Supporting Information

Simultaneous recording of multiple cellular events by FRET

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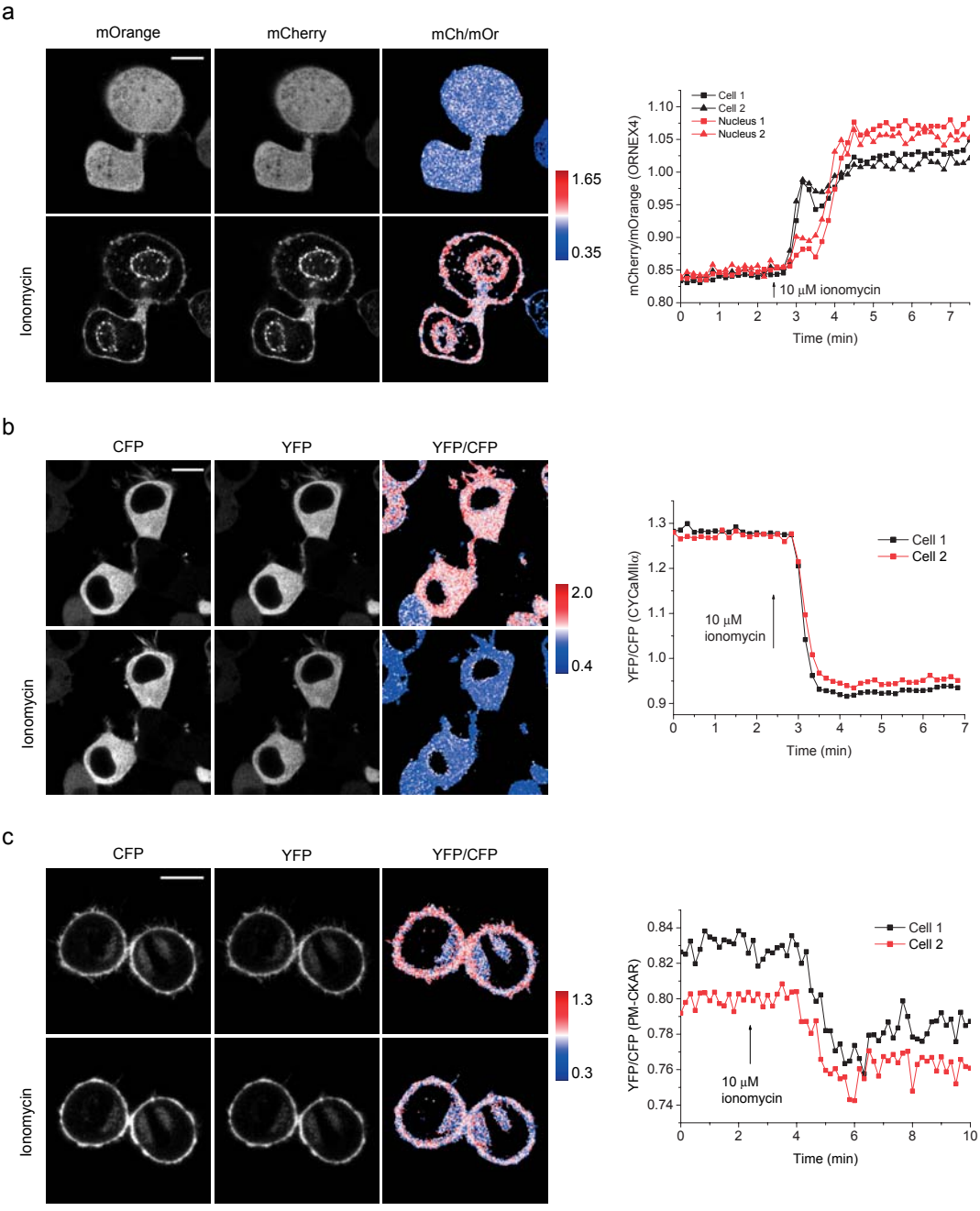
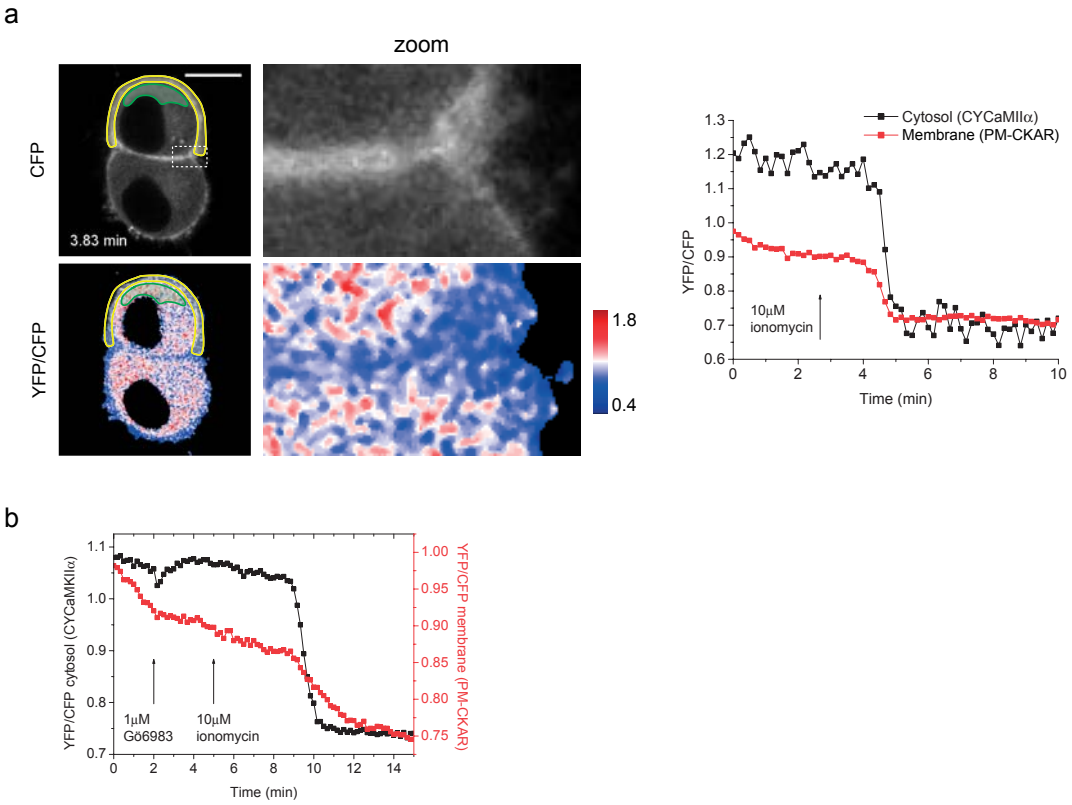
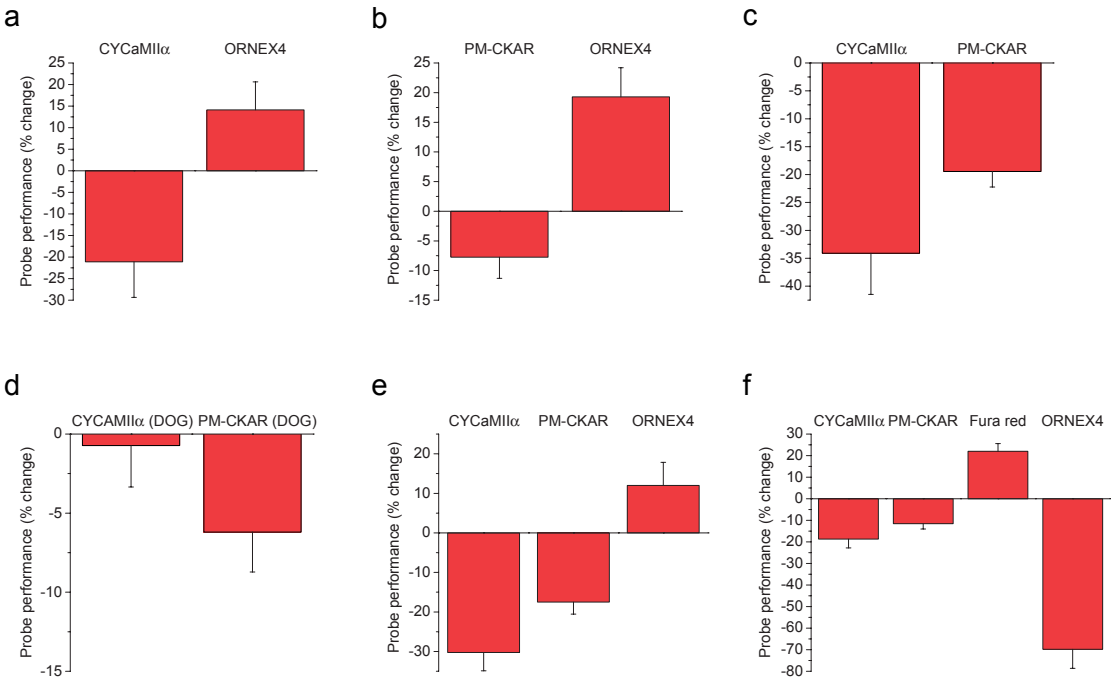
Supplementary Figures



Supplementary Figure 1. FRET sensors individually tested in N1E-115 cells. a) Cells expressing ORNEX4 are shown with corresponding ratio images before and after ionomycin addition and a graph showing ratio changes over time. ORNEX4 translocates and self-associates on the inner leaflet of the plasma and the nuclear membrane after calcium elevation similar to CYNEX4. However, it should be noted that maximal ratio change observed with ORNEX4 is about five times smaller than that observed with CYNEX4 (30% vs. 150% ratio change). This demonstrates that the mOrange-mCherry FRET pair is far inferior to the commonly used cyan-yellow FRET pairs. This may be true for other sensors as well. Therefore, only those cyan-yellow sensors that exhibit large FRET changes may be successfully converted to useful mOrange-mCherry variants. b) Cells expressing CYCaMIIα are shown as well as ratio images before and after ionomycin addition. The sensor exhibits a drop in emission ratio following a rise in calcium. Note that the sensor is only localized in the cytosol. Ratio changes over time for two cells are depicted in the graph. c) Images show N1E-115 cells expressing PM-CKAR. The sensor localizes primarily to the plasma membrane. It is excluded from the cytosol. Ratio images with corresponding curves demonstrate a small FRET decrease following the calcium rise. All bars, 15 μm.



Supplementary Figure 2. Spatial resolution of FRET probes utilizing the same or spectrally overlapping fluorescent proteins. a) N1E-115 cells expressing CYCaMIIα and PM-CKAR. CYCaMIIα is localized in the cytosol, PM-CKAR in the plasma membrane (see enlargement). YFP/CFP ratio images demonstrate different starting FRET values of the two sensors. PM-CKAR is exhibiting less FRET than CYCaMIIα in unstimulated cells. The ratio change is measured over time in the indicated regions and plotted without normalization. The graph depicts a difference in starting FRET values and the difference in ratio change of the two sensors. Bar, 15 μm. b) When PKC inhibitor Gö6983 is applied to the cells prior to ionomycin, PM-CKAR ratio change is significantly slowed, while CYCaMIIα activation remains unaffected. This further confirms that PKC and CaMKIIα activities are effectively separated.



Supplementary Figure 3. The performance of probes (FRET ratio or fluorescence change) in individual experiments. a) CYCaMIIα and ORNEX4. Shown is the mean ratio change and SD (n=8 cells). b) PM-CKAR and ORNEX4 (n=5). c) CYCaMIIα and PM-CKAR (n=6). d) CYCaMIIα and PM-CKAR stimulated with 1,2-di-*O*-octanoyl-*sn*-glycerol (DOG) (n=6). e) Three-parameter experiments involving CYCaMIIα, PM-CKAR and ORNEX4 (n=10). e) FRET ratio change of CYCaMIIα and PM-CKAR and fluorescence intensity change of Fura red and ORNEX4 in four-parameter experiments (n=4). Fura red fluorescence is increasing due to excitation at 405 nm. Annexin A4 translocation is measured as loss of mOrange (ORNEX4) fluorescence in the cytosol.