**Supplementary Text**

Other donor-acceptor pairs such as different versions of cyan (ECFP, CyPet, mTFP1, Cerulean, mTurquoise2) and yellow (EYFP, Citrine, Venus, SYFP2, YPet) fluorescent proteins are also suitable for FRET measurements19-21. The advantage of these pairs is a greater spectral overlap between the emission spectrum of the cyan and the absorption spectrum of the yellow protein (as compared to GFP-Cherry) resulting in somewhat higher R0 values and larger FRET efficiencies. However, for the same reason, the number of non-negligible crosstalk factors and their magnitudes are also larger. For exciting the donor e.g. 405- or 458-nm, for the acceptor 488- or 514-nm laser lines are often used. The most general form of the FRET equations for cyan-yellow FRET pairs is given below17.



For simplicity, all *Ix* fluorescence intensities in the equations are corrected for autofluorescence (*Bx* values based on non-labeled cells are subtracted). The spectral cross-talk factors *S1* and *S3* are calculated from cells expressing the cyan protein alone:



*S2* and *S4* are measured using cells expressing the yellow protein alone:



Depending on the actual excitation wavelengths and emission filters used, *S3* or *S4* may be negligible. This can be directly measured, or checked by comparing the absorption and emission spectra of the dyes with the laser wavelengths and transmission ranges of the emission filters. The terms *ε2* and *ε4* are ratios of the extinction coefficients of cyan and yellow proteins at the wavelengths used for exciting the donor (*λD*, e.g. 405 or 458 nm) and the acceptor (*λA*, e.g. 488 or 514 nm):

 

Depending on the excitation wavelengths used, the value of *ε4* may be negligible (e.g., for *λA*=514 nm because cyan proteins do not absorb at this wavelength; however, they do at 488 nm).

The α factor can be determined using a fusion protein expressing the cyan and yellow proteins (e.g. ECFP-EYFP) at a 1:1 ratio as



The mean pixelwise FRET efficiencies are calculated as



Note that the above formulas become simpler if any of the *S3*, *S4* or *ε4* factors are negligible in the given microscope setup.