



Supplementary Figure 1. Spectra of fluorophores used in ratiometric FRET multiplex experiments. A) EBFP/ECFP and mOrange/mCherry; B) ECFP/EYFP and mOrange2/mCherry; C) mAmetrine/tdTomato and mTeal/YFP; D) T-Sapphire/DsRed and ECFP/EYFP. The shaded boxes marked “ex” and “em” indicate suggested excitation and emission wavelengths. Spectral data adapted from sources described in McNamara et al.¹

Supplementary Table 1: FRET-based multiplexing studies

Reference	Fluorophores Used	Methods Used for Imaging	Biological Insights
2-4	CFP/YFP/mRFP1	Experimental: Three-color, Ratiometric FRET x2 on same probe	Identification of trimeric molecular complexes; Analysis of caspase activities
5, 6	CFP/DsRed with YFP/DsRed	Experimental: Intensity-based FRET x2	Analysis of multiple caspase activities upon TNF α -induced cell death
7	CFP/YFP	Experimental: Spatial separation, Ratiometric FRET x2	Analysis of cAMP signaling in multiple cellular compartments simultaneously
8	CFP/YFP	Experimental: Ratiometric FRET x2 on same probe	Simultaneous monitoring of PKA and PKC activities
9	CFP/YFP, mOrange/mCherry, Fura Red	Experimental: Ratiometric FRET x2, Spatial separation, Ratiometric FRET x2 + Intensiometric FRET + Calcium Sensor	Relative timing relationships of CaMKII and PKC activation and Annexin A4 assembly in response to Ca ²⁺ stimulation
10	mTFP1/YFP, mAmetrine/tdTomato	Experimental: Ratiometric FRET x2	Dual caspase-3 sensors monitor delay between cytoplasmic and nuclear caspase-3 activity
11	Sirius/mseCFP, Sapphire/DsRed	Experimental: Ratiometric FRET x2	Relative timing of Ca ²⁺ signals and caspase-3 activity in response to TNF α
12	mSapphire/RFP CFP/YFP	Experimental: Single wavelength excitation, Ratiometric FRET x2	Simultaneous imaging of cGMP and cAMP, or simultaneous imaging of Ca ²⁺ and cAMP in contracting cardiomyocytes
13, 14	CFP/YFP, Dyes: Mero87/Alexa750 pair	Experimental and Computational: Ratiometric FRET + Ratiometric dye change	Relative timing and position of Cdc42, Rac1, and RhoA during cellular protrusion
15	mTagBFP/sREACH, CFP/YFP, Fura Red	Experimental: Ratiometric FRET x2 + Ca ²⁺	Simultaneous imaging of cGMP, cAMP, and Ca ²⁺ using spectrally separable probes
16	CFP/YFP, mOrange2/mCherry	Experimental: Ratiometric FRET x2	Simultaneous analysis of the relative timing of Src and MMP-MT1 activities in response to EGF stimulation
17	FLIM: CFP/tHcRed and YFP/tHcRed	Experimental: FLIM FRET	Simultaneous analysis of K-ras and H-ras activities in different cellular compartments in response to EGF stimulation using a common binding domain
18	CFP/YFP and FLIM: mTagRFP/mPlum	Experimental: Ratiometric FRET + FLIM FRET	Simultaneous imaging of Ca ²⁺ signals and H-ras activity in response to EGF stimulation
19	FLIM: mRFP/GFP, mStrawberry/GFP, mRFP/Venus, mStrawberry/Venus, and mDarkVenus/mGFP	Experimental: FLIM FRET	Analysis of CaMKII activity in neuronal synapses and evaluation of a variety of FLIM fluorescent protein pairs with potential for multiplex imaging

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