Fluorescence Microscopy

Chelsea Aitken
Peter Aspinall

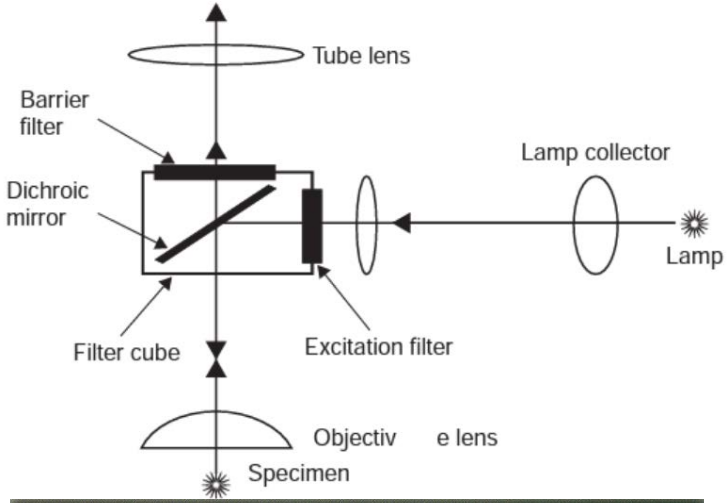
Advantages Over Light Microscopy

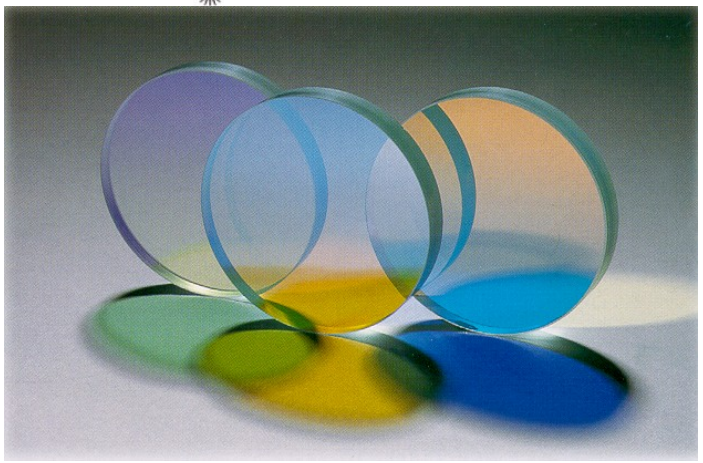
Method	Resolving power	Probed volume
Conventional microscopy	$\lambda/2$	10 μl
Confocal microscopy	$\lambda/3$	Less than 1fl
4Pi microscopy	$\lambda/3$	About 1fl or less
Standing wave illumination	$\lambda/4$–$\lambda/5$	About 1fl or less
Stimulated emission depletion microscopy	$\lambda/6$	Less than 1 al

- Resolution of light microscopy limited by Rayleigh Criterion
  - If two objects cannot be seen as distinct structures, then they may be considered coincident in space
  - Unable to determine whether there are molecular associations
- Fluorescence microscopy can determine the distance between two molecules to 20 – 100 Å

Wide-Field Fluorescence Microscope

- Molecule (fluorophore) absorbs a photon and then quickly reemits a lower energy photon
  - Change in energy allows us to filter out incident light
- Uses epi-illumination
  - Light source goes into a filter cube and is reflected into the sample
  - Emission returns through same objective and filter cube
  - Because of its longer wavelength, it passes through the dichroic mirror and is read

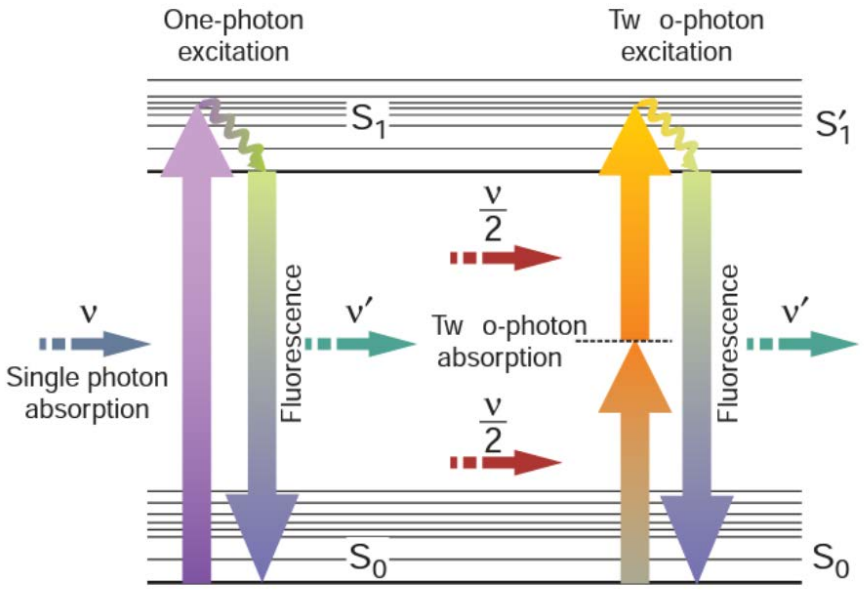




http://upload.wikimedia.org/wikipedia/commons/4/49/Dichroic_filters.jpg

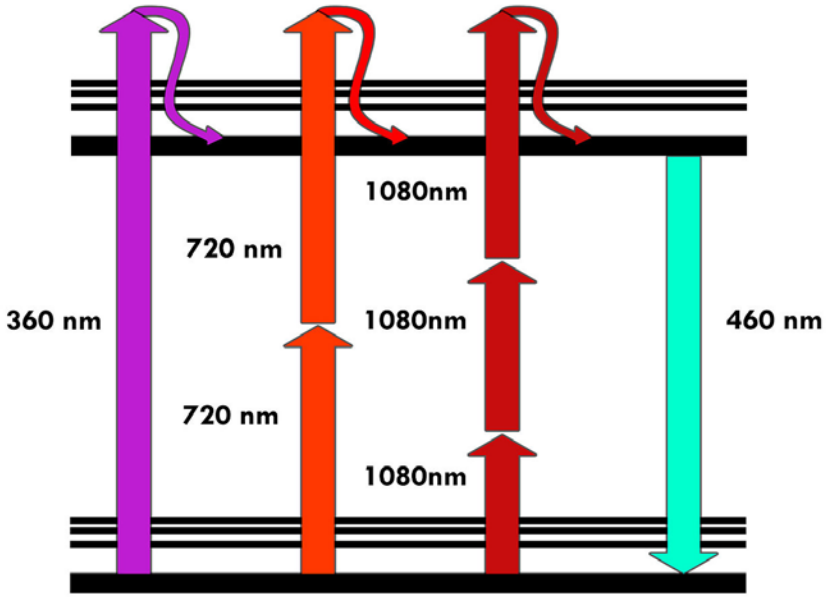
Two-Photon Excited Microscopy

- Simultaneous absorption of two photons causing the fluorophore to emit a higher energy (2x) photon
  - Simultaneous = ~ 10^{-18} s
- However to generate the same number of two-photon events, the laser needs to be ~10^6 times more powerful than for one-photon events
  - Use mode-locked (pulsed) lasers
    - Intensity at peak is great enough to cause two-photon events



Three-Photon Excited Microscopy

- Three-photon events
- Photon density needed is only ten times that needed for two-photon events
- Useful to excite fluorophores that fluoresce at very short wavelengths (that can be difficult to produce)



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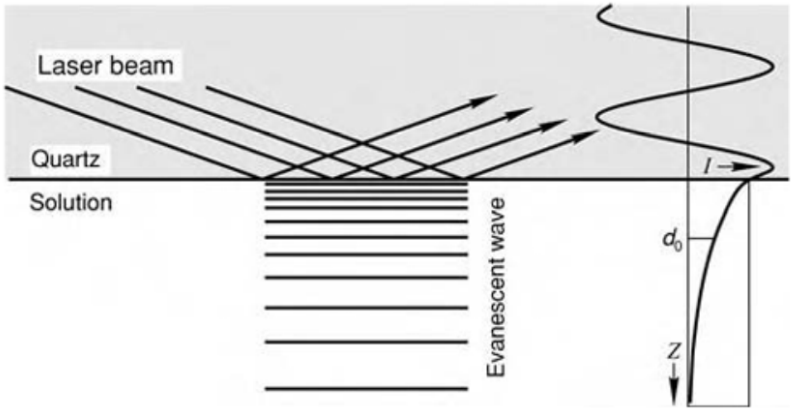
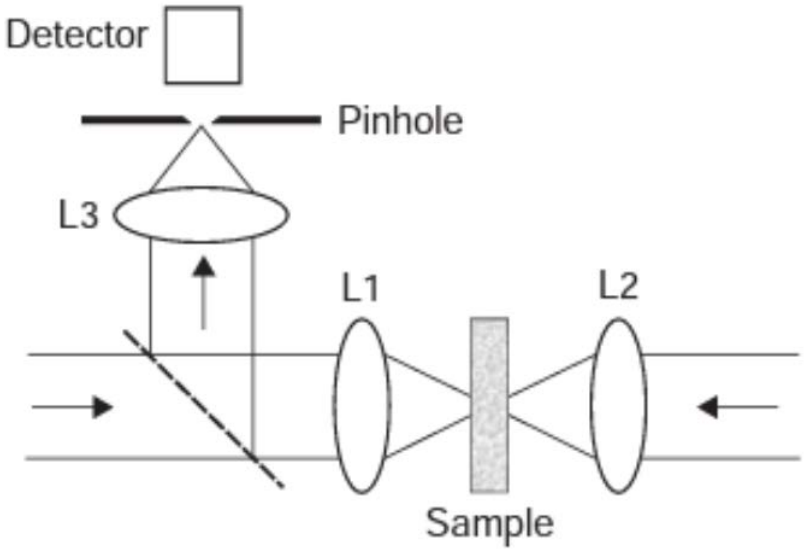
Total Internal Reflectance Fluorescence Microscopy (TIRFM)

- Laser is pointed through one medium in contact with the medium of interest
- Because of differences in refractive indices the light only reaches a short distance into the solution
- $d_p = \frac{\lambda_0/n_2}{4\pi\sqrt{sin^2\theta-(n_2/n_1)}}$
- Allows us to image cellular binding structures



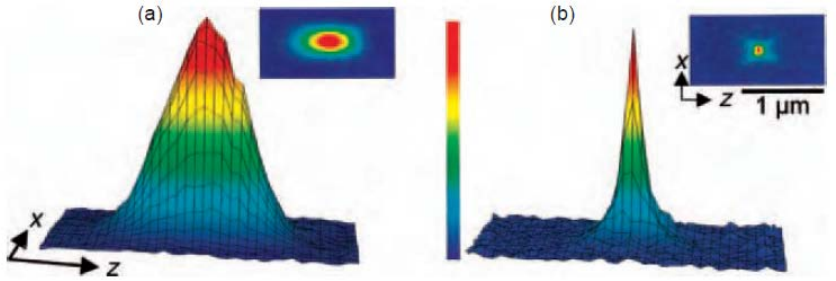
4Pi-Confocal Microscopy

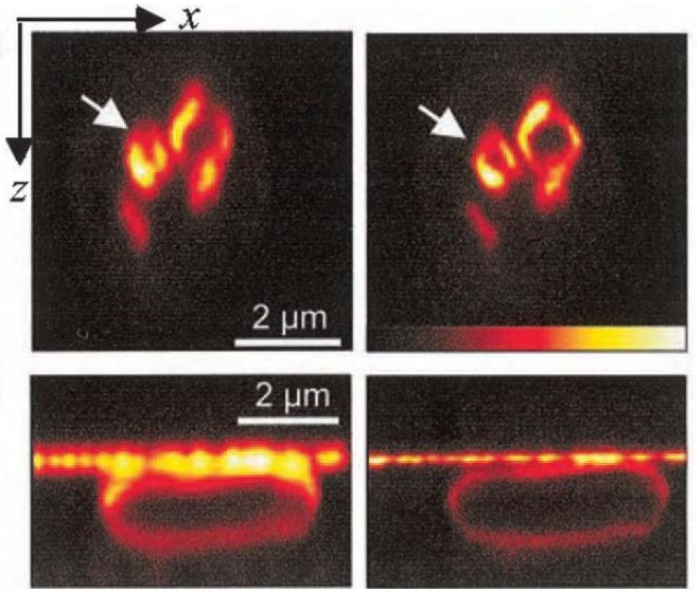
- Uses two objective lens
  - One to illuminate
  - One to observe
- This doubles the aperture angle making it possible to have an aperture angle of 4pi
- Produces clearer more detail images
- Can be further improved by combining this setup with standing wave microscopy



Stimulated Emission Depletion Microscopy (STED)

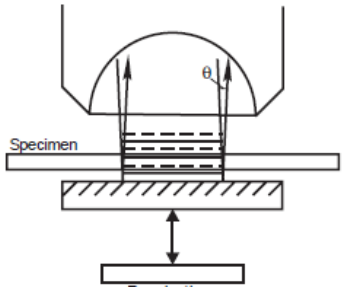
- Fluorophores in a small area are excited by a short pulse of light (200 fs)
- Then fluorophores around this area are forced back to ground state by a second longer pulse (40 ps)
- This creates a very sharp peak of fluorescence and increases resolution

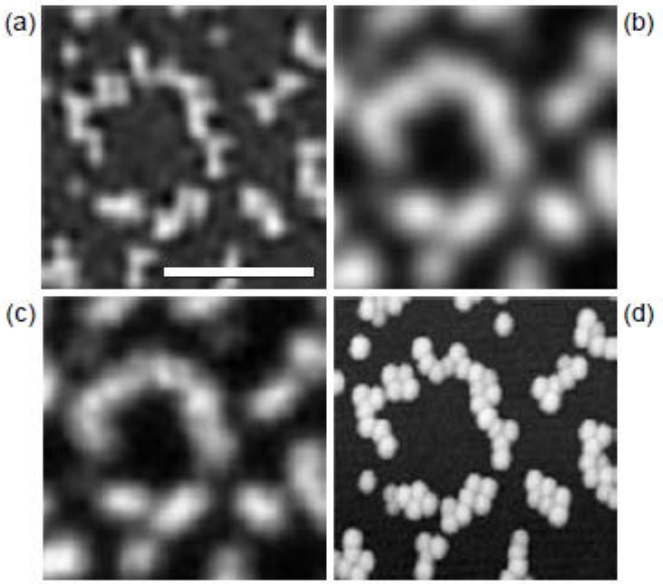




Standing-Wave Illumination Fluorescence Microscopy (SWFM)

- Two laser planes cross in the solution and creates an interference pattern
  - The nodes have spacing:
    - $\Delta s = \lambda/(2n\cos\theta)$
  - Theta (the angle between the two planes) can be varied to reduce the node spacing to $\lambda/2n$
- This allows it to have a better resolution than other methods



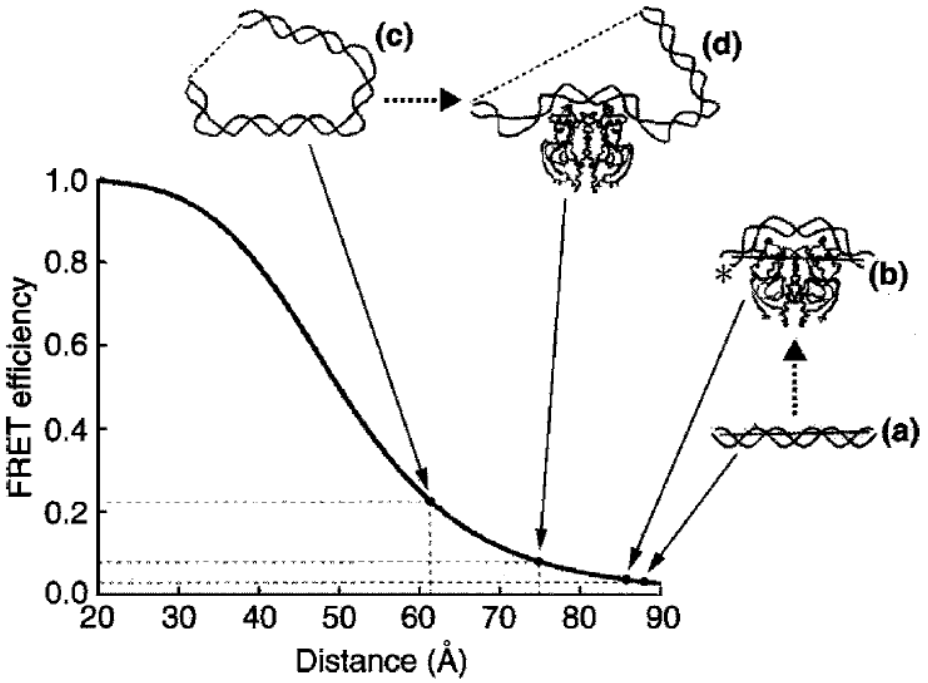


Fluorescence Resonance Energy Transfer (FRET)

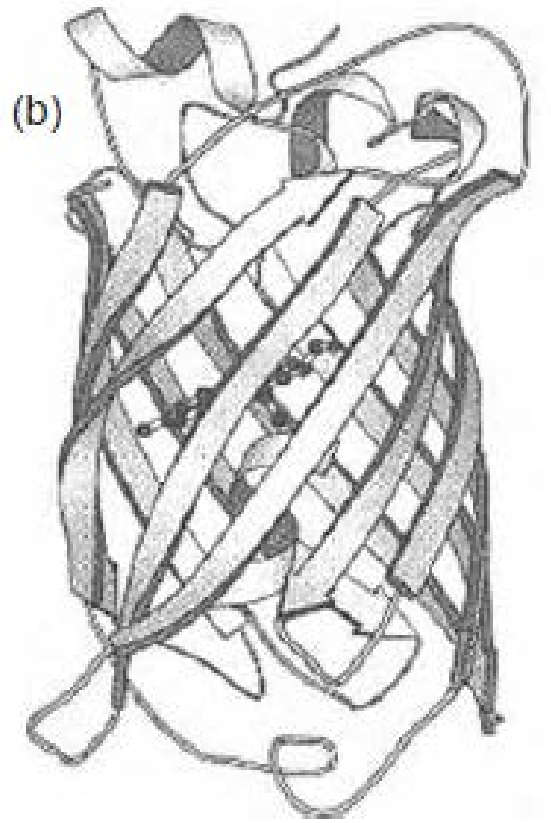
- A donor fluorophore is excited and then transfers its energy to an acceptor (if its close enough) through dipole-dipole interactions
- Measures molecule interactions efficiency (E_T)
  - $E_T = \frac{R_o^6}{R_o^6 + R^6}$
    - R_0 is the distance at which 50% of the energy is transferred
    - $R_o = [k^2 \times J(\lambda) \times n^{-4} \times Q_D]^{1/6} \times 9.7 \times 100$
      - k^2 – relative position of dipoles
      - $J(\lambda)$ – integral of the overlap between the acceptor and donor spectra
      - Q_D – quantum yield of the donor
- This method can be used as a quantum ruler (solve for R,) since ET can be measured and R_0 can be calculated

Applications of FRET

- Very useful for studying how DNA's form changes when introduced to certain proteins
- Label both ends of DNA and then can measure how the distance changes
  - Much better at measuring changes in distance than absolute distance
- Very good spectroscopic ruler for 20 – 100 Å range
  - However cannot detect dynamic events



Green Fluorescent Protein (GFP)

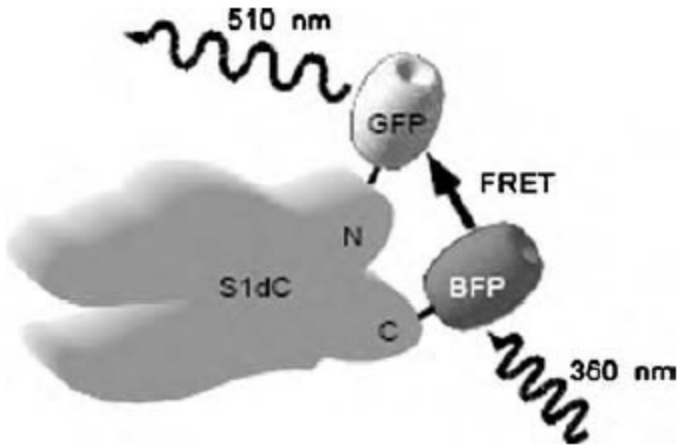


- Not all molecules fluoresce, so to use fluorescence microscopy they need to be fluorescently labeled
- Dye molecules have to bind to specific location and not interfere with the reaction being monitored or the cell in general
- Use GFP from *Aequoria Victoria* (type of jellyfish)

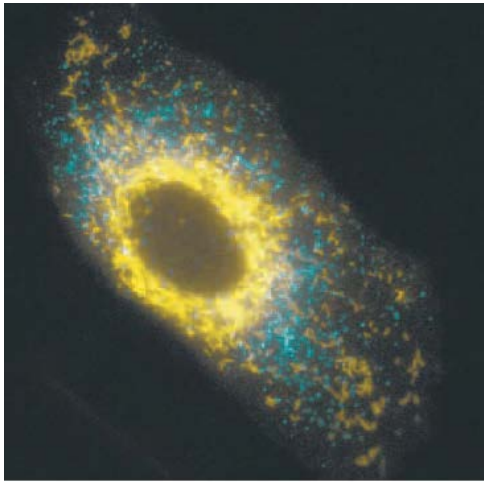
Pros/Cons of GFP

- Pros
  - When expressed is spontaneously fluorescent
  - Doesn't interfere with bound protein function
  - Can target specific organelles
  - Mutants have varying fluorescent properties
- Cons
  - Limited sensitivity
  - Very large -> limits resolution
  - Can undergo color changes from irradiation independent from FRET
  - Takes hours to fold into its fluorescent shape

Applications of GFP



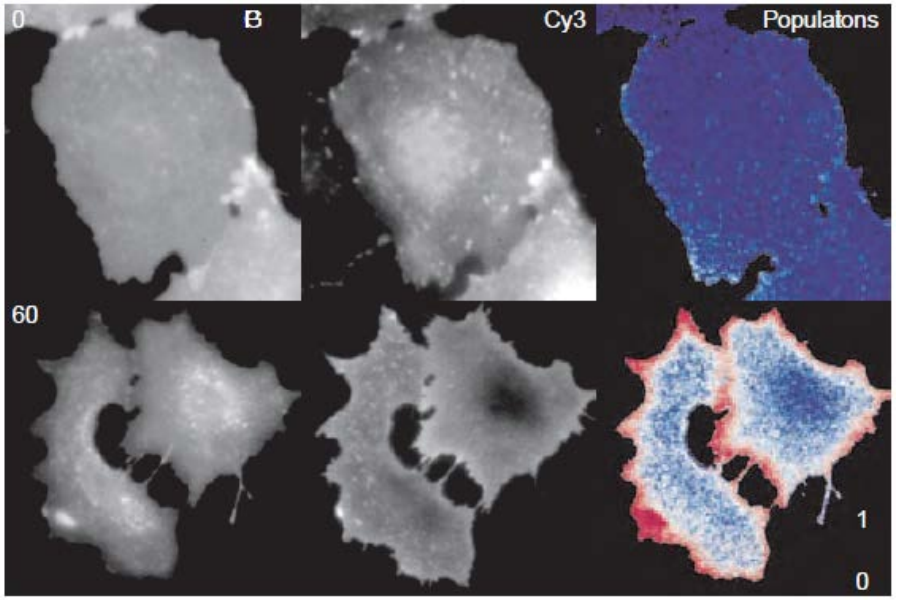
- Conformational Sensor
  - Uses FRET between GFP and BFP to measure distance
  - Position varies as the bound protein undergoes structure changes
  - Can use reemitted wavelength of light to determine distance



- Cellular Reporter
  - Can image living cells *in vitro*
    - Picture uses CFP (cyan) and YFP (yellow)
  - Can again use FRET principles with two different dyes
    - Measure conformational changes when two complexes interact

Fluorescence Lifetime Imaging Microscopy (FLI)

- Image the fluorescence lifetime of all fluorophores in a sample
- Can image live cells this way
- Also possible to calculate FRET efficiencies at every pixel:
  - $E_T^i = 1 - \left(\frac{\tau_{DA}^i}{\tau_D'}\right)$
  - Where τ_D and τ_{DA} are the donor and acceptor lifetimes respectively
- Can use this technique to visualize the locations of GFPs in a living cell in real time
  - Allows us to see cellular events in real time



Sources

- Serdyuk, Igor N., Nathan R. Zaccai, and Joseph Zaccai.*Methods in Molecular Biophysics: Structure, Dynamics, Function*. New York: Cambridge University Press, 2007. Print.