Quantification

Quantifying Fluorescence Intensity

Table II. **Protocol for quantitation of fluorescence intensity values**

1. Acquire optical images

- Set up specimen and imaging system for optimal signal detection, low background, and low noise (Table I)

2. Acquire digital images

- Use software to monitor intensity values in the image to choose the best acquisition settings[a]
- Use full dynamic range of the camera for fixed specimens[a]
- For live-cell work, it is often necessary to sacrifice SNR to minimize specimen exposure to light and maintain cell health and viability[a]
- Consider binning to increase SNR[a]
- Avoid high camera gain when a large dynamic range is needed[a]
- Avoid saturating pixels in the image[a]
- Eliminate or minimize exposure of specimen to fluorescence excitation light prior to image acquisition[a]
- Focus carefully, preferably with phase or DIC[b]

3. Store images

- Always save the raw images[c]
- Use either no compression or lossless compression[c]

4. Process images

- Use flat-field correction to correct for uneven illumination[d]
- Be sure any other image processing used prior to quantitation preserves relative intensity values[c,d]

5. Analyze images

- Subtract local background value from intensity measurements[e]
- Do not measure intensity values on compressed or pseudo-colored images[c]
- Validate image segmentation and analysis method[f]
- Calculate and report the error in your measurements[d,g]

Waters, JCB, 2009 7(185)

Quantifying Intermolecular FRET—Sensitized Emission

Predetermined factors with pure samples of donor and acceptor:

Donor cross-talk : R_D

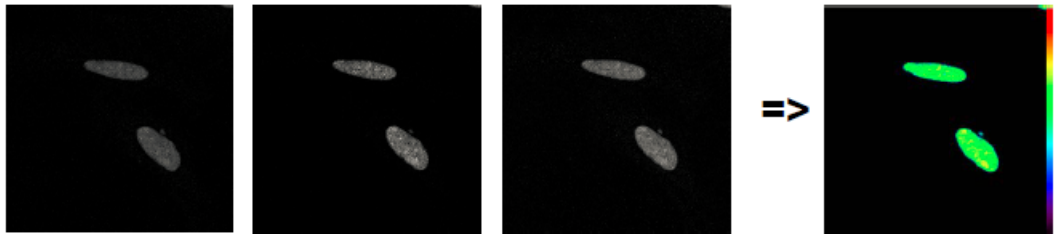
Acceptor cross-excitation: R_E

Required images:

Donor channel
Donor excitation
F^D

Acceptor channel
Donor excitation
F^{DA}

Acceptor channel
Acceptor excitation
F^A



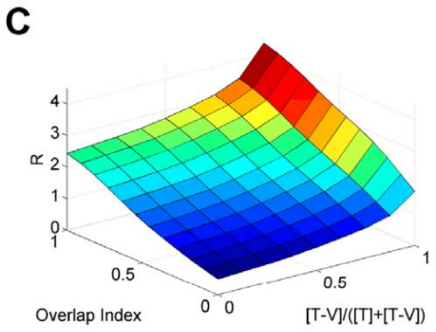
=>

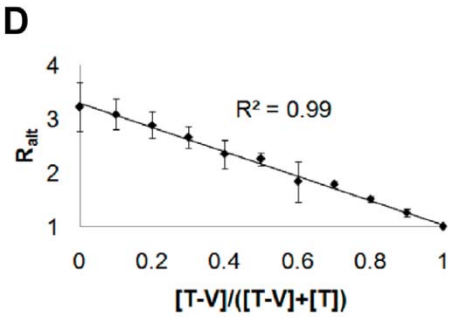
$F^{DA}corr/F^A$

$$E_a = \frac{F^{DA} - \underbrace{F^D \cdot R_D}_{\text{Donor cross-talk correction}} - \underbrace{F^A \cdot R_E}_{\text{Acceptor cross-excitation correction}}}{F^A} = C \cdot E \cdot \alpha_A$$

$$\Rightarrow E_a = \frac{F^{DA}_{corr}}{F^A}$$

Quantifying Intramolecular FRET





Birtwistle et al., PLoS One, 2011

- Because the stoichiometry is known and fixed (typically 1 to 1) a simple ratio can be used
- Typically, it is the Donor Ex/Acceptor Em channel divided by the Donor Ex/Donor Em channel (denoted R)
- However this can have a non-linear relationship to FRET efficiency (Birtwistle et al., 2011)
- Something which may solve that is using Donor Ex/Donor Em channel divided by Acceptor Ex/Acceptor Em channel (denoted R_{alt})
- Another way to quantify FRET, either intra or inter, is by fluorescence lifetime (FLIM)…we won't cover that here