Co-localization, FRET

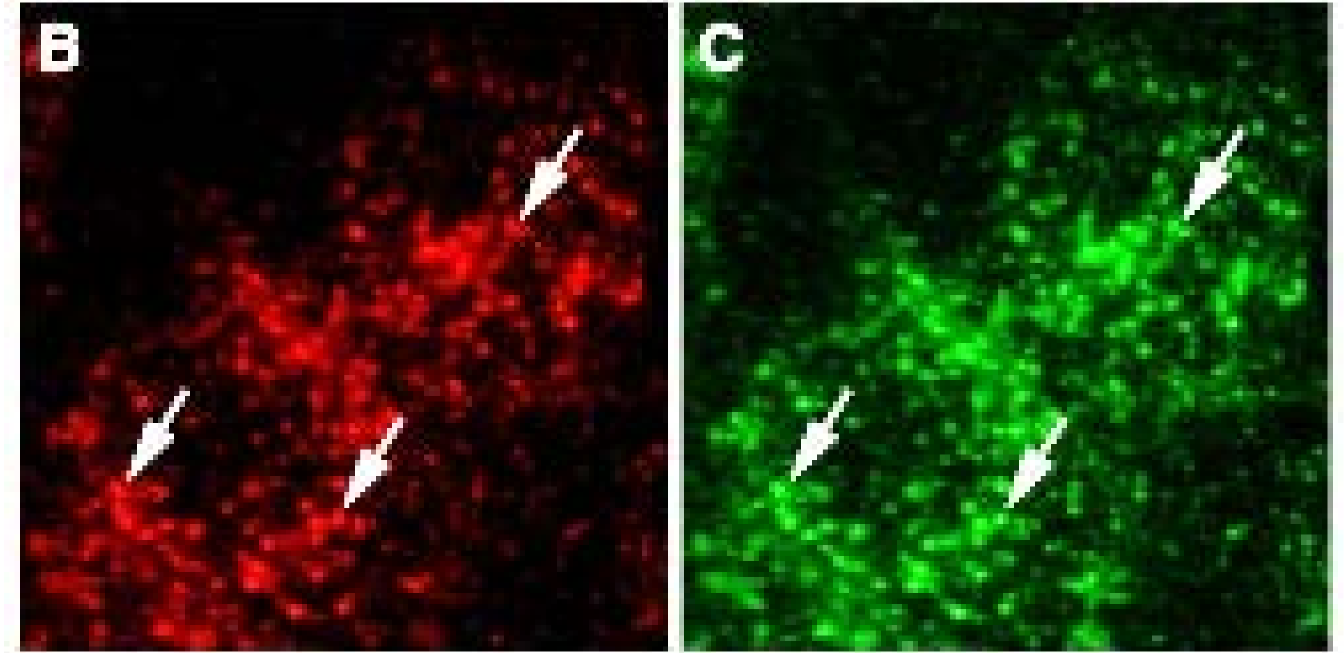
- Last class
  - FRAP
  - Diffusion
- This class
  - Co-localization
  - Correlation
  - FRET

Co-localization

- Can you infer function of protein from it’s intracellular location
- How do you measure if two colors are localized

Co-localization – Are two fluorophores in the same region

- Very common to label two proteins with two colors, and ask where they go in the cell
- How do you determine if they go to the same place?
- Note – colocalization DOES NOT imply interaction
- Used to determine if molecules associate with same structure



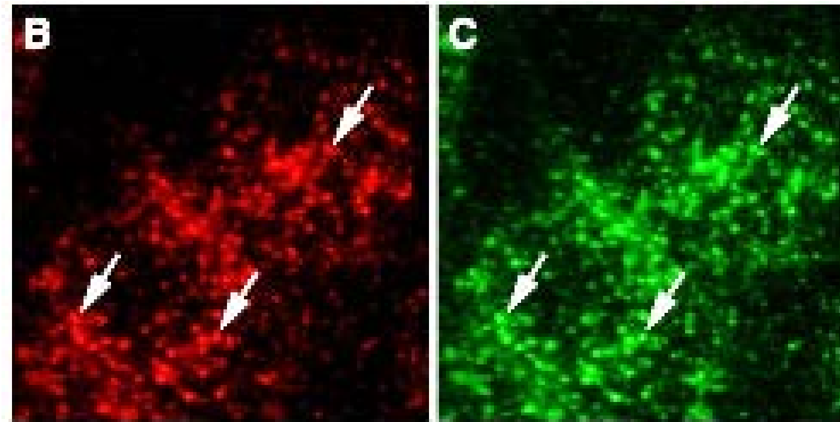
Transferrin labeled with Alexa488 or Texas Red

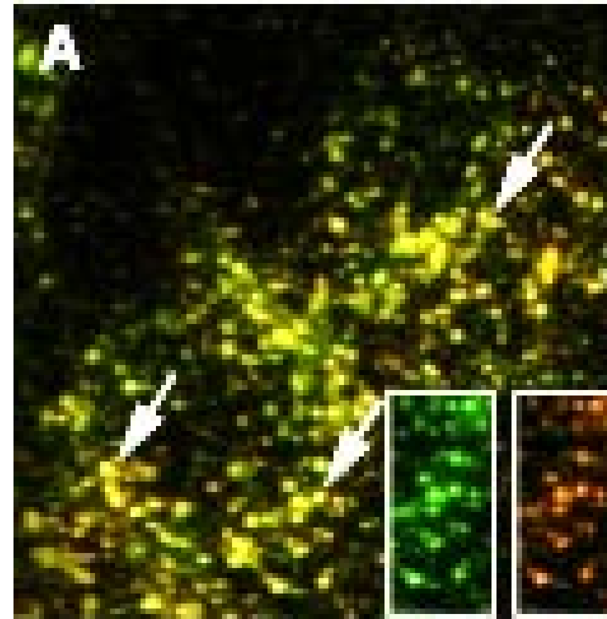
Distance scales:
Colocalization, confocal -> 200 nm
Colocalization, peak fitting -> 30 nm
Electron microscopy -> ~0.5 nm

Defining and testing colocalization

- Have to think about both spatial distribution of colors (co-occurance), as well as the proportion (correlation)
- Some experiments may suggest their should be defined ratios of colors, but others might have no static proportions

Easiest thing to do is overlay the two images



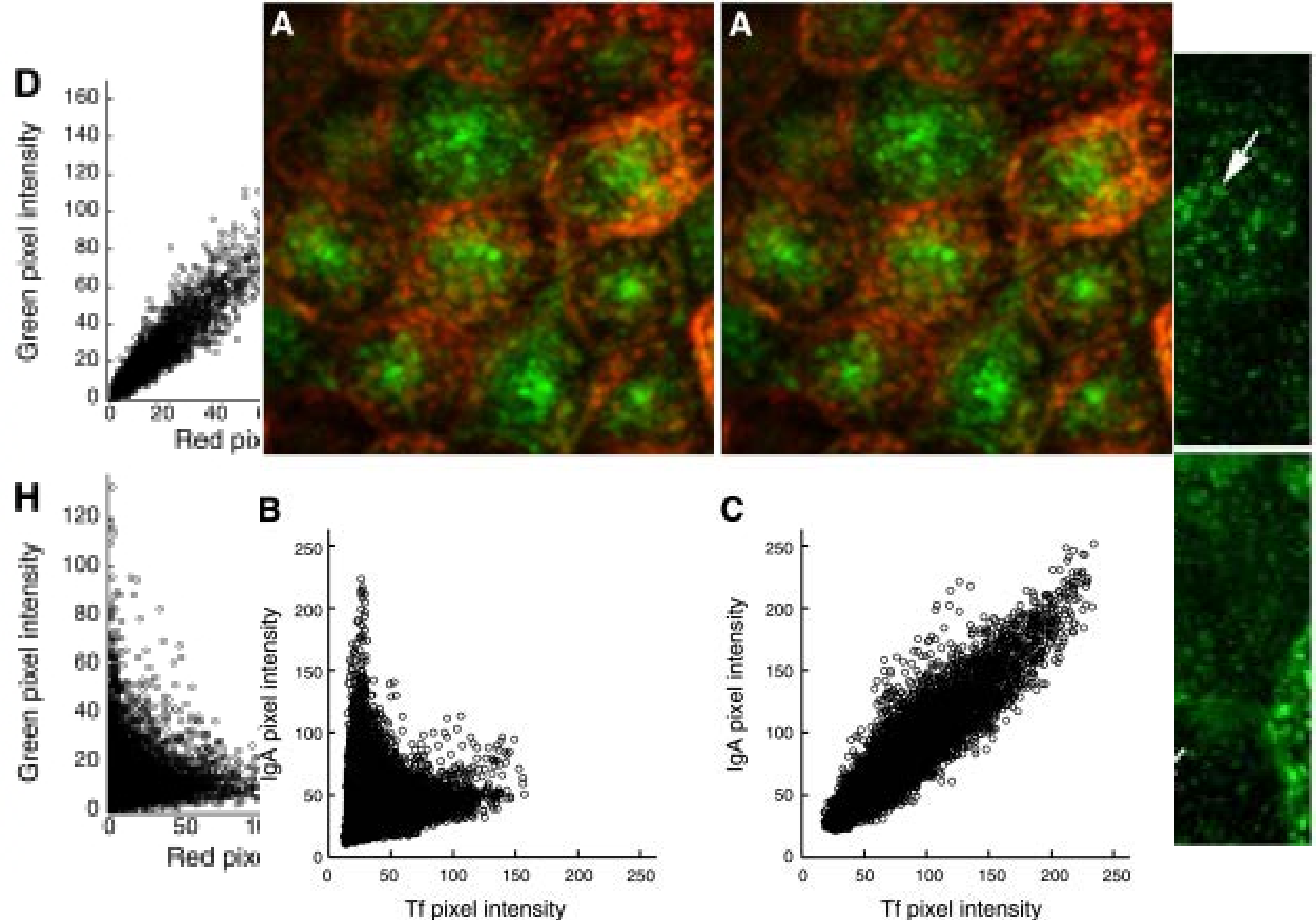


This suffers from the fact that image brightness will determine overlap

Side-by-side visual inspection is typically more accurate

Next easiest definition of co-localization

- Scatter plots of individual pixels with each intensity
- Colocalized images will appear as lines on the scatter plot, the slope is the proportion
- It is also useful for revealing separate populations



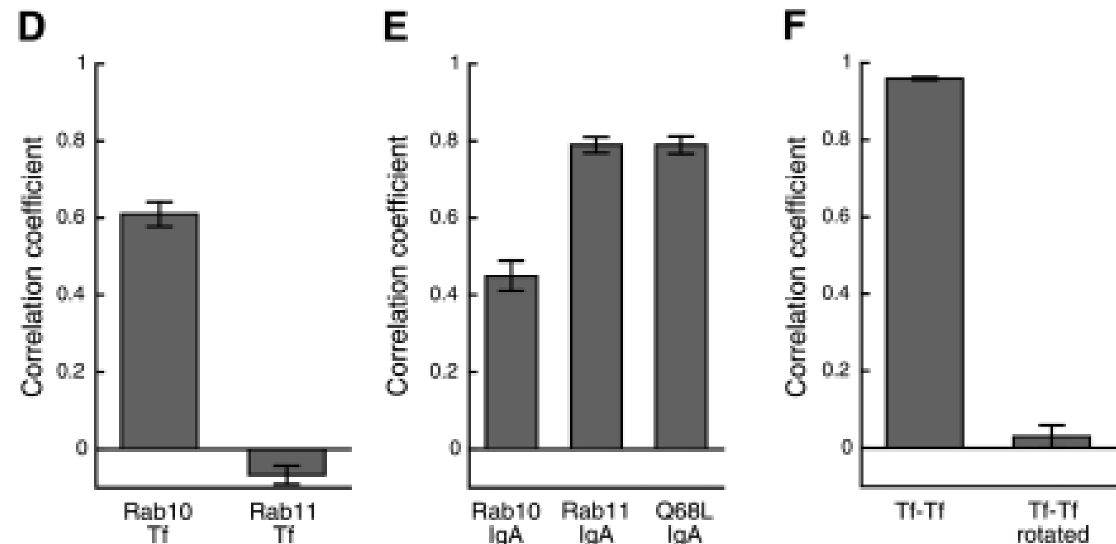
Quantitating correlation

- A widely used definition is the Pearson's correlation coefficient
- Varies between 1 and -1
- PCC = 1: Perfectly correlated
- PCC = 0: Zero correlation (random distributions)
- PCC = -1: Perfectly anticorrelated
- PCC assumes a linear correlation, won't work if you expect non-linear distribution

$$PCC = \frac{\sum_i \left((R_i - \overline{R}) * (G_i - \overline{G}) \right)}{\sqrt{\sum_i (R_i - \overline{R})^2 * \sum_i (G_i - \overline{G})^2}}$$

R_i is the red intensity value at pixel I

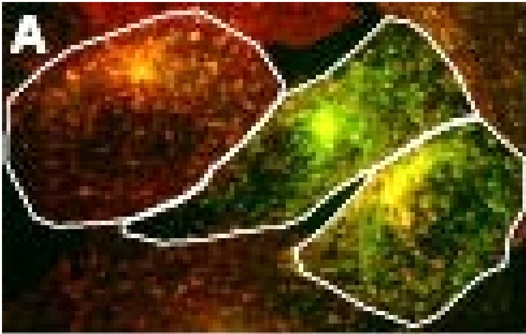
$\overline{R}$ is the average red intensity of the image

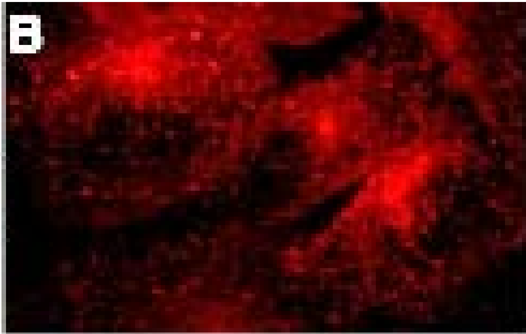


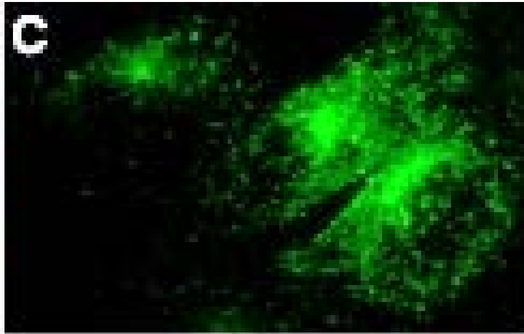
Care with PCC, varying expression

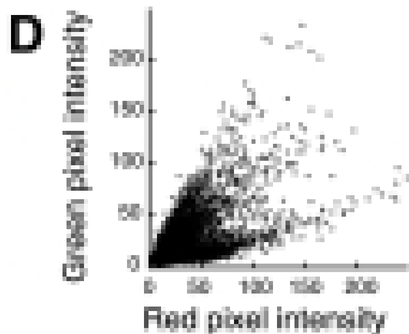
- If different cells express different amounts of receptors, it can lead to an apparent reduction in correlation
- Best to measure cell by cell

PCC_{cell} = .88, .85, .89
PCC_{all3} = .66

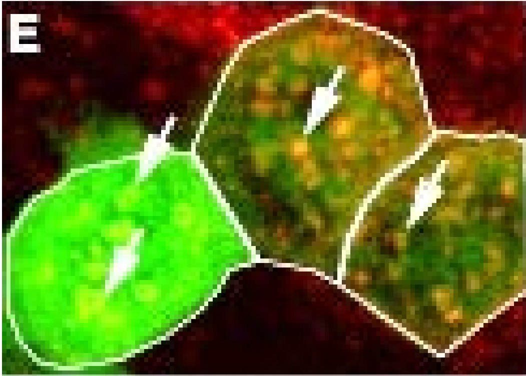


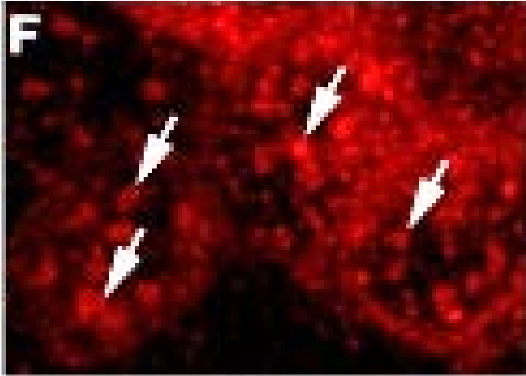


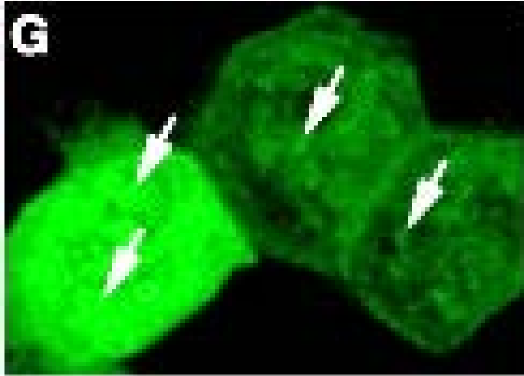


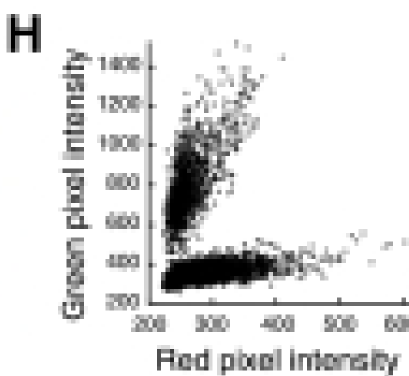


PCC_{cell} = .69, 56, .57
PCC_{all3} = .07







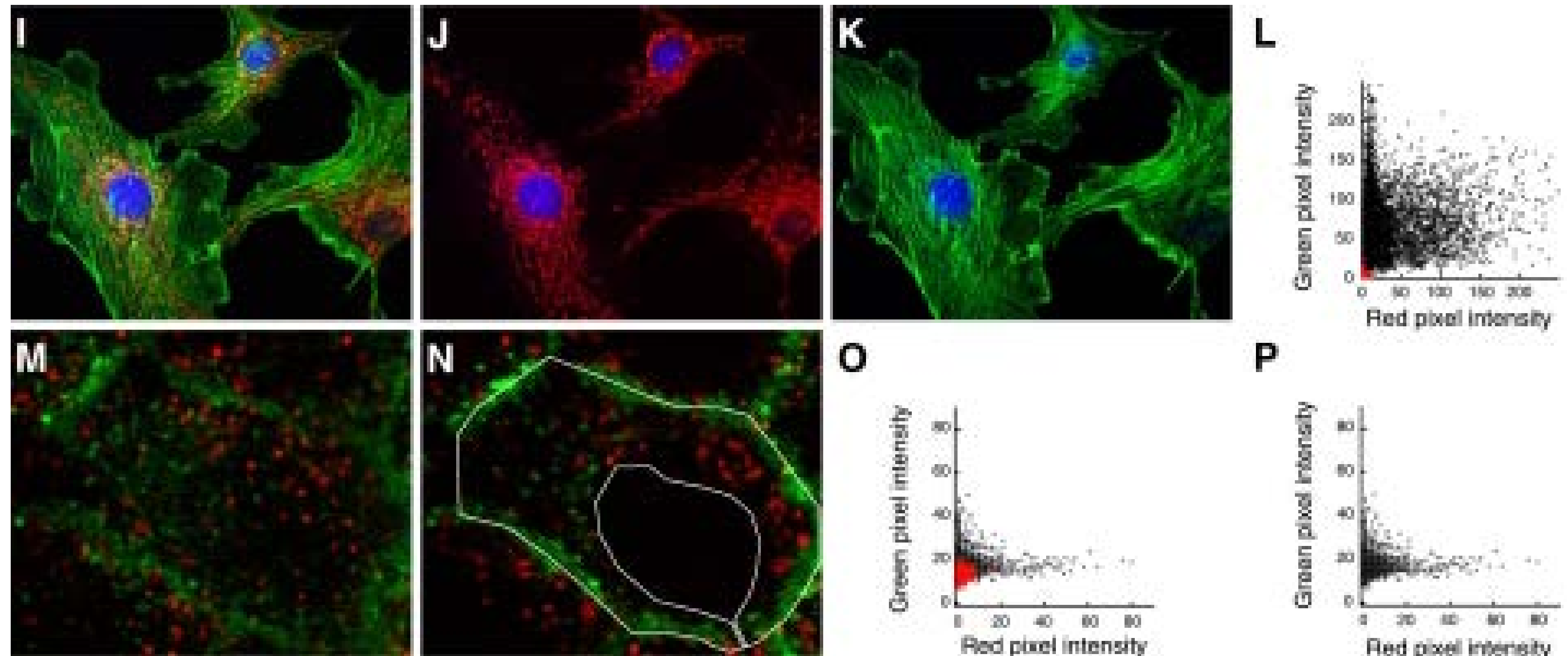


Care with PCC, background pixels

- Background pixels can artificially inflate PCC
- Other fluctuations in your recording can seem to be correlated

PCC_{cells} = .16
PCC_{imag} = .39

PCC_{-nuc} = .16
PCC_{+nuc} = .39



Quantitating correlation

- Mander's overlap coefficient (MOC)

$$MOC = \frac{\sum_i (R_i x G_i)}{\sqrt{\sum_i (R_i^2) x \sum_i (G_i^2)}}$$

- Available in imageJ, Imaris
- No negative correlation, but it can be hard to disentangle positive vs negative correlation

- Manders Colocalization Coefficients

$$M1 = \frac{\sum_i (R_{i,colocal})}{\sum_i (R_i)} \qquad M2 = \frac{\sum_i (G_{i,colocal})}{\sum_i (G)}$$

- Requires you to set manual thresholds on what is a peak
- Very easy to count, and easy to interpret

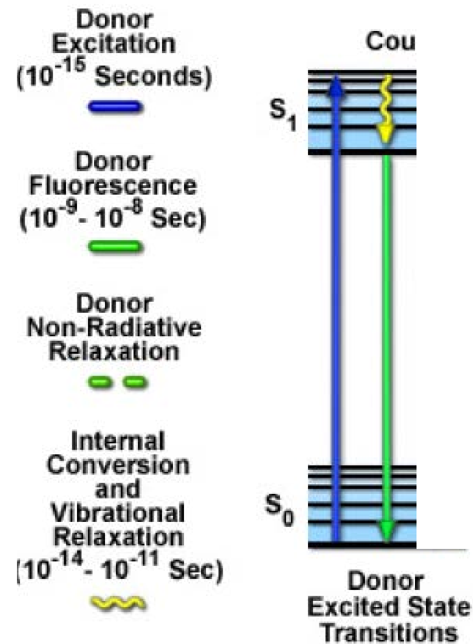
FRET

- Ability to measure nanometer distances
- Useful for determining if two things physically interact

Distance scales:
Colocalization, confocal -> 200 nm
Colocalization, peak fitting -> 30 nm
FRET -> 5 nm
Split GFP -> 3 nm
Electron microscopy -> ~0.5 nm

FRET – Forster resonant energy transfer

- Consider two fluorophores with different spectra.
- If the energy of fluorescent photon corresponds to absorption of second fluorophore, the can switch excited states
- Non-radiative energy transfer



FRET Transfer efficiency

F_d = fluorescence of donor
k_f = rate of fluorescence
k_{nr} = rate of non-radiative decay
k_T = rate of energy transfer

- FRET competes with all the other pathways of decay
- The presence of a FRET acceptor will reduce the fluorescence of the donor
- Our goal is to experimentally determine E by intensity or lifetime measurements

$$QY_d = \frac{k_f}{k_f + k_{nr}}$$

$$\tau_d = \frac{1}{k_f + k_{nr}}$$

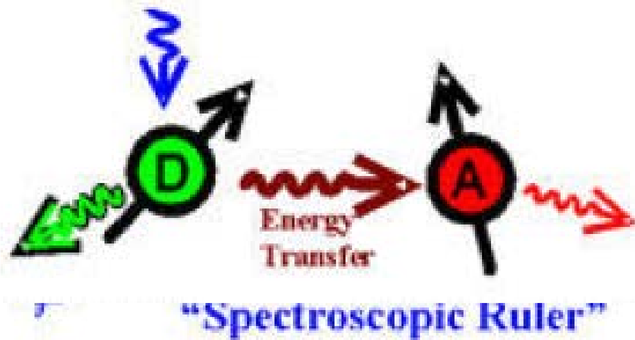
$$QY_{d,FRET} = \frac{k_f}{k_f + k_{nr} + k_T}$$

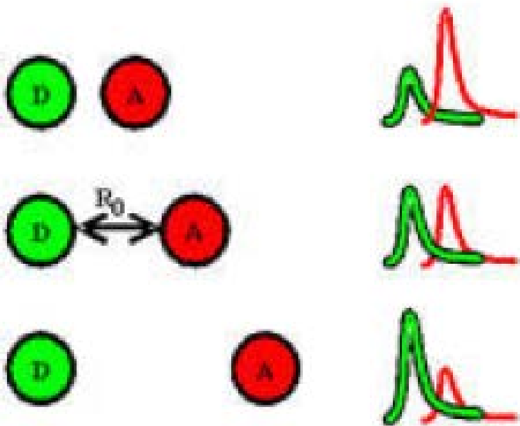
$$\tau_{d,FRET} = \frac{1}{k_f + k_{nr} + k_T}$$

$$E = 1 - \frac{QY_{d,FRET}}{QY_d}$$

FRET efficiency

- Likelihood of FRET is highly dependent on fluorophore distance





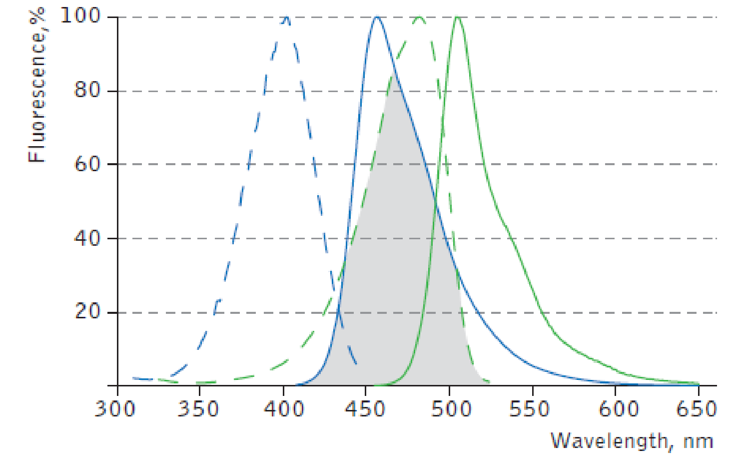
$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{R}\right)^6$$

$$E = \frac{k_T}{k_T + k_D} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

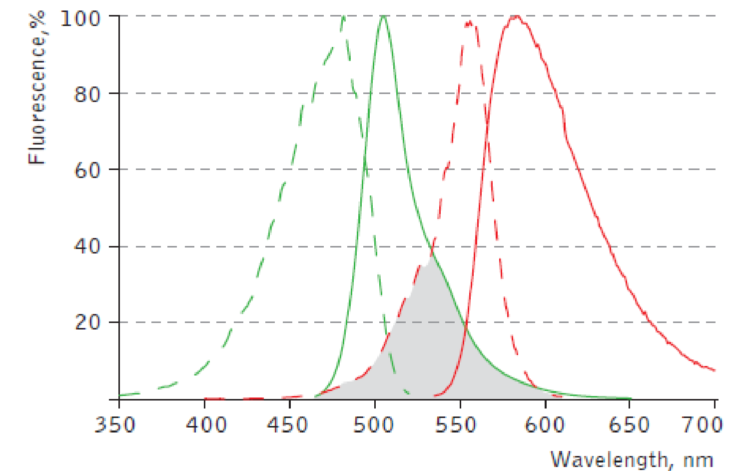
$$R_0^{\,6} = \frac{9\,Q_0\,(\ln 10)\,\kappa^2\,J}{128\,\pi^5\,n^4\,N_A}$$

$$J = \int f_D(\lambda)\,\epsilon_A(\lambda)\,\lambda^4\,d\lambda$$

Q_o = QY of donor
κ = orientation factor
J = J(λ) = spectral overlap
n = index of refraction
N_A = avagardro's number



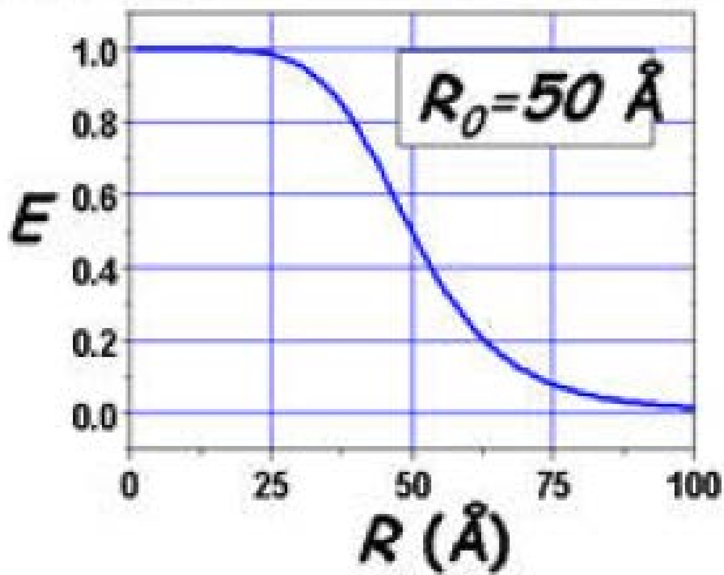
Excitation (dashed lines) and emission (solid lines) spectra of TagBFP (blue) and TagGFP2 (green) are shown individually. Spectral overlap is filled with grey.

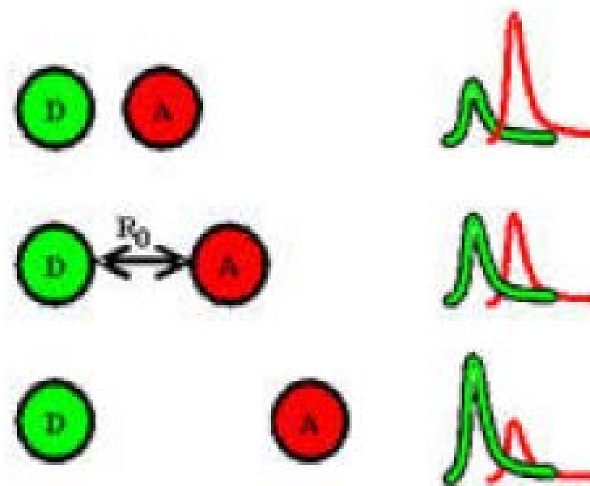


Calculating distances

$$r = R_0[\left(\frac{1}{E}\right) - 1]^{1/6}$$

You have to look up R_0 for your FRET pair of interest





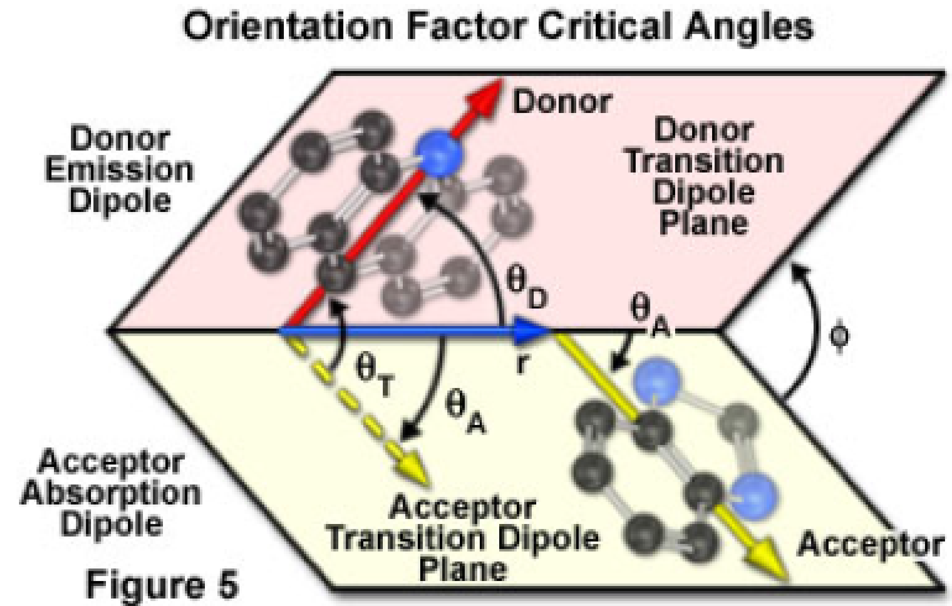
Donor	Acceptor	R_0 (Å)
Fluorescein	TRITC	55
Cy3	Cy5	>50
CFP	YFP	50
BFP	GFP	40
CFP	GFP	48
GFP	YFP	57

Distances can be measured from ~0.5 to 1.5 R_0

Orientation factor (κ)

- Dipoles don't emit isotropically in all directions
- Orientation of donor and acceptor will influence FRET ration

$\kappa^2 = 1$ – parallel dipoles
$\kappa^2 = 4$ – parallel and linear
$\kappa^2 = 0$ – perpendicular
$\kappa^2 = 2/3$ – freely diffusing



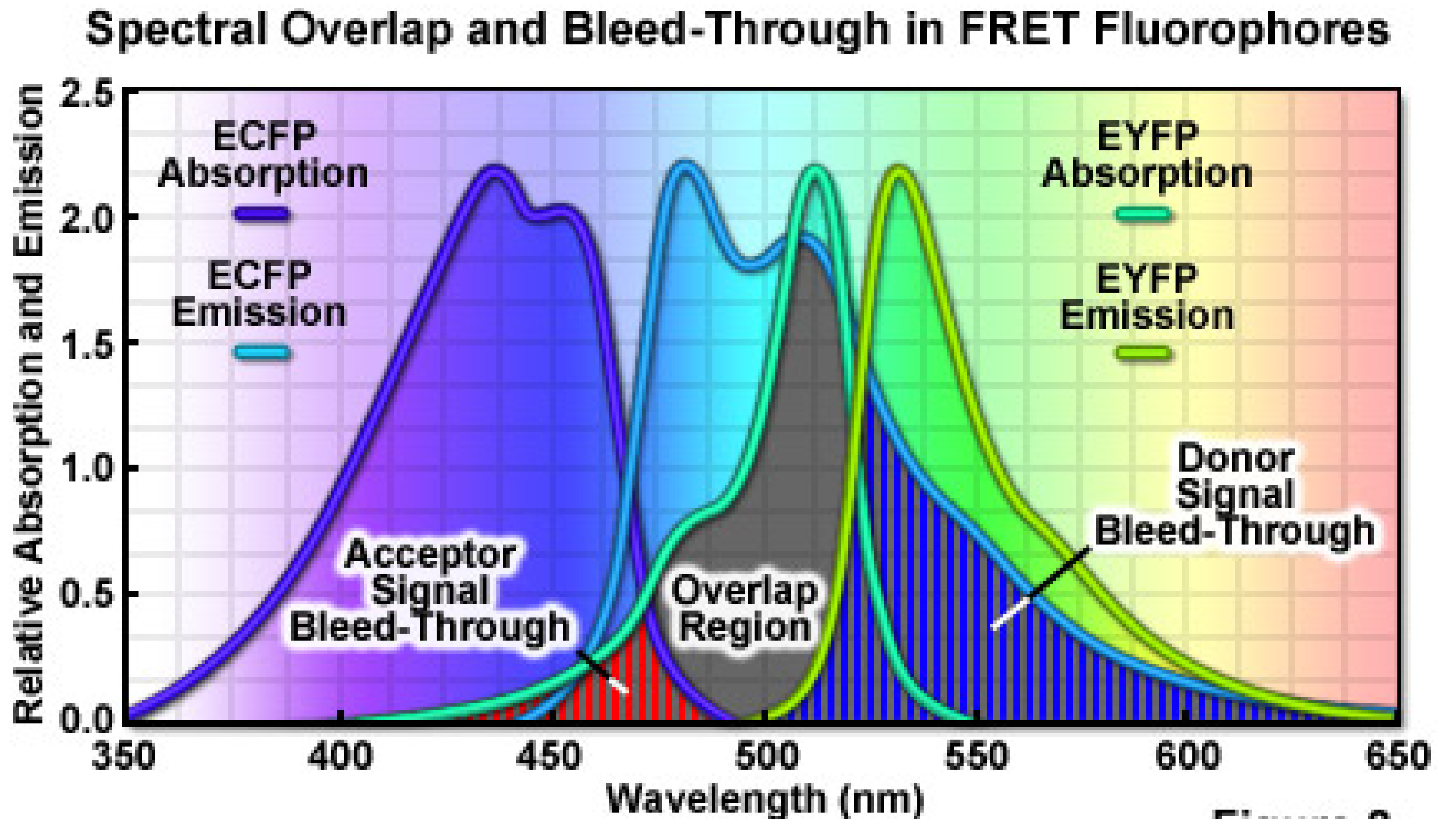
$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$
$$= (\sin\theta_D \sin\theta_A \cos_{\Phi} - 2\cos\theta_D \cos\theta_A)^2$$

FRET Bleedthrough and artifacts

Acceptor emission: How much is acceptor directly excited

Donor leakage: Does donor spectrum emit into acceptor band?

Photobleaching, gamma factor



And on to Matlab…