, CyRFP1, and mEGFP. Dotted line indicates approximate 2p excitation (920 nm). (b) Normalized emission spectra of mCyRFP1, CyRFP1, and mEGFP. Dotted rectangles indicate transmission of green and red emission filters. (c) Fluorescence lifetime decay curves of coexpressed mEGFP and mCyRFP1 in HEK293 cells, fitted with a single exponential decay, alongside experimental instrument response function (IRF). (d) A dendritic segment of a CA1 pyramidal neuron in organotypic hippocampal slice transfected with CyRFP1 and GCaMP6s imaged with 2p microscopy. The spine indicated with the arrowhead was stimulated with 2p glutamate uncaging. Images before uncaging, 64 ms after the indicated number of uncaging pulses, and 30 s after the final uncaging are presented. (e) Quantification of changes in spine volume (Δ volume) and changes in the ratio of GCaMP6 and CyRFP fluorescence (Δ ratio for) the spine shown in panel d during structural plasticity induced with glutamate uncaging. (f) *In vivo* 2p images of a dendritic segment of a layer 2/3 neuron in the motor cortex transfected with

GCaMP6s and CyRFP1. The top panels show a spontaneous calcium elevation in spine 2. The bottom panels show a dendrite calcium transient. Merge, overlay of CyRFP1 and GCaMP6s images. (g) Timecourses of change in green-to-red fluorescence ratio in the two spines and the dendritic shaft in the image f. Inset shows time courses of CyRFP1 fluorescence. Scale bars, 2 μm .

Figure 2. Characterization of the mCyRFP1-mMaroon1 FRET pair for FLIM
 (a) Normalized (norm.) spectra of absorptions and excitations (ex, excitation; em, emission) of mCyRFP1 and mMaroon1. (b) Fluorescence lifetime decay curves of dominant negative (DN) and constitutive active variants of a red-shifted RhoA sensor, RhoA-CyRM. (c) Schematics of a red-shifted Cdc42 sensor, Cdc42-CyRM, and representative fluorescence lifetime images of HEK293 cells expressing the sensor (WT) or the sensor with dominant negative (DN) or constitutively active (CA) mutations, acquired with 2pFLIM. (d) Binding fraction of Cdc42-CyRM mutants (80, 50, and 59 cells for DN, WT, and CA, respectively). (e) Schematics of RhoA-CyRM and representative fluorescence lifetime images of HEK293 cells expressing the sensor (WT) or its variants with DN or CA mutations. (f) Binding fraction of RhoA-CyRM mutants expressed in HEK293 cells (184, 190, and 181 cells for DN, WT, and CA, respectively). Scale bars, 20 μ m. Error bars represent s.e.m.; statistical difference was measured using one-way ANOVA followed by Dunnett multiple comparison test.

Figure 3. Simultaneous FLIM measurements of RhoA and CaMKII activation in single dendritic spines undergoing structural plasticity

(a) Schematics of CaMKII- α sensor (Green-Camuia) and RhoA-CyRM used for experiments. (b,c) Representative fluorescence lifetime images of RhoA (b) and CaMKII (c) activation in a dendritic segment of a CA1 pyramidal neuron in an organotypic hippocampal slice, acquired with 2pFLIM. Structural plasticity was induced in a single spine using 2p glutamate uncaging (marked with arrowhead). Scale bar, 2 μ m. (d) Average changes in fluorescence lifetime of RhoA and CaMKII sensors during spine structural plasticity in the stimulated spines and their adjacent dendritic shafts (ten spines, eight neurons). (e) Quantifications of transient (top panel; 16–64 s for CaMKII and 16–76 s for RhoA) and sustained (bottom panel; 20–30 min) fluorescence lifetime changes in stimulated spines during simultaneous CaMKII-RhoA imaging under the following conditions: control, without extracellular Ca²⁺ (no Ca²⁺), replacing Green-Camuia with mEGFP-dimVenus

fusion (+mEGFP–dimVenus) and replacing acceptor of RhoA–CyRM with acceptor for Cdc42–CyRM (+RhoA false acceptor). (10/8, 10/8, 7/6, and 8/6 spines/cells, respectively; Error bars represent s.e.m.; statistical difference was measured using one-way ANOVA followed by Dunnett multiple comparison test).

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