Electronic Supplementary Material (ESI) for Integrative Biology.

SUPPLEMENTARY DATA

Fully Quantified Spectral Imaging Reveals in Vivo Membrane Protein Interactions

Christopher King, Michael Stoneman, Valerica Raicu, and Kalina Hristova

Noise Analysis

Straightforward spectral decomposition of every pixel of an image without considerations of noise, can lead to decreased accuracy in the calculated values of F^{DA} and F^{AD}, from which concentrations and thermodynamic parameters of interest are calculated. Specifically, the best-fit k-values can become negative when unmixing "dim" pixels. In this case, a large positive k-value for the other spectral component can provide a fit that basically adds up to a linear background contribution. This gives erroneous calculated pixel-level FRET efficiency and fluorescence information. Furthermore, negative k-values are not physically realizable (there is no such thing as negative fluorescence) and they need to be properly corrected.

With this goal in mind, approximately 500,000 pixels of PBS buffer-only scans at 840 nm and 960 nm laser excitation were analyzed in order to determine the typical k-values that occur in a signal comprised only of noise. The spectra were decomposed as a sum of donor and acceptor fluorescence and the k^{AD} and k^{DA} values were analyzed as shown in Supplementary Figure 1.

As a result of this noise-only analysis, we found that the magnitude of the k-values from the unmixing of noise-only pixels was generally less than five. We decided on a minimum k value = 10, which is twice the value corresponding to noise. All pixels in which the k-values for both fluorophores are less than 10 are rejected as not having a useable signal and excluded from any further analysis.

In order to not bias the data in terms of erroneously rejecting pixels with no FRET (the FRET scan $k_{\lambda 1}^{AD}$ could be low or zero), or very low donor excitation levels (Acceptor scans at 960 nm can give a $k_{\lambda 2}^{DA}$ near zero), the minimum k value and negative k value corrections must be carefully applied as shown in Table 1, below. In the case of a correction, the negative k value is set to zero and the pixel is unmixed again, but with a composite signal lacking the negative fluorescence component. For example, if the k^{AD} value of a pixel in the FRET scan is less than zero, but the k^{DA} value is greater than 10, then the pixel will be decomposed as containing only the donor signal, with zero acceptor signal. This prevents the negative k-value from affecting the true value of the remaining component in the "best" fit.

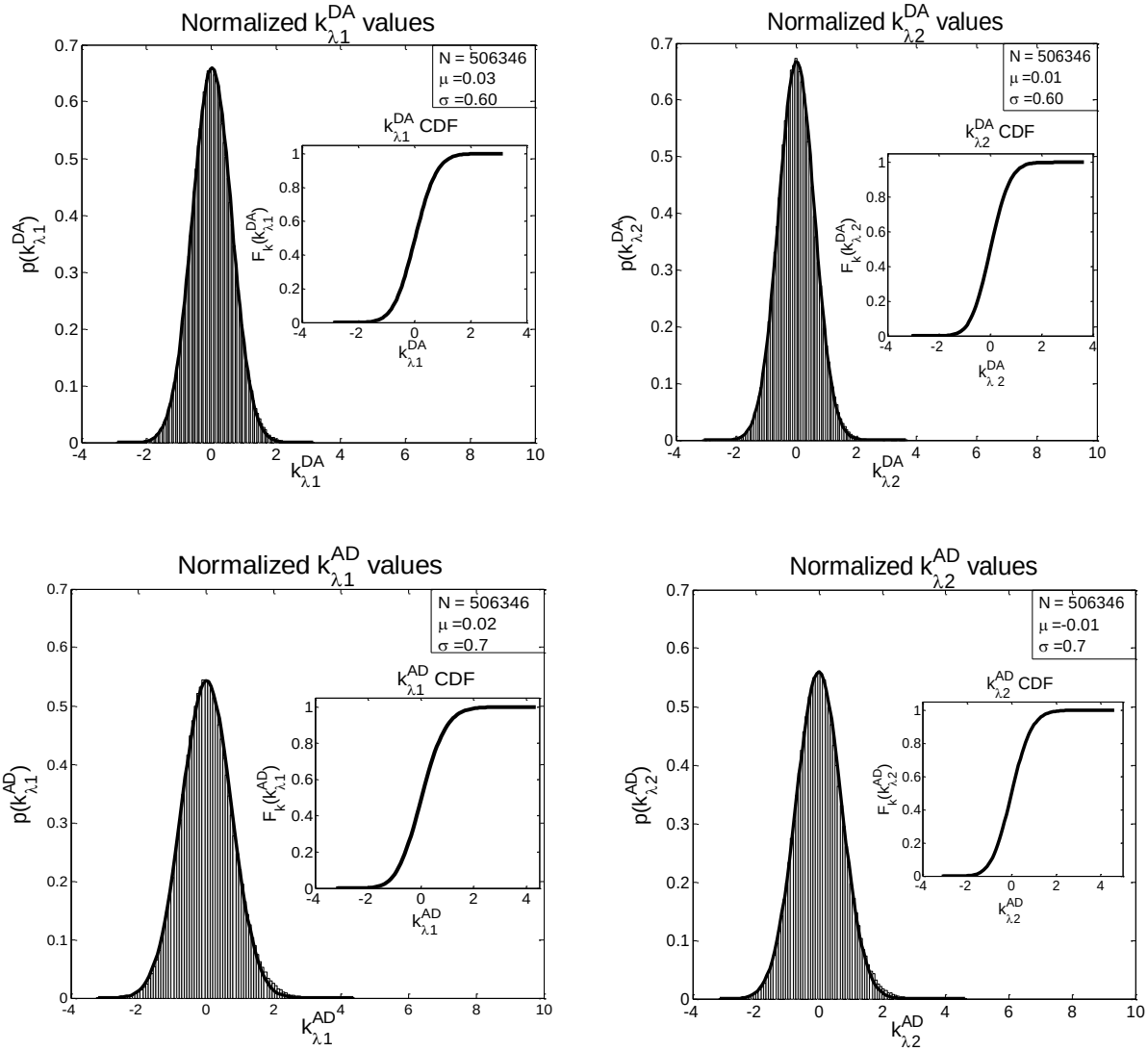
The results of applying these conditions to an image are shown in Supplemental Figure 3. We see that these criteria are able to reject most pixels that do not exhibit any fluorescence.

Image Registration

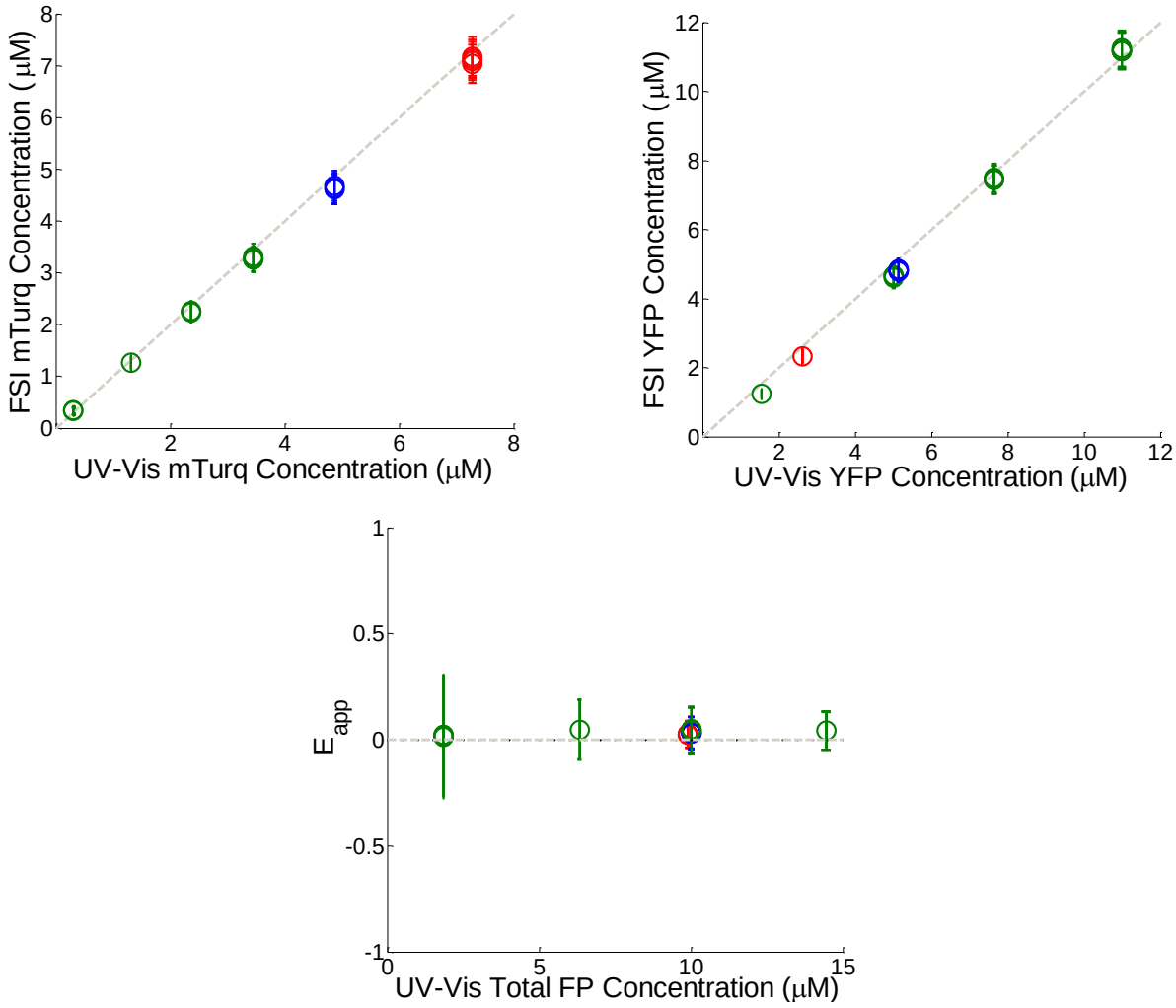
During the time required to tune the laser for the FRET and Acceptor Scans, a stage drift corresponding to one to two pixels is sometimes observed. To correctly align the cells in both images, a subsection of the full field image is selected for analysis and an image registration algorithm is applied to align the Acceptor Scan with the FRET scan (30). This ensures that the same regions are selected in both scans. Images with a correction greater than two pixels are excluded from analysis.

FRET Scan	$k_{\lambda 1}^{DA} > 10$	$k_{\lambda 2}^{DA} > 10$	ACCEPT
	$k_{\lambda 1}^{DA} > 10$	$0 < k_{\lambda 2}^{DA} < 10$	ACCEPT
	$k_{\lambda 1}^{DA} > 10$	$k_{\lambda 2}^{DA} < 0$	CORRECTED
	$k_{\lambda 1}^{DA} < 10$	$k_{\lambda 2}^{DA} < 10$	REJECT
Acceptor Scan	$k_{\lambda 1}^{AD} > 10$	$k_{\lambda 2}^{AD} > 10$	ACCEPT
	$0 < k_{\lambda 1}^{AD} < 10$	$k_{\lambda 2}^{AD} > 10$	ACCEPT
	$k_{\lambda 1}^{AD} < 0$	$k_{\lambda 2}^{AD} > 10$	CORRECTED
	$k_{\lambda 1}^{DA} < 10$	$k_{\lambda 2}^{DA} < 10$	REJECT

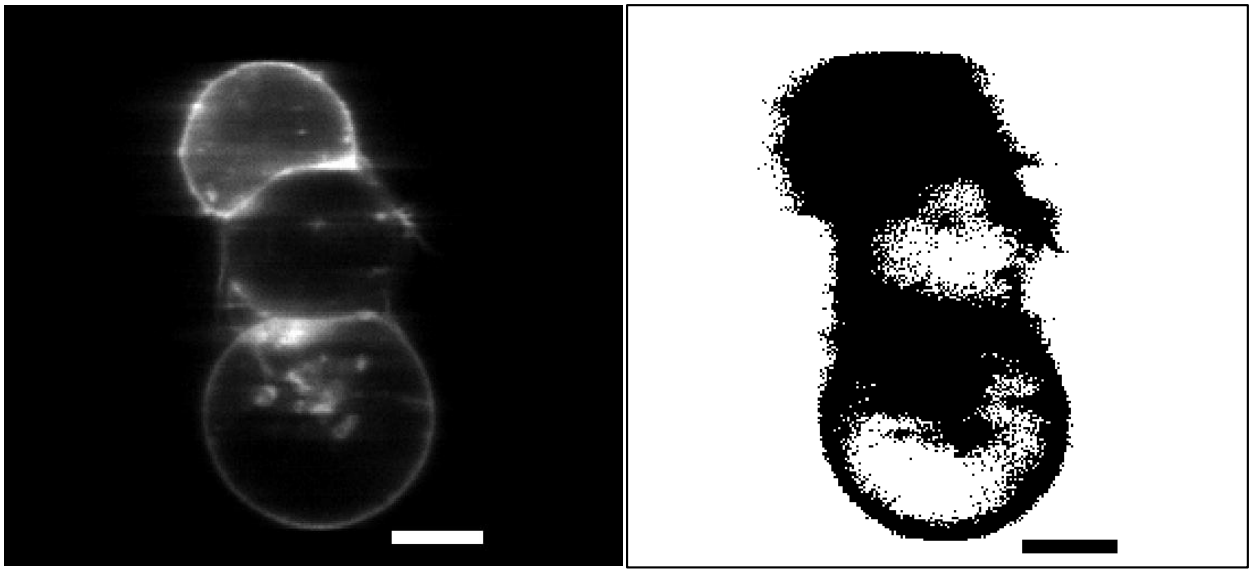
Supplementary Table 1. During unmixing, pixels are either accepted, corrected, or rejected based on the minimum k-value criteria described.



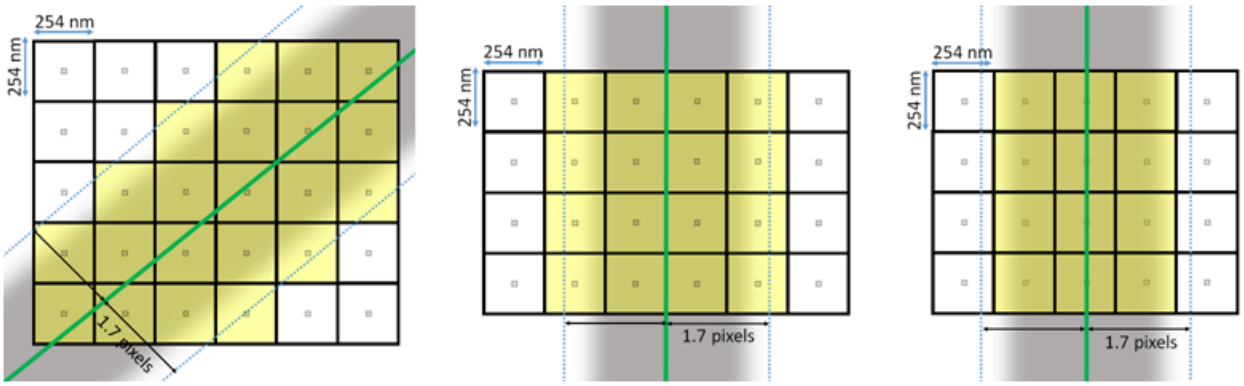
Supplementary Figure 1. *Noise analysis.* Control buffer-only scans are unmixed in order to determine the typical signal contained within pixels that lack fluorescence. The normalized histograms of k-values were integrated to create cumulative distribution functions, which revealed that pixels lacking measurable fluorescence yield k values < 5.



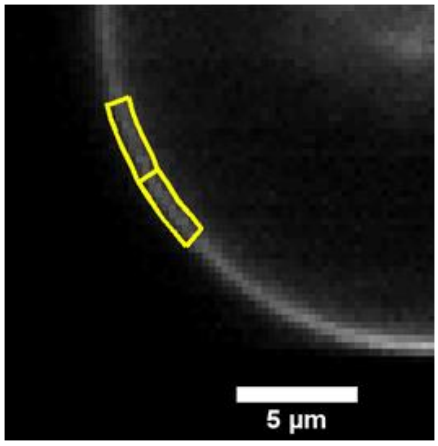
Supplementary Figure 2. Verification of the FSI method for $\lambda_1 = 800$ nm and $\lambda_2 = 960$ nm.



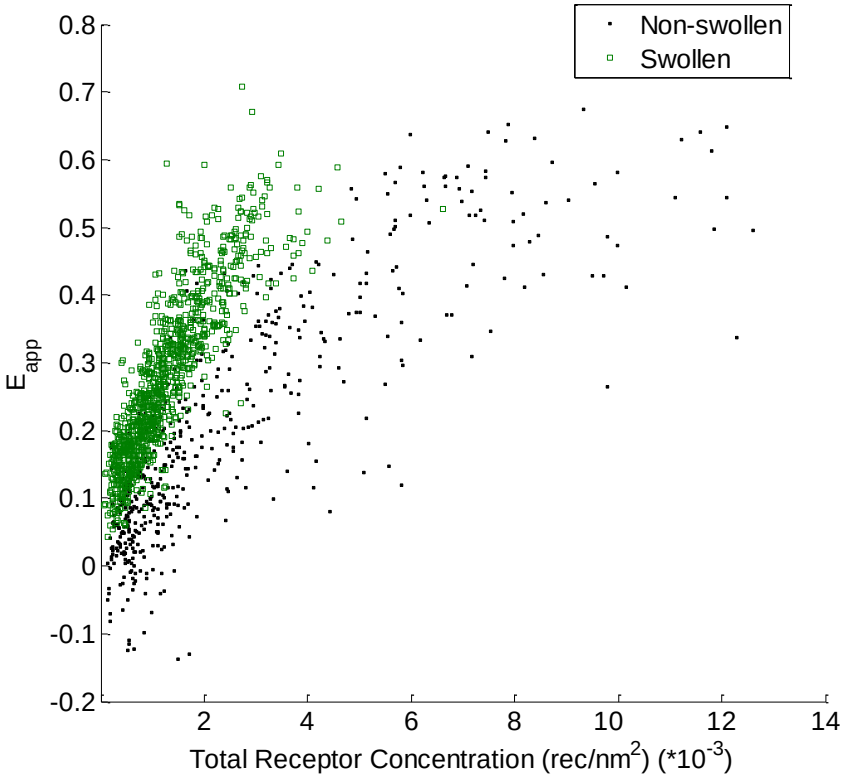
Supplementary Figure 3. *Pixel rejection based on noise.* Left: HEK293T cells co-expressing VEGFR2 EC+TM-mTurq and VEGFR2 EC+TM-YFP. Right: The spectra of every pixel in the image is unmixed and pixels that meet the criteria of Table 1 are shown in black, while pixels that failed to meet the unmixing criteria are shown in white. Pixels that do not meet the unmixing requirements are rejected from further analysis.



Supplementary Figure 4. *Selection of Membrane Regions: principles.* Shown here are three idealized representations of the orientation of the diffraction-limited line of membrane fluorescence that can be encountered in a cell image (light grey): diagonal to the pixels (left), in the center of a line of pixels (center) and between two lines of pixels (right). The line connecting points of fluorescence, chosen by the user, is shown in green. The region selection utilizing a 1.7 pixel region-half width will select regions three to four pixels across, depending on the orientation of the membrane with respect to the pixels of the CCD. Only pixels with centers that falls within the polygon outlining the fluorescence (blue dashed lines) are selected and used for further analysis (yellow).



Supplementary Figure 5. *Selection of membrane regions: experiment.* A cell under reversible osmotic stress is shown, with two ~3 µm regions selected. A 1.7 pixel half-width is used to outline the path of the membrane**.** Pixels with centers that fall within the outlined membrane region are selected and utilized for analysis.



Supplementary Figure 6. Measured FRET efficiencies versus total receptor concentrations, for cells under reversible osmotic stress (green) and intact cells (black). The calculated two dimensional concentrations for the intact cells are erroneous (see Figure 2 and main text), and thus association constants cannot be calculated. The range of measured FRET efficiencies in similar.