

Supplementary information S2 (Table) | **FRET-based multiplexing studies**

Reference	Fluorophores used	Methods used for imaging	Biological insights
1-3	CFP/YFP/mRFP1	Experimental: Three-color, Ratiometric FRET x2 on same probe	Identification of trimeric molecular complexes; Analysis of caspase activities
4, 5	CFP/DsRed with YFP/DsRed	Experimental: Intensity-based FRET x2	Analysis of multiple caspase activities upon TNF α -induced cell death
6	CFP/YFP	Experimental: Spatial separation, Ratiometric FRET x2	Analysis of cAMP signaling in multiple cellular compartments simultaneously
7	CFP/YFP	Experimental: Ratiometric FRET x2 on same probe	Simultaneous monitoring of PKA and PKC activities
8	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
9	mTFP1/YFP, mAmetrine/tdTomato	Experimental: Ratiometric FRET x2	Dual caspase-3 sensors monitor delay between cytoplasmic and nuclear caspase-3 activity
10	Sirius/mseCFP, Sapphire/DsRed	Experimental: Ratiometric FRET x2	Relative timing of Ca ²⁺ signals and caspase-3 activity in response to TNF α
11	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
12, 13	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
14	mTagBFP/sREACH, CFP/YFP, Fura Red	Experimental: Ratiometric FRET x2 + Ca ²⁺	Simultaneous imaging of cGMP, cAMP, and Ca ²⁺ using spectrally separable probes
15	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
16	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
17	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
18	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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