Bi177 – Lecture 10

"Quantitative" Fluorescence

- Fluorescence linearity (non-linearity)
  - Dye, microscope, camera
- Flat-fielding to linearize
- Quantitating the image
- Multispectral imaging
- FRET

Our discussion of fluorescence has made hidden assumption that dyes have an ideal behavior

How true is this?

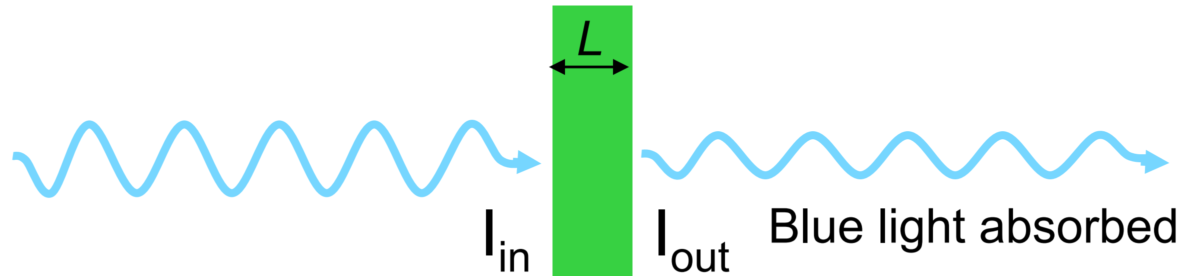
Fluorescent Dye

Dipole antenna

Delocalized electrons

Longer dipole, longer λ

Fluorophore absorption



Beer-Lambert law

$I_{out} = I_{in} \exp(-\varepsilon L c)$

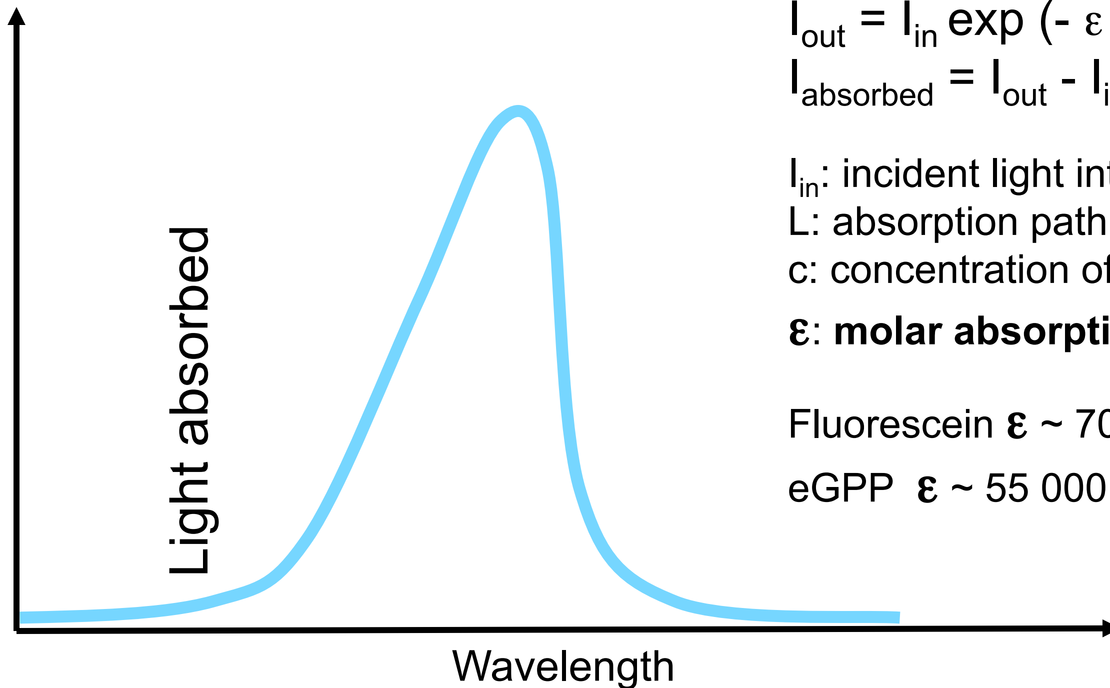
$I_{absorbed} = I_{out} - I_{in}$

I_{in}: incident light intensity (in $W.cm^{-2}$)

L: absorption path length (in cm)

c: concentration of the absorber (in M or $mol.L^{-1}$)

ε: molar absorption coefficient (in $M^{-1}cm^{-1}$ or $mol^{-1}.L.cm^{-1}$)



Fluorescein **ε** ~ 70 000 $M^{-1}.cm^{-1}$

eGPP **ε** ~ 55 000 $M^{-1}.cm^{-1}$

Fluorophore absorption

Other expressions of the Beer-Lambert law:

$I_{out} = I_{in} \exp(-\varepsilon L c)$
$I_{out} = I_{in} \exp(-\sigma L N)$
$I_{out} = I_{in} \exp(-\mu_a L)$

I_{in}: incident light intensity in $W.cm^{-2}$
L: absorption path length in cm
c: concentration of the absorber in M or $mol.L^{-1}$
N: density of the absorber in $molecule.cm^{-3}$
ε: molar absorption coefficient in $M^{-1}cm^{-1}$ or $mol^{-1}.L.cm^{-1}$
σ: absorption cross section in cm^2 or $cm^2.molecule^{-1}$
μ_a: absorption coefficient in cm^{-1}

$N = N_{Avogadro} \cdot 10^{-3} c$ ($1L = 10^3 cm^3$)
$\varepsilon = N_{Avogadro} \cdot 10^{-3} \sigma = 6.022\ 10^{20} \sigma$

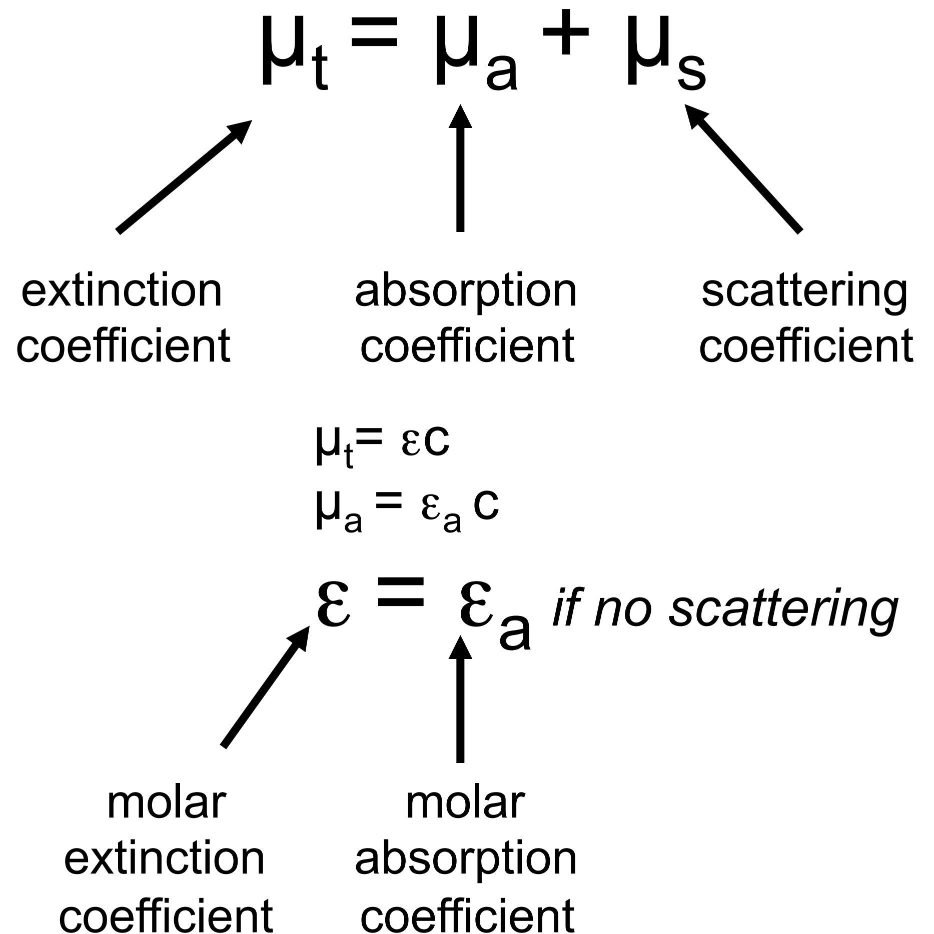
eGPP
$\varepsilon = 55\ 000\ M^{-1}.cm^{-1}$
$\sigma = 9.13\ 10^{-17}\ cm^2.molecule^{-1}$

Fluorophore absorption

In the literature…

The "extinction coefficient" is usually given in tables.
confusions:
- "extinction coefficient" used for "absorption coefficient" (it assumes the scattering coefficient is negligible)
- "extinction coefficient" used for "molar extinction coefficient" (check the unit!)

$\varepsilon(\lambda)$!
The maximum is given in tables, or the excitation wavelength is indicated.



Fluorophore absorption

Example: properties of fluorescent protein variants

it is the *molar absorption coefficient*

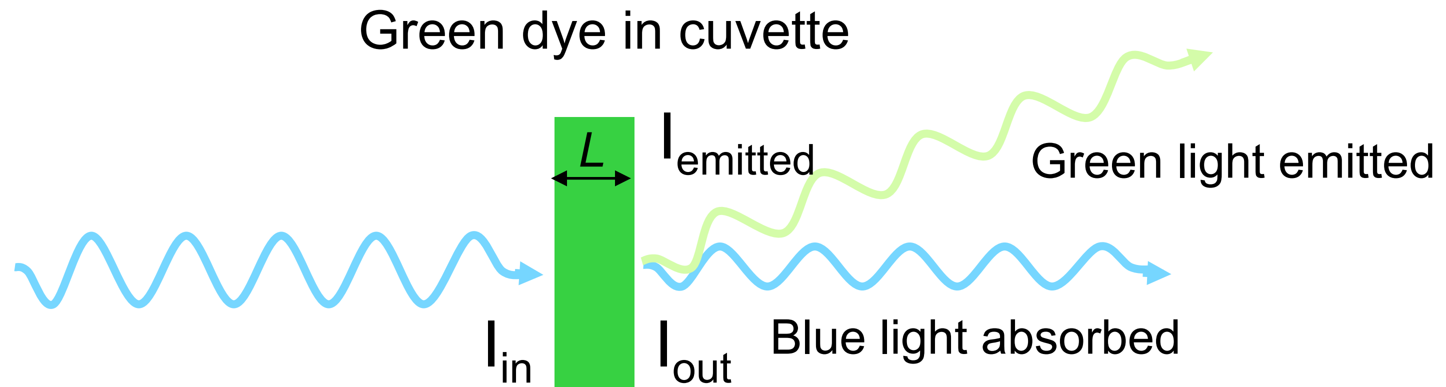
Table 1 Properties of novel fluorescent protein variants

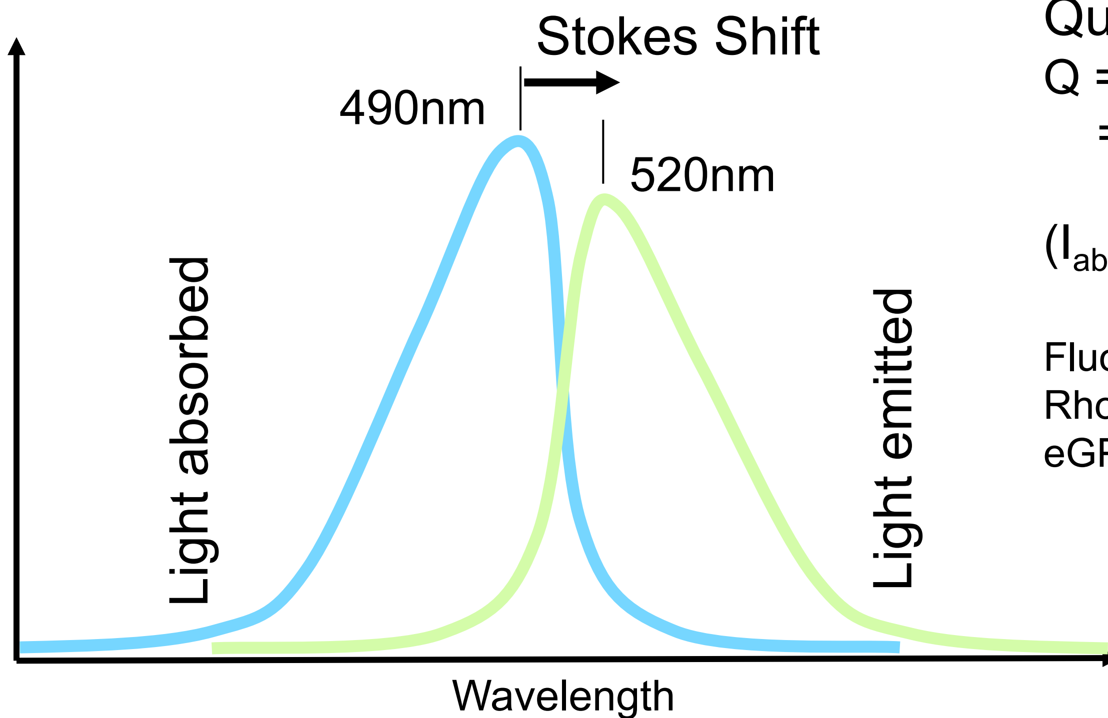
Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient per chain[a] ($M^{-1}cm^{-1}$)	Fluorescence quantum yield	Brightness of fully mature protein (% of DsRed)	pKa	$t_{0.5}$ for maturation at 37 °C	$t_{0.5}$ for bleach[b], s
DsRed	558	583	75,000	0.79	100	4.7	~10 h	ND
T1	555	584	38,000	0.51	33	4.8	<1 h	ND
Dimer2	552	579	69,000	0.69	80	4.9	~2 h	ND
mRFP1	584	607	50,000	0.25	21	4.5	<1 h	6.2
mHoneydew	487/504	537/562	17,000	0.12	3	<4.0	ND	5.9
mBanana	540	553	6,000	0.70	7	6.7	1 h	1.4
mOrange	548	562	71,000	0.69	83	6.5	2.5 h	6.4
dTomato	554	581	69,000	0.69	80	4.7	1 h	64
tdTomato	554	581	138,000	0.69	160	4.7	1 h	70
mTangerine	568	585	38,000	0.30	19	5.7	ND	5.1
mStrawberry	574	596	90,000	0.29	44	<4.5	50 min	11
mCherry	587	610	72,000	0.22	27	<4.5	15 min	68

[a]Extinction coefficients were measured by the alkali denaturation method[8,30] and are believed to be more accurate than the previously reported values for DsRed, T1, dimer2 and mRFP1[7].
[b]Time (s) to bleach to 50% emission intensity, at an illumination level that causes each molecule to emit 1,000 photons/s initially, that is, before any bleaching has occurred. See Methods for more details. For comparison, the value for EGFP is 115 s, assuming an extinction coefficient of 56,000 $M^{-1}cm^{-1}$ and quantum efficiency of 0.60 (ref. 30). ND, not determined.

Shaner et al, Nature Biotechnology, 2004

Fluorophore quantum yield Q





Quantum Yield

$Q = I_{emitted} / I_{absorbed}$

= # photons emitted / # photons aborbed

$(I_{absorbed} = I_{out} - I_{in})$

Fluorescein Q ~ 0.8
Rhodamine B Q ~ 0.3
eGPP Q ~ 0.6

Fluorophore brightness = εQ

Example: properties of fluorescent protein variants

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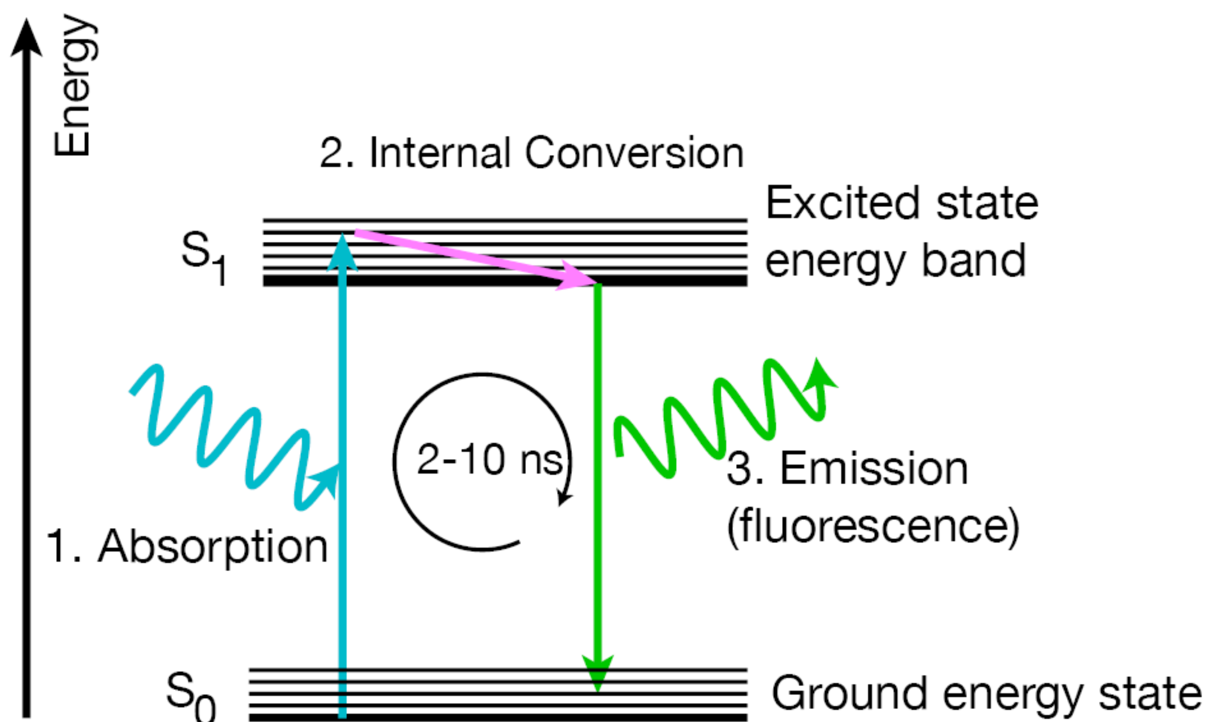
Shaner et al, Nature Biotechnology, 2004

DsRed	$\varepsilon Q \sim 0.79 \times 75\,000 \sim 59\,250\ M^{-1}.cm^{-1}$	(100%)
mRFP1	$\varepsilon Q \sim 0.25 \times 50\,000 \sim 12\,500\ M^{-1}.cm^{-1}$	(21%)
eGPP	$\varepsilon Q \sim 0.6 \times 55\,000 \sim 33\,000\ M^{-1}.cm^{-1}$	(56%)
Fluorescein	$\varepsilon Q \sim 0.8 \times 70\,000 \sim 56\,000\ M^{-1}.cm^{-1}$	(95%) (dye!)

The dilute limit

Extinction coefficient and quantum yield corresponds to “well behaved” dye in the dilute limit: dilute photon and dilute dye



Note:

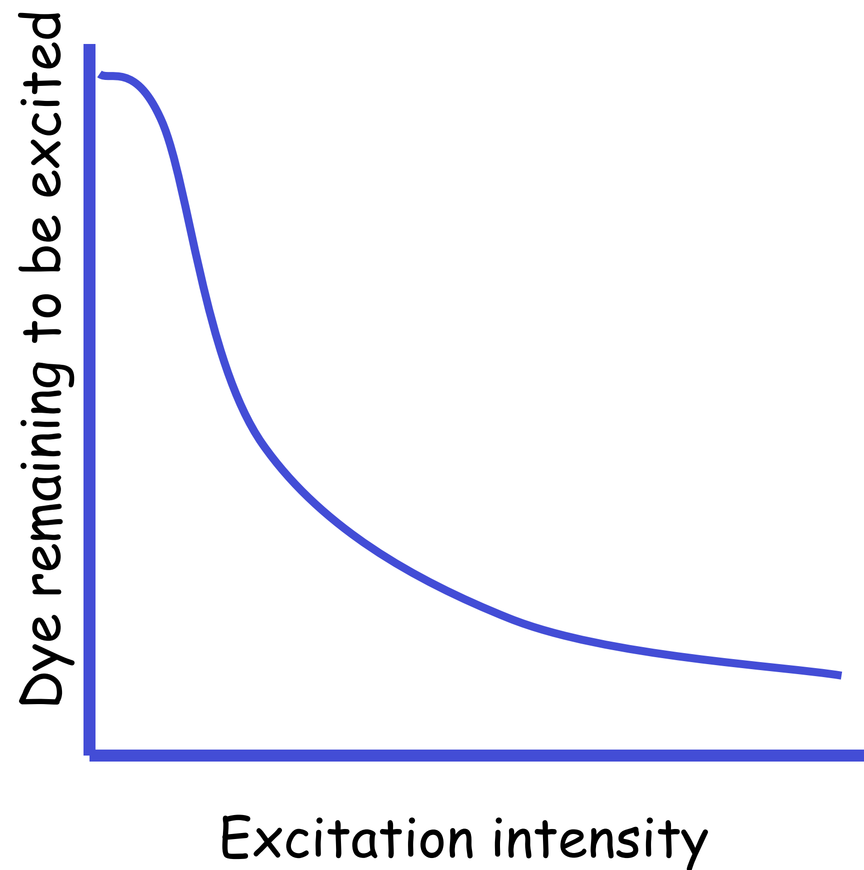
Fraction Q: Emission occurs

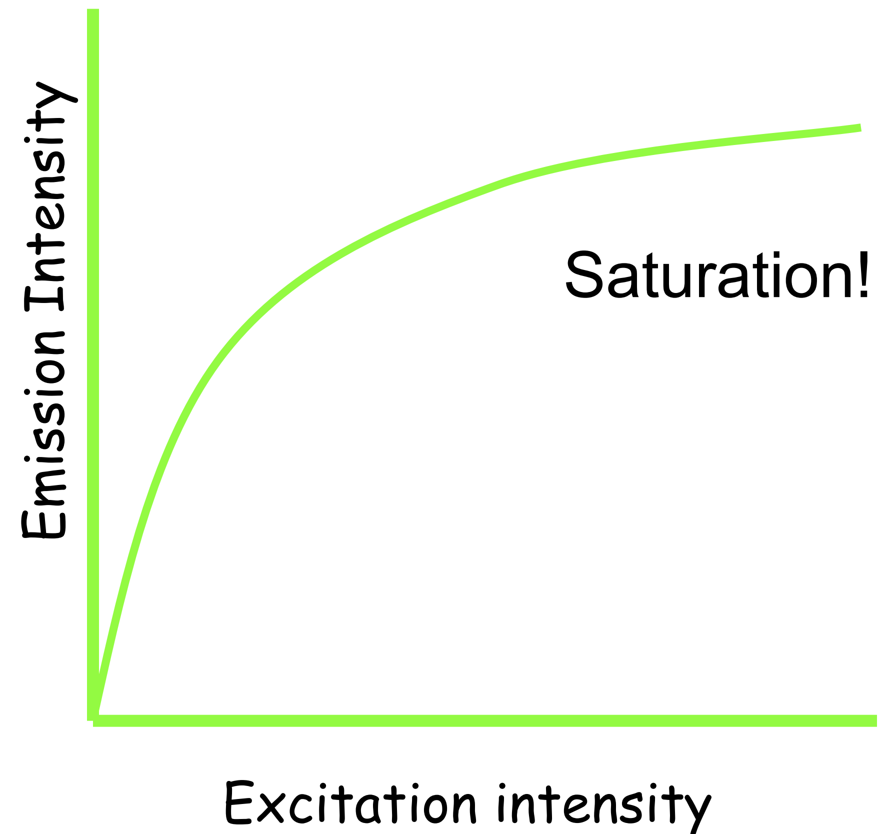
Fraction $1 - Q$: Other losses (heat and (non-radiative) energy transfer)

From Michael Liebling, UCSB

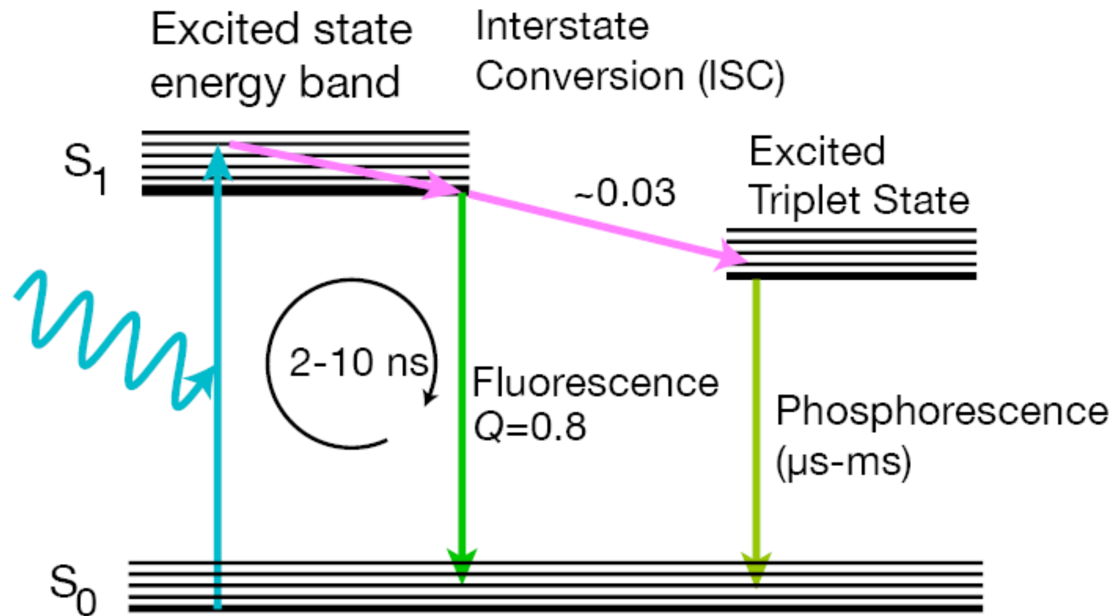
The dilute limit: dilute photons

As photons hit specimen: dye molecules excited and less dye left unexcited





Interstate crossing (ISC)



Triplet state is long lived

Even low probablility can deplete active dye.

Probability of molecule in triplet state after N cycles: $(1 - 0.03)^N = 0.97^N$

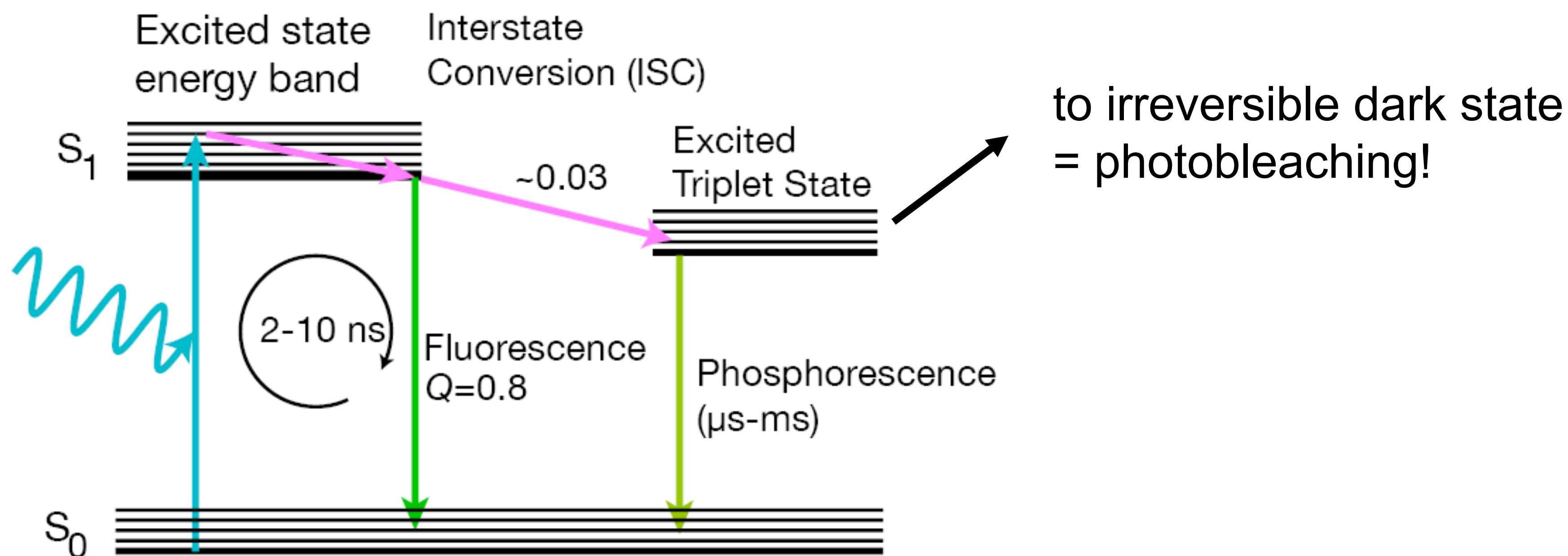
(N=10: 74%) Steady state reached in about 200ms

if 80-90% of molecules in triplet state: image is 5–10 fold dimmer

CLSM: can have major impact (about 5 fold less throughput)

From Michael Liebling, UCSB

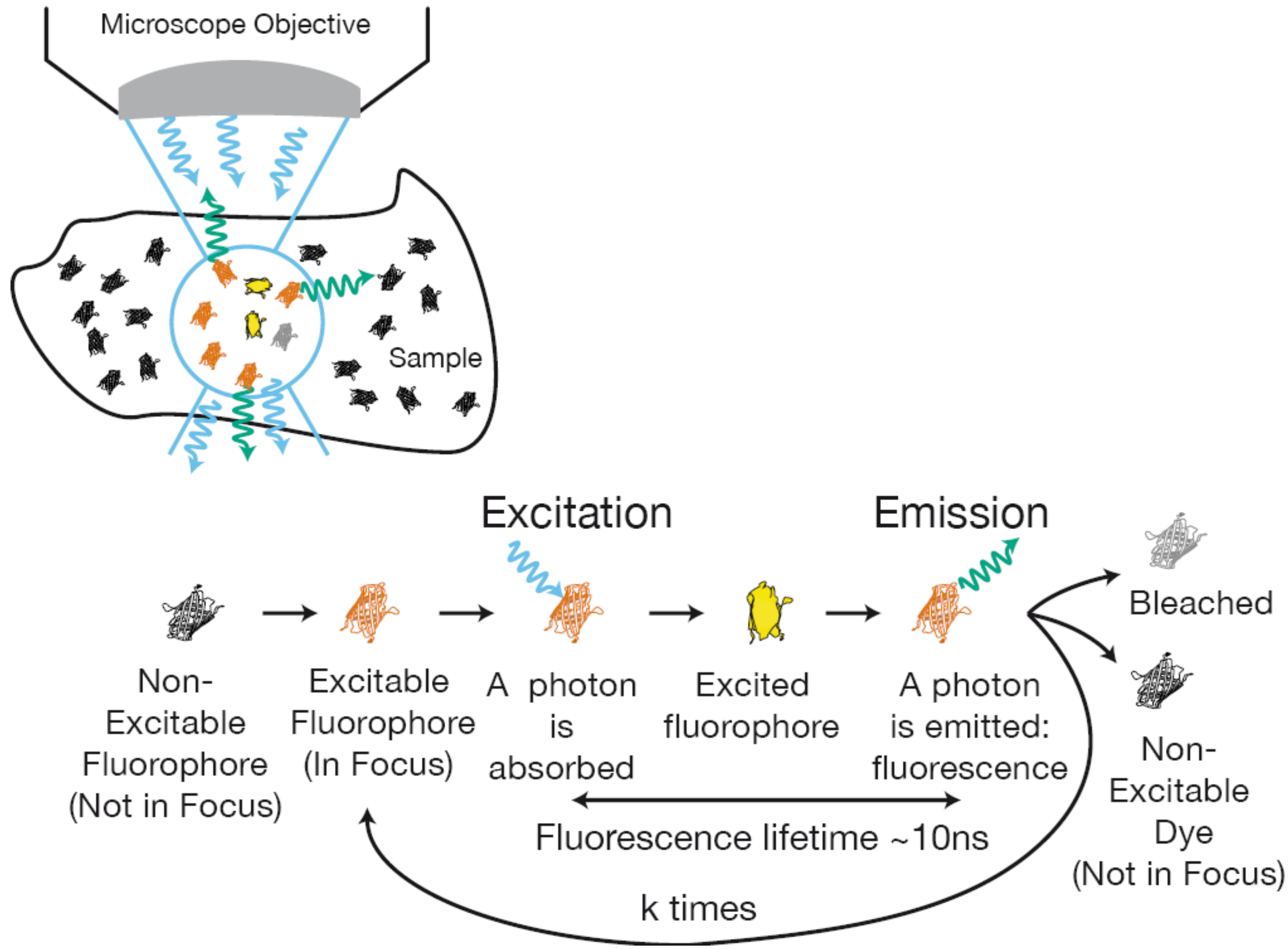
Interstate crossing (ISC) and photobleaching



As ISC takes place: less dye molecules available and unexcitable dye accumulates

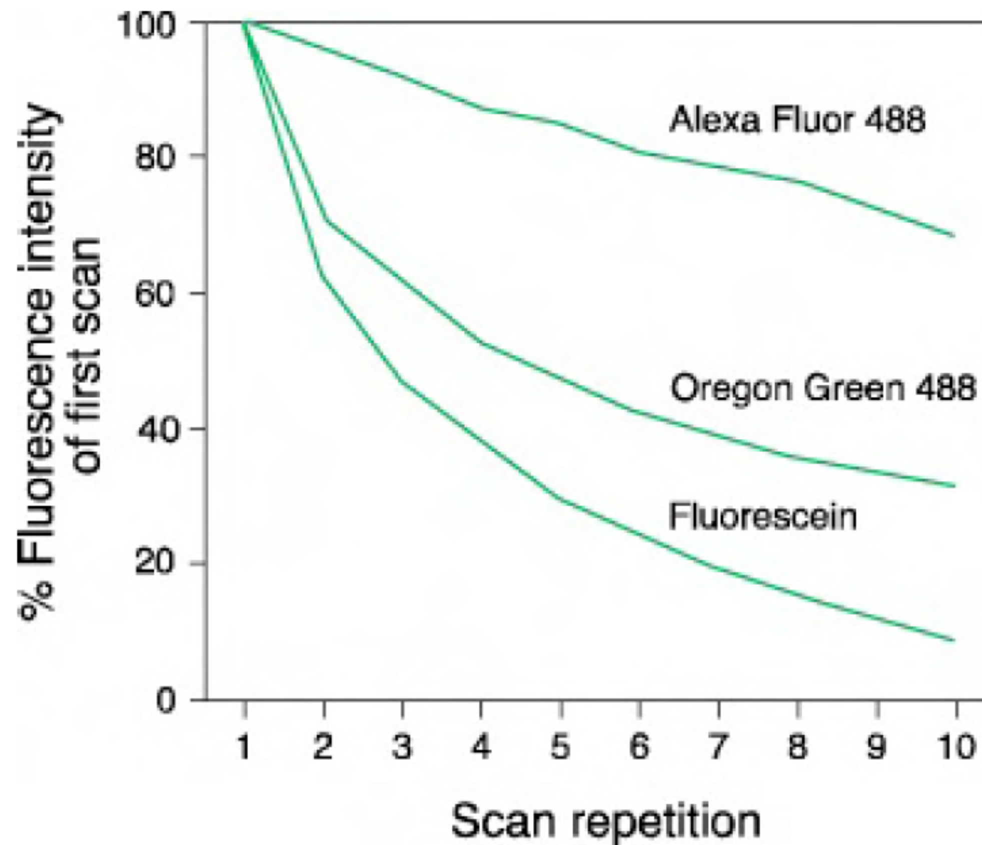
From Michael Liebling, UCSB

Cycle of a fluorophore



From Michael Liebling, UCSB

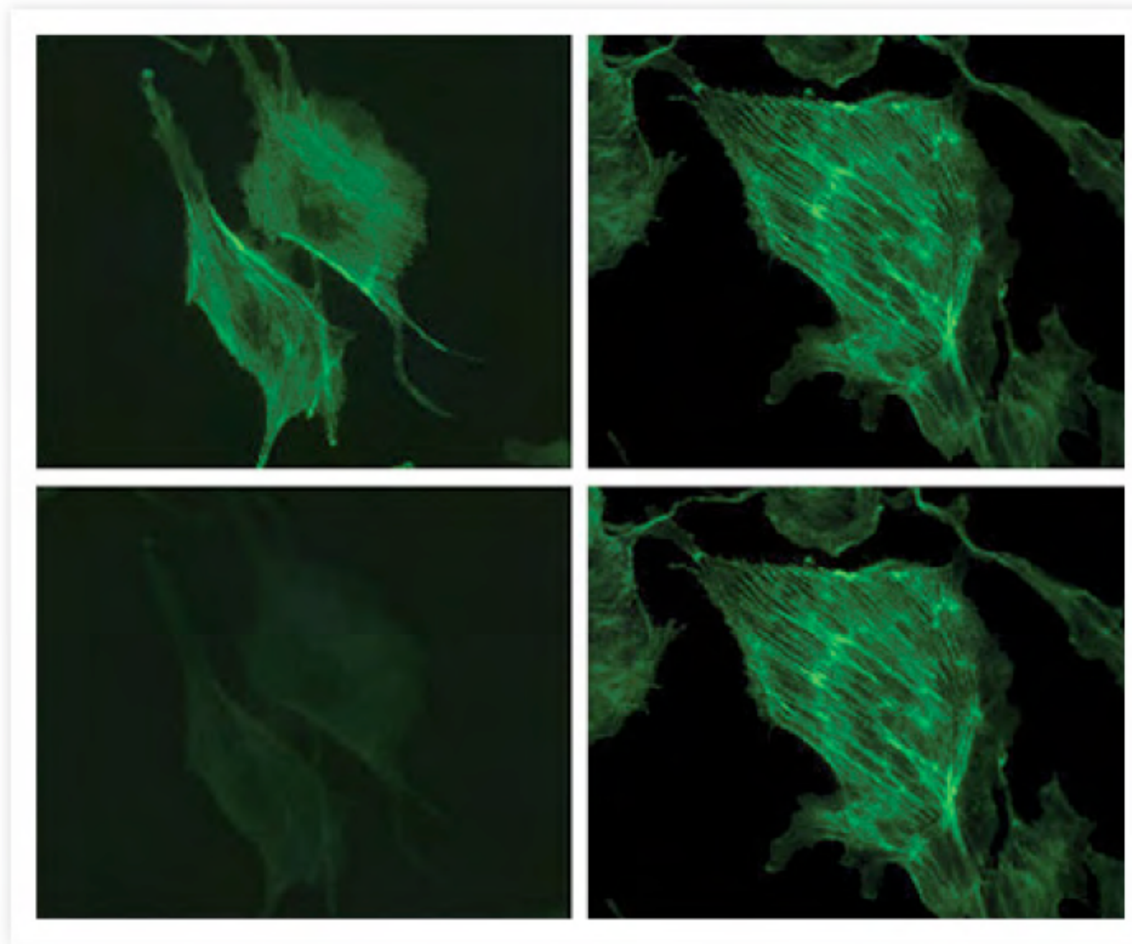
Interstate crossing (ISC) and photobleaching



Photobleaching resistance of the green-fluorescent Alexa Fluor 488, Oregon Green 488 and fluorescein dyes, as determined by laser-scanning cytometry. EL4 cells were labeled with biotin-conjugated anti-CD44 antibody and detected by Alexa Fluor 488 (S11223), Oregon Green 488 (S6368) or fluorescein (S869) streptavidin (Section 7.6). The cells were then fixed in 1% paraformaldehyde, washed and wet-mounted. After mounting, cells were scanned 10 times on a laser-scanning cytometer; laser power levels were 25 mW for the 488 nm spectral line of the argon-ion laser. Scan durations were approximately five minutes apiece, and each repetition was started immediately after completion of the previous scan. Data are expressed as percentages derived from the mean fluorescence intensity (MFI) of each scan divided by the MFI of the first scan. Data contributed by Bill Telford, Experimental Transplantation and Immunology Branch, National Cancer Institute.

A good dye is more photostable (less photobleaching)

Interstate crossing (ISC) and photobleaching



after 30 seconds

Bovine pulmonary artery endothelial cells (BPAEC) were labeled with fluorescein phalloidin (left panels, F432), or Alexa Fluor 488 phalloidin (right panels, A12379), which labels filamentous actin, and mounted in PBS. The cells were placed under constant illumination on the microscope with an FITC filter set using a 60 objective. Images were acquired at one-second intervals for 30 seconds. Under these illumination conditions, fluorescein photobleached to about 20% of its initial value in 30 seconds; the fluorescence of Alexa Fluor 488 phalloidin stayed at the initial value under the same illumination conditions.

Source: `http://probes.invitrogen.com/servlets/photo?fileid=g001270&company=probes`

Photobleaching characterization

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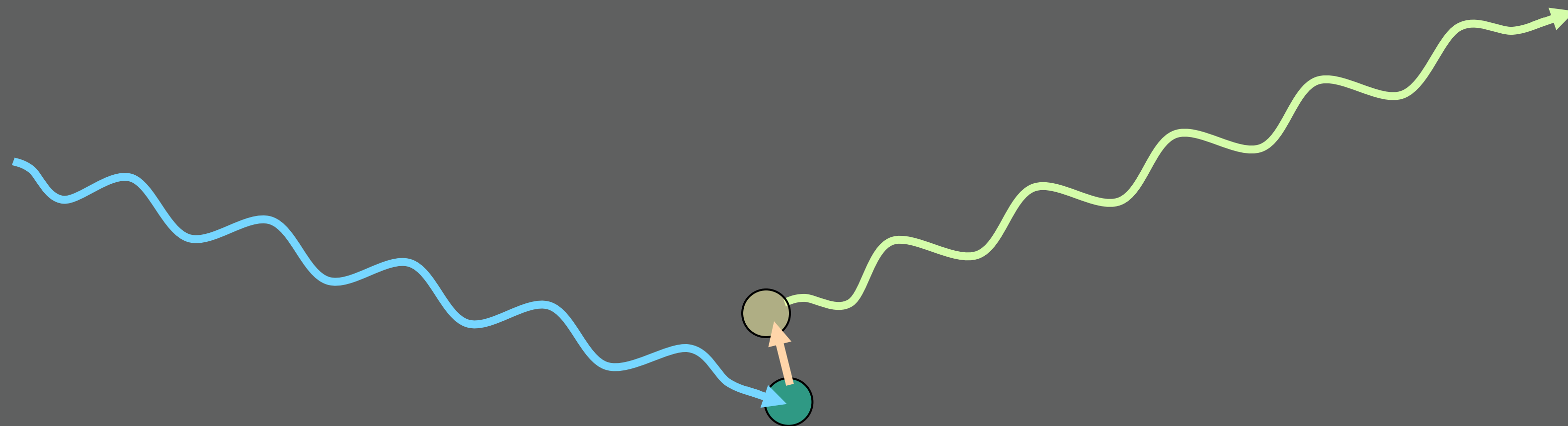
Shaner et al, Nature Biotechnology, 2004

Resonance Energy Transfer (non-radiative)



Transfer of energy from one dye to another

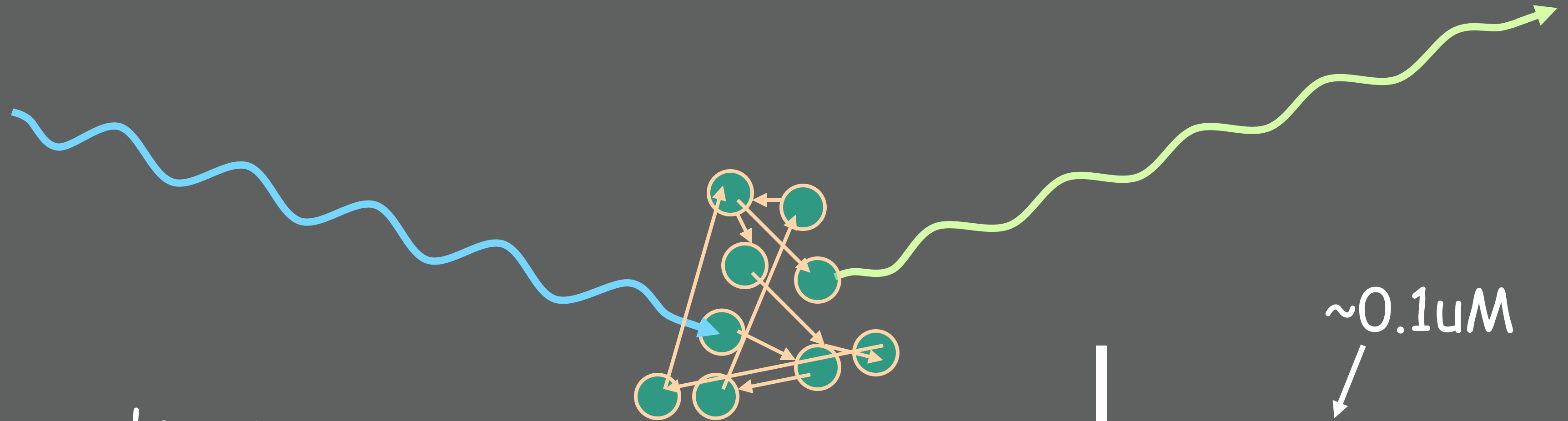
Depends on:

- Spectral overlap
- Distance
- Alignment

FRET is not always between dissimilar dyes

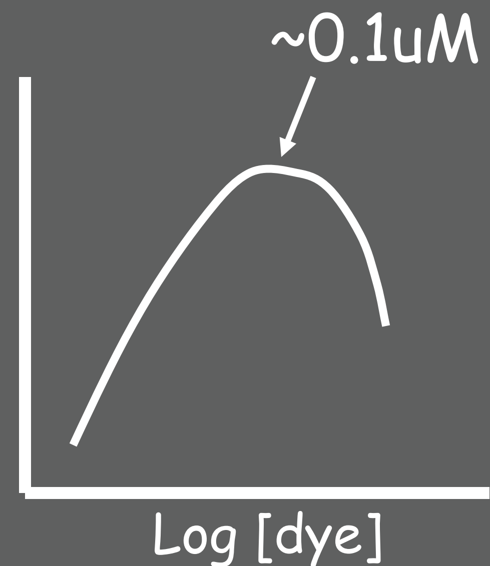
"Self-quenching" of dye

("hot-potato" the energy until lost)



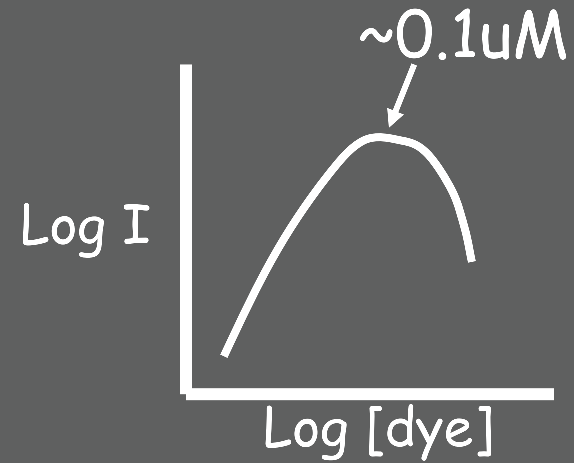
Depends on:

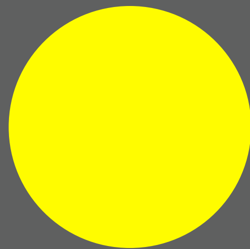
- Dye Concentration
- Geometry
- Environment



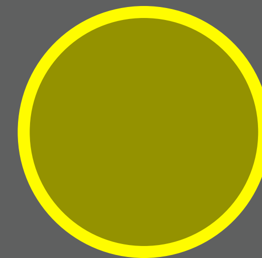
"Self-quenching" of dye

Depends on:
- Dye Concentration
- Geometry



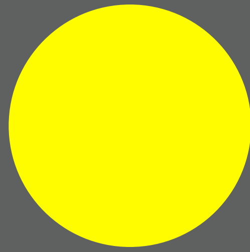


A uniformly dyed structure

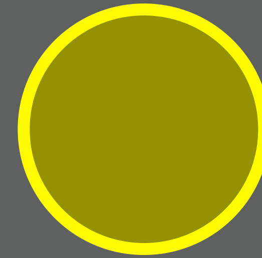


Instead, looks "hollow"

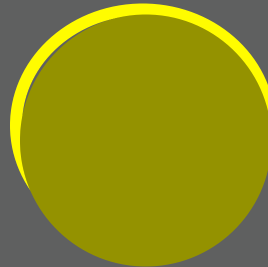
If structure large enough, collection efficiency not equivalent for top, side, bottom



A uniformly dyed structure

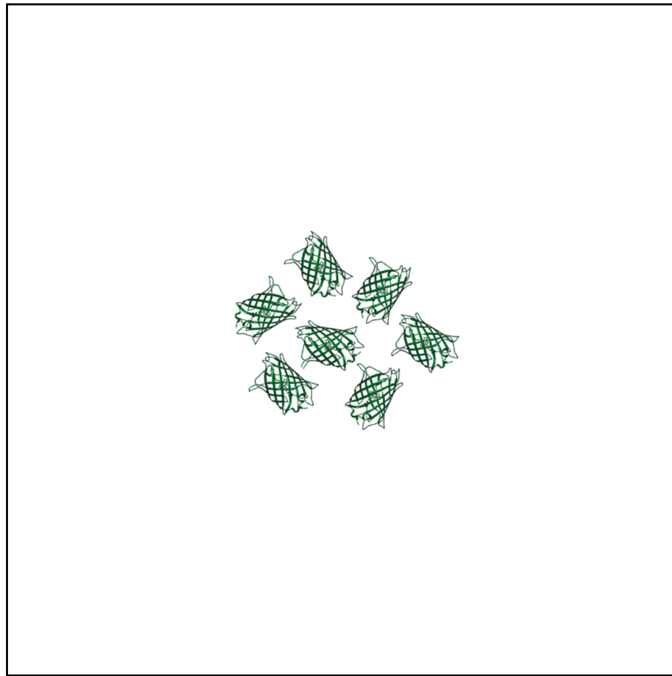


Instead, looks “hollow”

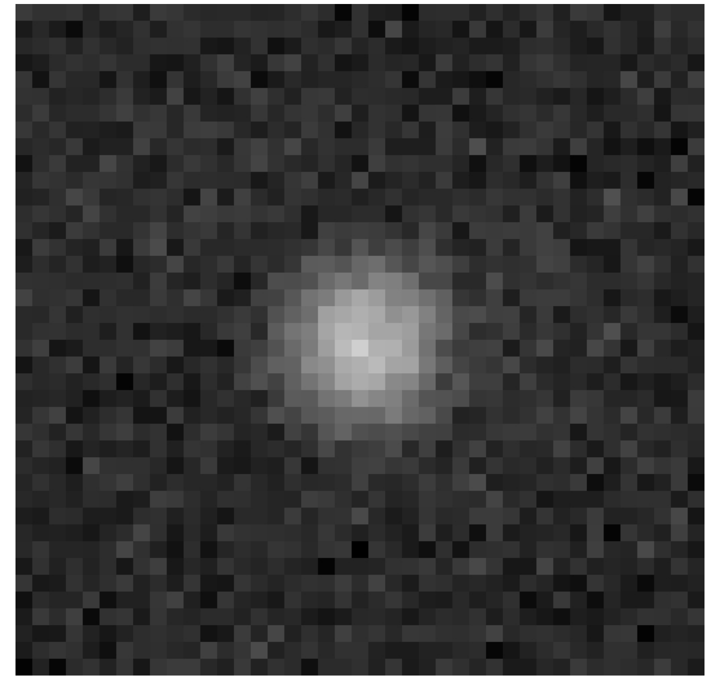


Can even become a crescent

Fluorescence quantification based on signal intensity



input: [fluorophore]

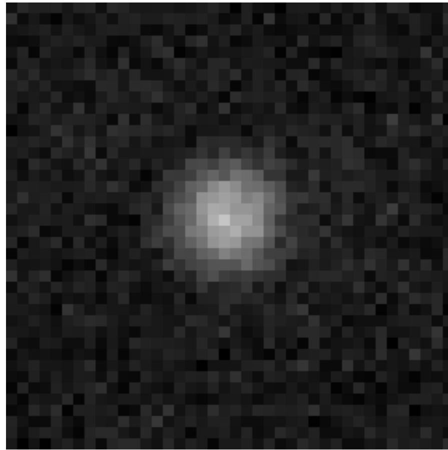


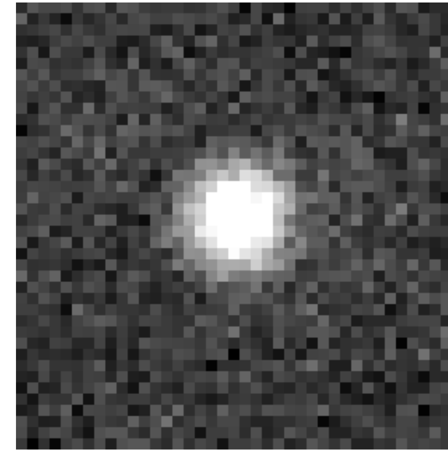
Output: pixel grey levels

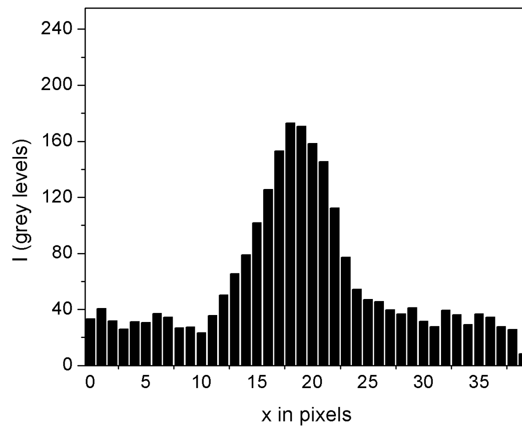
Example:
in = level of expression of a fluorescent protein
out = fluorescent signal and grey level of pixel on an image.

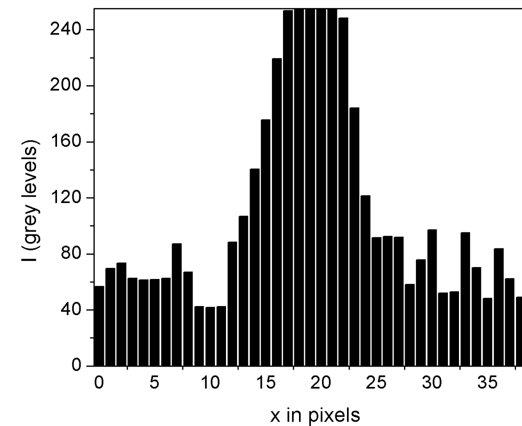
Example of nonlinearity: pixel saturation

(detector or digital contrast)



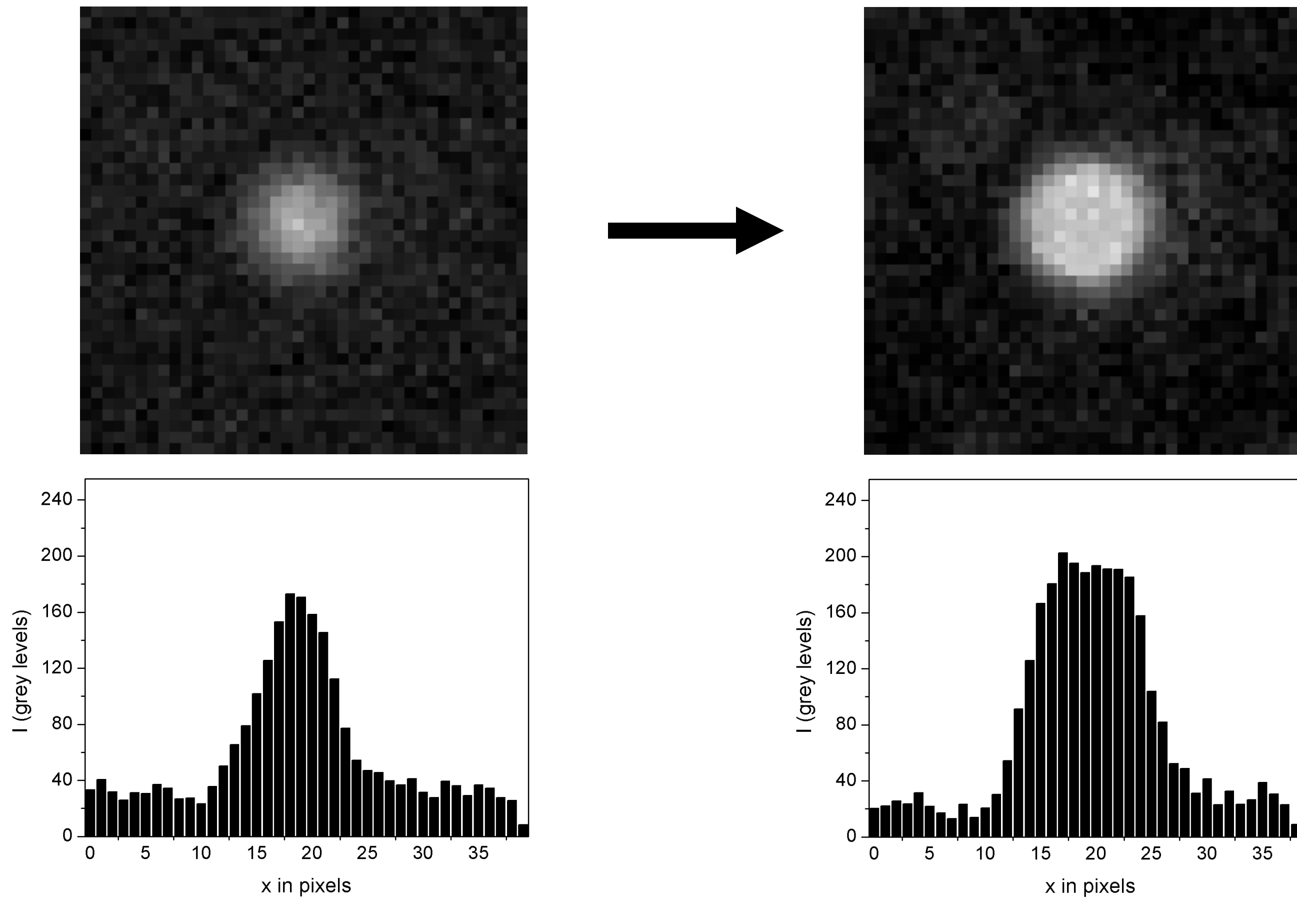






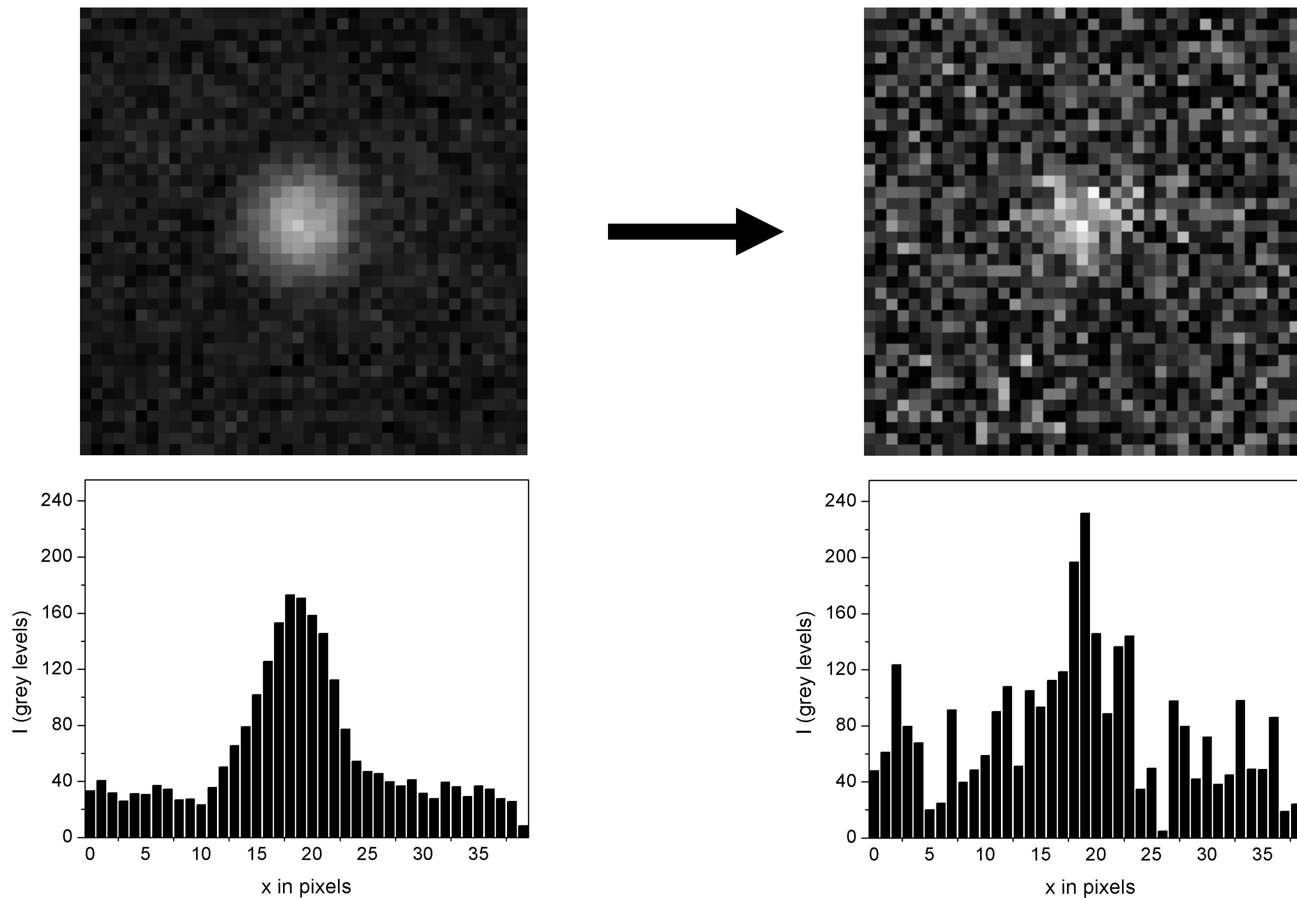
Solution(s): use less power!!!, decrease the acquisition time, decrease [fluorophore],…

Example of nonlinearity: fluorophore saturation



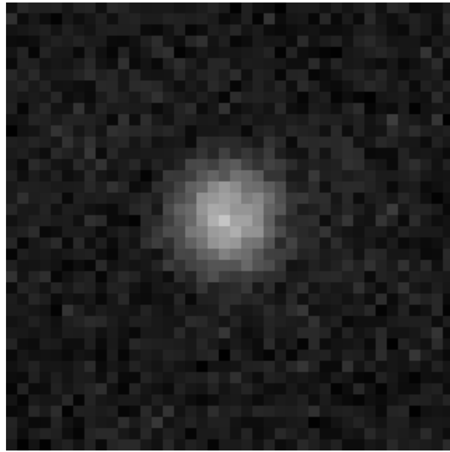
Solution(s): use less power!!!, decrease the acquisition time,…

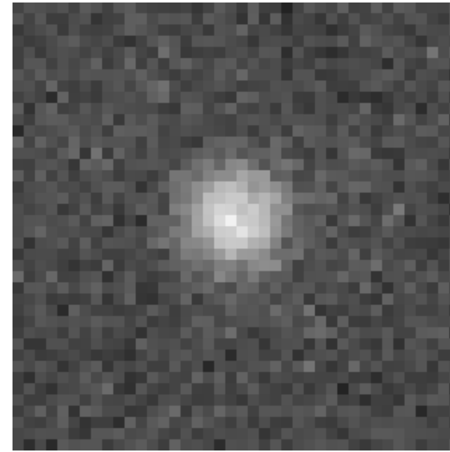
Example of nonlinearity: noise

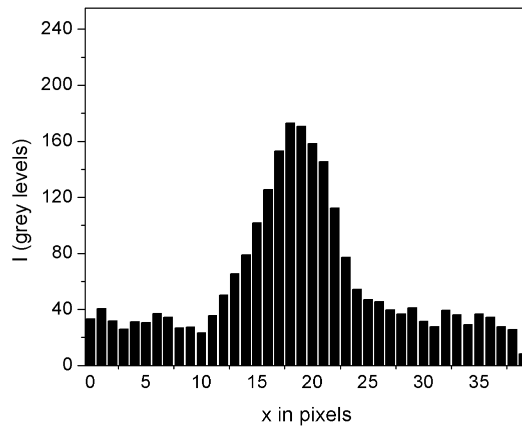


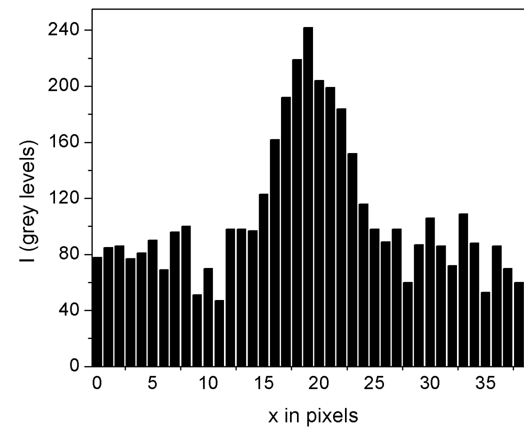
Solution(s): optimize the excitation wavelength, increase the acquisition time, use more power, use a stronger fluorophore, increase [fluorophore],…

Example of nonlinearity: photo-induced fluorescence





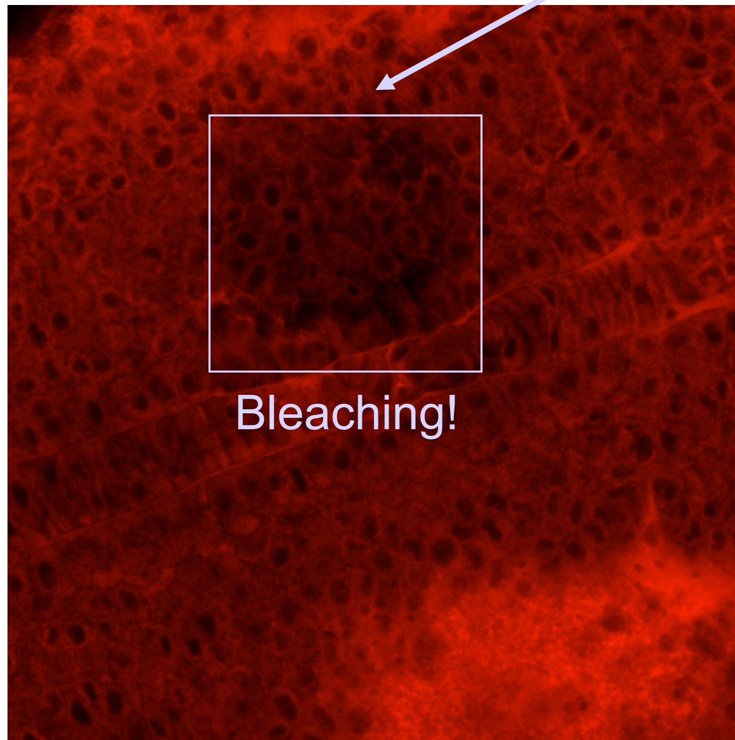




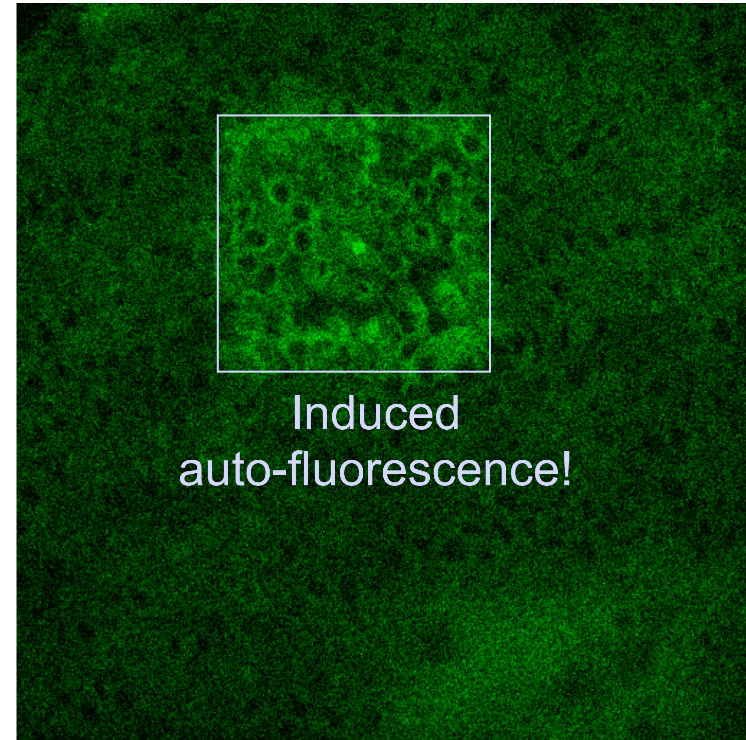
Solution: use less power!

Example of nonlinearity: photo-induced fluorescence

zoom out after imaging in this area



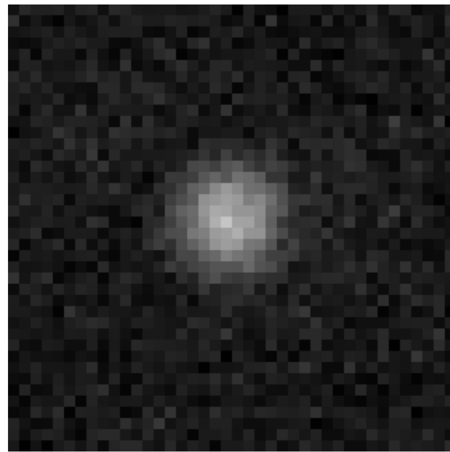
Red channel

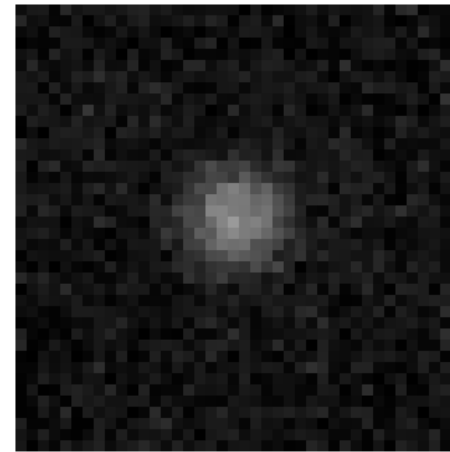


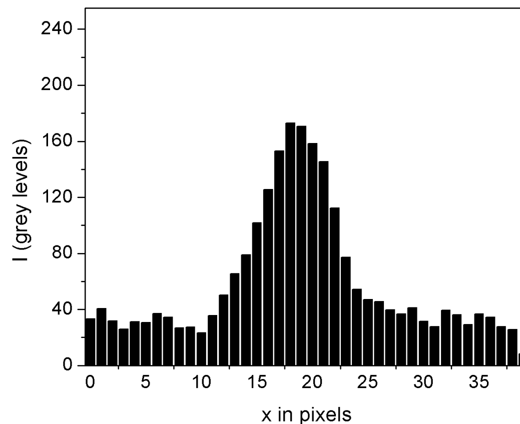
Green channel

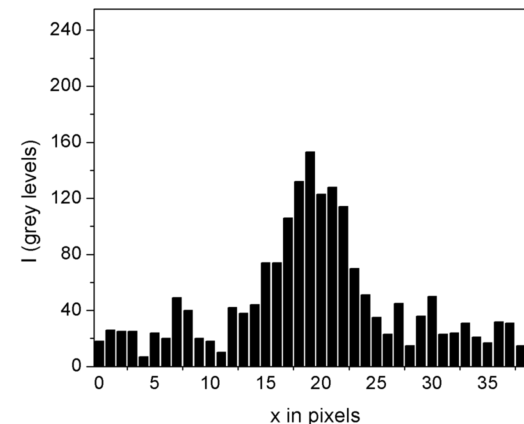
Compare what is comparable: imaging depth

Same object imaged at different tissue depth…









The fluorescence level depends on
the depth of imaging and the optical properties of the the tissue
(variation from one sample to another)…

All dyes look redder as you look deeper in tissue

How to protect yourself from non-linearities?

You can't - but you can look for diagnostic defects
- Edges to structures
- Asymmetries in intensity

Test: reduce laser; does image reduce proportionately?

Avoid over-labeling
Avoid over-stimulating

"When in doubt, reduce intensity of stimulation"

Microscope has non-linearities

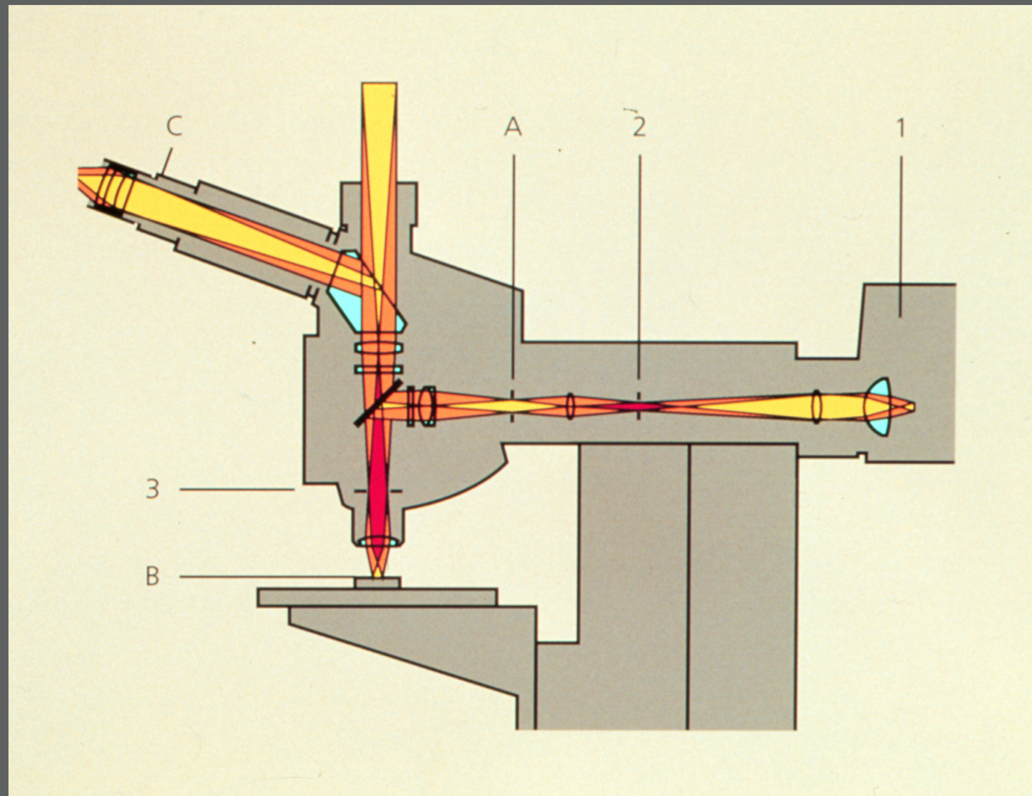
Camera

Relay optics

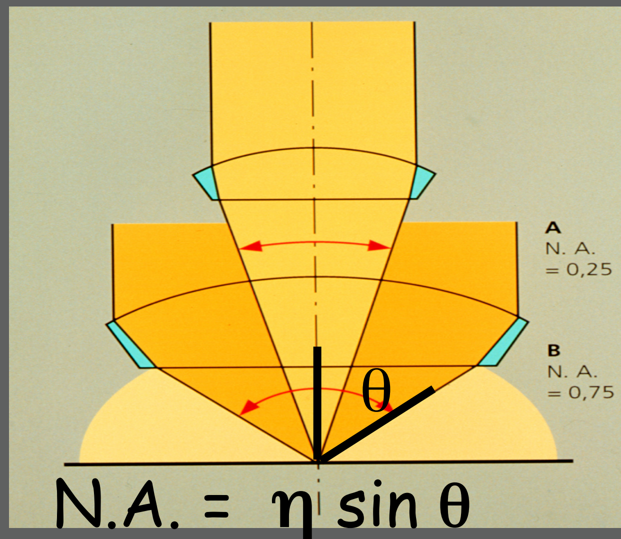
Eyepieces

light source
(image of arc)

Objective



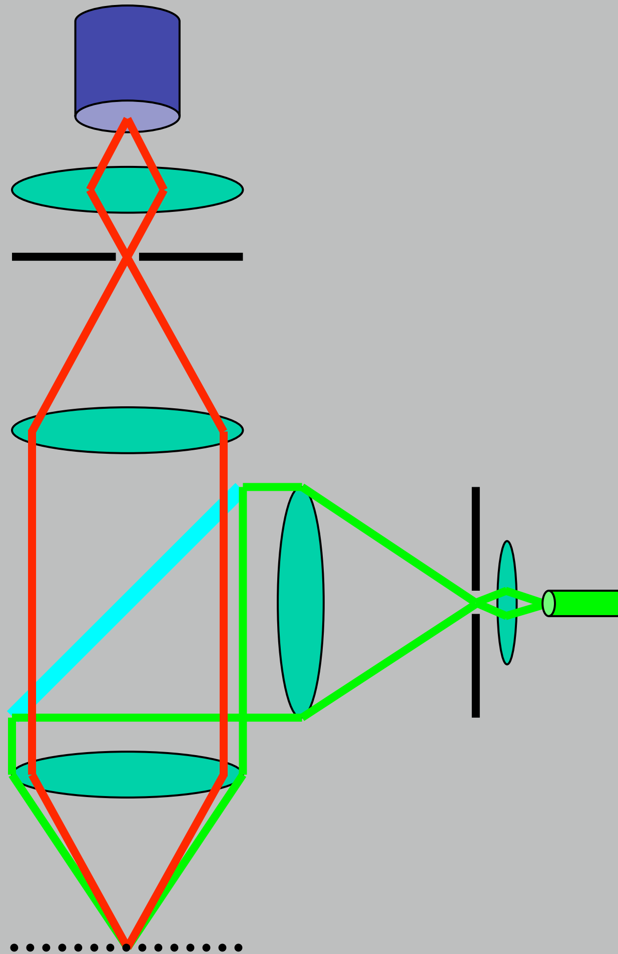
Objective lens better at collecting light near center



No free lunch from Confocal

Good: Single detector

Good: Single light source



Bad: very sensitive to optical aberrations

Bad: Easy to saturate dye (less excitation, ISC)

Spherical Aberration

The image cannot be displayed. Your computer may not have enough memory to open the image, or the image may have been corrupted. Restart your computer, and then open the file again. If the red x still appears, you may have to delete the image and then insert it again.

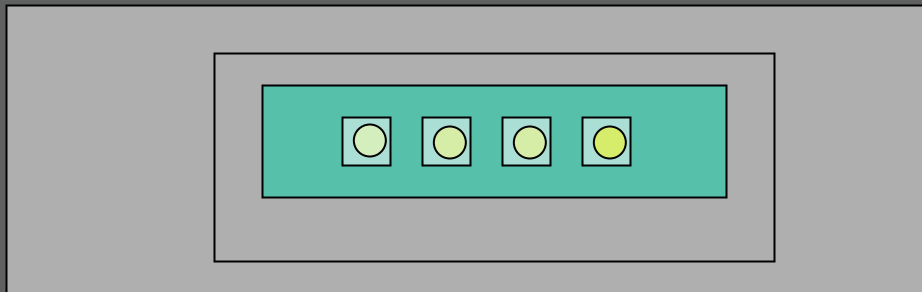
Lateral Chromatic Aberration

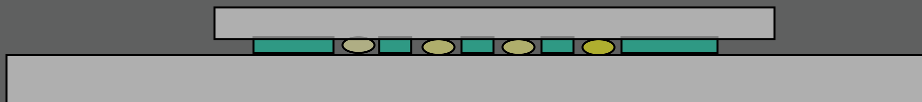
The image cannot be displayed. Your computer may not have enough memory to open the image, or the image may have been corrupted. Restart your computer, and then open the file again. If the red x still appears, you may have to delete the image and then insert it again.

Solution: Flat Fielding (pixel by pixel correction)

Requirements:

- Specimen of uniform intensity
- Set of specimens of different known brightness

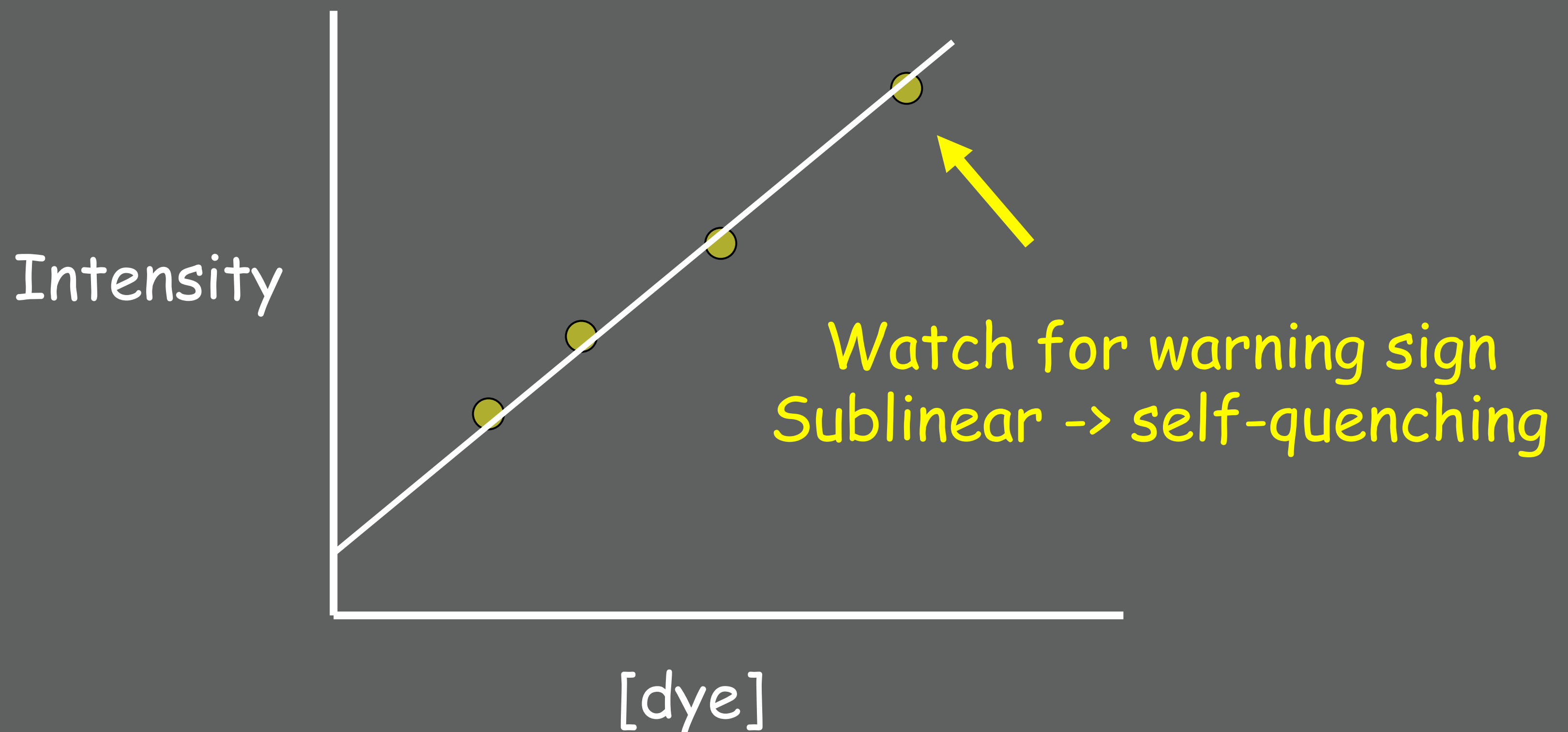




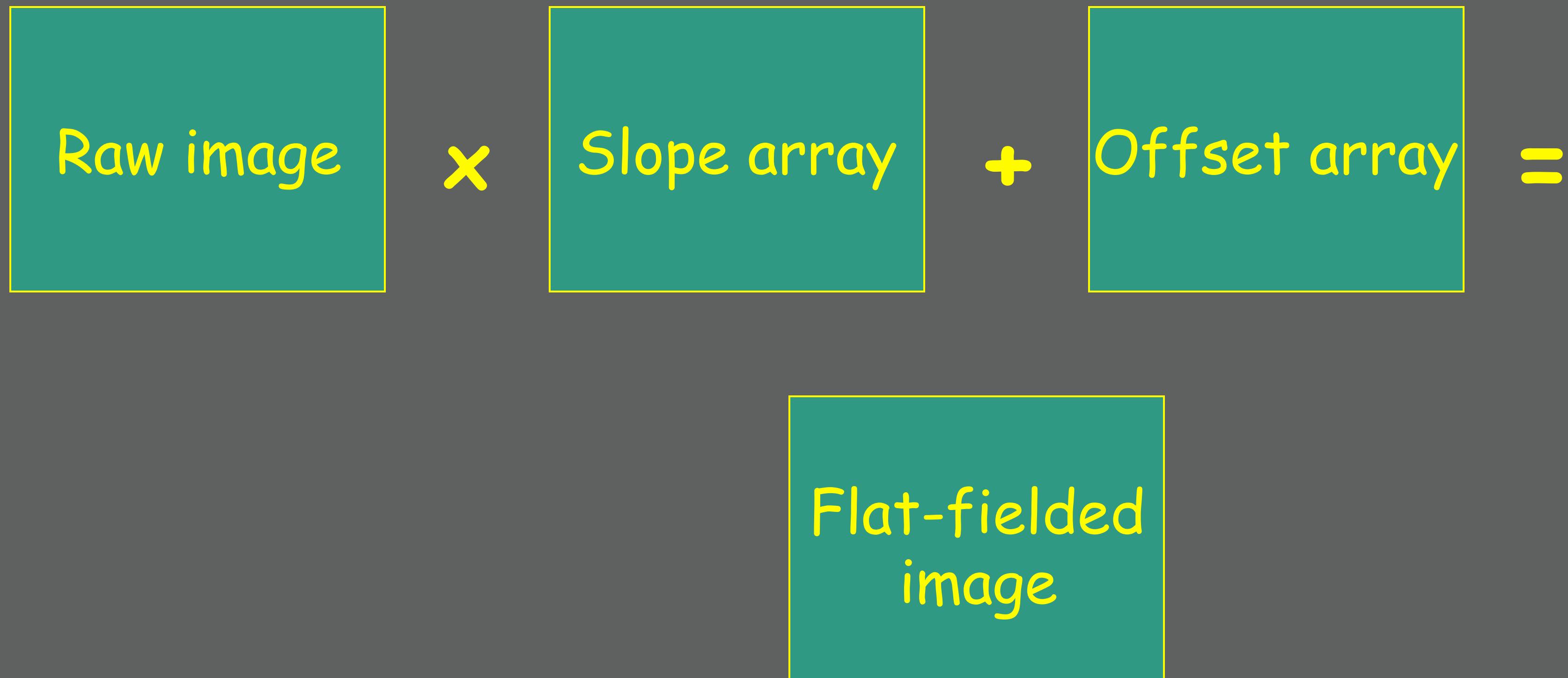
Slide with double-stick tape
Cut holes in tape
Drop dye in holes
- Different [dye] in each hole

Coverslip over the top

On pixel by pixel basis
Plot I vs [dye]
Calculate slope & intercept



References:
Kindler & Kennedy (1996) J Neurosci Methods 68:61-70
Stollberg & Fraser (1988) *J Cell Biol* **107**: 1397–1408.



References:
Kindler & Kennedy (1996) J Neurosci Methods 68:61-70
Stollberg & Fraser (1988) *J Cell Biol* **107**: 1397–1408.

So how many fluors does a given intensity equal?

Single-molecule measurements calibrate green fluorescent protein surface densities on transparent beads for use with "knock-in" animals

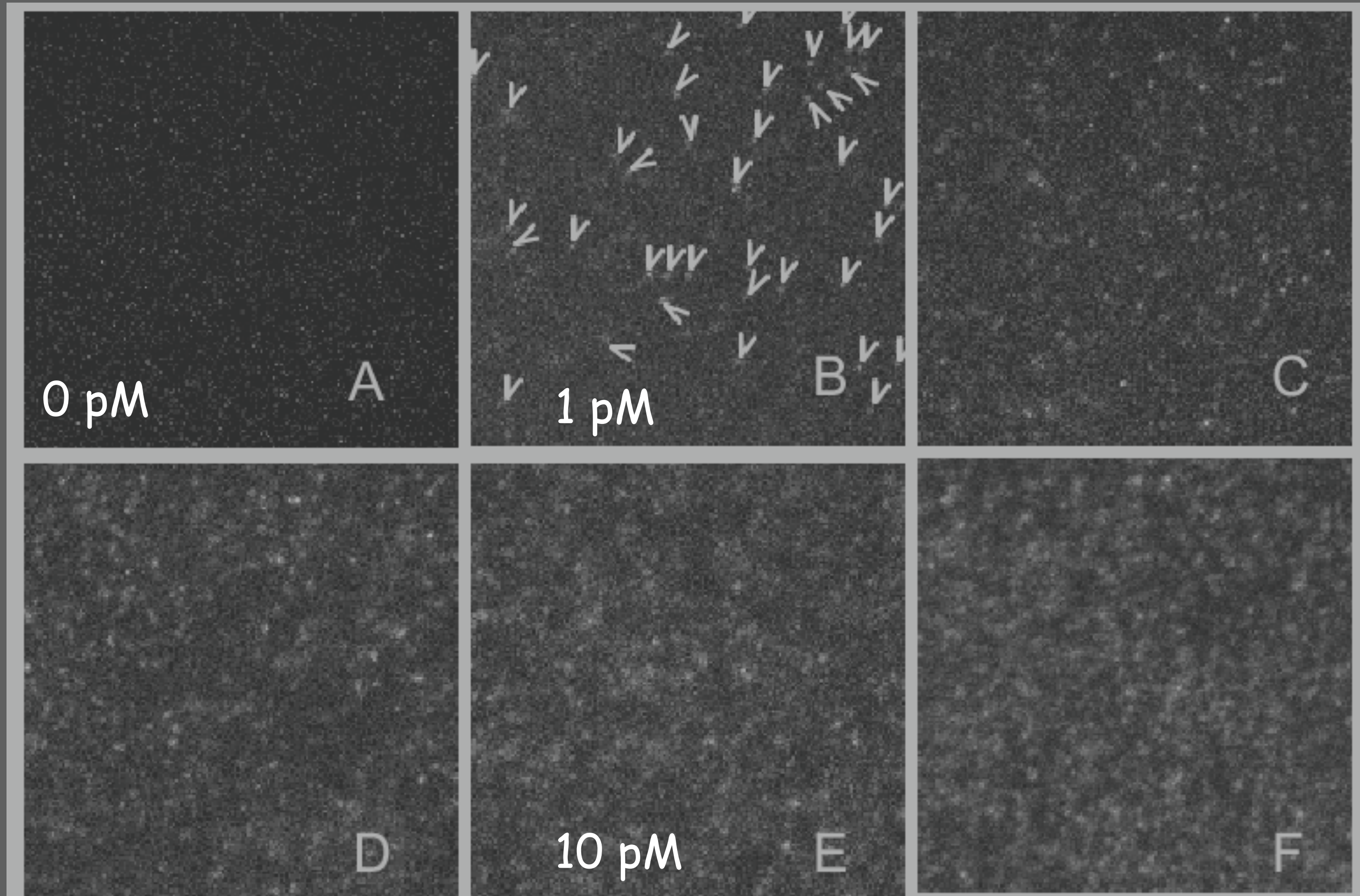
Chi-Sung Chiu[1], Emil Kartalov[2], Marc Unger[2], Stephen Quake[2], and Henry A. Lester[1]

Divisions of [1]Biology and [2]Applied Physics, California Institute of Technology, Pasadena CA 91125

Running title: Beads with calibrated GFP surface densities

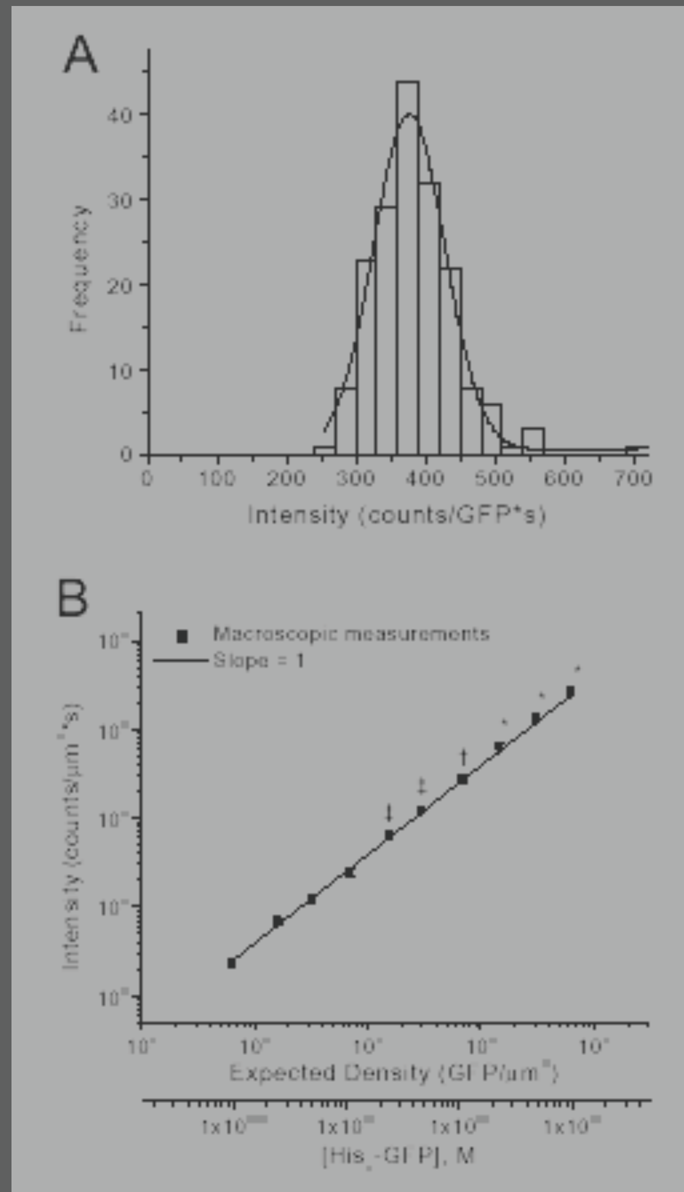
J Neurosci Methods 105:55-63 (2001)

Beads with Ni-NTA; GFP::His_6



J Neurosci Methods 105:55-63 (2001)

Alternative: use viruses with defined numbers of GFP's



J Neurosci Methods 105:55-63 (2001)

Spectral image dataset

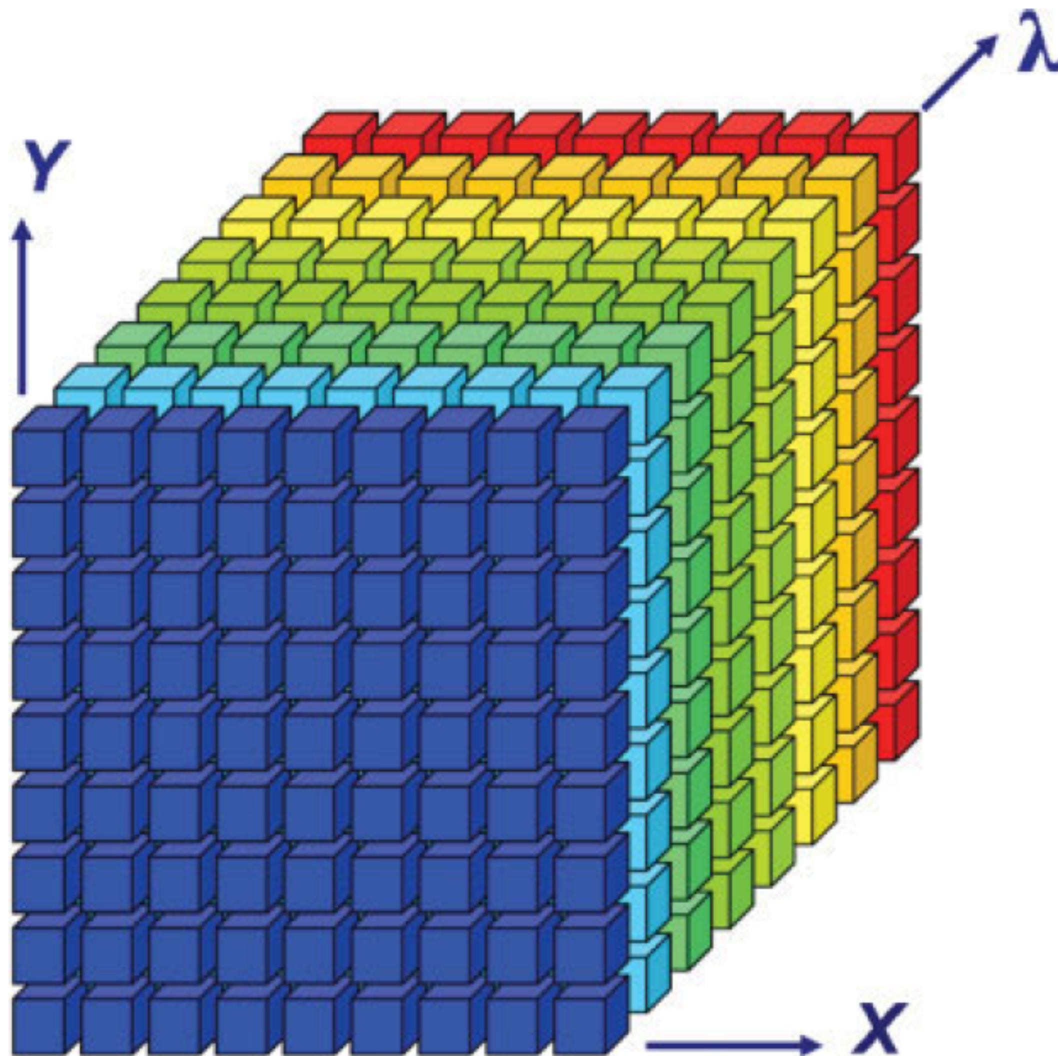


FIG. 2. Description of a spectral image data set. Each point in the cube represents a single number and the spectral image is described as $I(x,y,\lambda)$. It can be viewed either as an image $I(x,y)$ at each wavelength λ, or as a spectrum $I(\lambda)$ at every pixel (x,y).

λ-stack

λ can be:

(i) $\lambda_{excitation}$
images acquired in a single channel at different $\lambda_{excitation}$

(ii) $\lambda_{emission}$
images acquired at a single $\lambda_{excitation}$ *in several channels at different* ($\lambda_{emission}$)

Garini et al, Cytometry Part A, 2006

Spectral image dataset

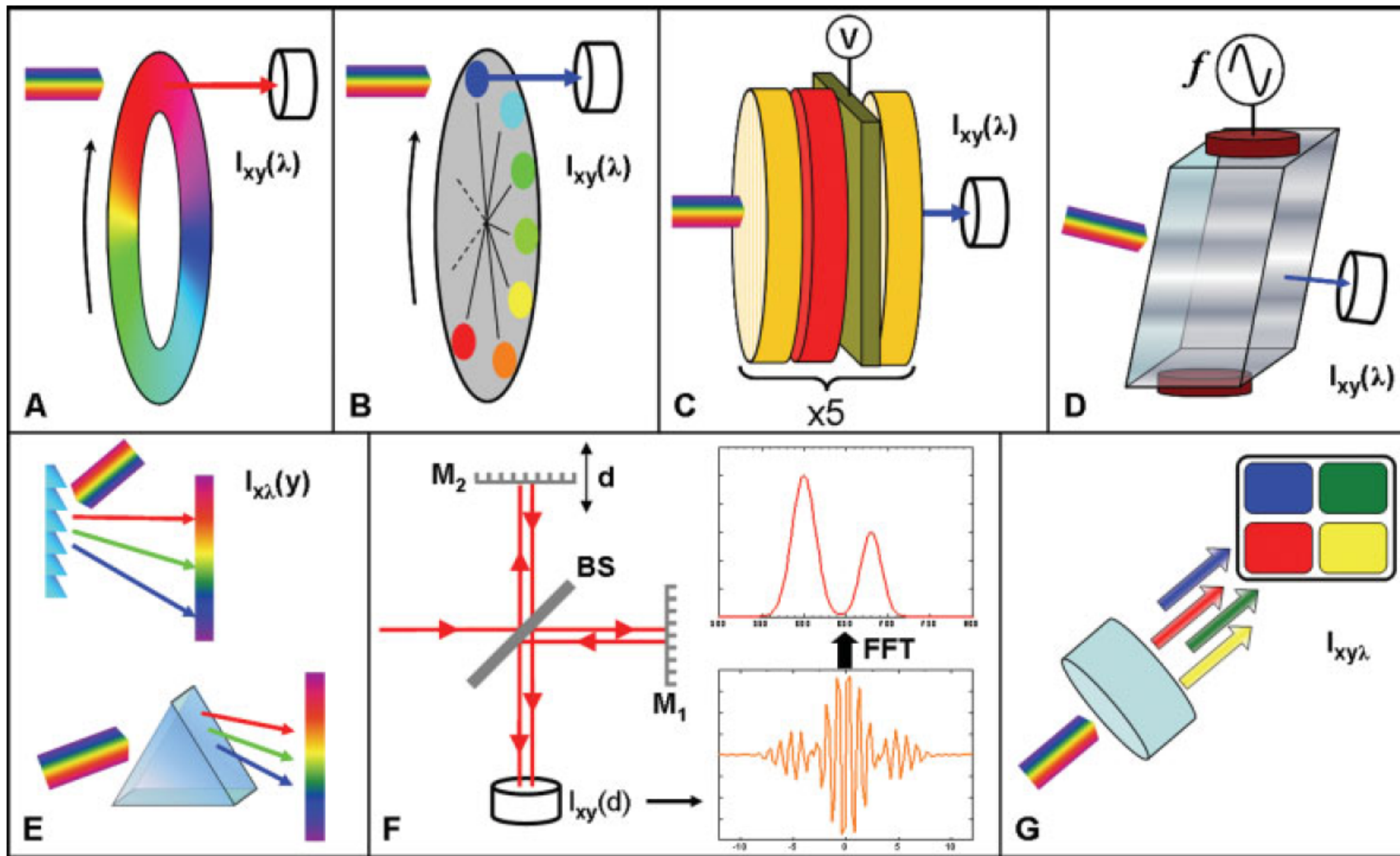
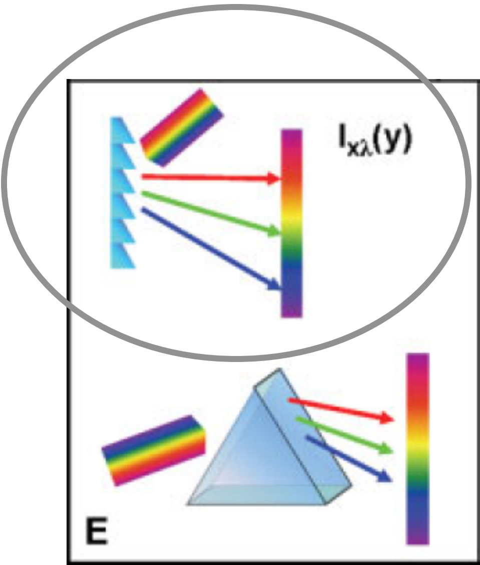
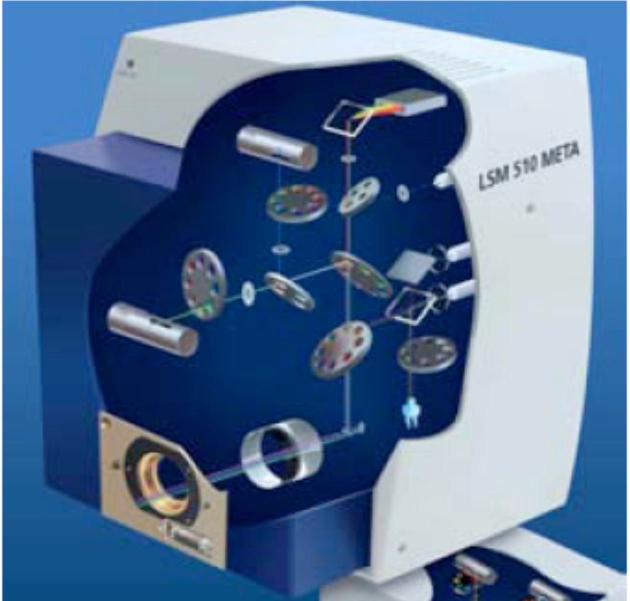


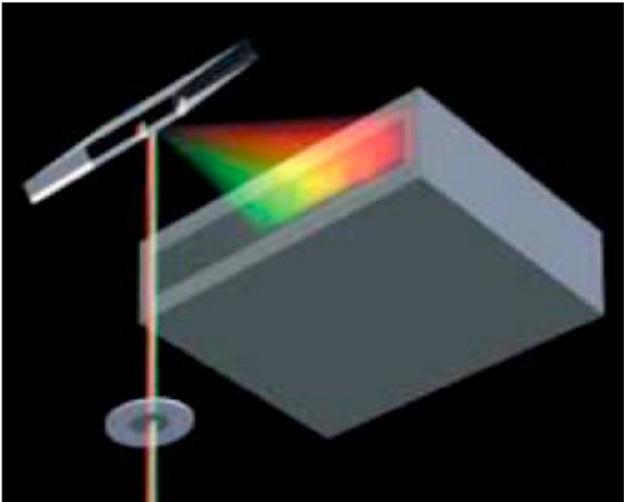
Fig. 3. Various methods of spectral imaging systems. They can be divided into four main methods: wavelength-scan (**A–D**), spatial scan (**E**), time scan (**F**) and "compromise" methods (**G**). In wavelength-scan methods, the whole image is measured one wavelength at a time. This can be realized using either a circular variable filter (A), a set of filters (B), a liquid crystal variable filter (C) or an acousto-optic variable filter (D). Spatial-scan methods use a dispersion element, either a grating or prism (E) and the image has to be scanned along at least one axis. There are also confocal microscopes that use a dispersive element and scan the image point by point. In time-scanning method (F), the whole image is measured after passing through an interferometer (or other optical elements). In order to calculate the spectrum at each pixel a mathematical transformation has to be carried out, for example, a Fourier transform. In "compromise" methods (G) only a few spectral ranges are measured and the FOV is limited, but the measurement is fast.

Garini et al, Cytometry Part A, 2006

Spectral imaging with a grating

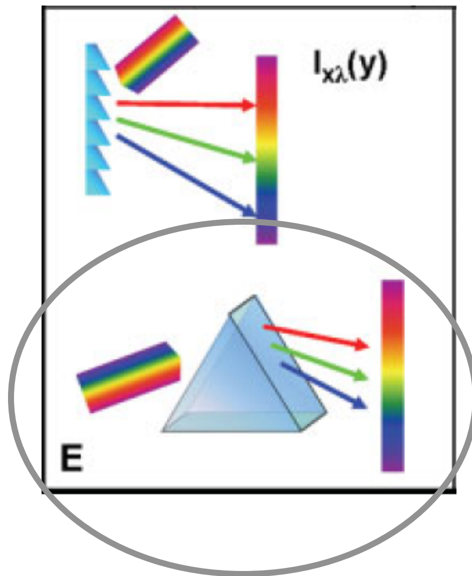


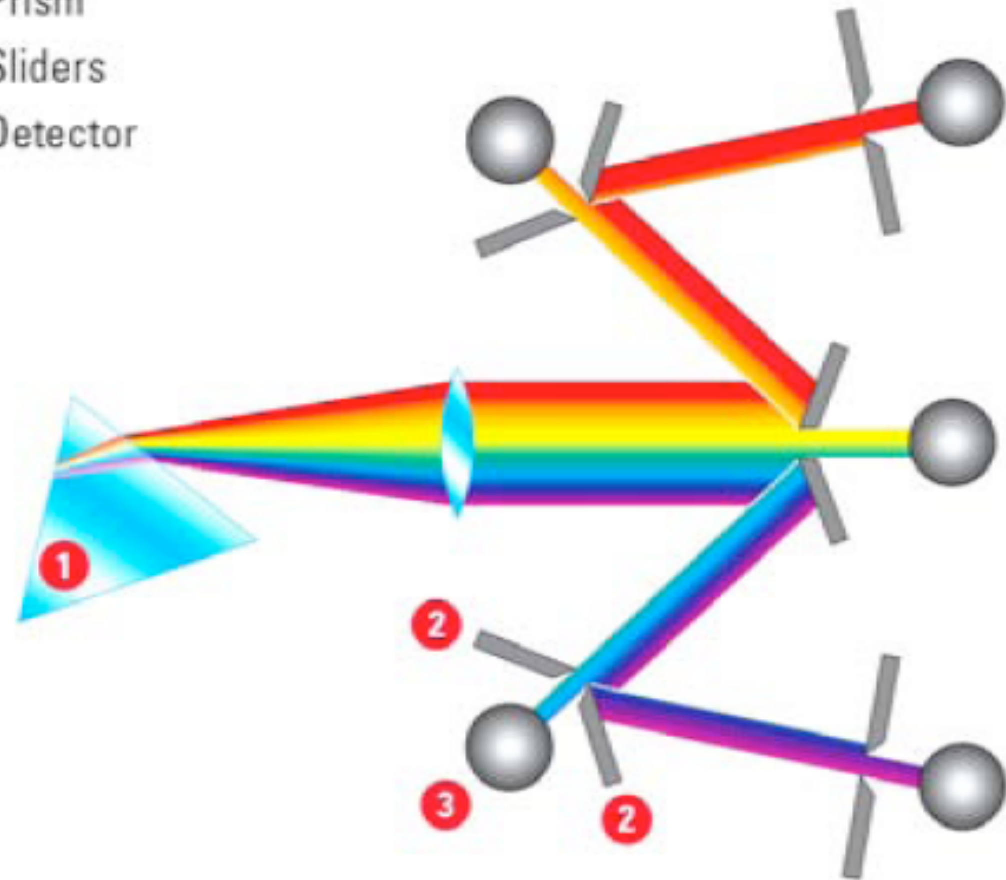




Source: Carl Zeiss, GmBH

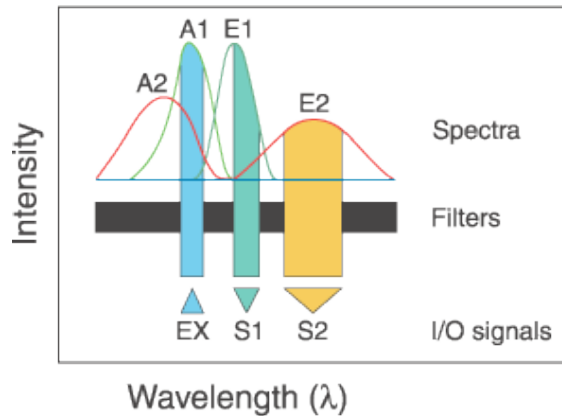
Spectral imaging with a prism and mirrors

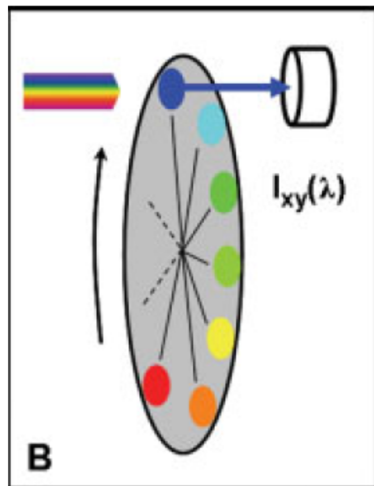


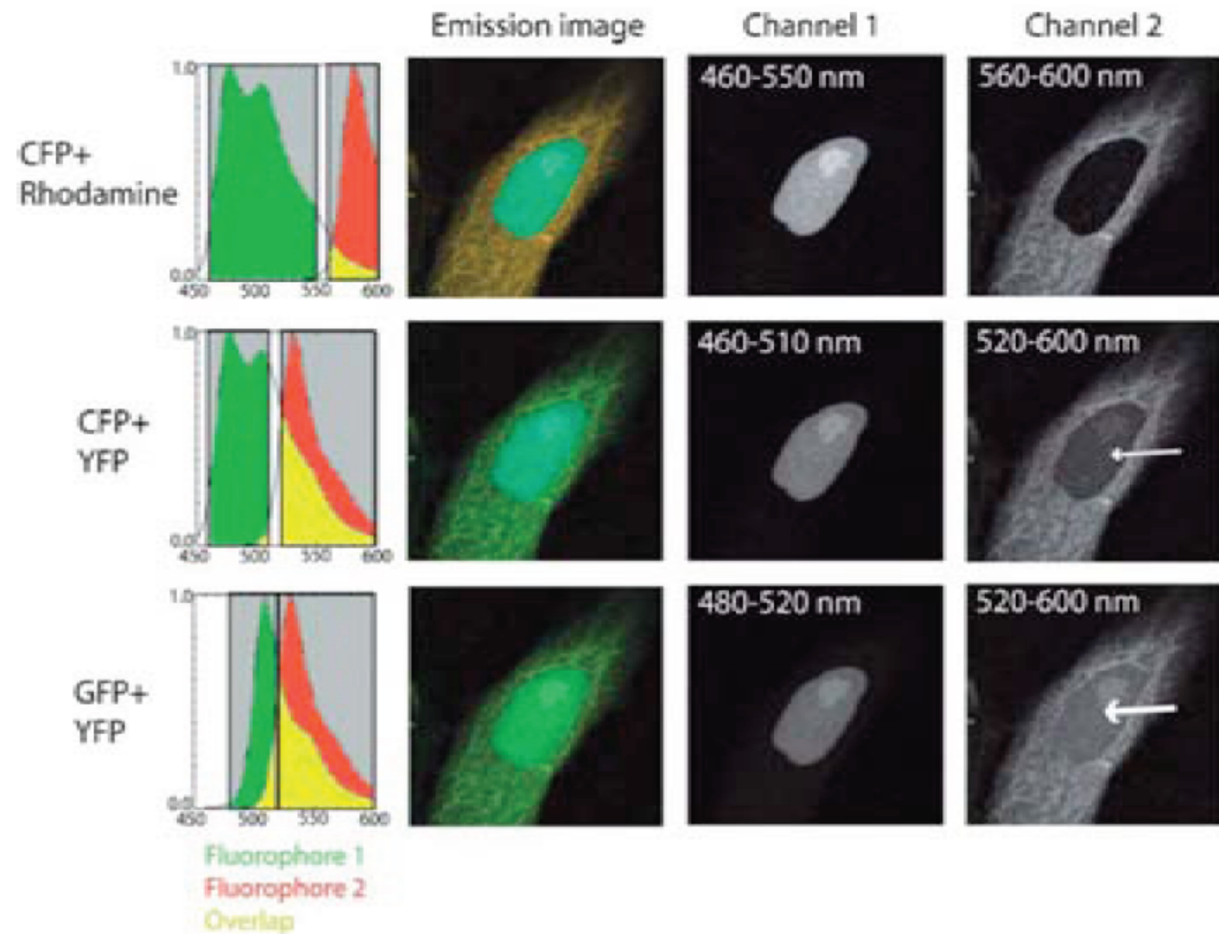


Source: Leica

Choose spectrally well-separated dyes





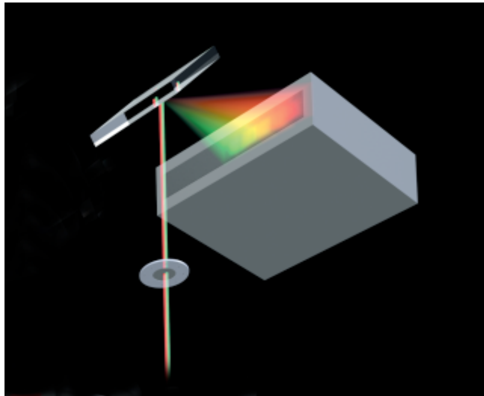


Source: T. Zimmermann, "Spectral Imaging and Linear Unmixing," Adv Biochem Engin/Biotechnol (2005) 95: 245–265

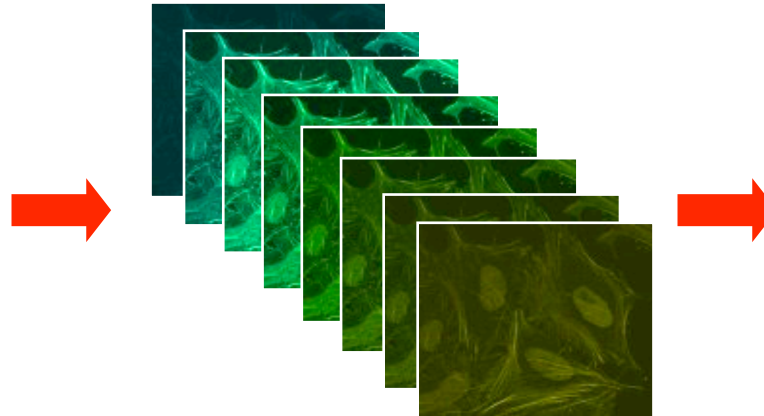
if not possible: use spectral unmixing!

Spectral unmixing: general concept

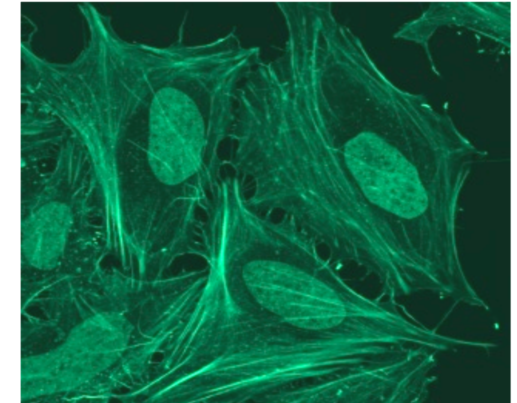
Multi-channel Detector

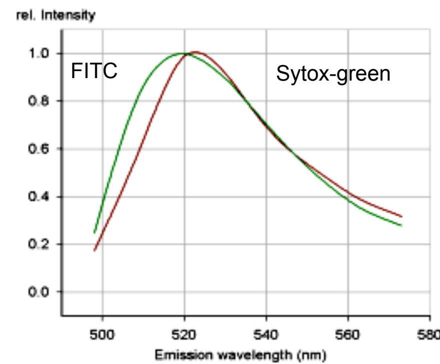


Collect Lambda Stack

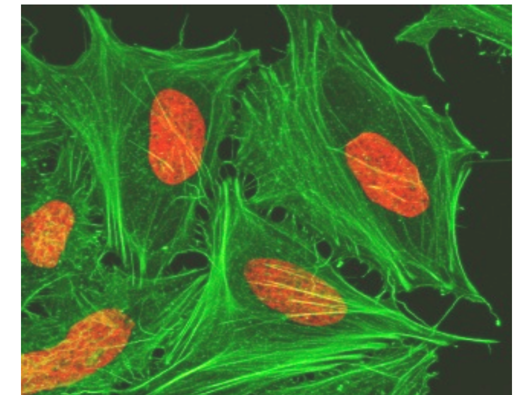


Raw Image





Derive Emission Fingerprints



Unmixed Image

Linear spectral unmixing: principle

Signal of a sample containing N fluorophores:

$$S(\lambda) = \sum_{i=1}^{N} A_i R_i(\lambda)$$

where A_i: relative contribution by specific fluorophore i with reference spectrum $R_i(\lambda)$.
If spectrum is known for M wavelengths (or integration over M wavelength intervals $\Delta\lambda$):

$$\underbrace{\begin{pmatrix} S(\lambda_1) \\ \vdots \\ S(\lambda_M) \end{pmatrix}}_{S} = \underbrace{\begin{pmatrix} R_1(\lambda_1) & \cdots & R_N(\lambda_1) \\ \vdots & \ddots & \vdots \\ R_1(\lambda_M) & \cdots & R_N(\lambda_M) \end{pmatrix}}_{R} \underbrace{\begin{pmatrix} A_1 \\ \vdots \\ A_N \end{pmatrix}}_{A}$$

To solve and obtain A_i for each pixel

From Michael Liebling, UCSB

Linear spectral unmixing: principle

2 possibilities:

Emission:
Single excitation wavelength, multi-spectral measurement (can be parallel)

Excitation:
Multiple excitations wavelengths, one detection channel (can't be parallel)

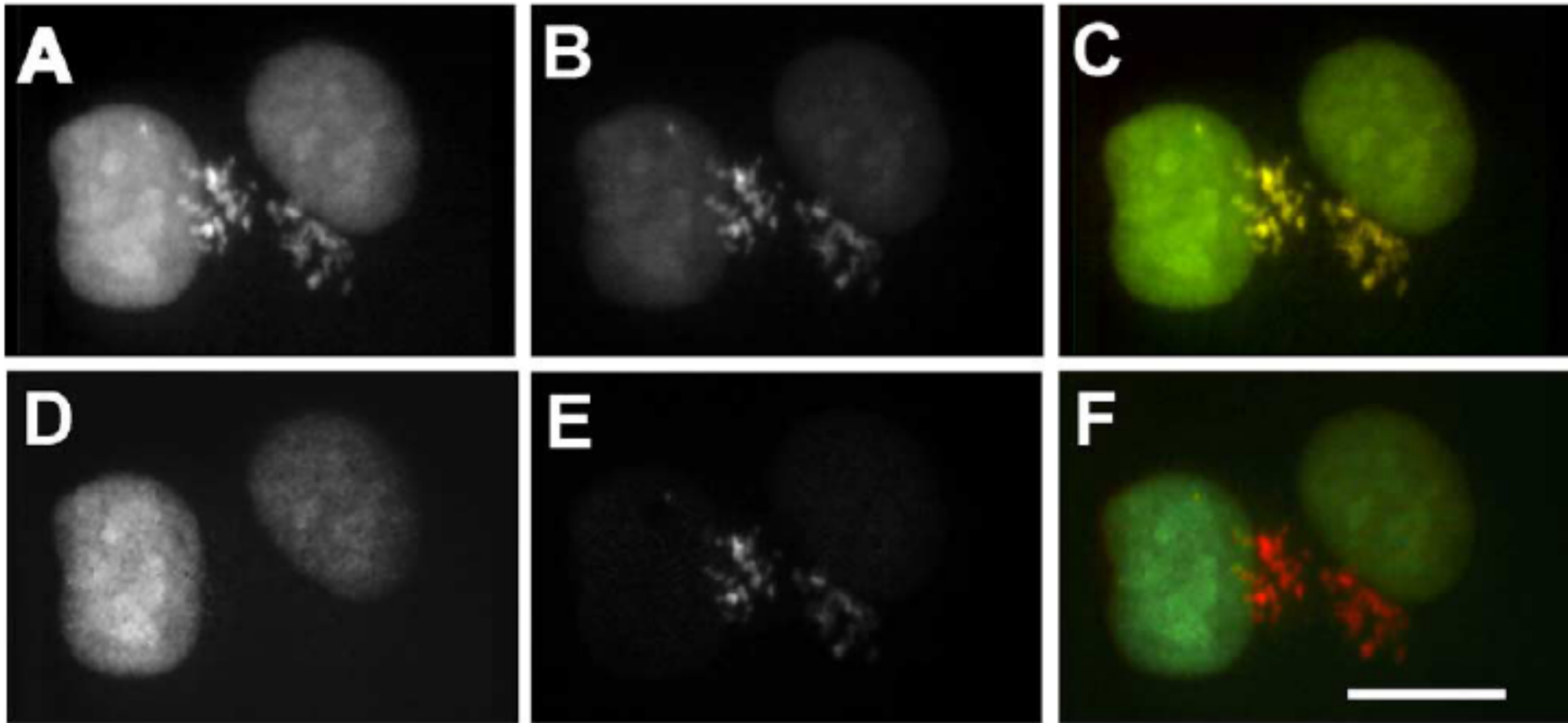
Hybrid: Multiple excitation wavelengths, multiple detection channels (can be partially parallel)

Criteria for Successful Linear Unmixing

- Number of channels should be at least equal to the number of fluorophores, $M \geq N$
- All fluorophores present in the sample have to be considered for the unmixing (don't forget autofluorescence).
  Note: unmixing is not affected by taking into account fluorophore spectra in addition to the ones present in the sample.

From Michael Liebling, UCSB

Spectral unmixing: GFP/YFP

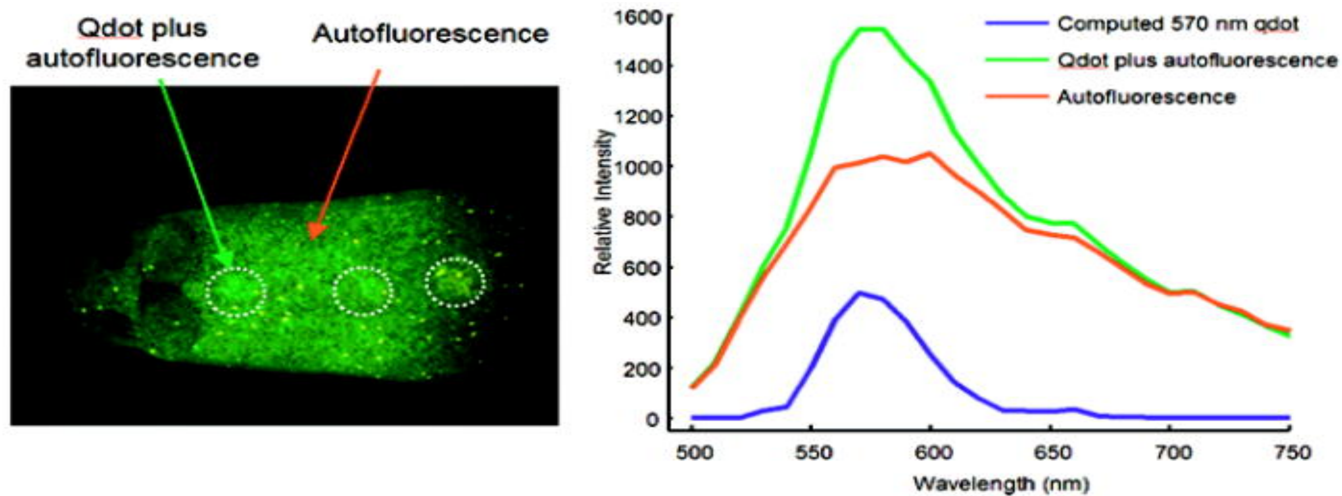


488 nm excitation, two channel detection (505–530nm, 530–565nm).

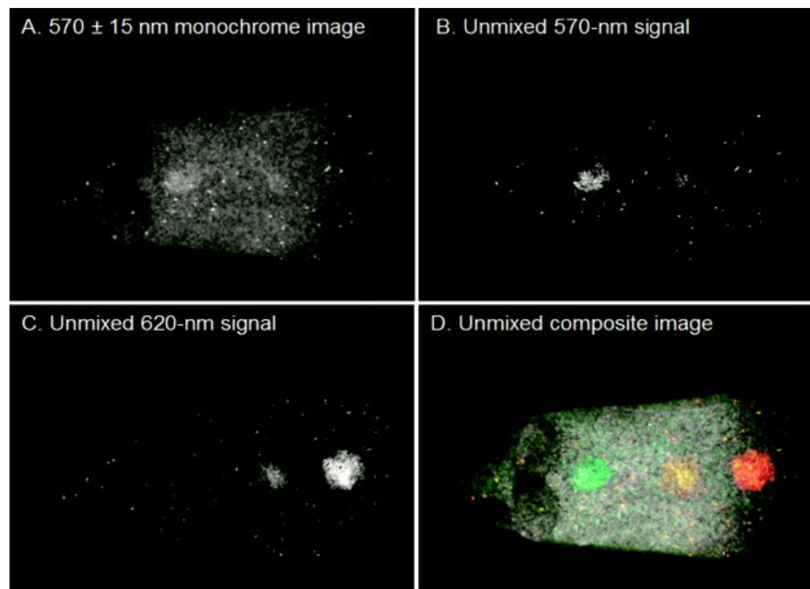
Histone-GFP (nucleus), YFP (Golgi)

From: Zimmermann T, Rietdorf J, Pepperkok R. Spectral imaging and its applications in live cell microscopy. FEBS Lett 2003;546:87–92.

Spectral unmixing of autofluorescence



Red and green arrows indicate regions from which sample spectra were obtained.
Blue = computed spectrum



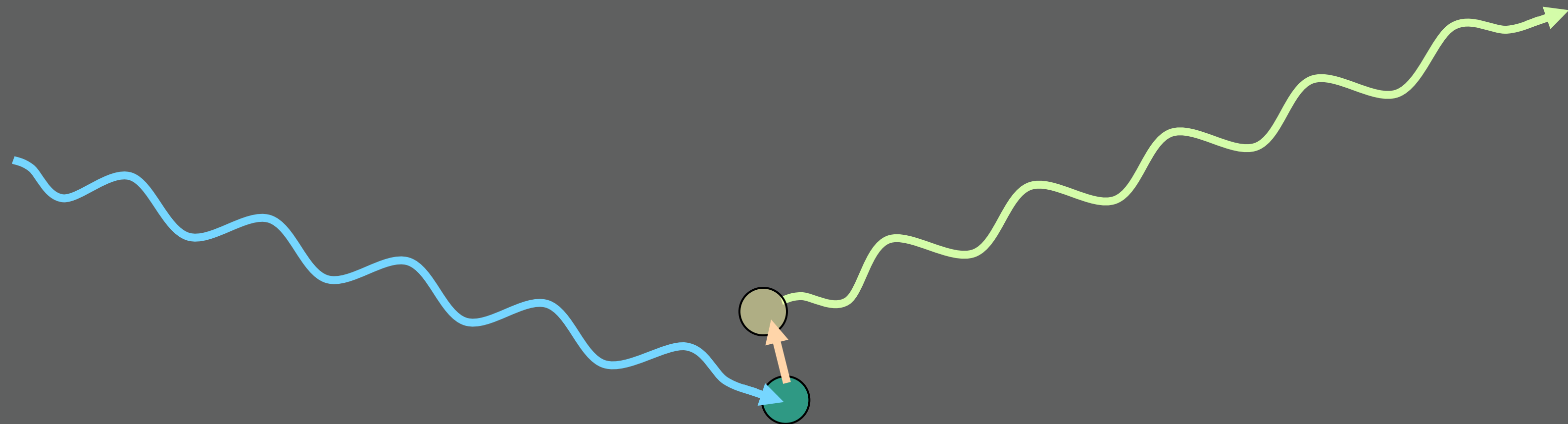
(a) Image obtained at the peak of one of the quantum dots.
(b) Unmixed image of the 570-nm quantum dot.
(c) Unmixed image of the 620-nm quantum dot.
(d) Combined pseudocolor image of (b) (green), (c), and autofluorescence channel (in white).

Mansfield et al, Journal of Biomedical Optics (2005)

FRET:

Resonance Energy Transfer (non-radiative)

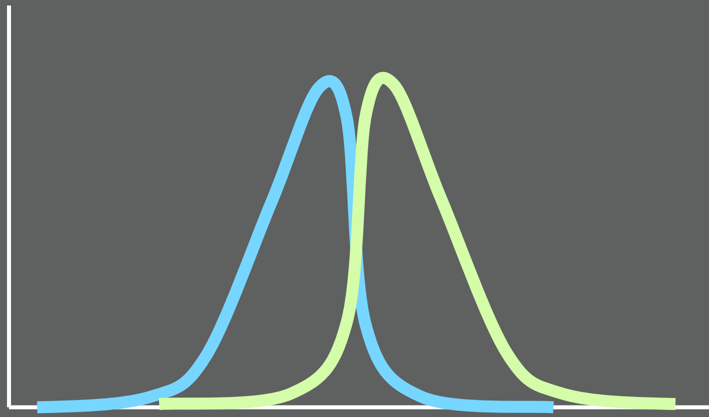
The Good: FRET as a molecular yardstick

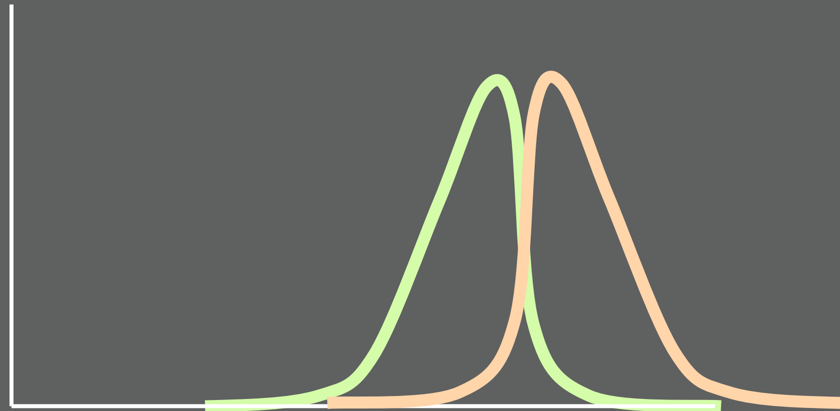


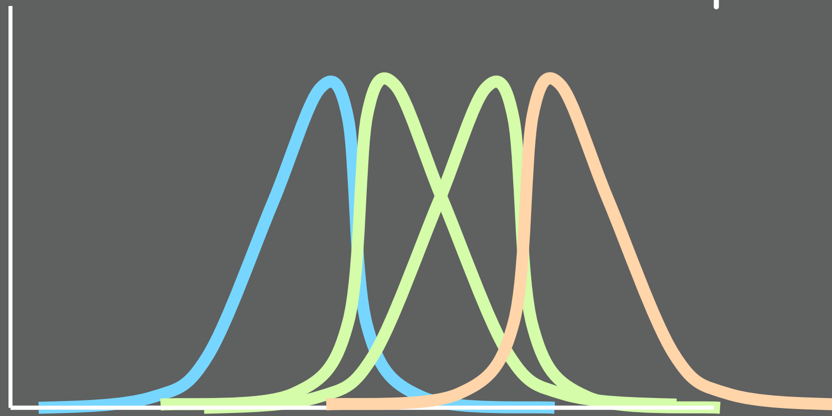
Transfer of energy from one dye to another

Depends on:

- Spectral overlap
- Distance
- Alignment







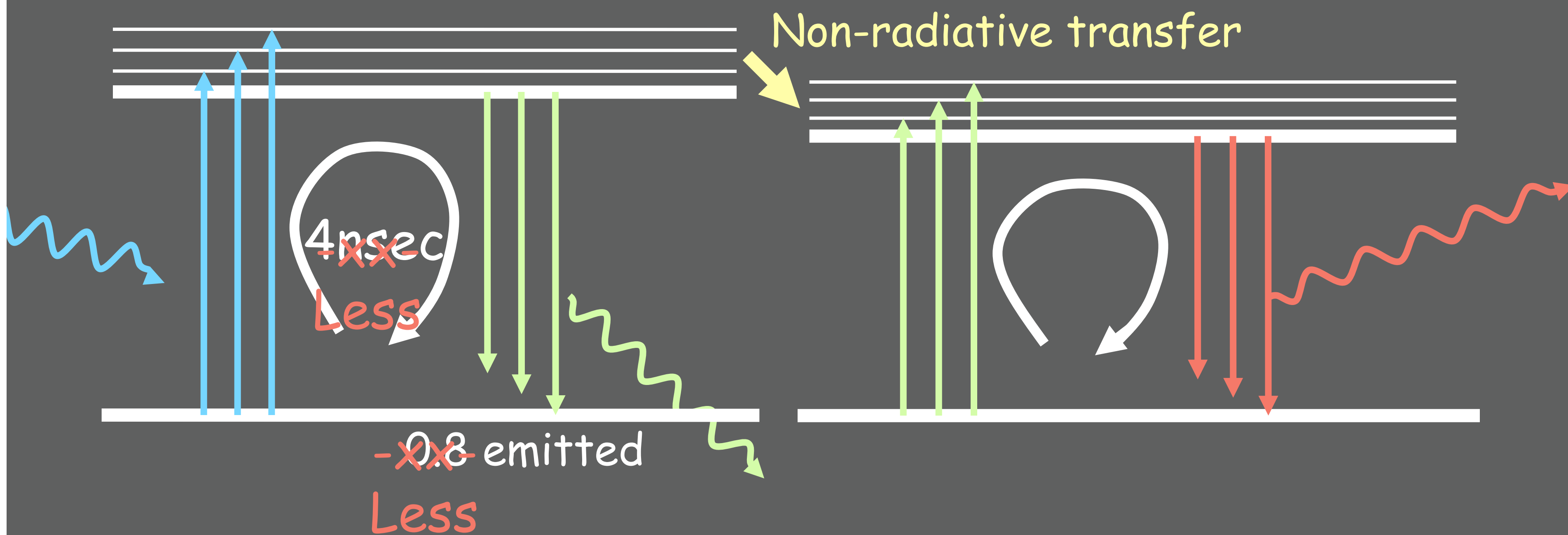
FRET:

Optimize spectral overlap

Optimize κ^2 -- alignment of dipoles

Minimize direct excitement of the acceptor
(extra challenge for filter design)

FRET Diagram



The Förster Equations.

$$k_t = (R_0/r)^6 (1/\tau_d)$$

$$E = [1 + (r/R_0)^6]^{-1}$$

$$R_0 = [8.79 \times 10^{-25} J(\lambda)\, \kappa^2 Q_0 n^{-4}]^{-6}$$

$$J(\lambda) = \int f(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda$$

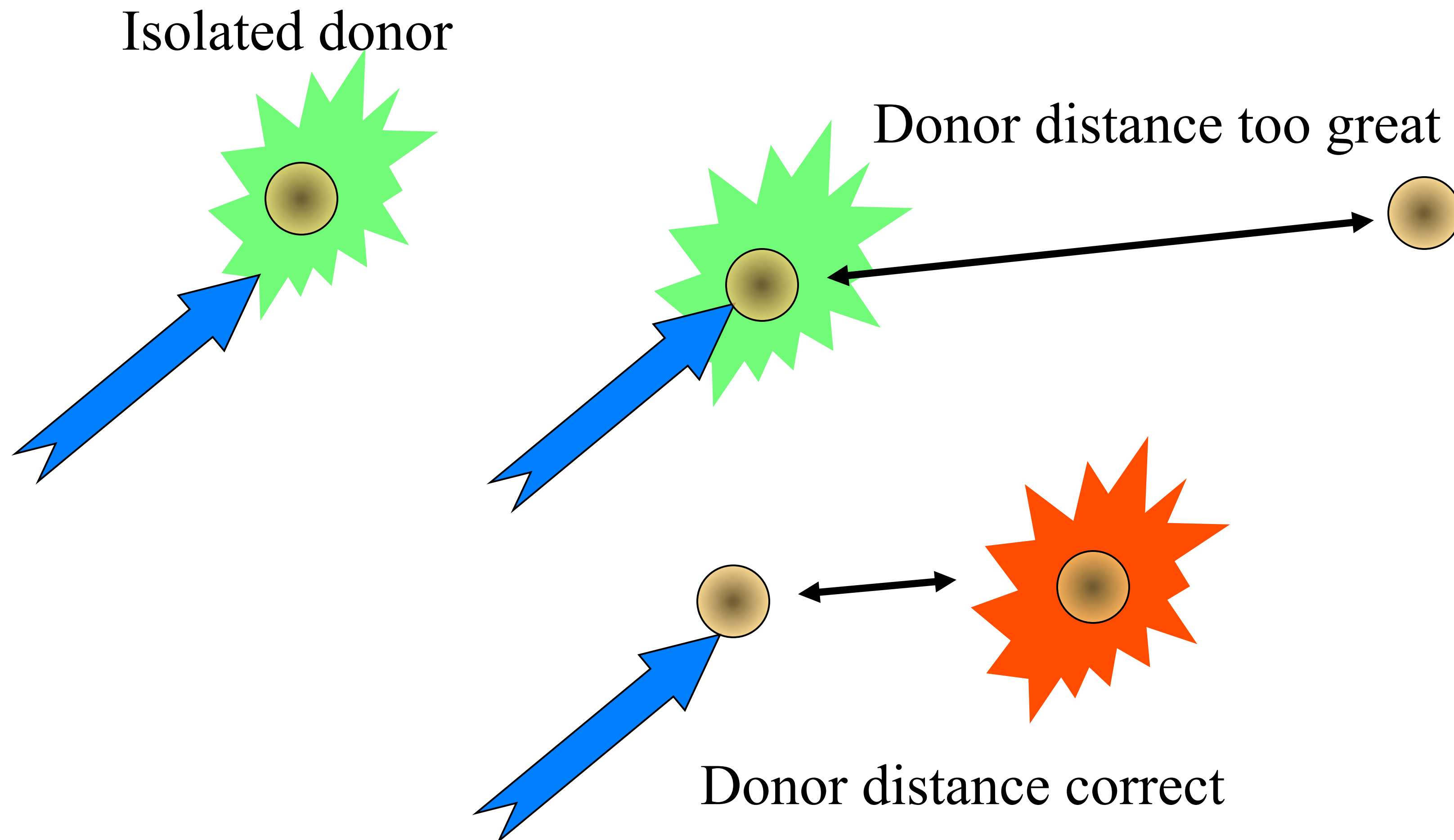
$$f(\lambda) = F_D(\lambda)\, d\lambda / \int F_D(\lambda)\, d\lambda$$

r is the center-to-center distance (in cm) between the donor and acceptor
τ_d is the fluorescence lifetime of the donor in the absence of FRET
κ^2 is the dipole-dipole orientation factor,
Q_0 is the quantum yield of the donor in the absence of the acceptor
η is the refractive index of the intervening medium,
F_D (λ) is the fluorescence emission intensity at a given wavelength λ (in cm)
ε_A (λ) is the extinction coefficient of the acceptor (in cm $^{-1}$ M $^{-1}$).

The orientation factor κ^2 can vary between 0 and 4, but
typically κ^2 = 2/3 for randomly oriented molecules (Stryer, 1978).

When r = R_0, the efficiency of FRET is 50%
(fluorescein-tetramethylrhodamine pair is 55 Å)

More about FRET (Foster Resonance Energy Transfer)



Effective between 10-100 Å only

Emission and excitation spectrum must significantly overlap

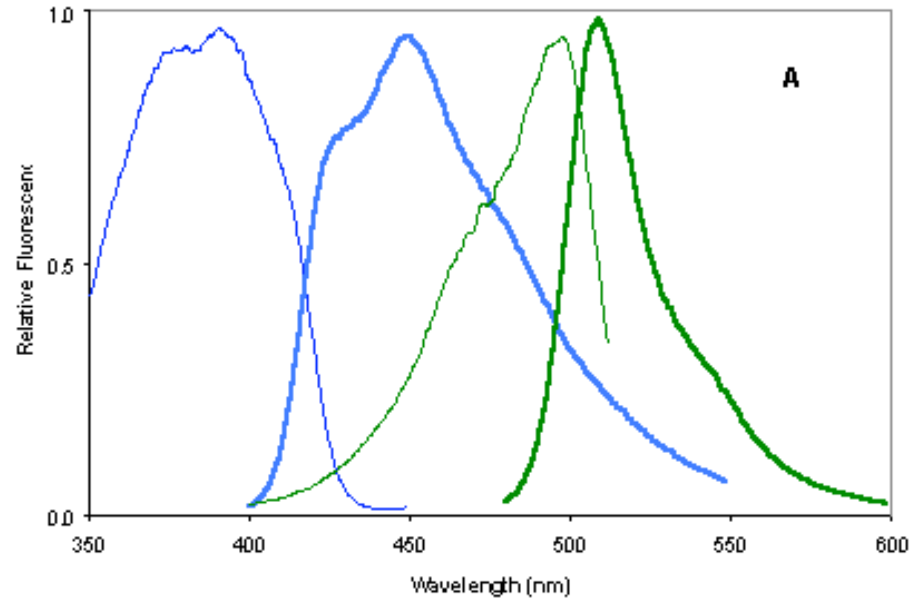
Note: donor transfers non-radiatively to the acceptor

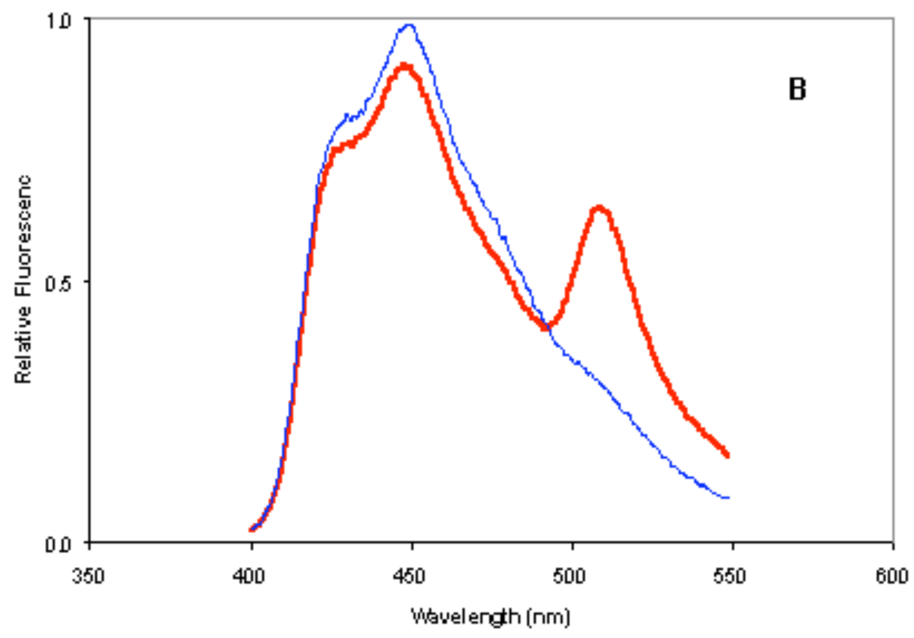
From J. Paul Robinson, Purdue University

Bi177 – Lecture 10

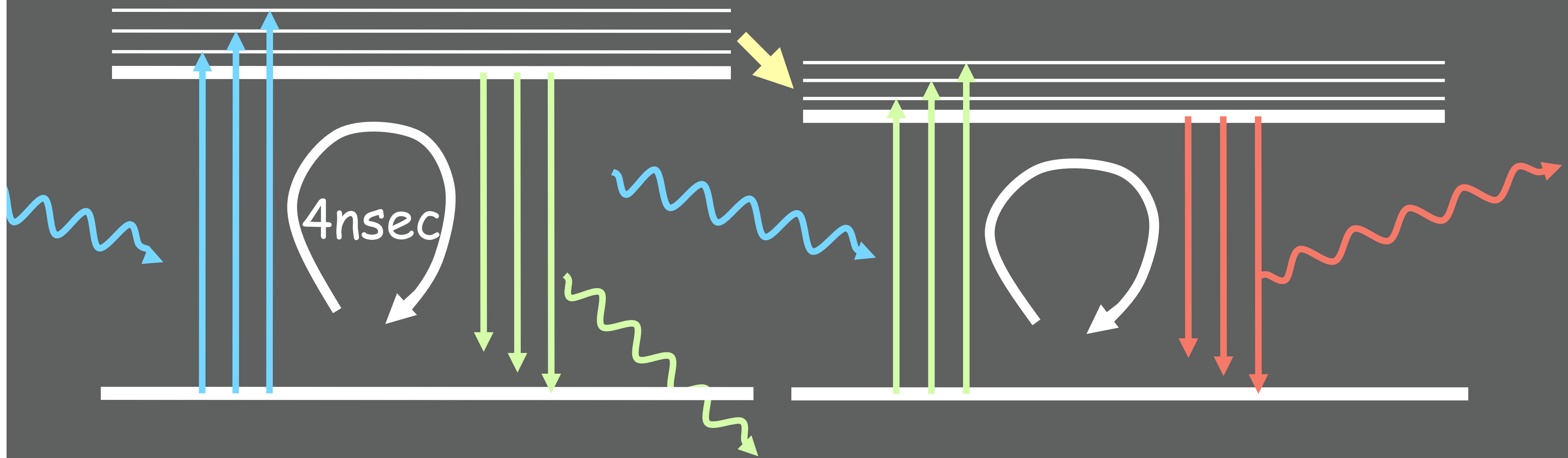
"Quantitative" Fluorescence

- Fluorescence linearity (non-linearity)
  - Dye, microscope, camera
- Flat-fielding to linearize
- Quantitating the image
- Multispectral imaging
- FRET


A
Relative Fluorescenc
Wavelength (nm)
1.0
0.5
0.0
350
400
450
500
550
600


B
Relative Fluorescenc
Wavelength (nm)
1.0
0.5
0.0
350
400
450
500
550
600

The realities strike



1. The acceptor excited directly by the exciting light
   - “FRET” signal with no exchange
   - Increased background
   - Decreases effective range for FRET assay

The realities strike

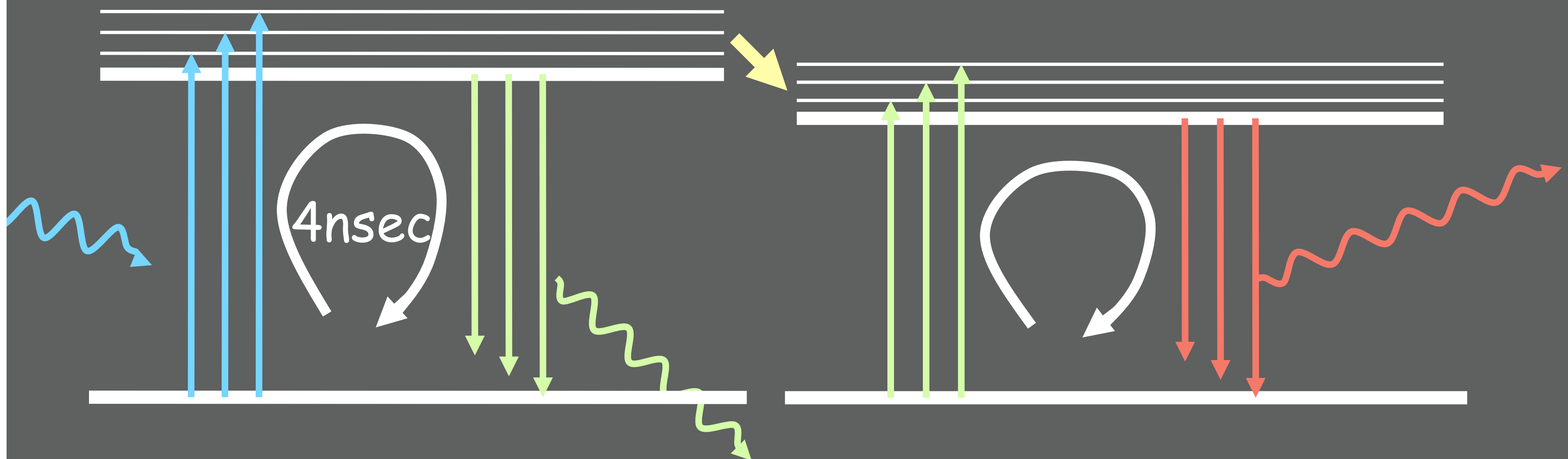
2. Hard to really serve as a molecular yardstick*
   - Orientation seldom known
     assume $\kappa^2 = 2/3$ (random assortment)

   - Exchange depends on environment of dipoles

   - Amount of FRET varies with the lifetime of the donor fluorochrome

* $r = R_0$, the efficiency of FRET is 50%
(fluorescein-tetramethylrhodamine pair is 55 Å)

Amount of FRET varies with the lifetime of the donor fluorochrome



Longer lifetime of the donor gives longer time to permit the energy transfer (more for longer)

Added Bonus: Allows lifetime detection to reject direct excitement of the acceptor (FRET=late)

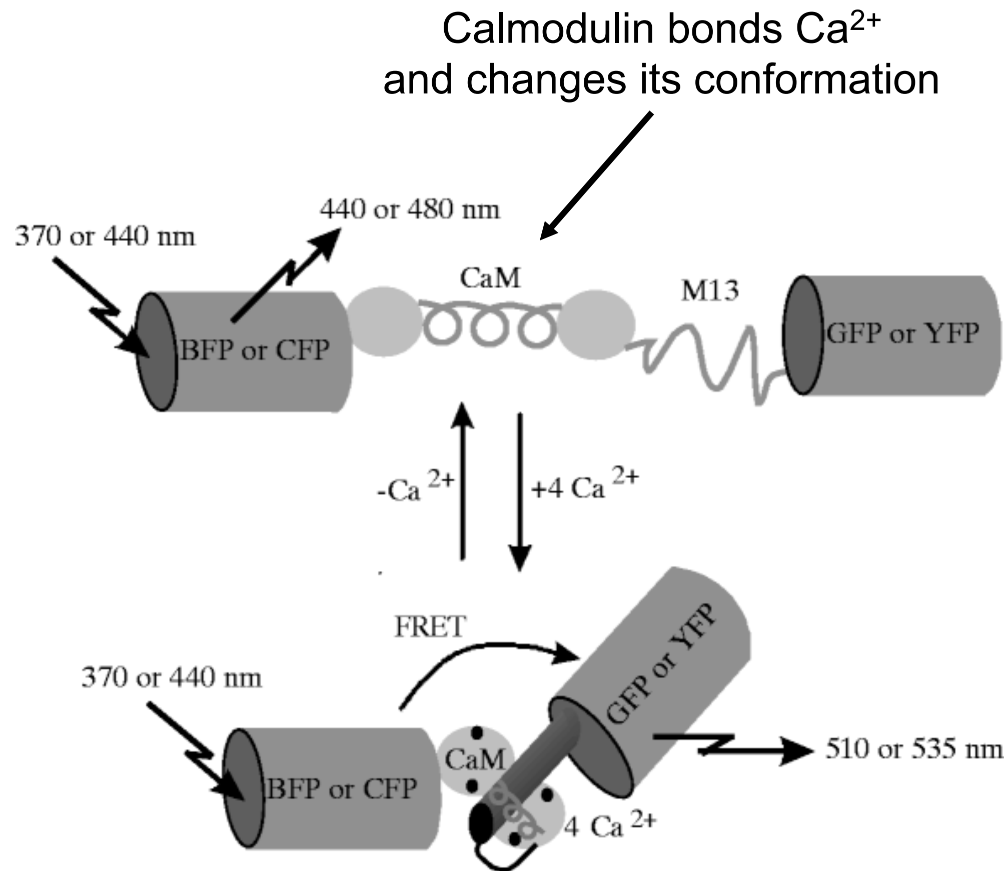
These drawbacks can all be used to make sensors

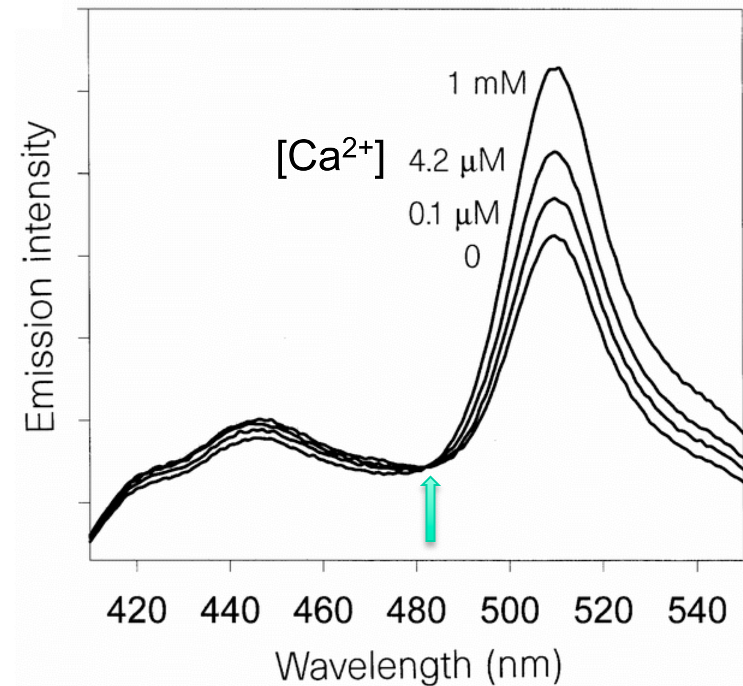
Change in FRET for changes in:

- Orientation
  - cameleon dye for Ca^{++}
- Local environment
  - Phosphate near fluorochrome
  - Membrane voltage (flash)
- Change in lifetime of donor
  - Binding of molecule displacing water

Cameleon: FRET-based and genetically-encoded calcium probe

Calmodulin bonds Ca^{2+}
and changes its conformation





Miyawaki et al, Nature, 1997

Cameleon family: calmodulin-based indicators of $[Ca^{2+}]$ using FRET

isosbestic point

Cameleon: FRET-based and genetically-encoded calcium probe

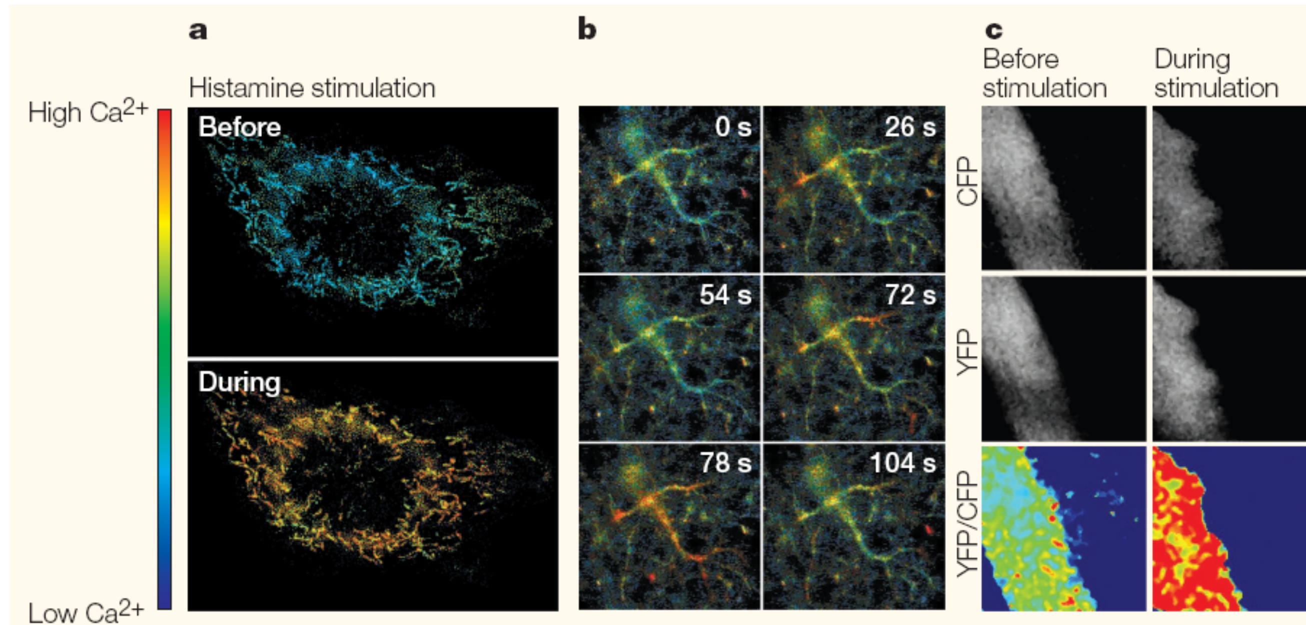
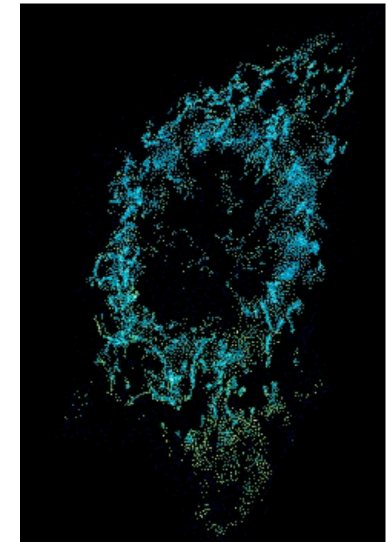
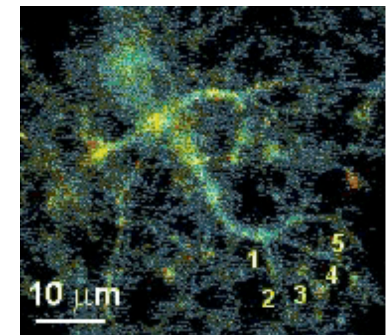


Figure 3 | **Examples of Ca^{2+}-concentration signalling in living cells. a** | Pseudo-colour-coded ratio-images obtained by conventional epifluorescence micrographs of a HeLa cell transfected with ratiometric pericam. Ca^{2+} concentrations are colour coded with a basal Ca^{2+} concentration in blue and a high Ca^{2+} concentration in yellow and red. Note the strong increase in mitochondrial Ca^{2+} concentration on histamine-induced release from the endoplasmic reticulum (see also Online Video 1). **b** | The pseudo-colour-coded ratio-images (as in FIG. 3a) of indo-1-loaded acute brain slices were obtained by confocal microscopy and show the Ca^{2+} waves in astrocytes that were induced by stimulation of the CA1 nerve fibres that contact the CA3 region of the hippocampus (known as the 'Schaffer collateral pathway') with an extracellular electrode, which results in the depolarization of the nerve terminals and the release of neurotransmitter. The panels show sequential frames of Online Video 2. Note that the waves are sweeping from one process to another, traversing the whole cell. The numbers indicate the time after stimulation. **c** | Two-photon confocal micrographs of a muscle fibre from tibialis anterior muscle transfected with cytoplasmic cameleon in live mouse. The upper four panels show the yellow (YFP) and cyan fluorescence channel micrographs (CFP) from the cameleon probe that are responsible for the pseudo-colour-coded ratio-images (lower 2 panels) as in FIG. 3a. Note the increase in the intracellular Ca^{2+} concentration in the muscle on stimulation of the sciatic nerve.





Rudolf et al, Nature Reviews Molecular Cell Biology, 2003

Ratiometric dyes offer easiest quantitation

uncertainty from amount of dye loaded:
- deltaF/F imaging can help
- isosbestic point is best

Making a ratio dyes by standardizing with a second dye:
- F from indicator / F from standard
- problem of different bleach rate
- problem of different ISC to dark state

Folding and bleaching of fluorescent proteins

Example: properties of fluorescent protein variants

Table 1 Properties of novel fluorescent protein variants

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient per chain[a] ($M^{-1}cm^{-1}$)	Fluorescence quantum yield	Brightness of fully mature protein (% of DsRed)	pKa	$t_{0.5}$ for maturation at 37 °C	$t_{0.5}$ for bleach[b], s
DsRed	558	583	75,000	0.79	100	4.7	~10 h	ND
T1	555	584	38,000	0.51	33	4.8	<1 h	ND
Dimer2	552	579	69,000	0.69	80	4.9	~2 h	ND
mRFP1	584	607	50,000	0.25	21	4.5	<1 h	6.2
mHoneydew	487/504	537/562	17,000	0.12	3	<4.0	ND	5.9
mBanana	540	553	6,000	0.70	7	6.7	1 h	1.4
mOrange	548	562	71,000	0.69	83	6.5	2.5 h	6.4
dTomato	554	581	69,000	0.69	80	4.7	1 h	64
tdTomato	554	581	138,000	0.69	160	4.7	1 h	70
mTangerine	568	585	38,000	0.30	19	5.7	ND	5.1
mStrawberry	574	596	90,000	0.29	44	<4.5	50 min	11
mCherry	587	610	72,000	0.22	27	<4.5	15 min	68

[a]Extinction coefficients were measured by the alkali denaturation method[8,30] and are believed to be more accurate than the previously reported values for DsRed, T1, dimer2 and mRFP1[7].
[b]Time (s) to bleach to 50% emission intensity, at an illumination level that causes each molecule to emit 1,000 photons/s initially, that is, before any bleaching has occurred. See Methods for more details. For comparison, the value for EGFP is 115 s, assuming an extinction coefficient of 56,000 $M^{-1}cm^{-1}$ and quantum efficiency of 0.60 (ref. 30). ND, not determined.

Shaner et al, Nature Biotechnology, 2004

eGFP ~ 2h