Optical fluorescence: spectroscopy and microscopy

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https://www.photometrics.com/applications/imaging/fluorescenceimaging.php

Outline

1. Basic concepts and definitions

   Fluorescence, phosphorescence, transmittance, absorbance, lifetime

2. Spectroscopy tools

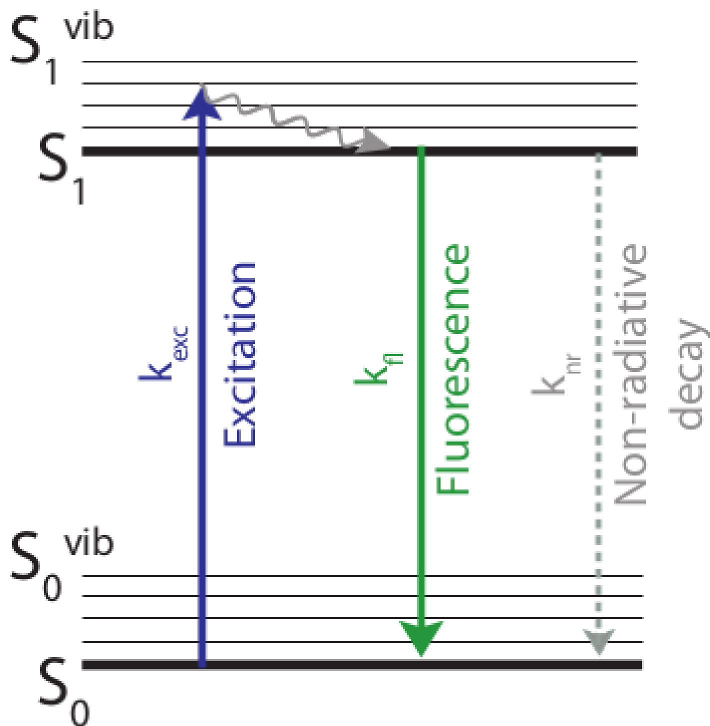
3. Fluorescence Imaging techniques

4. Applications

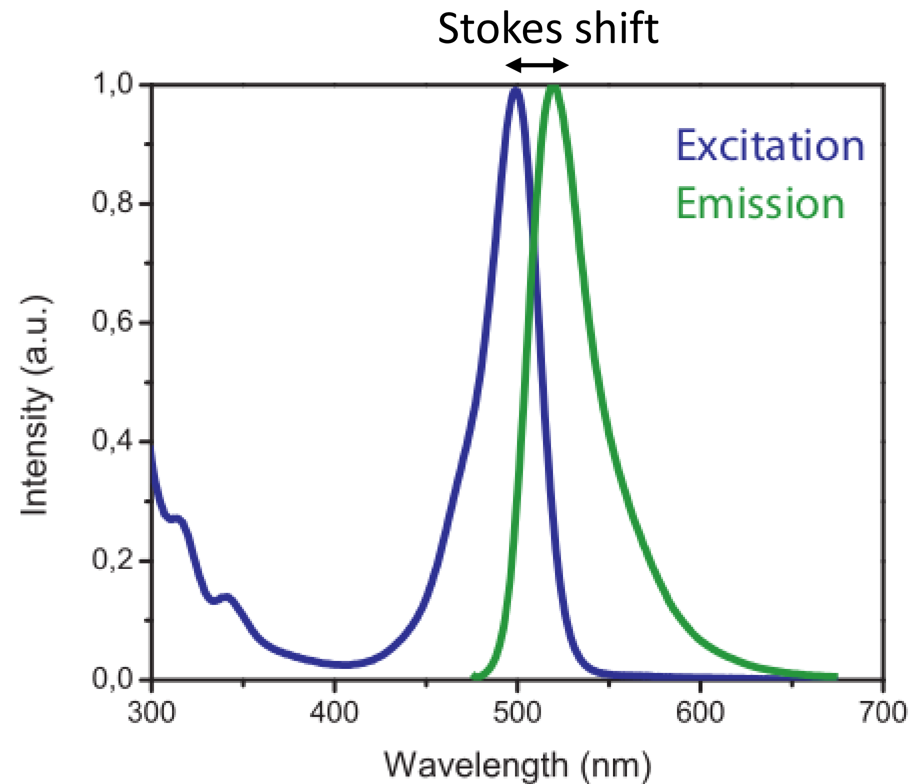
Fluorescence

Emission of photon during atomic transition to lower-energy state

$$E_\gamma = \frac{hc}{\lambda}, k = \frac{2\pi}{\lambda}$$

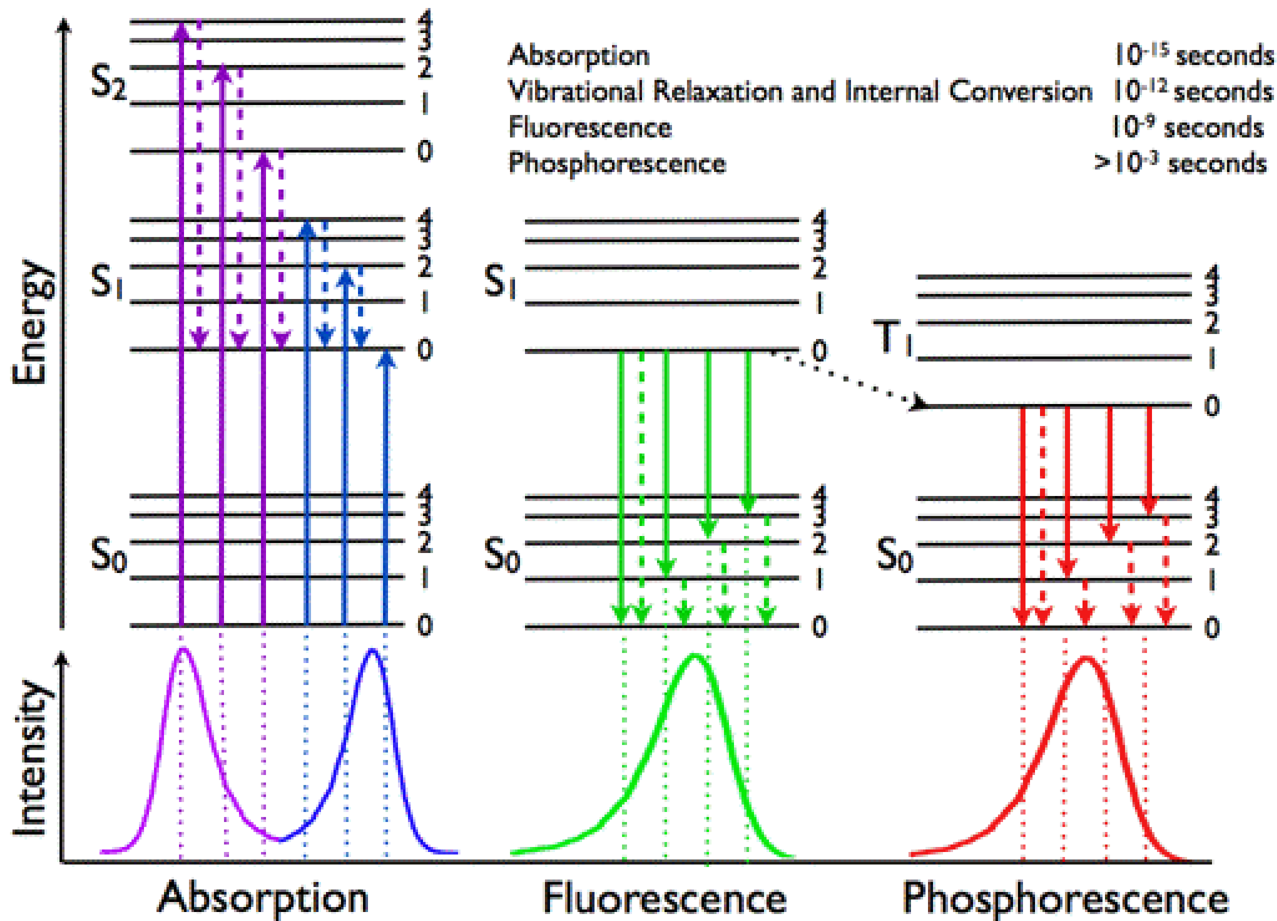


Stokes shift



https://svi.nl/FluorescenceMicroscope

Phosphorescence



Transition from excited triplet state to singlet ground state

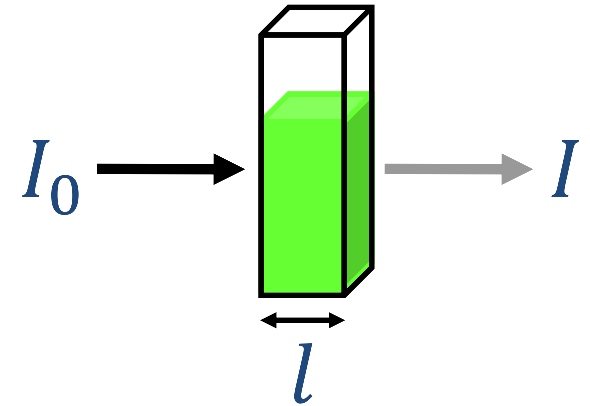
Much slower due to ‘forbidden’ spin transition

http://photobiology.info/Visser-Rolinski.html

Transmittance (T) and Absorbance (A)

Transmittance: $T = \frac{I}{I_0} = 10^{-A}$

Absorbance: $A = \varepsilon(\lambda) * cl$



$\varepsilon(\lambda)$: molar attenuation constant

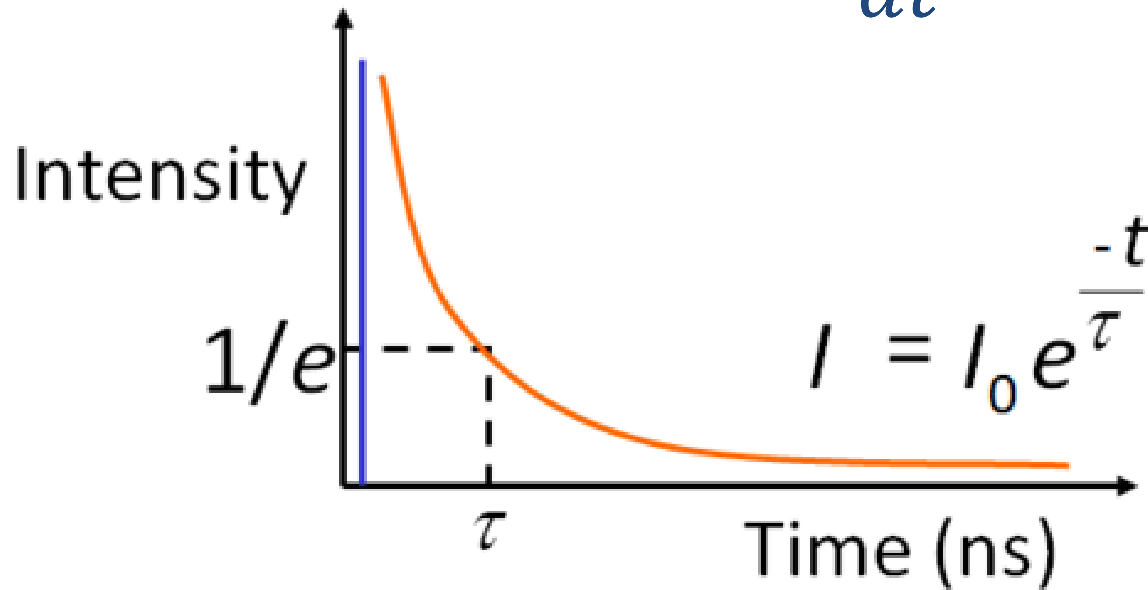
c: amount concentration

l: path length

Lifetime (τ)

$$\frac{dS_1}{dt} = -\frac{S_1}{\tau} \quad \Rightarrow \quad S_1(t) = S_1(0) * e^{-t/\tau}$$

$$I \propto \frac{dS_1}{dt} \propto S_1$$



https://www.lambertinstruments.com/technologies-1/2014/12/4/fluorescence-lifetime-imaging-microscopy

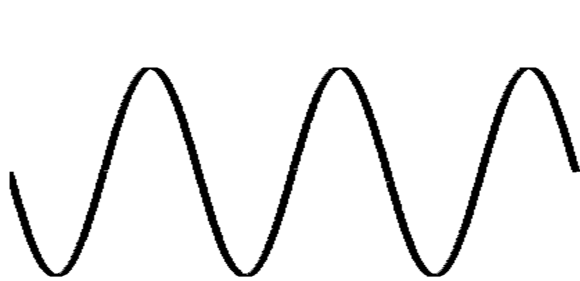
Fluorescence lifetime:

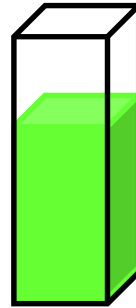
$\tau \sim ns$

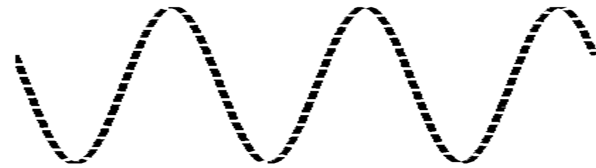
Phosphorescence lifetime:

$\tau \sim ms$

Frequency-domain Lifetime Measurement







$E(t) = E_0 + E_\omega \cos(\omega t + \varphi_e)$

$F(t) = F_0 + F_\omega \cos(\omega t + \varphi_e - \varphi)$

$$M = \frac{F_\omega / F_0}{E_\omega / E_0} = \frac{1}{\sqrt{1 + (\omega\tau)^2}}, \quad \tan(\varphi) = \omega\tau$$

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   Monochromator, diffraction grating, prism, spectrophotometer, fluorophores

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Monochromator

Czerny-Turner Monochromator:

A: Incoming light

B: Entrance slit

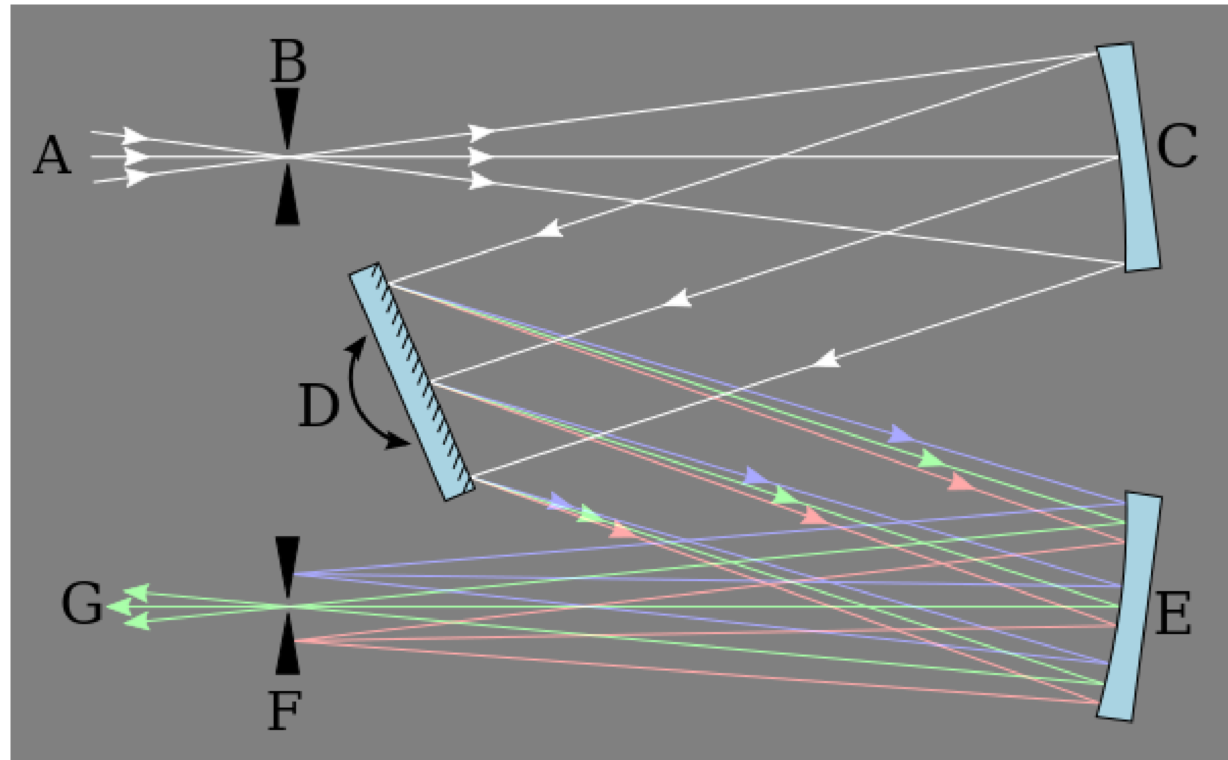
C: Collimating mirror

D: Diffraction grating / Refracting prism

E: Refocusing mirror

F: Exit slit

G: Outgoing light



https://en.wikipedia.org/wiki/Monochromator#/media/File:Czerny-Turner_Monochromator.svg

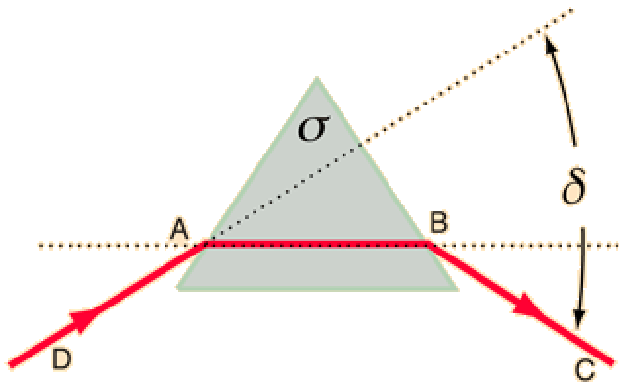
Light splitting

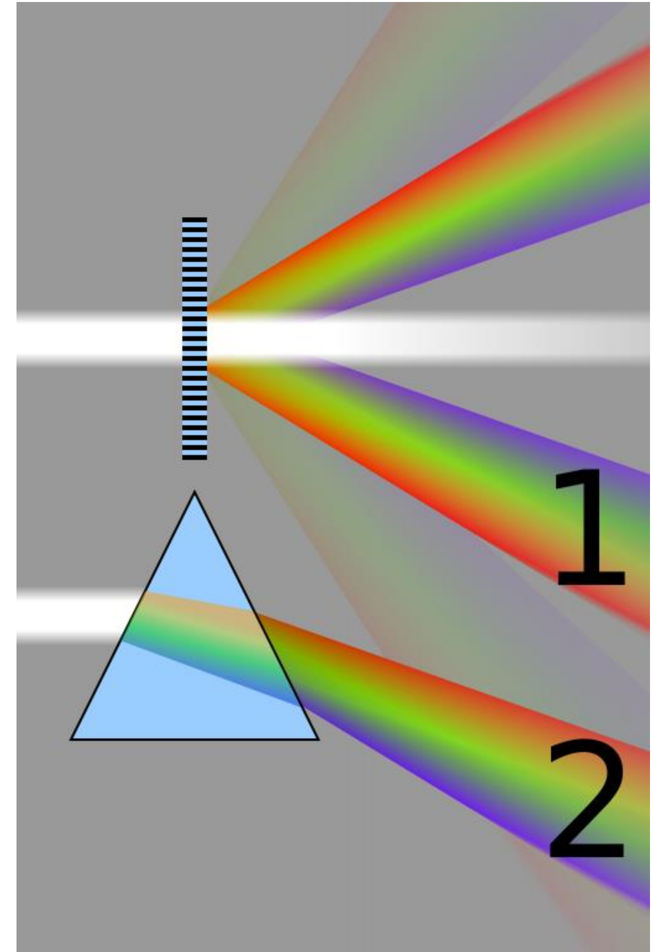
(1) Diffraction grating:

$$\theta = \sin^{-1}\left(\frac{\lambda}{d} - \sin\theta_i\right)$$

(2) Prism:

$$\delta = 2\sin^{-1}\left(\frac{n_p(\lambda)}{n_0}\sin\frac{\sigma}{2}\right) - \sigma$$

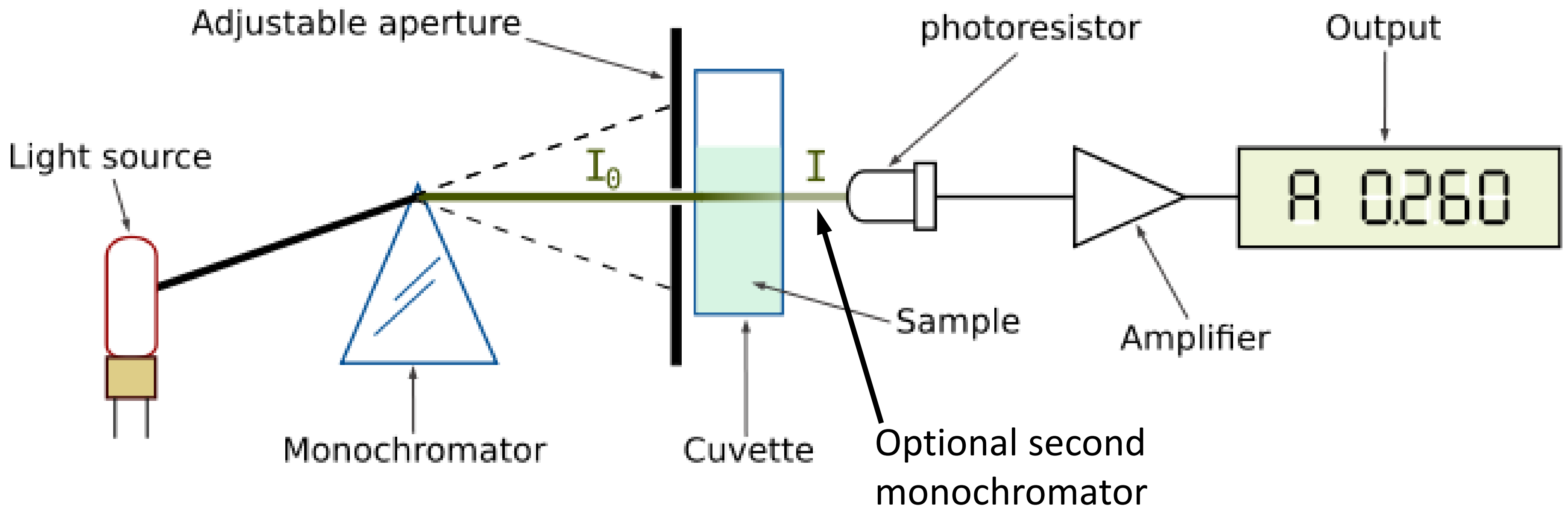




https://en.wikipedia.org/wiki/Diffraction_grating#/media/File:Comparison_refraction_diffraction_spectra.svg

Spectrophotometer

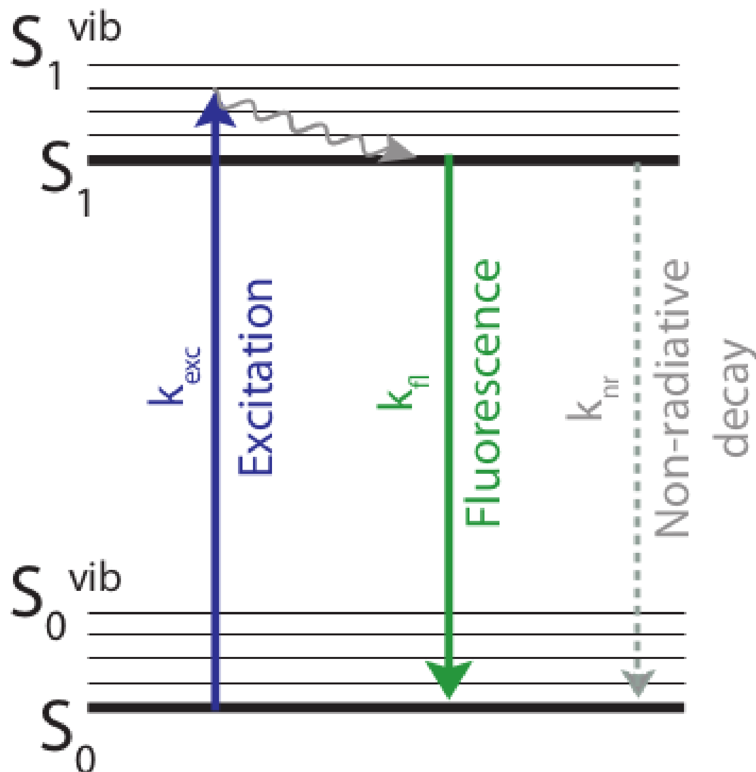
System for measuring absorption (and emission) spectra



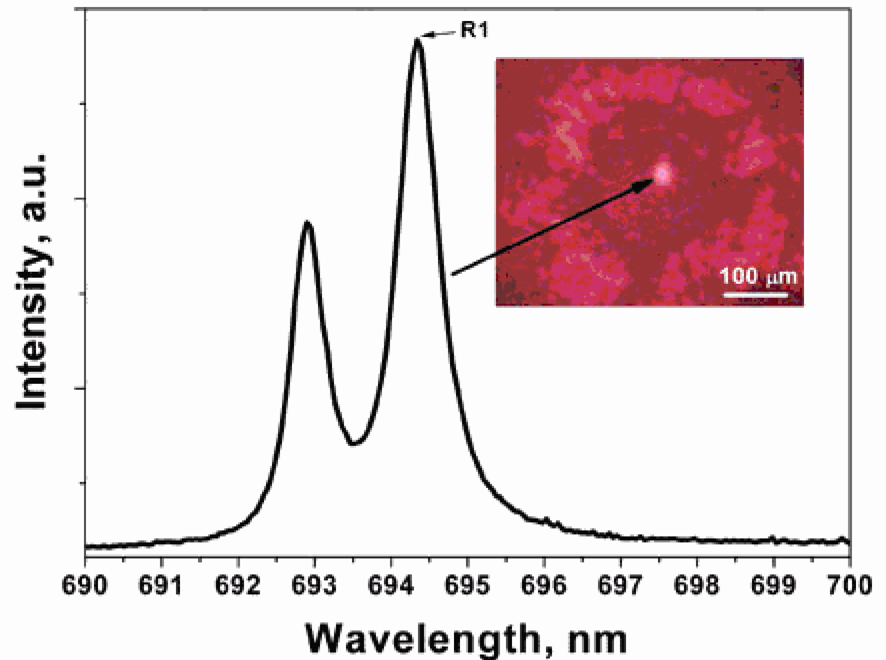
Fluorescence spectroscopy

- Can be used to measure energy differences between excited and ground states
- Can resolve fine structure

$$E_\gamma = \frac{hc}{\lambda}$$

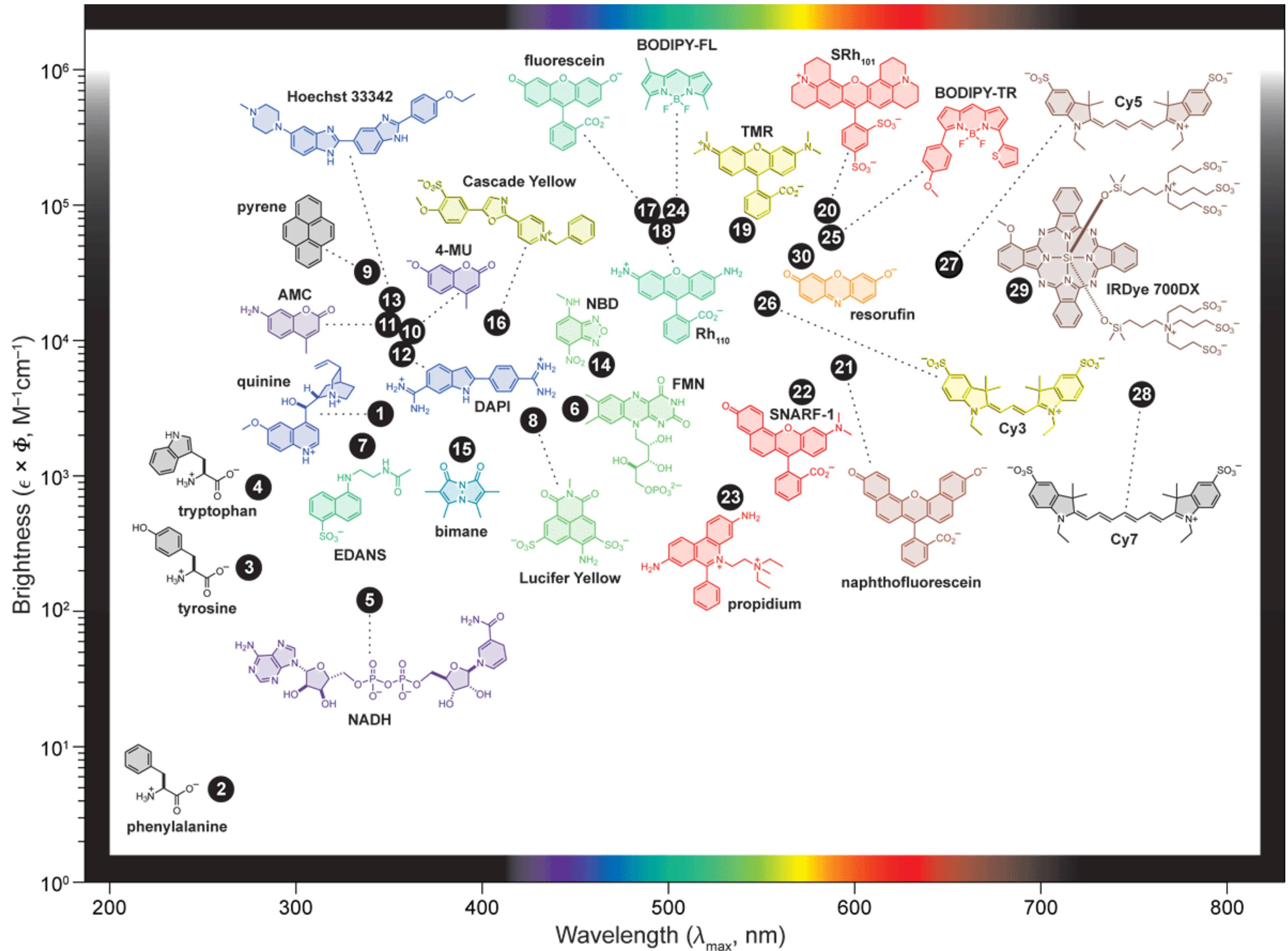


https://svi.nl/FluorescenceMicroscope



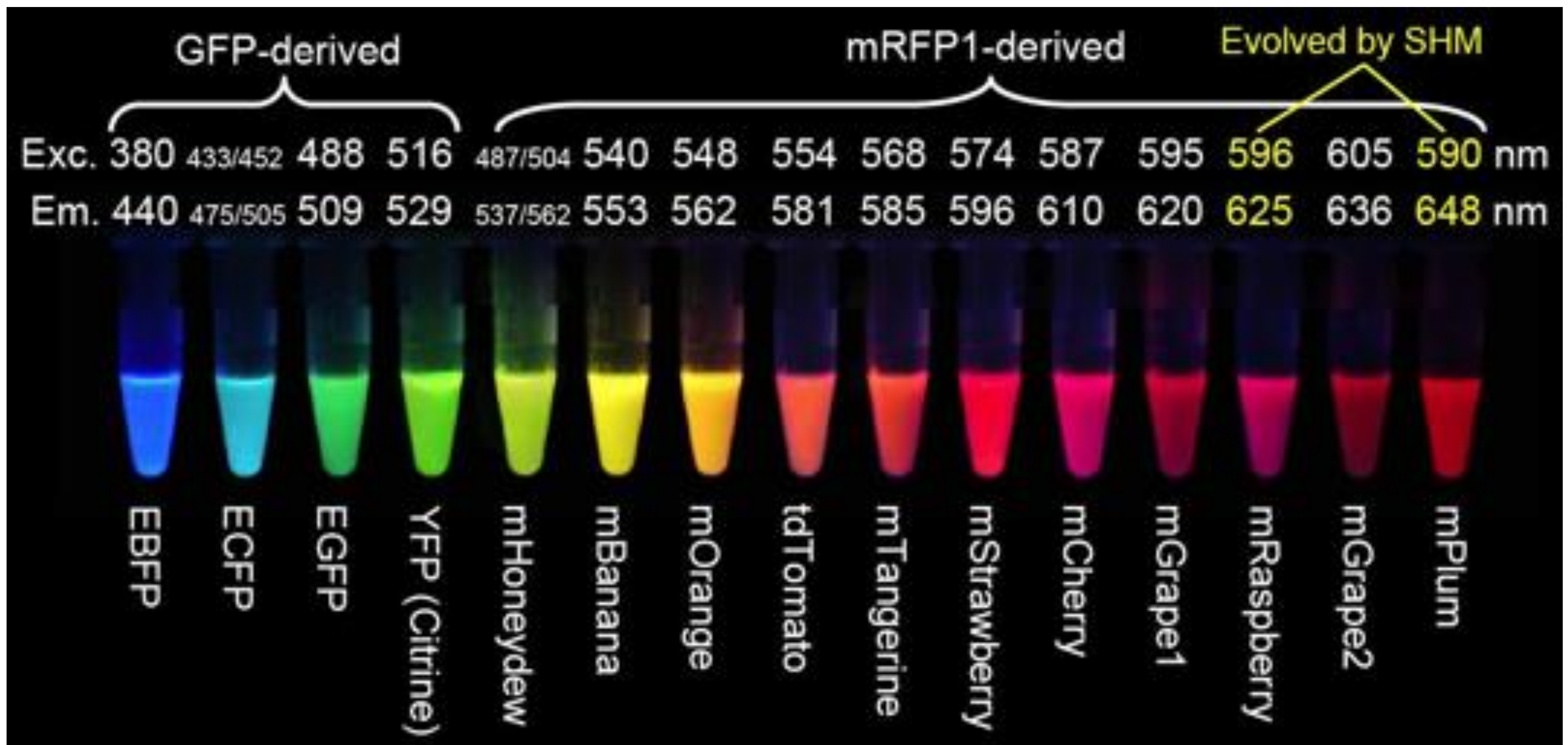
https://www3.aps.anl.gov/News/APS_News/Images/184209-2.gif

Fluorophores



Fluorescent Proteins

Derived from naturally-occurring fluorescent proteins in jellyfish, etc.



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   FLIM, FRET, FIONA

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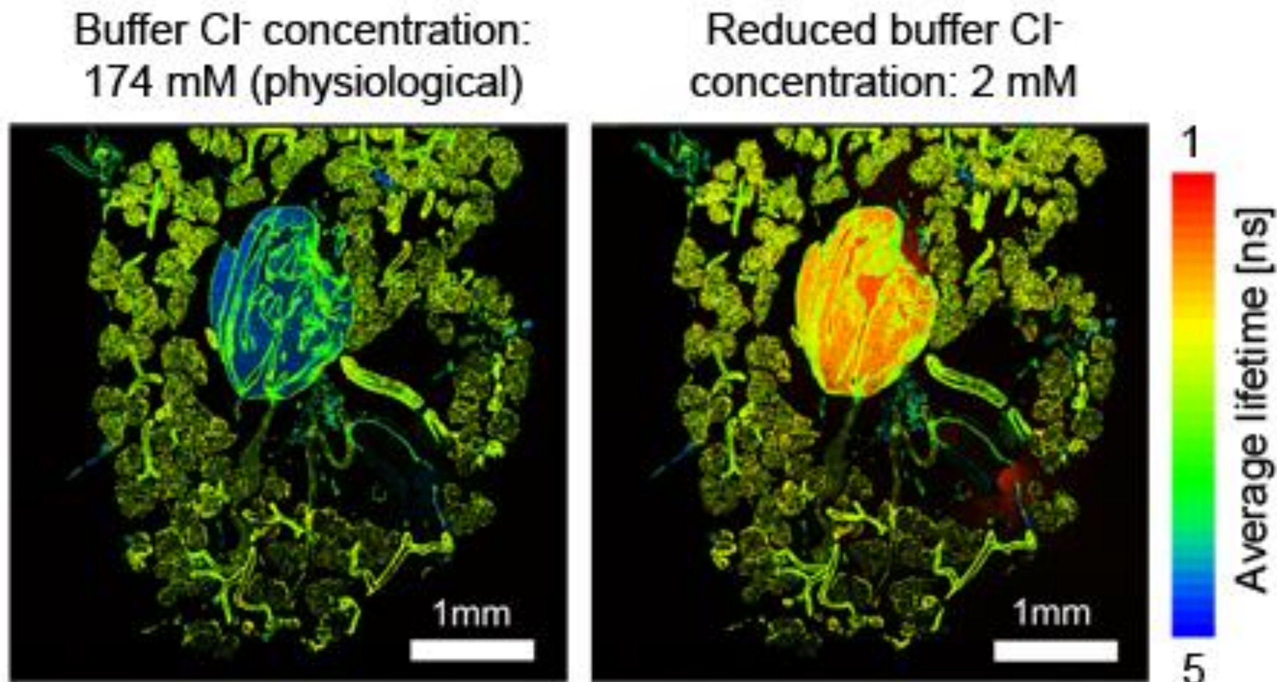
Fluorescence-lifetime imaging microscopy (FLIM)

- Spatial imaging by fluorescence lifetime
- Non-intensity based imaging technique



https://www.picoquant.com/applications/category/life-science/fluorescence-lifetime-imaging-flim#tab-5

FLIM in Urbana-Champaign

Robert Clegg – UIUC
Full-field FLIM

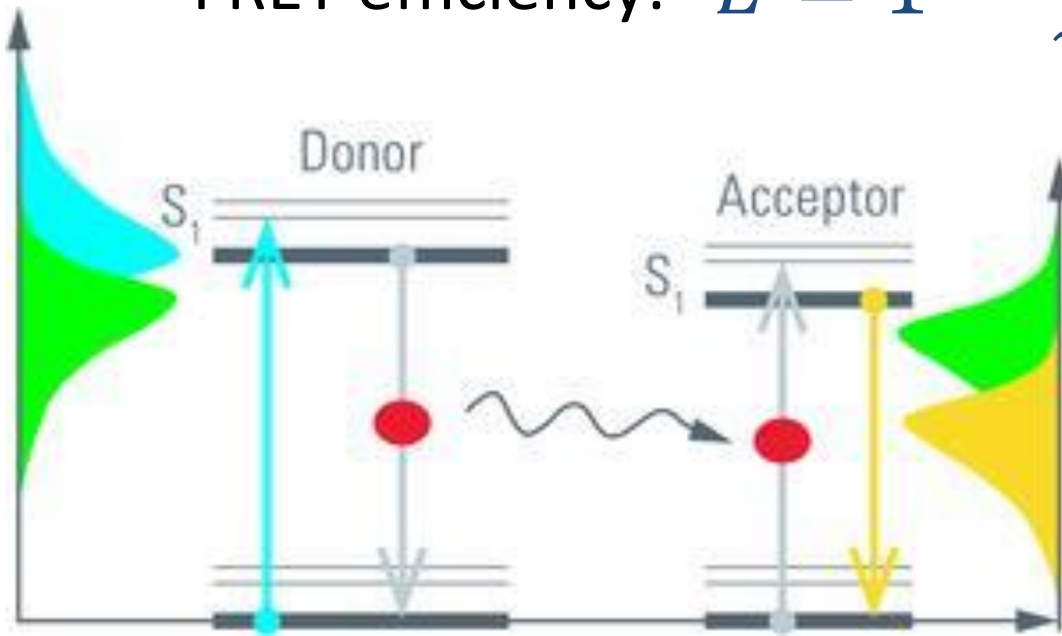
Enrico Gratton – UIUC
Scanning confocal FLIM

Beniamino Barbieri – ISS Inc.
Commercialization of FD FLIM

Förster resonance energy transfer (FRET)

- Donor and acceptor fluorophore attached to different parts of molecule or two bound molecules
- Emission energy of donor matches absorption energy of acceptor

FRET efficiency: $E = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{1}{1 + (r/R_0)^6}$

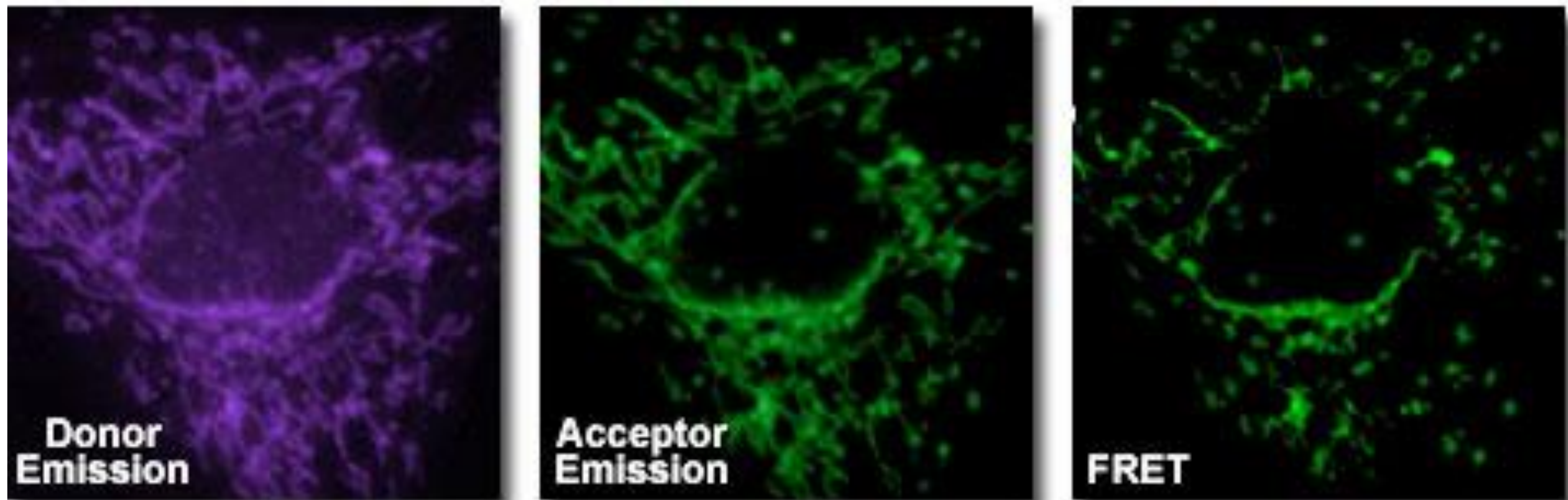


R_0: Förster distance – Property of fluorophores

http://www.leica-microsystems.com/science-lab/fret-with-flim/

Ratiometric FRET

$$E_{rel} = \frac{I_A}{I_A + I_D}$$



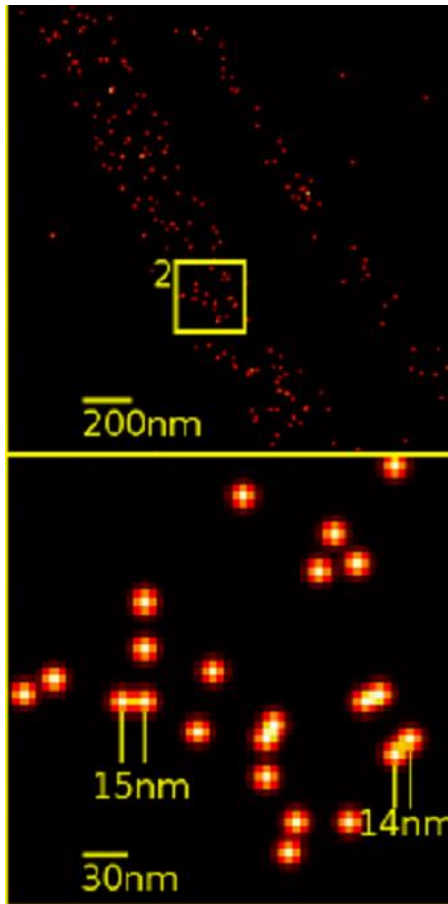
http://www.olympusmicro.com/primer/techniques/fluorescence/fret/fretintro.html

$E_{rel} \rightarrow E$ requires correction factors

Super-resolution microscopy

Optical microscopy limited by diffraction $d = \frac{\lambda}{2n \sin\theta}$

$d_{min} \approx 250\ \mathrm{nm}$



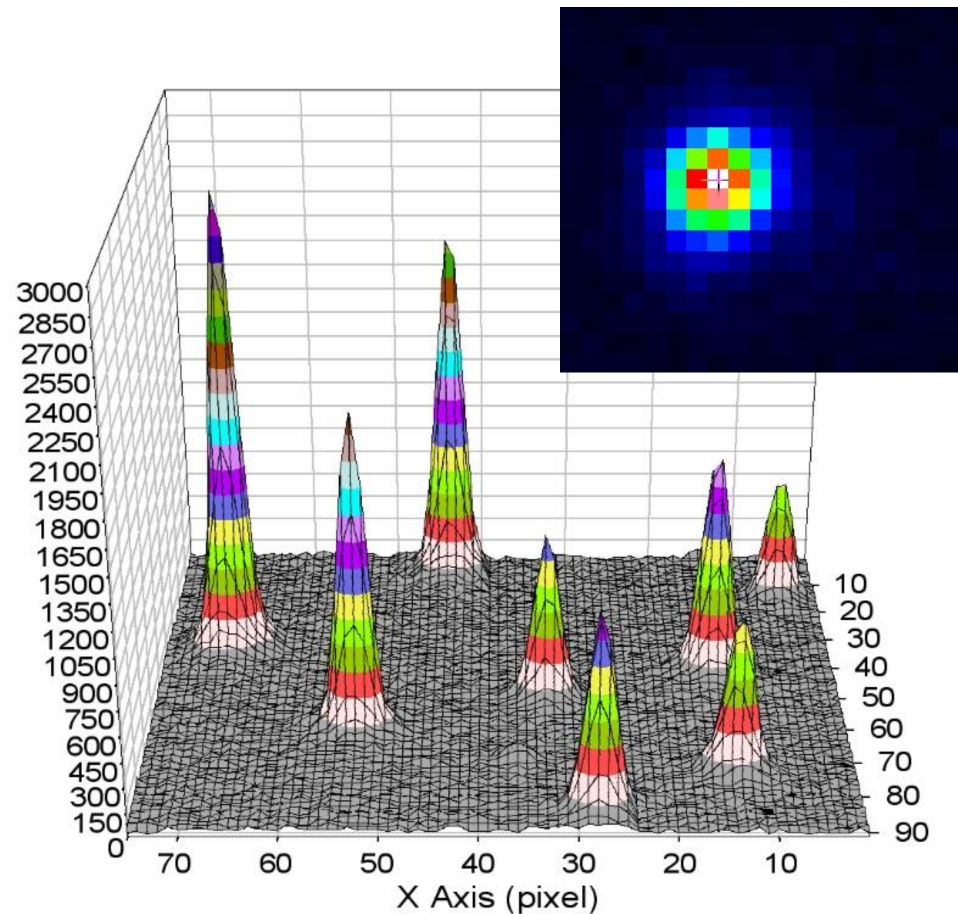
https://en.wikipedia.org/wiki/Super-resolution_microscopy#/media/File:Single_YFP_molecule_superresolution_microscopy.png

Super-resolution imaging relies on isolating individual fluorophores and fitting the intensity profile to find the center

Can achieve resolution several orders of magnitude finer than diffraction limite

Fluorescence imaging with one-nanometer accuracy (FIONA)

- Developed by Paul Selvin
- Extreme application of super-resolution imaging
- Requires efforts to maximize signal (use bright fluorophore), and minimize noise (use very sensitive detector)



https://valelab4.ucsf.edu/external/images/res-singlemolecule/Fig%203a.jpg

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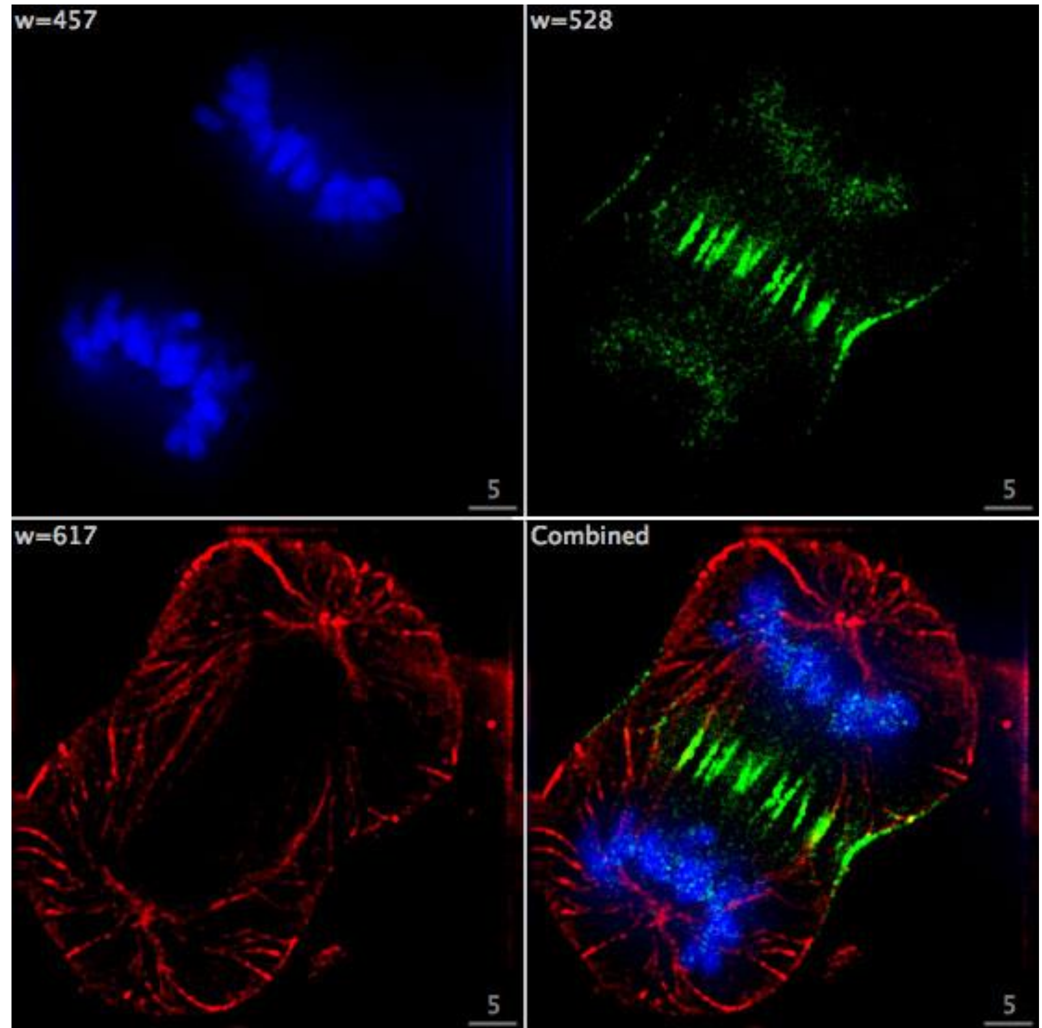
Biological applications

Dividing cancer cell:

Blue is DNA

Green is the protein INCENP

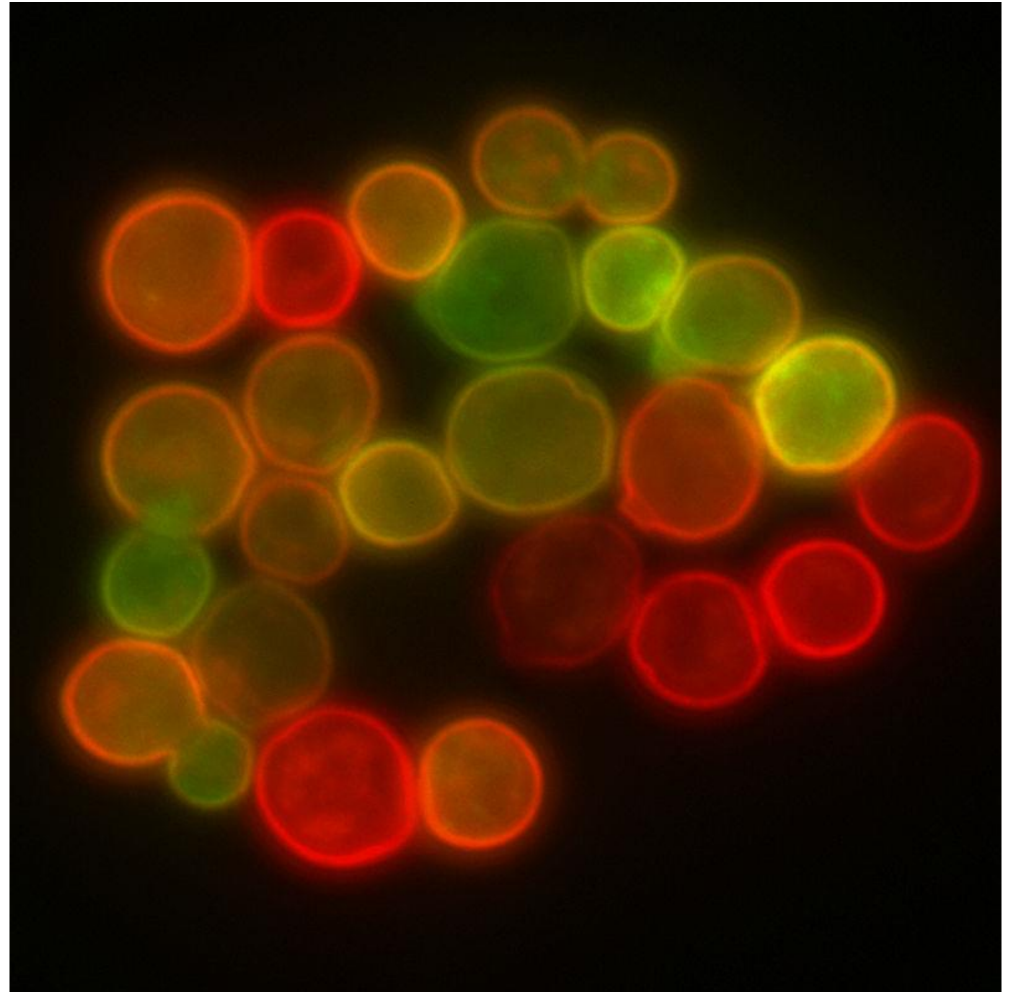
Red is microtubules



https://en.wikipedia.org/wiki/Fluorescence_microscope#/media/File:Dividing_Cell_Fluorescence.jpg

Biological applications

Yeast cell membranes labelled with red and green fluorescent proteins

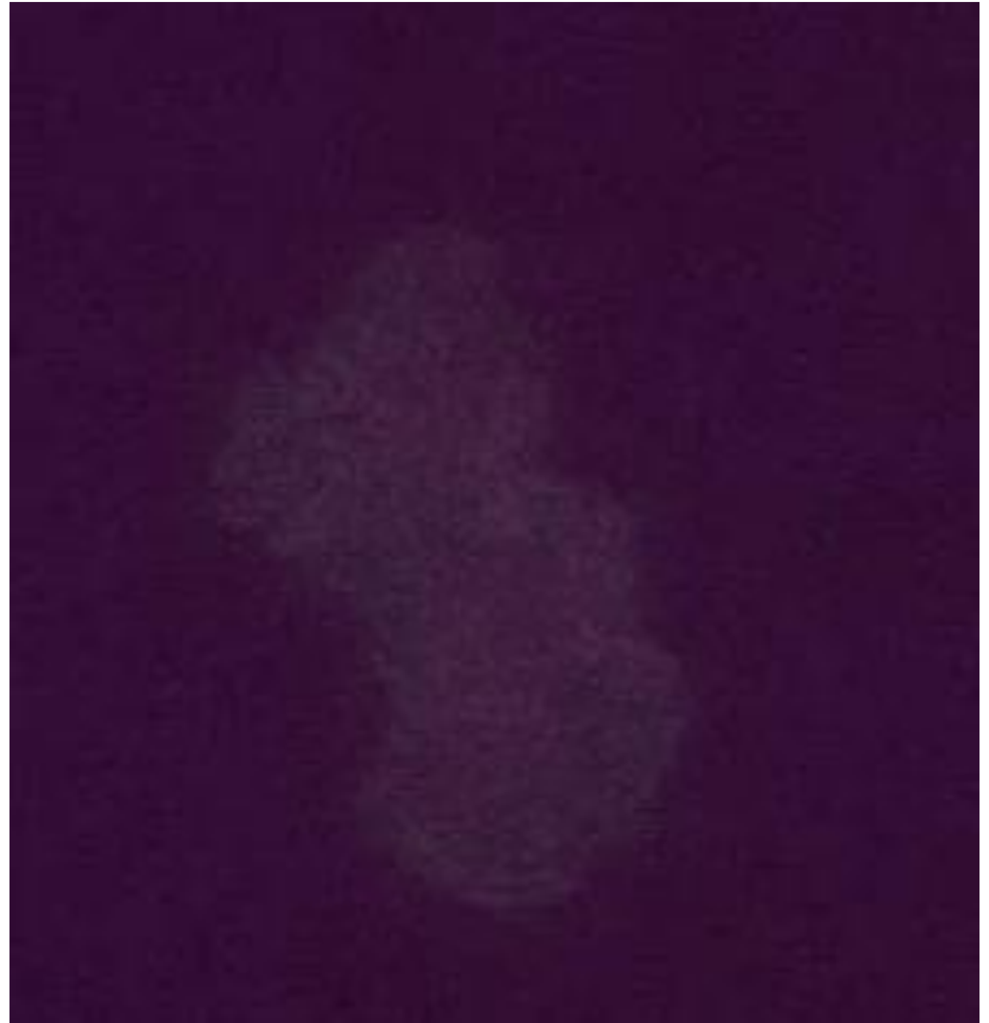


https://en.wikipedia.org/wiki/Fluorescence_microscope#/media/File:Yeast_membrane_proteins.jpg

Biological applications

From Thomas Kuhlman

Image of transposon jumping (green) and transposase (red) in E. coli cells



http://kuhlman.physics.illinois.edu/research_transposon_dynamics.html

Questions?