More FRET, TIRF

- Last class
  - Smoothing
  - Edge finding
  - Sharpening
- This class
  - Single molecule fluorescence
  - Applications and practicalities

FRET

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

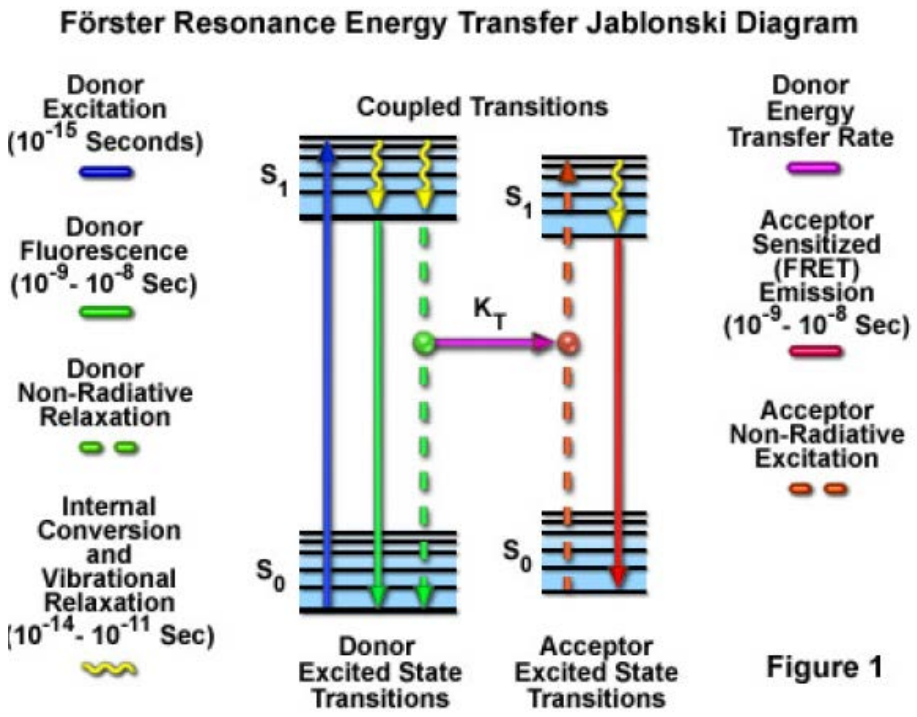


Figure 1

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

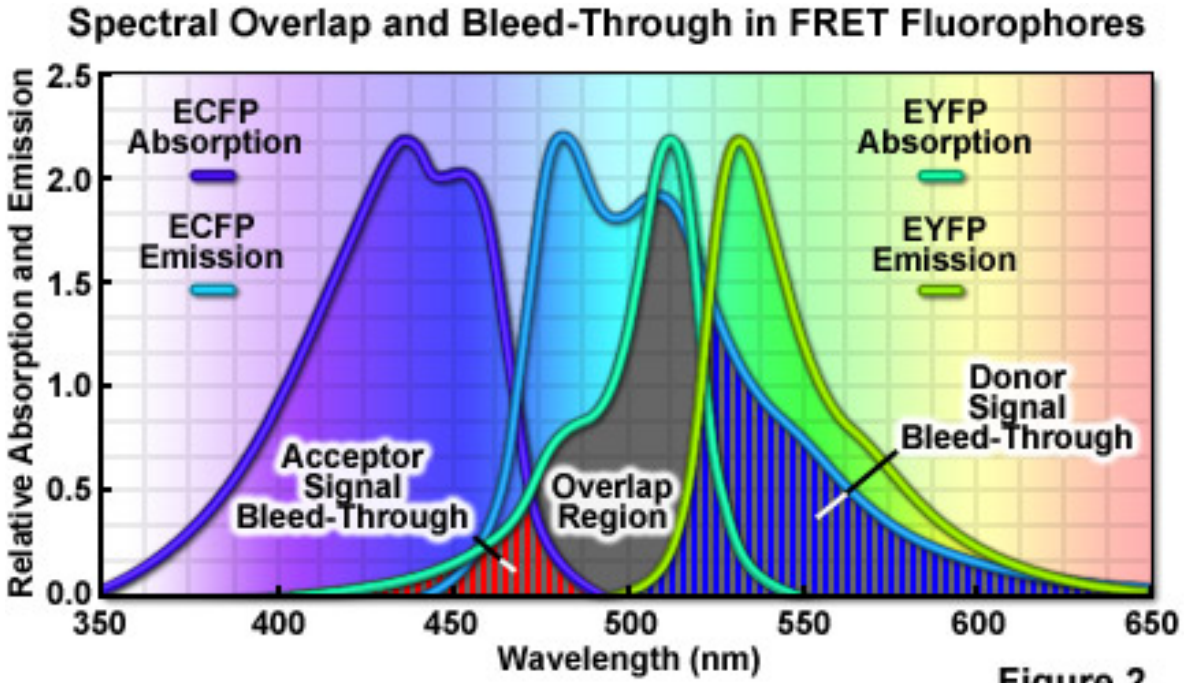
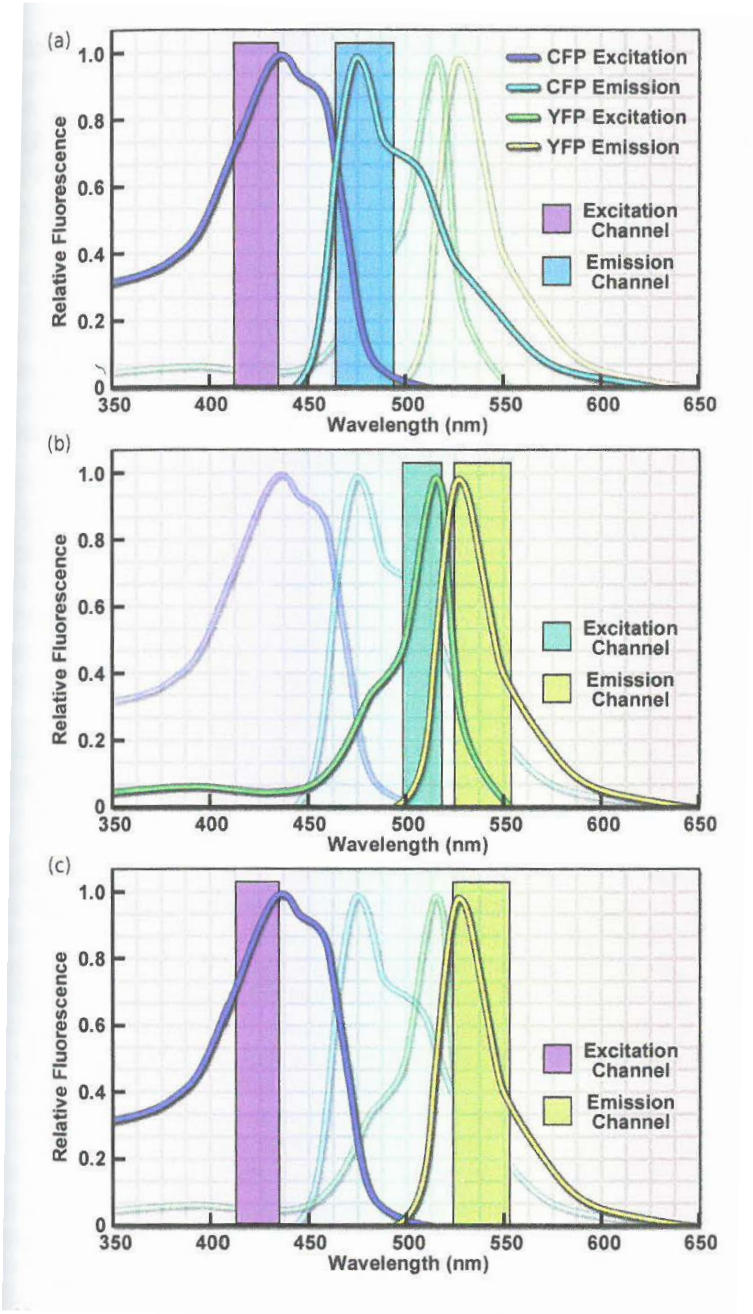


Figure 2

Sensitized emission FRET

- Widefield (image) detection of FRET
- 3 Cube FRET for single image
  - Donor Excitation/Donor emission
  - Acceptor Excitation/Acceptor emission
  - Donor Excitation/Acceptor emission
- Record, for your measurement conditions, leakage intensities and correct



Measurement of accurate FRET

Suitable to measure large changes in FRET (yes or no)

Best analysis comes if #donor < #acceptor

Quantitating in this way always adds noise

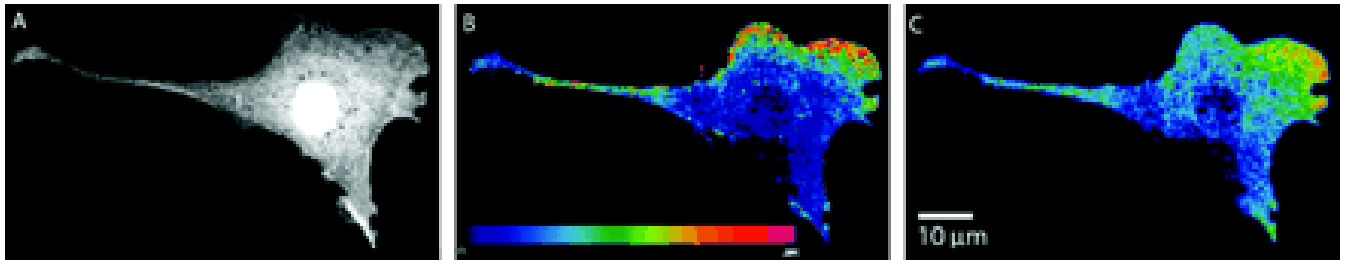
1. Subtract background separately from donor and acceptor channels
2. Measure the percentage of donor leakage (**β**) into acceptor channel by imaging a sample with donor only molecules.
3. Measure direct acceptor emission (**α**) by the donor excitation laser through imaging a sample with acceptor only molecules.
4. To determine actual FRET efficiency, one has to determine the correction factor, **γ**, which accounts for the differences in quantum yield and detection efficiency between the donor and the acceptor. γ is calculated as the ratio of change in the acceptor intensity, ΔI_A to change in the donor intensity, ΔI_D upon acceptor photobleaching ($\gamma = \Delta I_A / \Delta I_D$)

$$E = \frac{I_A}{I_A+I_D} \Rightarrow \frac{I_A-\beta I_D}{I_A+I_D} \Rightarrow \frac{(1-\alpha)I_A-\beta I_D}{(1-\propto)I_A+I_D} \Rightarrow \frac{(1-\alpha)I_A-\beta\gamma I_D}{(1-\propto)I_A+\gamma I_D}$$

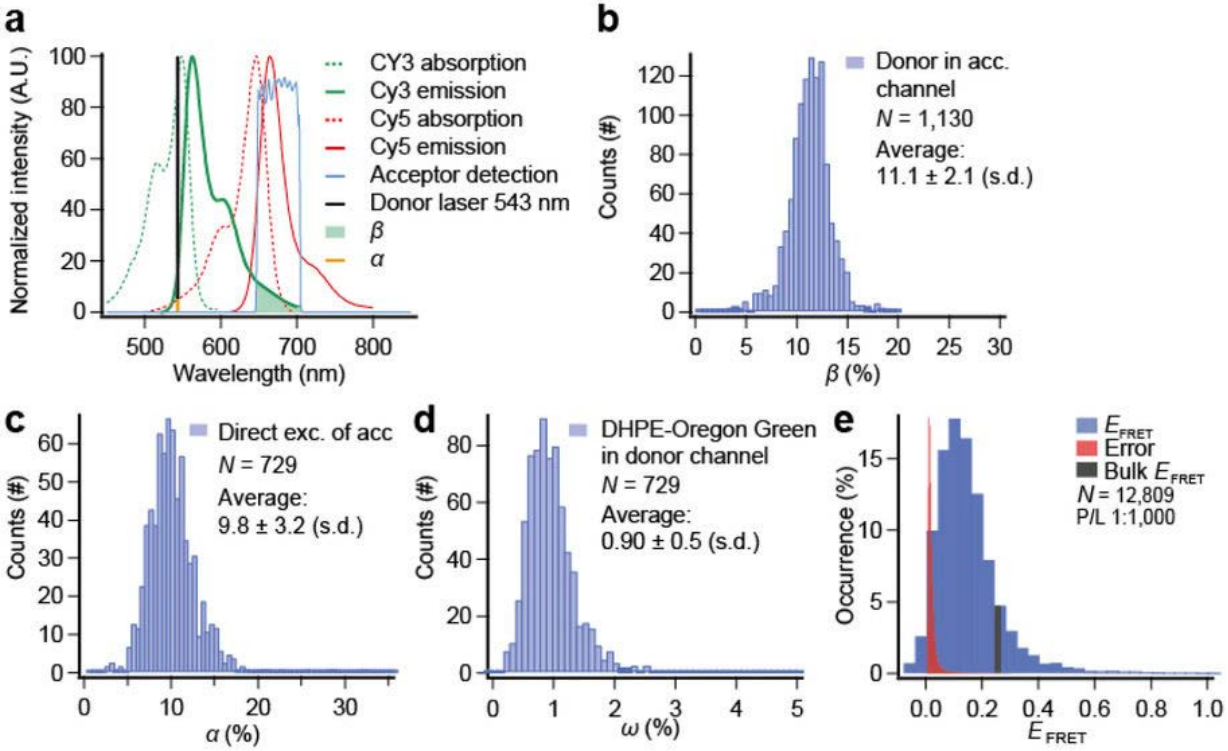
β α γ

Effects of correction

3T3 cells – V12 RAC GFP + AlexaPBD



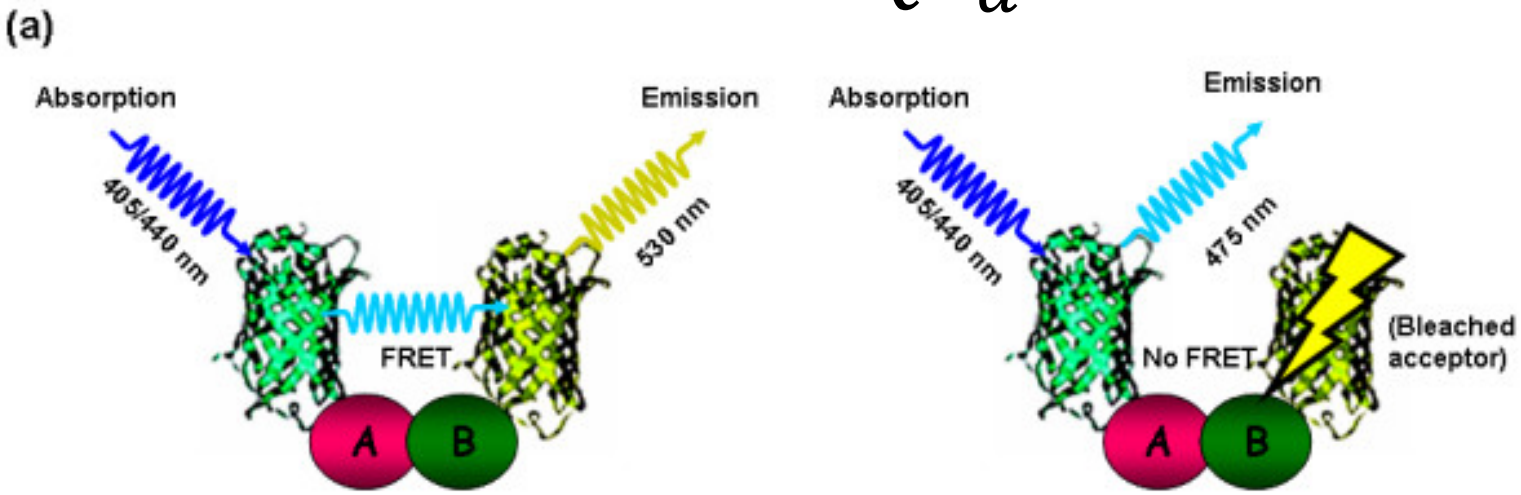
Donor alone | Uncorrected FRET | Corrected FRET



Photobleaching FRET

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

- Two images with one cube
  - Donor excitation/emission
  - Photobleach ONLY acceptor
  - Donor excitation/emission
- Can not photobleach donor
- Built in control because it's on the same molecule
- If there is movement, it can confound signal

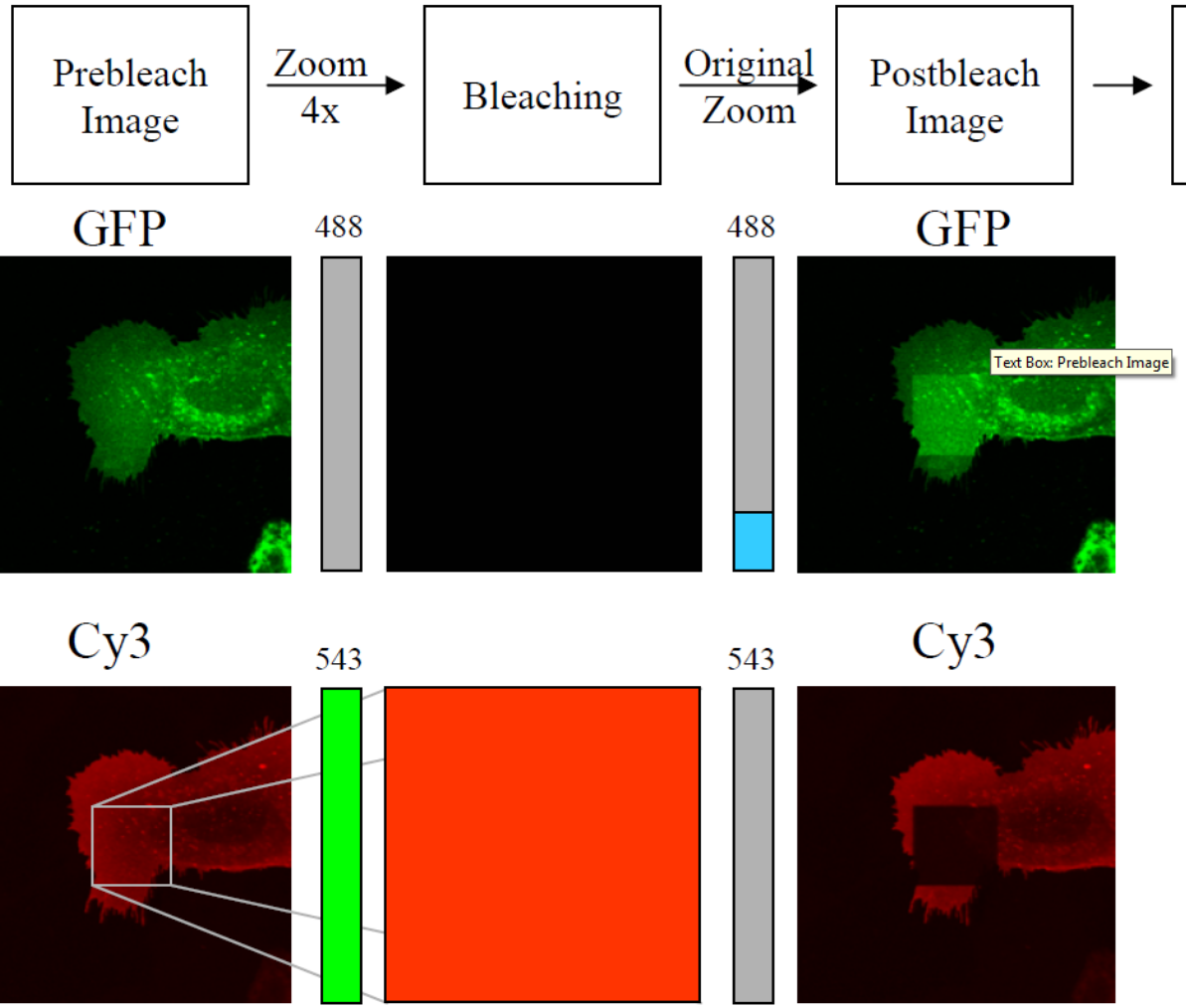


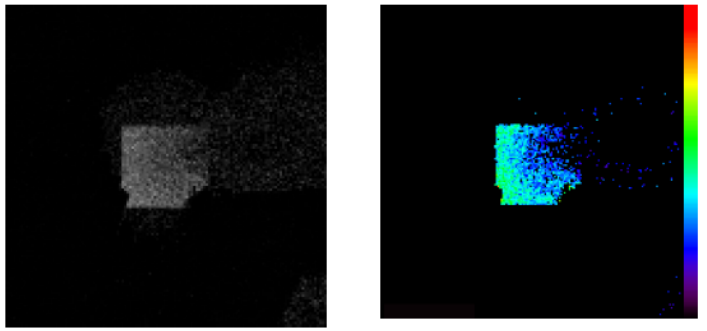
$$QY_{d,FRET} = \frac{k_f}{k_f + k_{nr} + k_T} \qquad \tau_{d,FRET} = \frac{1}{k_f + k_{nr} + k_T}$$

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

Scheme for photobleaching FRET

$$FRET = E = \frac{D_{post} - D_{pre}}{D_{post}}$$





$$E_A(i) = 1 - \frac{F^D(i)}{F^D_{pb}(i)} = E \cdot \alpha_D(i)$$

An apparent FRET efficiency (product of the efficiency of the FRET pair and the amount of interacting donor) can be calculated

Ensure that you do NOT photobleach donor

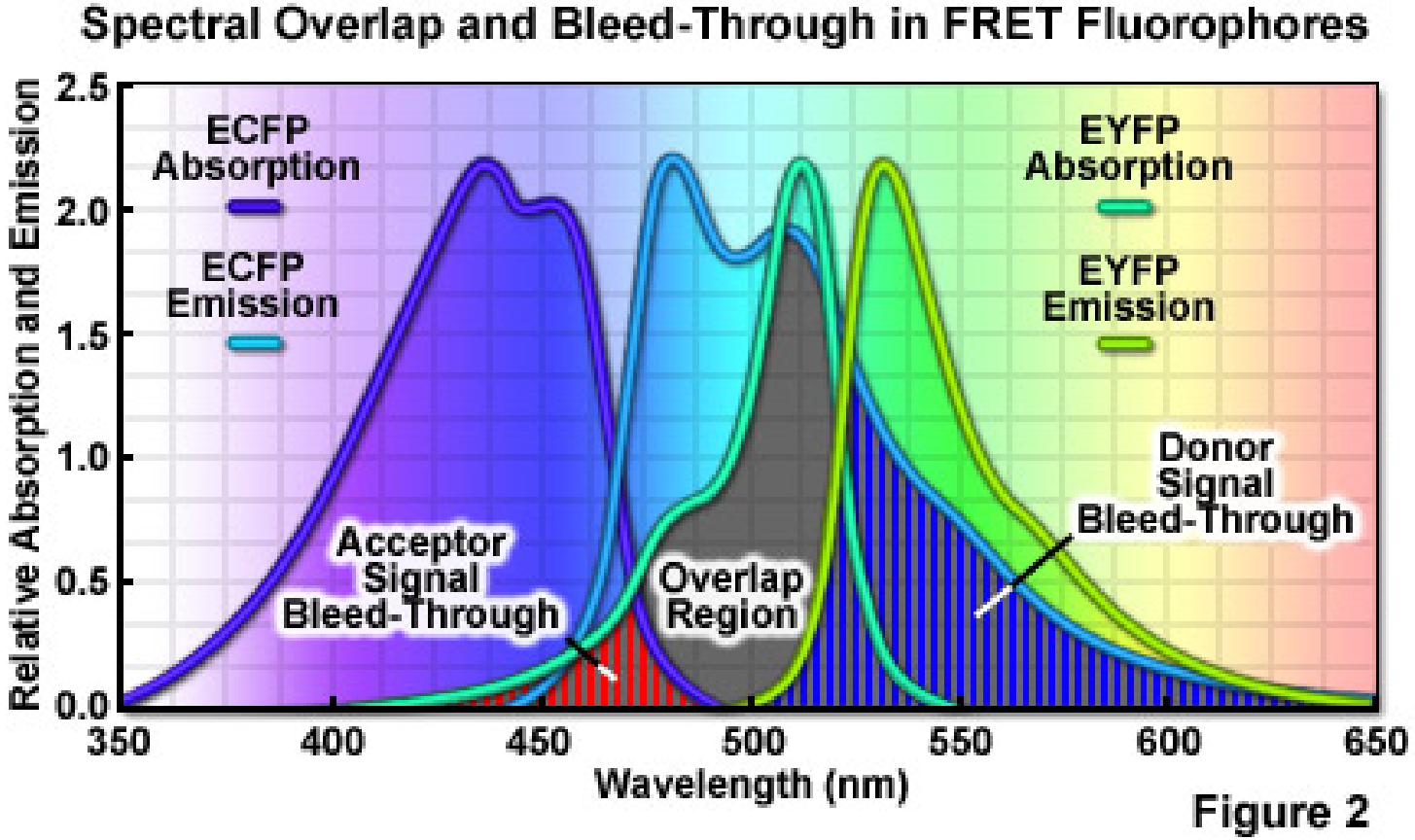


Figure 2

Limitations of FPs

- Fluorescence properties always worse than dyes
- Broader spectra -> more bleedthrough
- Due to their large size, high FRET signals can't be seen
- GFPs tend to oligomerize
- Organic dyes are far better in practice, but hard to get into cells
- Quantum dots can be used, but can't get into cells

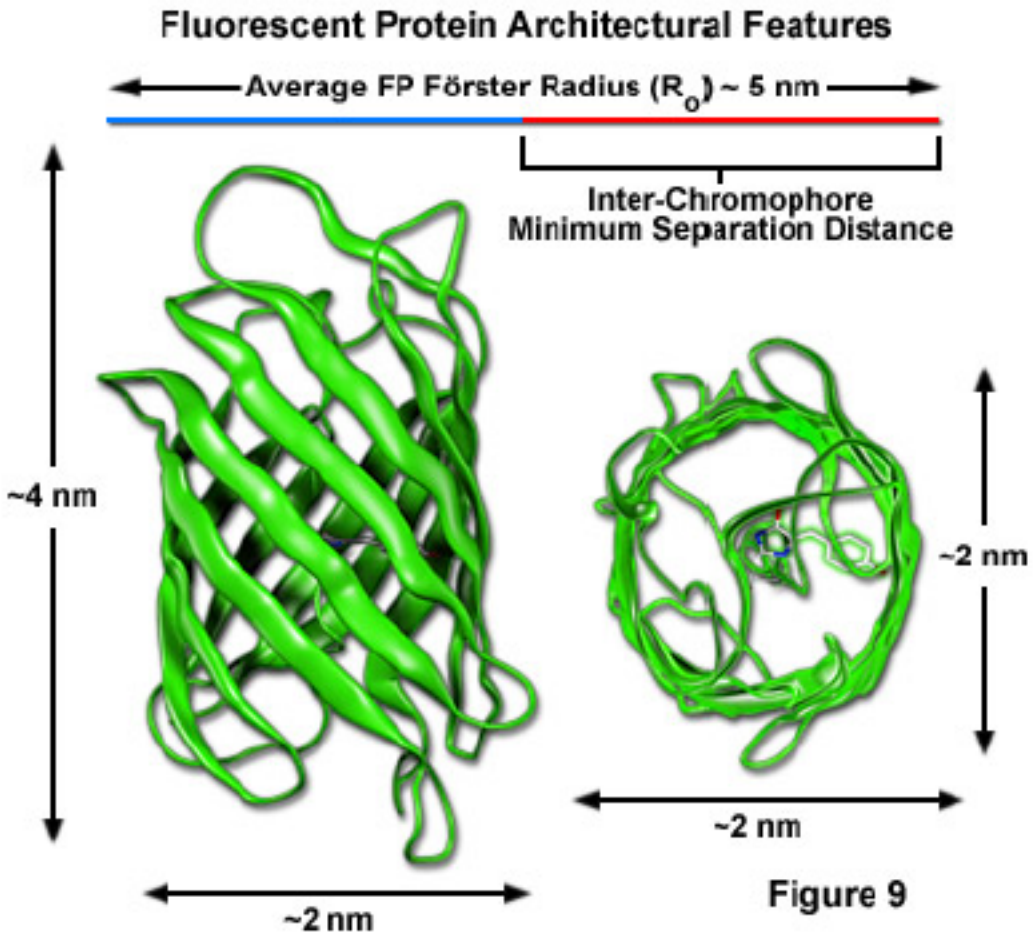


Figure 9

Applications of FRET

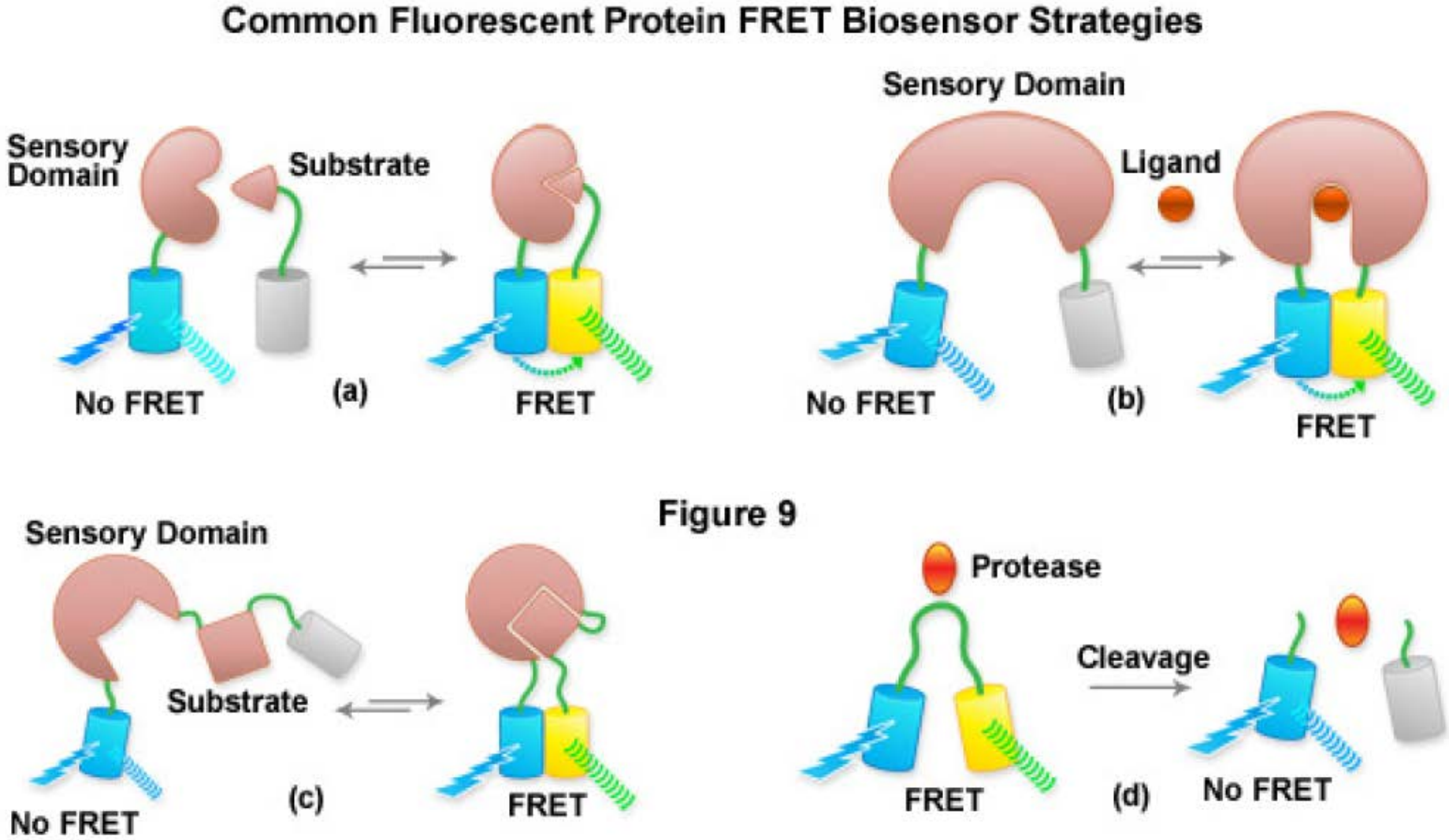
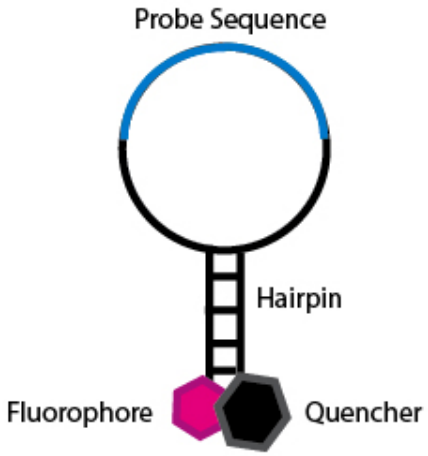


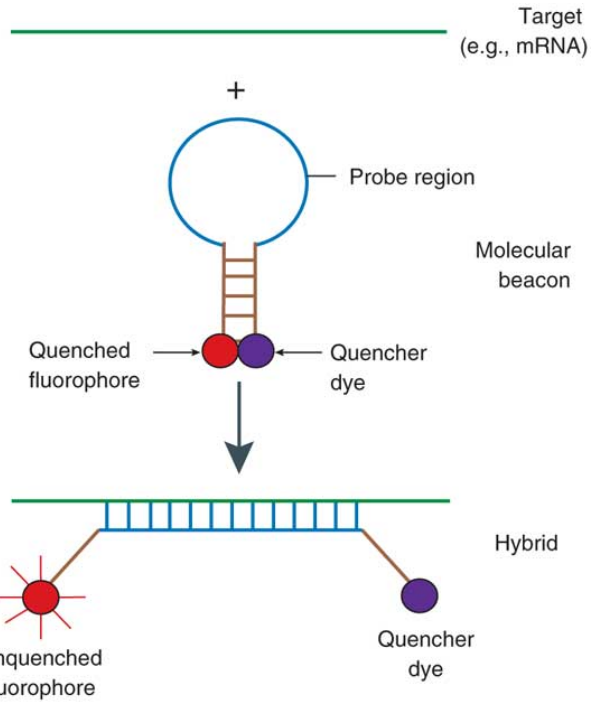
Figure 9

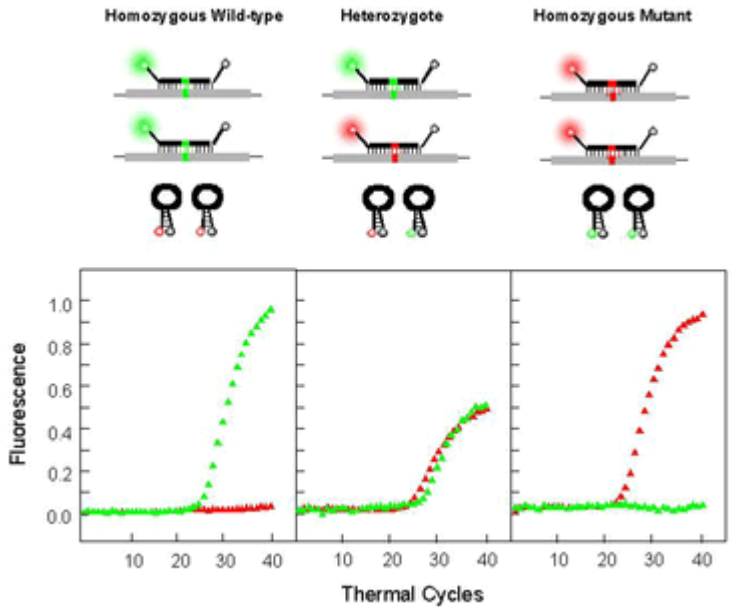
Idea of quenching goes beyond FRET

- Quenchers can absorb excited electrons, and then dissipate energy by vibrations
- Quenchers can be non-fluorescent dyes or metal particles

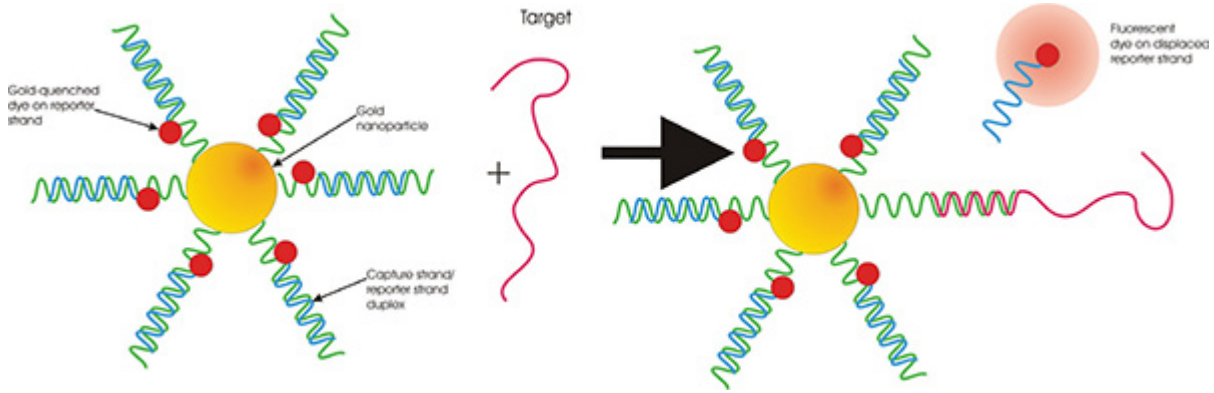
Molecular beacons

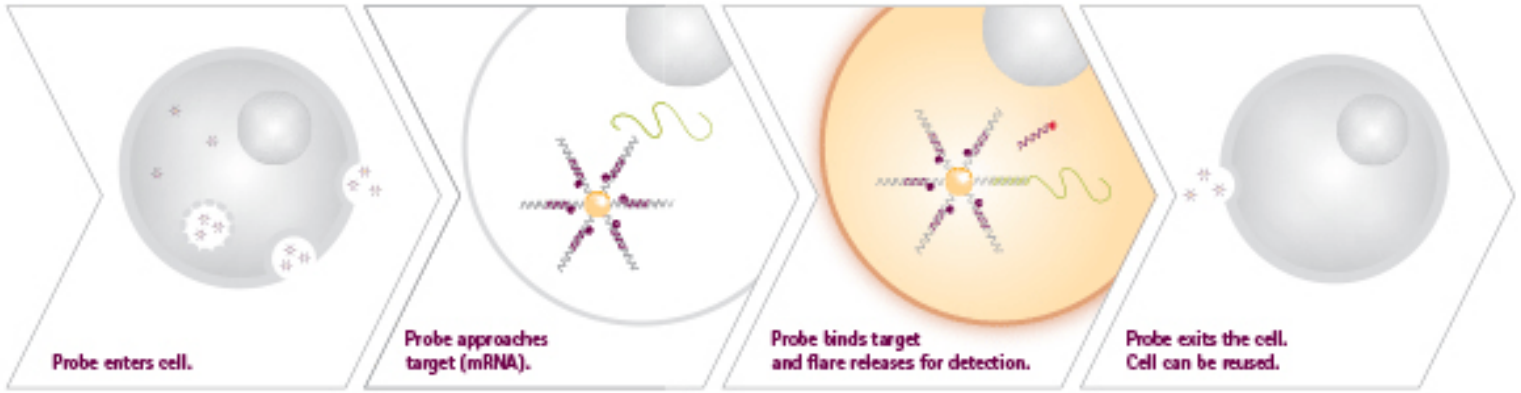


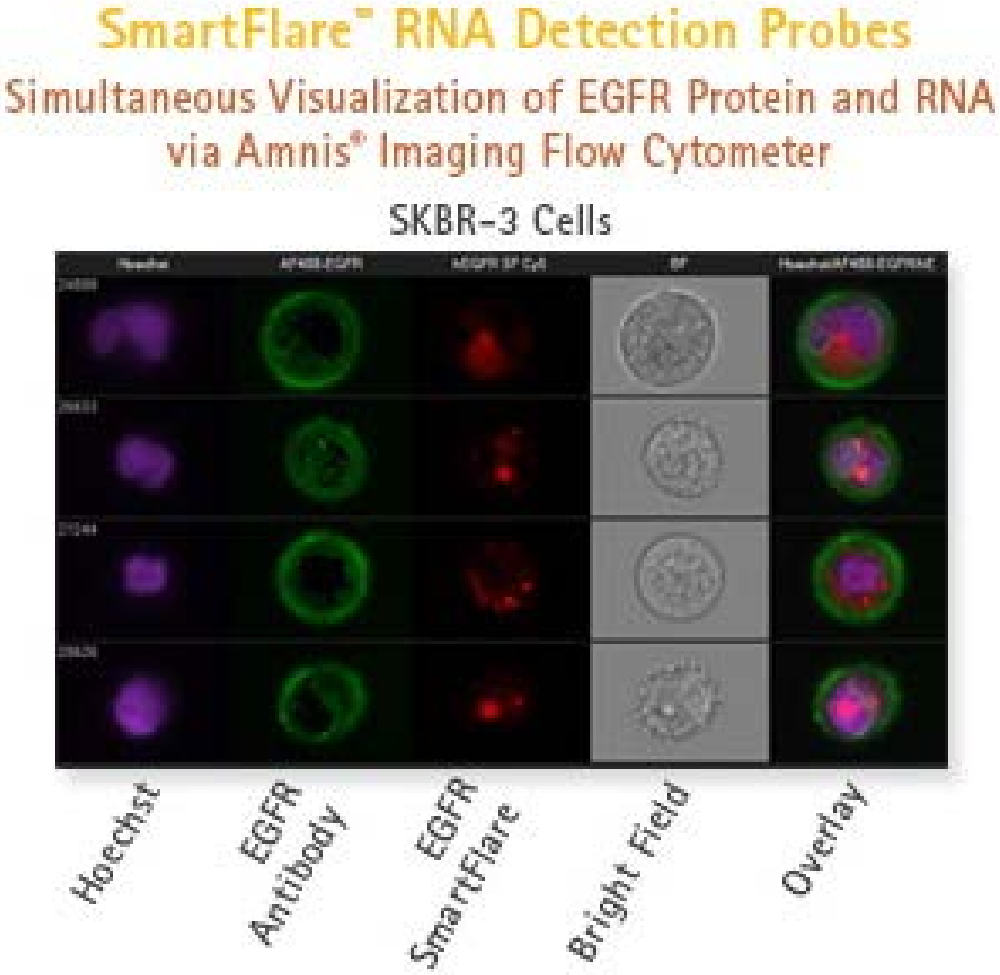




Smart Flares – imaging RNA in live cells







TIRF microscopy

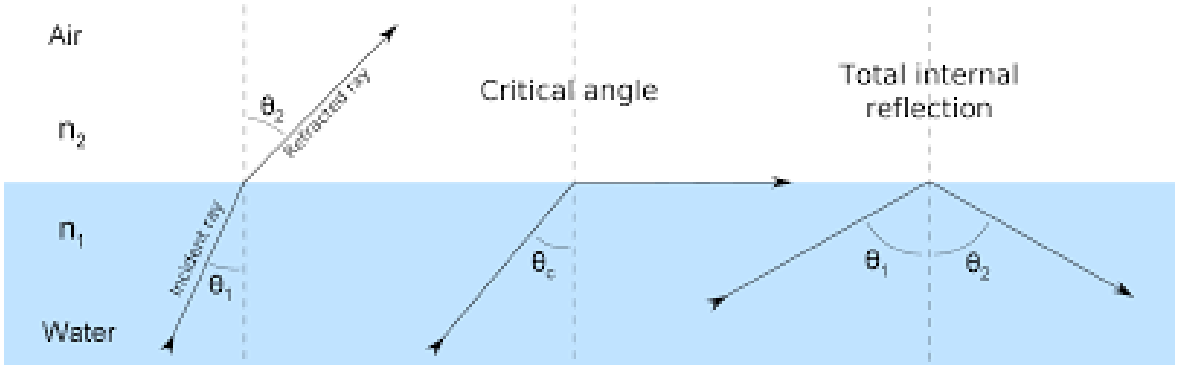
TIR review

- Snell's law governs the angle of reflection
- At the critical angle, the light travels along the surface of the interface
- As the angle increases, wave is totally reflected
- $E_{in} = E_{out} = E_{trans} + E_{refl}$

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{v_1}{v_2} = \frac{\lambda_1}{\lambda_2} = \frac{n_2}{n_1}$$

$$\theta_c = \theta_i = \arcsin\left(\frac{n_2}{n_1}\right),$$

n_1 = glass = 1.52
n_2 = water = 1.33

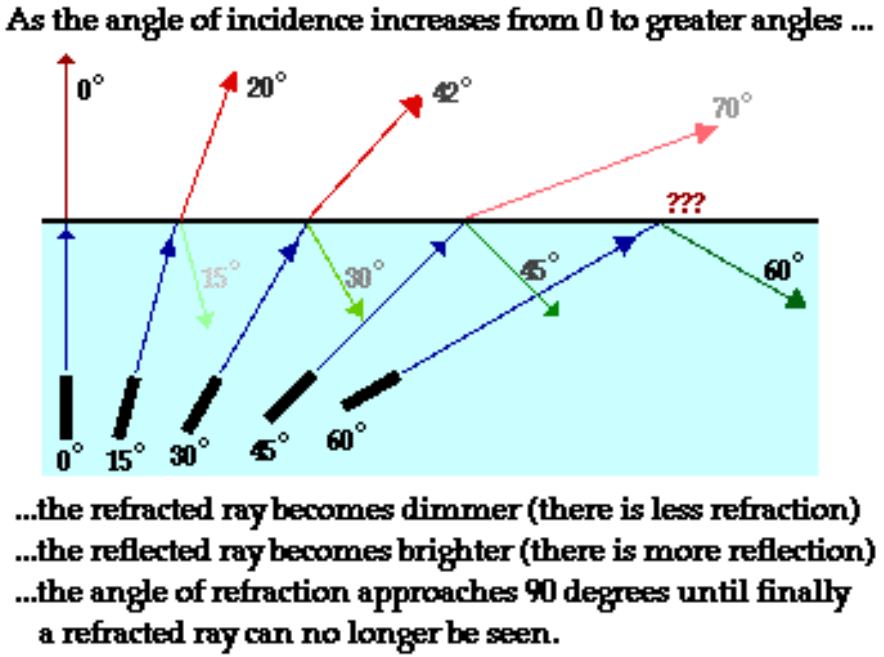


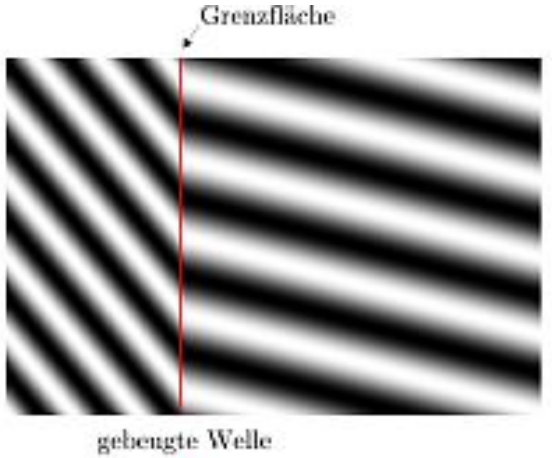
Total internal reflection fluorescence TIRF basics

- Transmission does not occur larger than the critical angle

$$\theta_c = \theta_i = \arcsin\left(\frac{n_2}{n_1}\right),$$

- No transmitted wave, but there is an evanescent wave





TIRF probes very close to surface

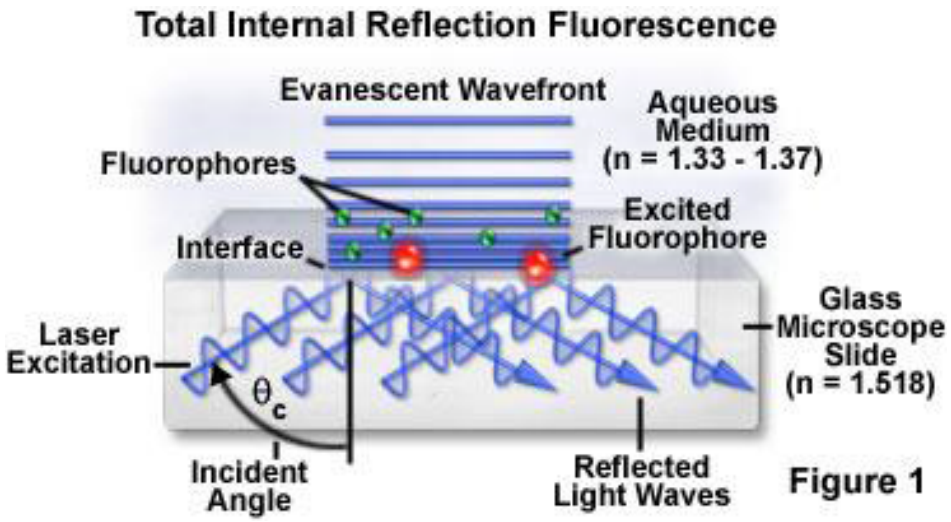
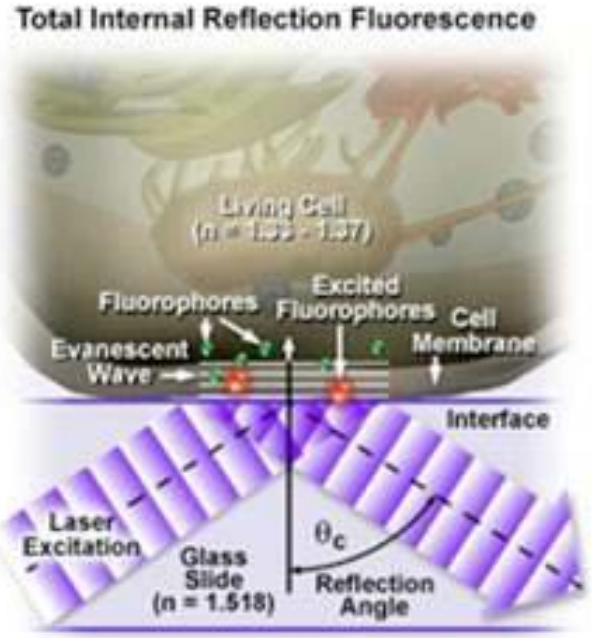


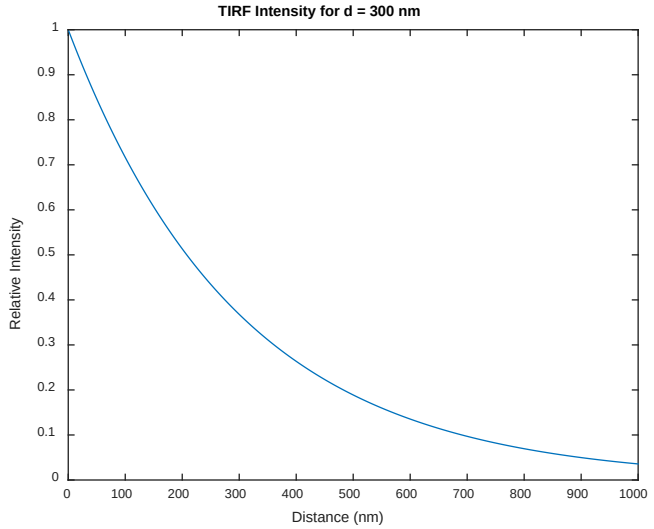
Figure 1

$$d_p = \lambda_0 / \left(2\pi \cdot n_1 \sqrt{\sin^2 \theta_1 - (n_2 / n_1)^2} \right)$$

$$I(z) = I_0 e^{-z/d}$$



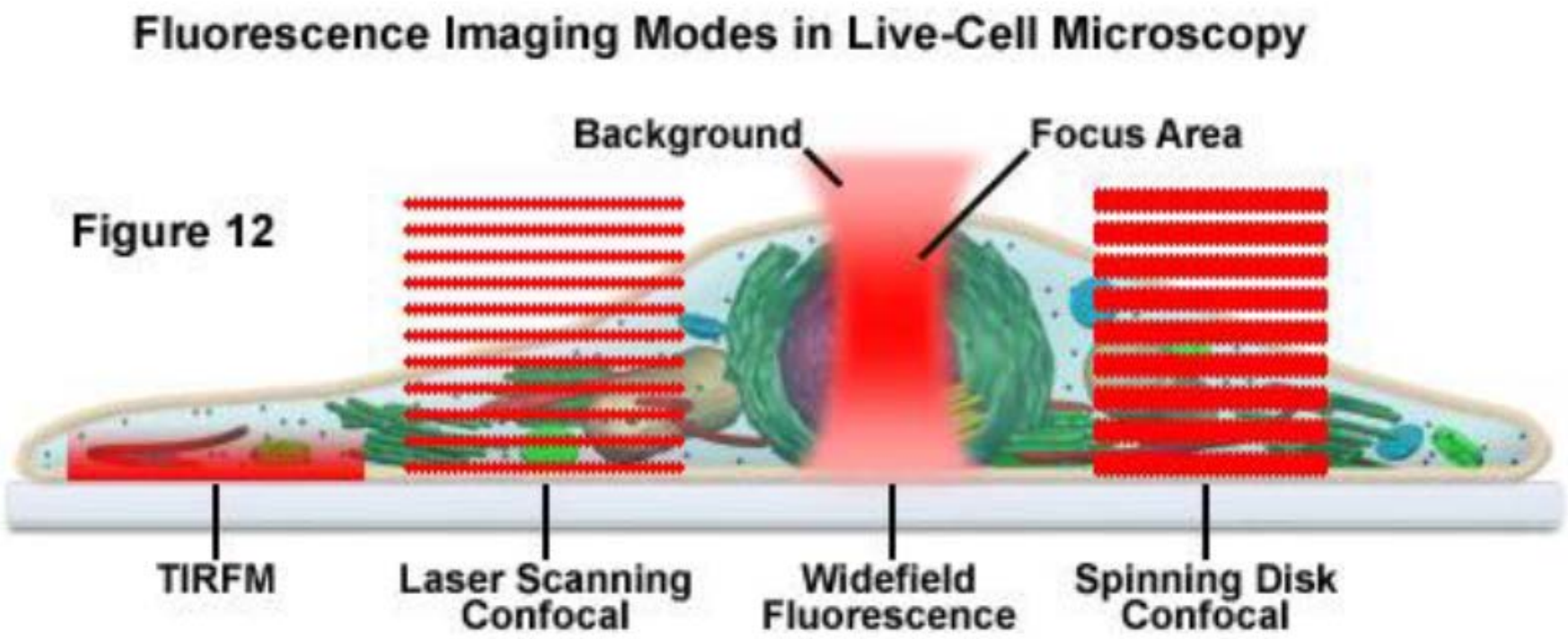
Distance (Nanometers)	Relative Intensity
1	0.99
10	0.92
100	0.43
1000	0.0002



Extremely useful for looking at objects in, or very near the plasma membrane attached to the glass

TIRF advantages

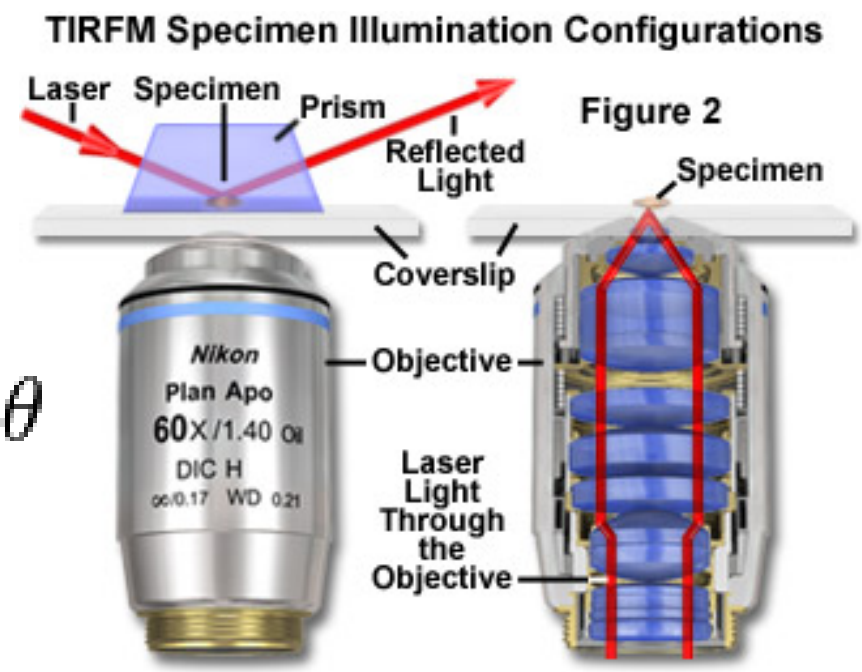
- TIRF is the first technique to use CONFINEMENT of illumination light
- Wide and thin illumination profile
- Reduced fluorescent background
- Allows high time resolution
- Ideal for thin and wide cell studies

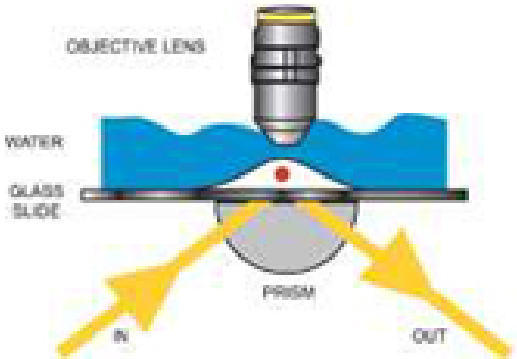


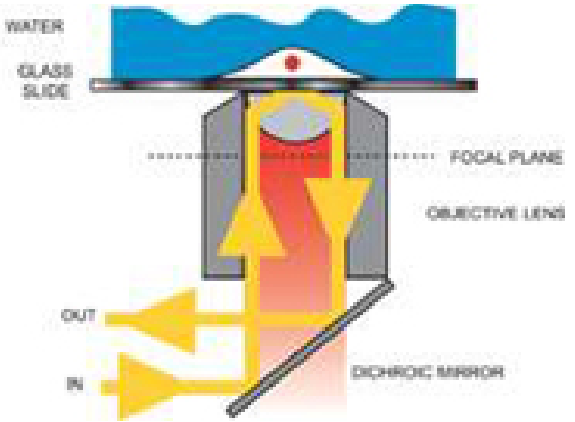
Two TIRF geometries

- Prism TIRF – Samples are under prism, lower background fluorescence, relatively easy to set up
- Objective TIRF– cells are easily placed, accessible to patch. Requires expensive objective (high NA)

$$\mathrm{NA} = n \sin\theta$$

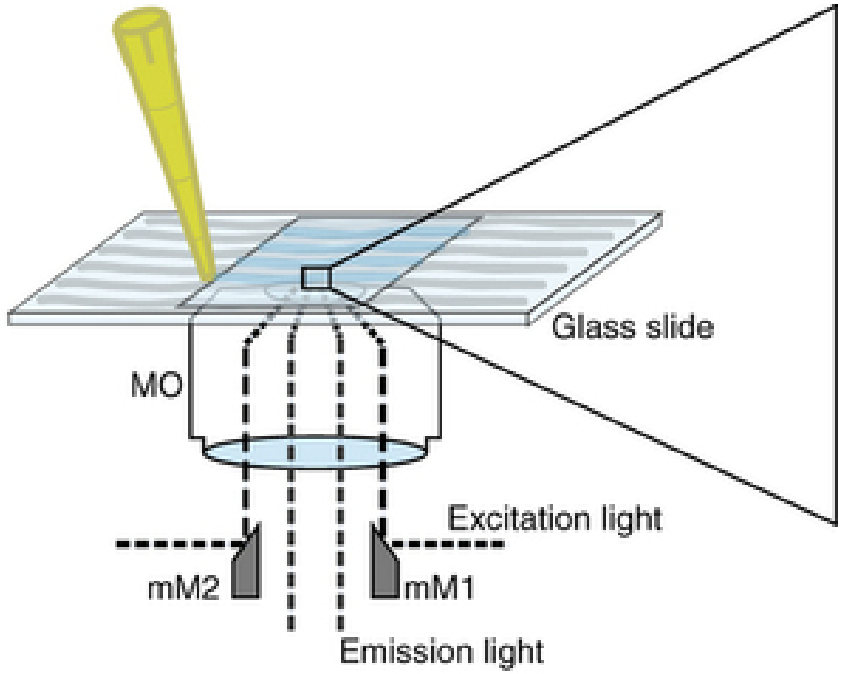


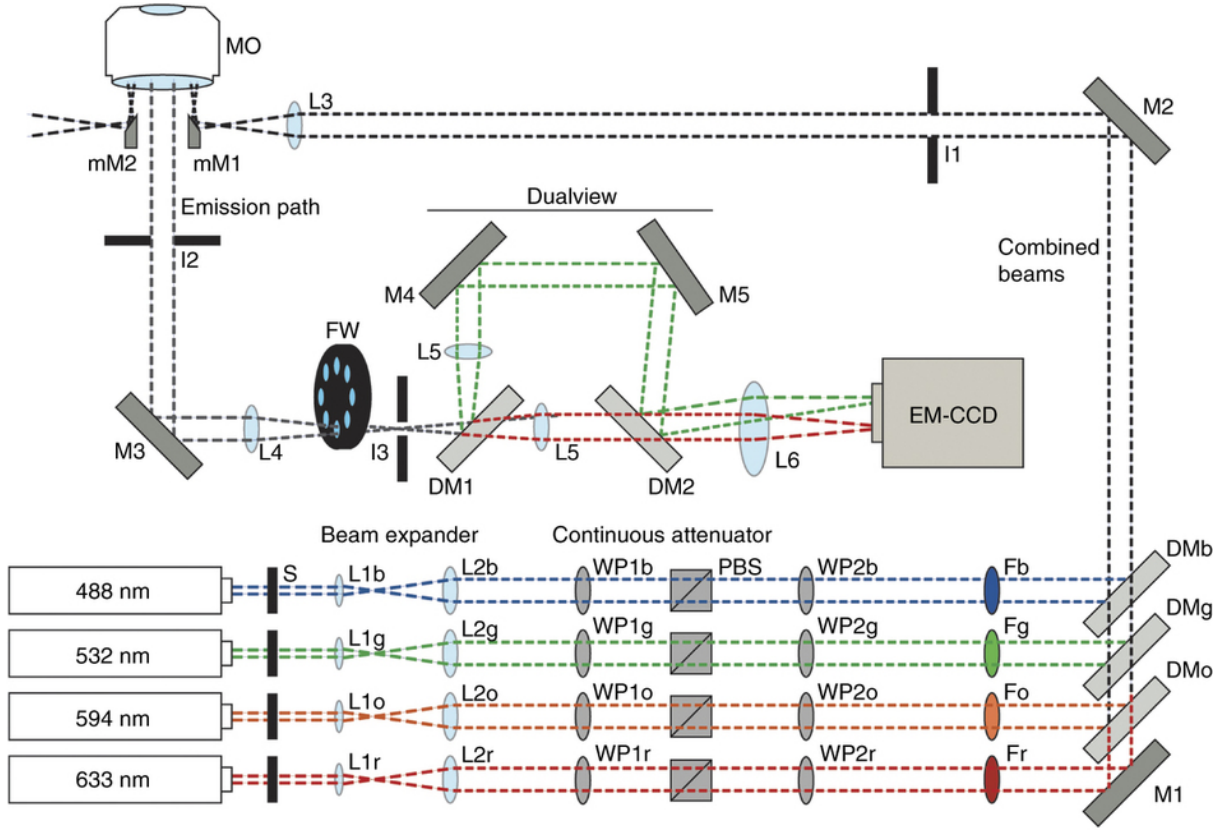




Micromirror TIRF – and dual emission

Prevents direct laser light from being imaged on camera





And on to Matlab…