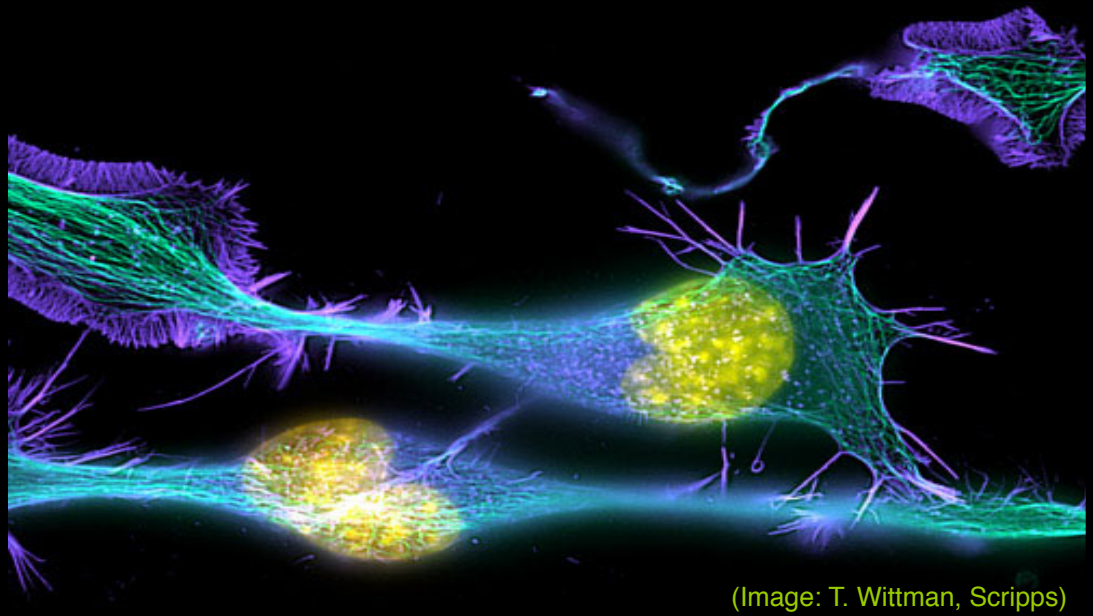
Principles & Practice of Light Microscopy 4



(Image: T. Wittman, Scripps)

Edited by: Zvi Kam, Weizmann
For Advance Light Microscopy course

OVERVIEW

- Principles of Fluorescence
- Fluorophores
- Fluorescence microscopes
- Operational Considerations

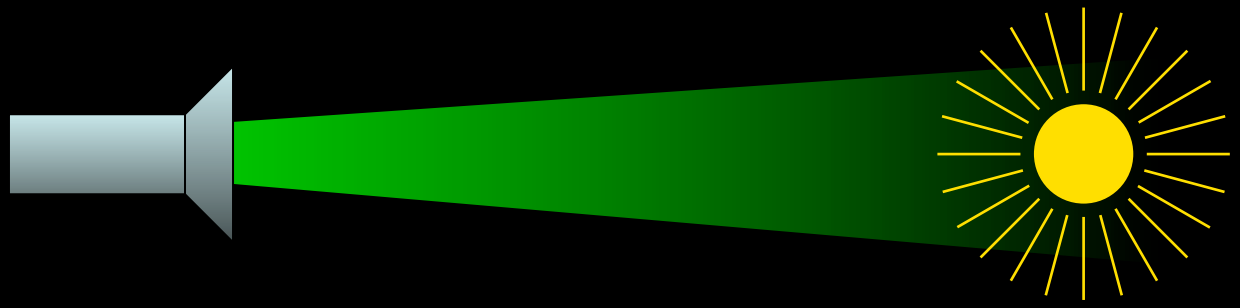
Why fluorescence?

- High contrast
  - Signal against dark background
- Highly specific, multi-color labeling
  - GFP etc.
  - Antibodies
- Live imaging
  - GFP etc.
- Quantitative
- Sensors for [Ca], pH, ...

Fluorescence

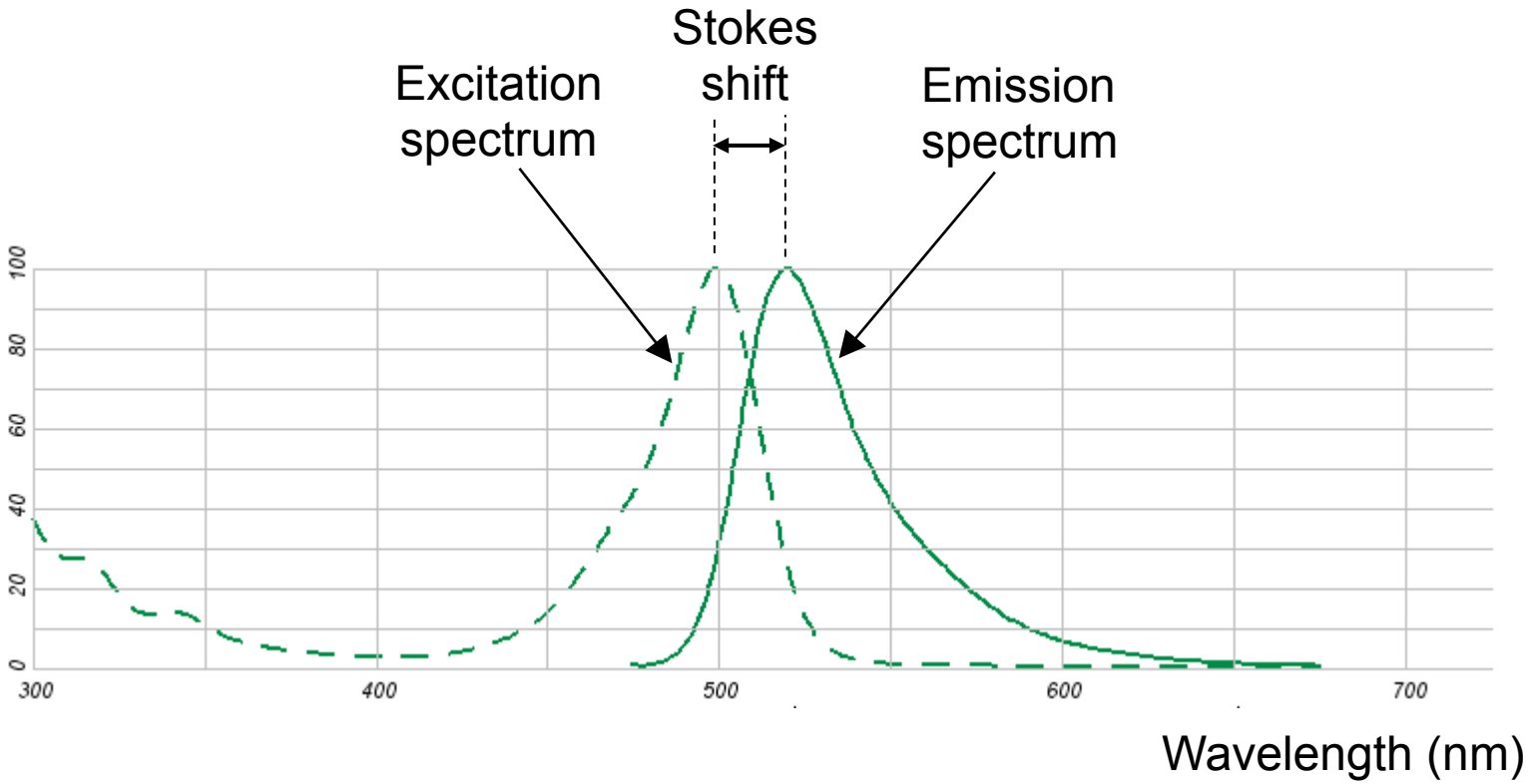
Some molecules, when illuminated by "excitation light" at a wavelength λ_{exc} ,...

...*emit* light at a longer wavelength λ_{em}

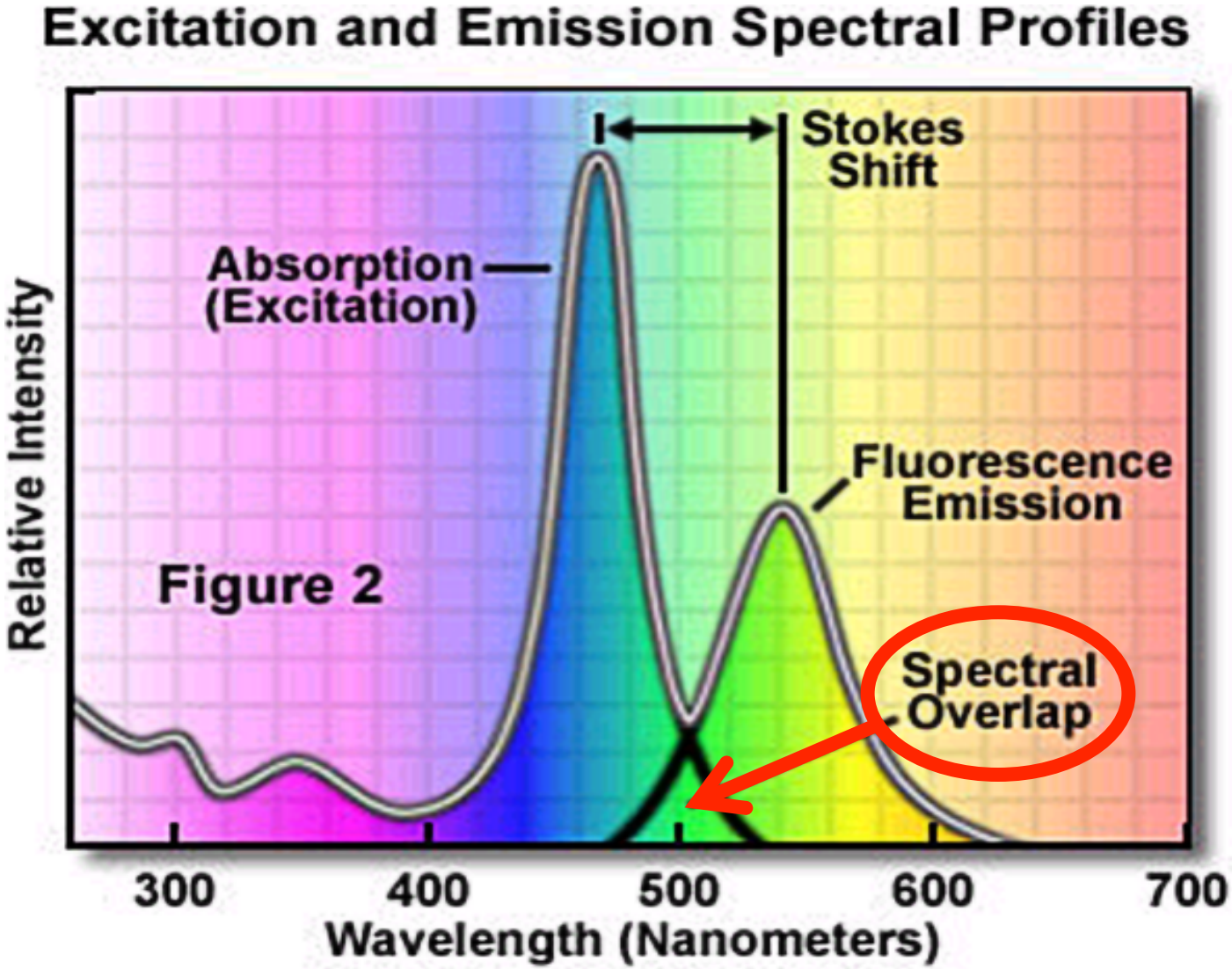


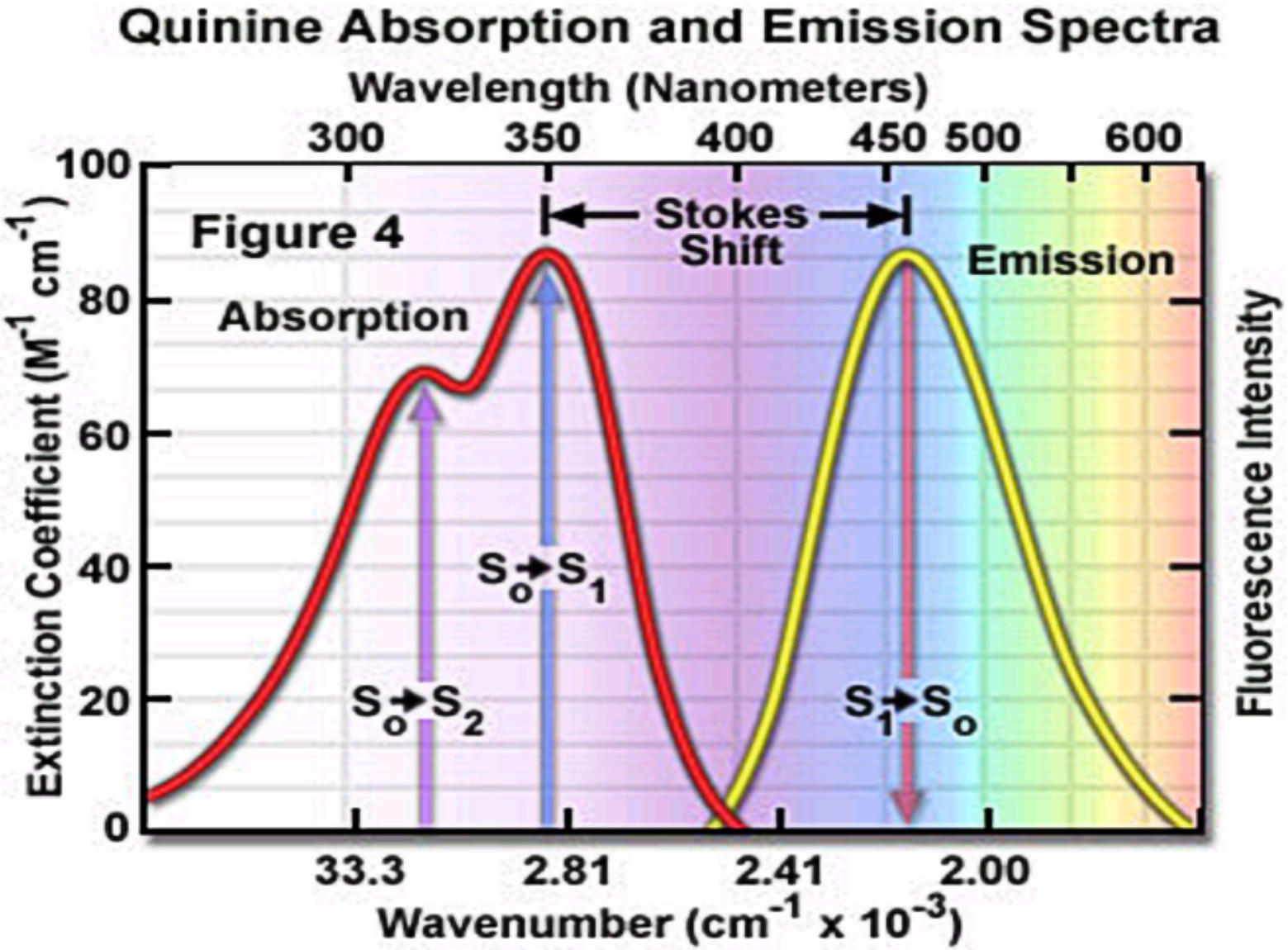
Why?
What kinds of molecules?

Fluorescence Spectra



(Alexa 488)


Excitation and Emission Spectral Profiles
Stokes Shift
Absorption (Excitation)
Fluorescence Emission
Figure 2
Spectral Overlap
Relative Intensity
300
400
500
600
700
Wavelength (Nanometers)


Quinine Absorption and Emission Spectra
Wavelength (Nanometers)
300
350
400
450
500
600
Figure 4
Stokes Shift
Absorption
Emission
$S_o \rightarrow S_1$
$S_o \rightarrow S_2$
$S_1 \rightarrow S_o$
Extinction Coefficient (M^{-1} cm^{-1})
Fluorescence Intensity
100
80
60
40
20
0
33.3
2.81
2.41
2.00
Wavenumber (cm^{-1} x 10^{-3})

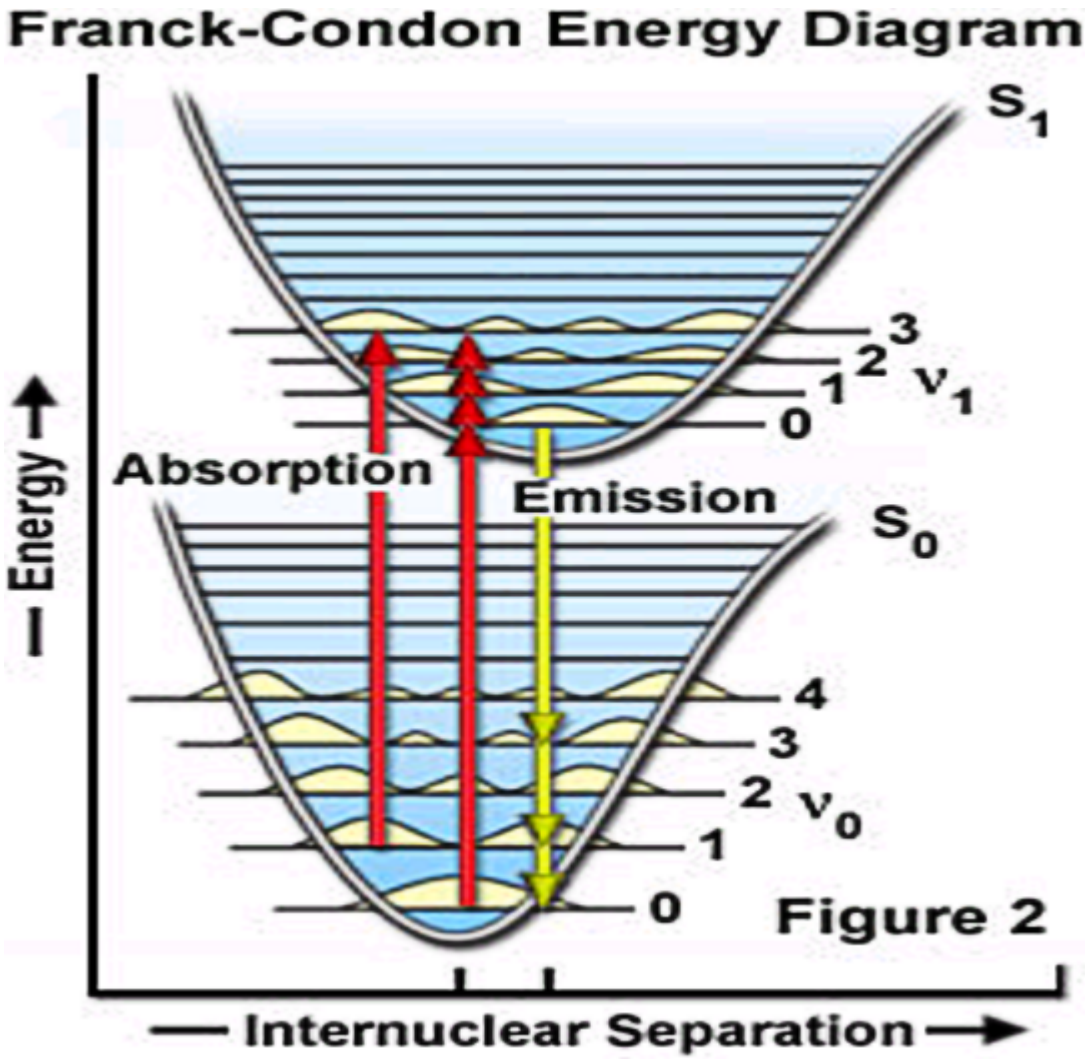

Franck-Condon Energy Diagram
S1
3
2
1
0
ν1
Absorption
Emission
S0
4
3
2
1
0
ν0
Energy
Internuclear Separation

Figure 2

EXCITED MOLECULES ALSO

- Get hot (increase vibrational energy)
- Emit a photon (usually plus some heat)
- Chemical reaction (photochemistry)
- Energy transfer (nondestructive, photosynthesis)
- Triplet conversion (nondestructive, but the cell may be very unhappy).

$$E = h\nu = hc / \lambda$$

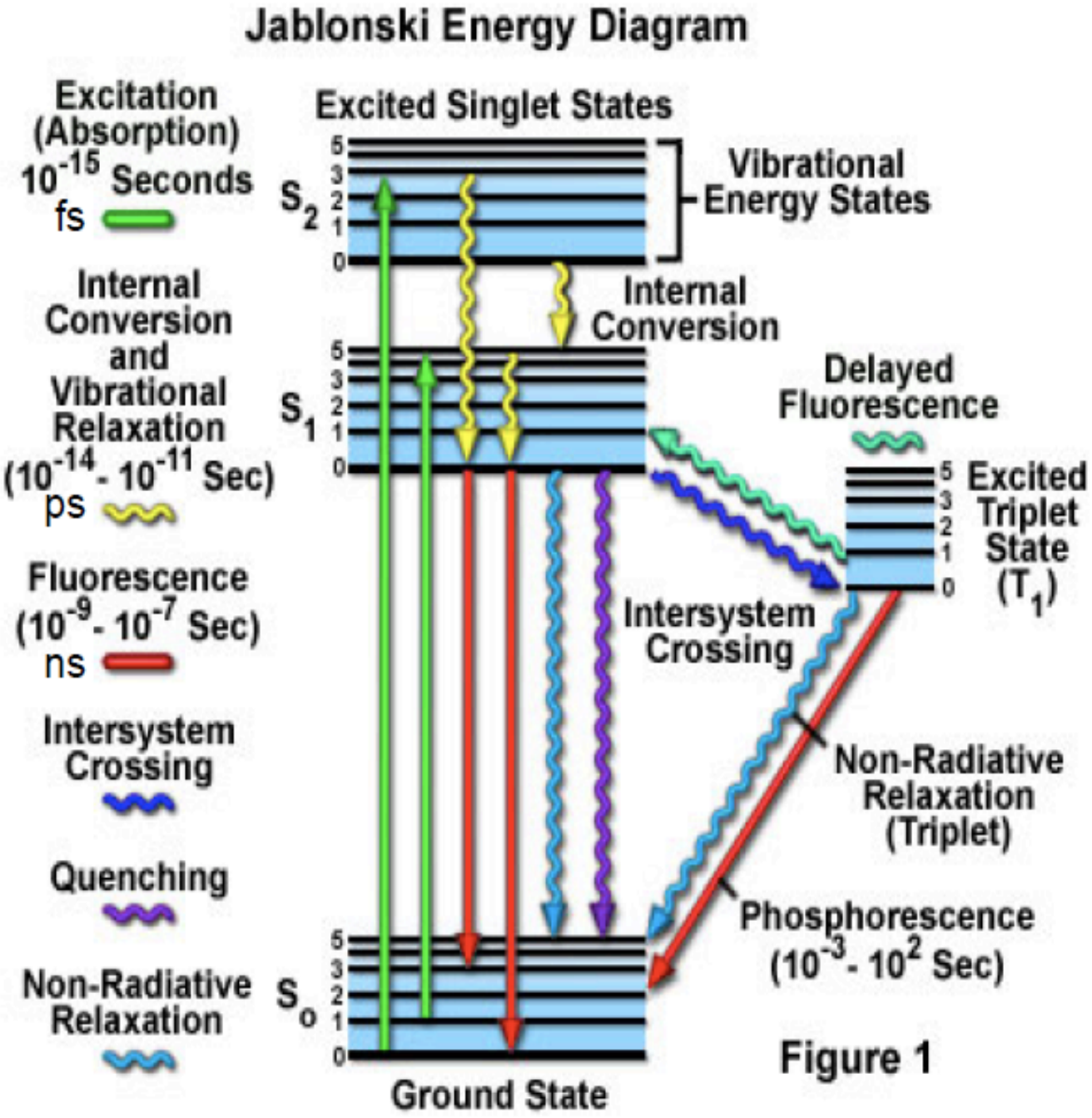
E – *energy*

ν – *frequency*

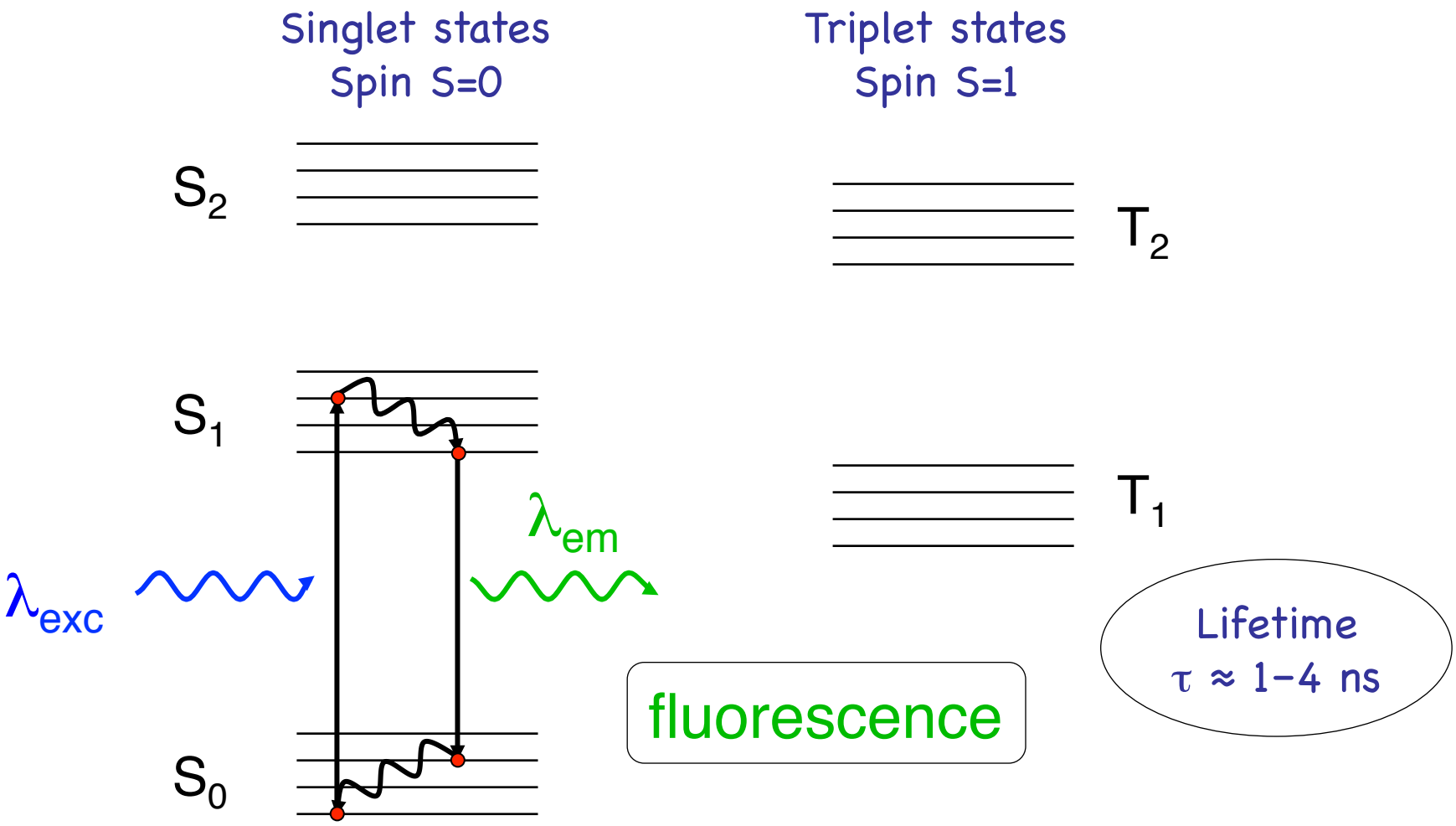
λ – *wavelength*

h – *plank*

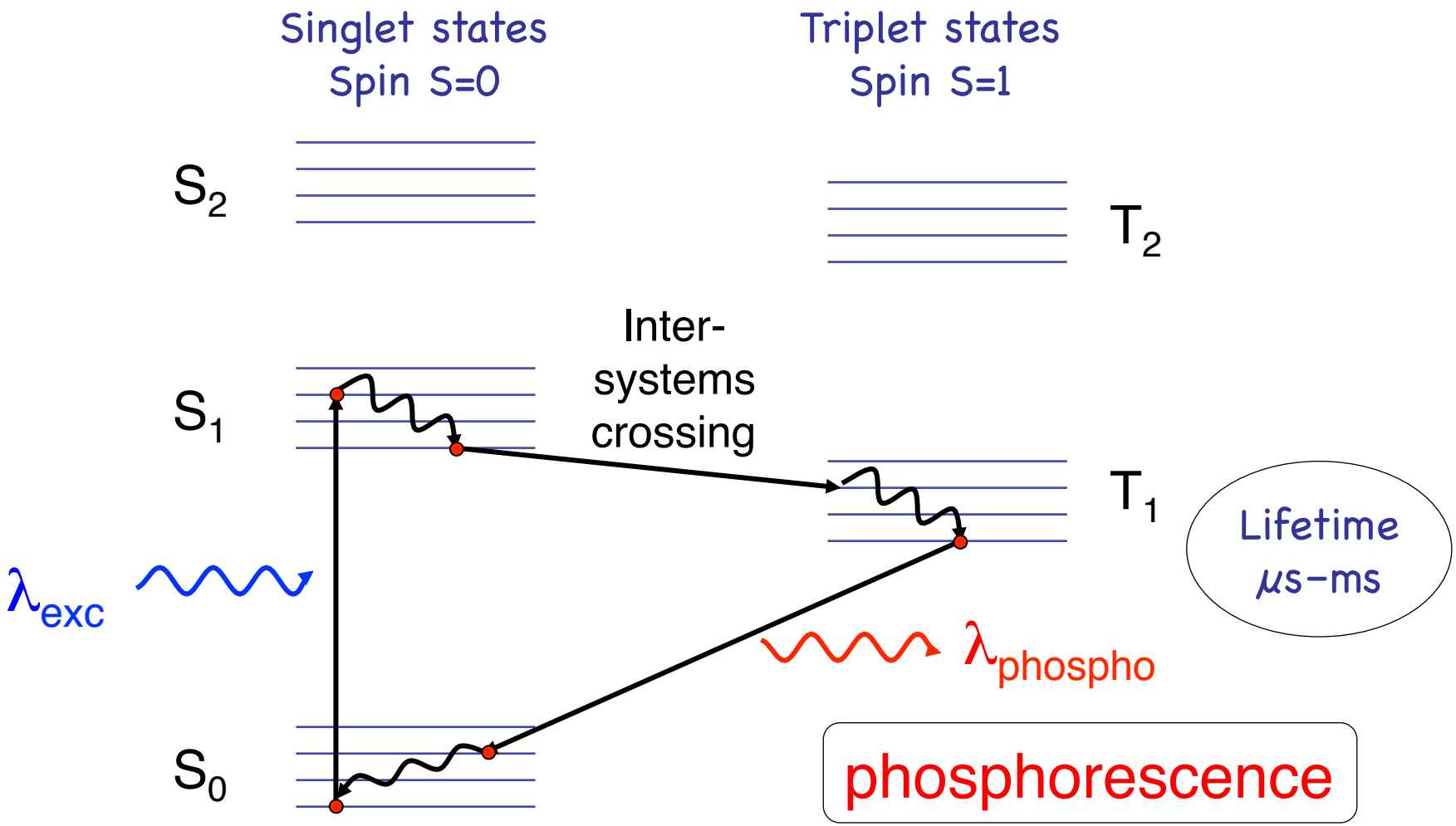
c – *speed*



Jablonski diagram
(Molecular energy diagram)

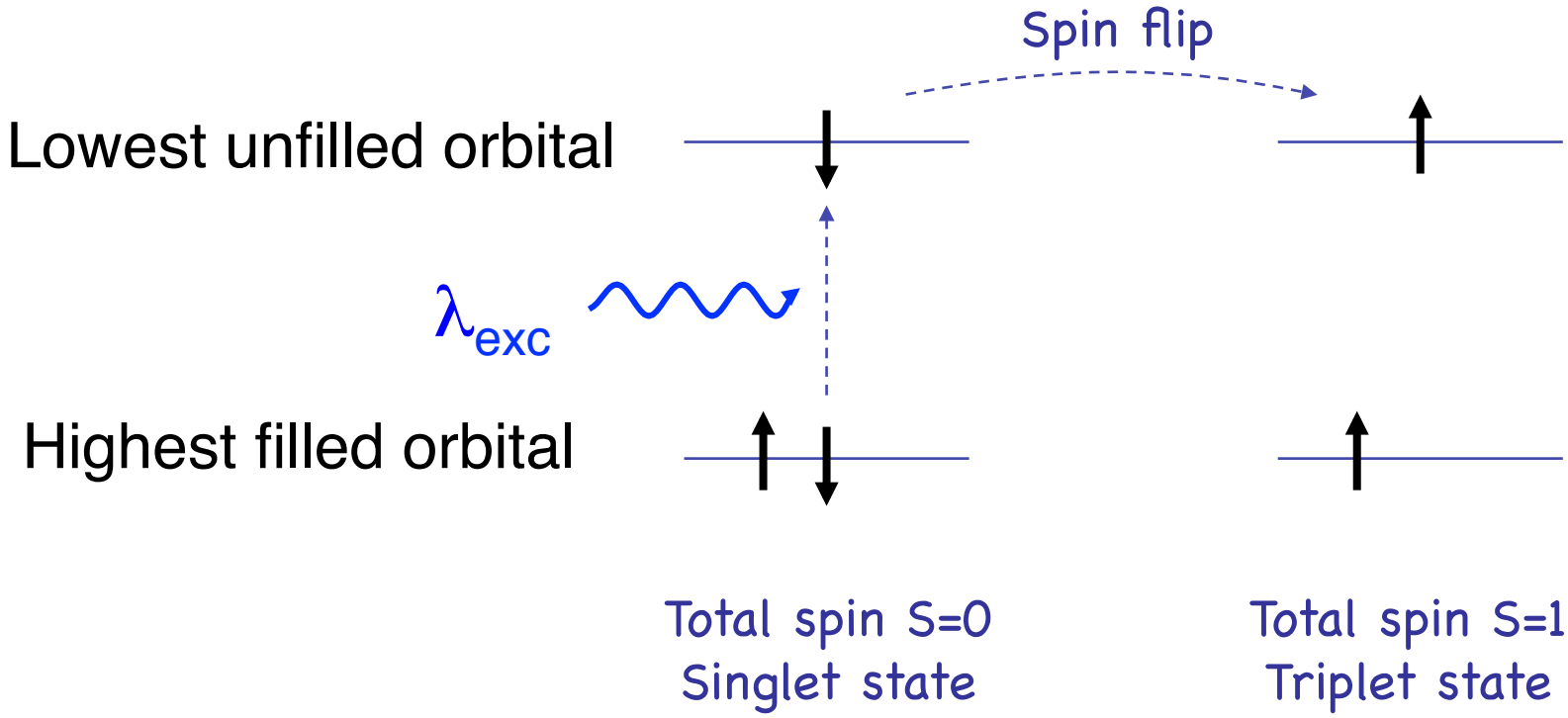


Jablonski diagram
(Molecular energy diagram)



Singlet and Triplet States

Orbital states of *each electron*



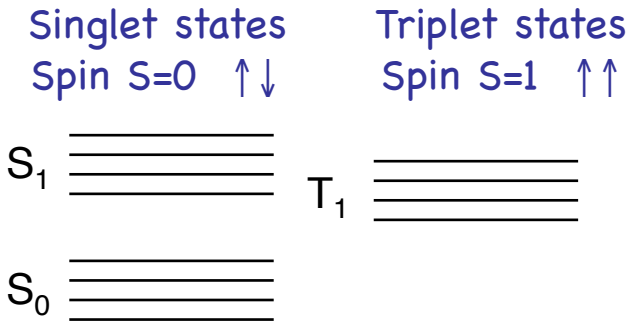
Spin flips are "dipole forbidden" → unlikely → long triplet lifetime

Why does the triplet state have lower energy?

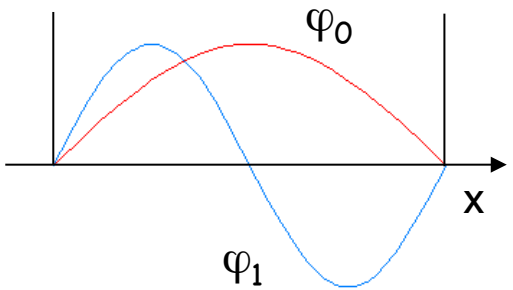
Hund's rule #1 (for atoms):
largest total spin → lowest energy

Electrons are Fermions
⇒ anti-symmetric wavefunction
$\Psi_r(r_1, r_2)\ \Psi_s(s_1, s_2) = -\Psi_r(r_2, r_1)\ \Psi_s(s_2, s_1)$

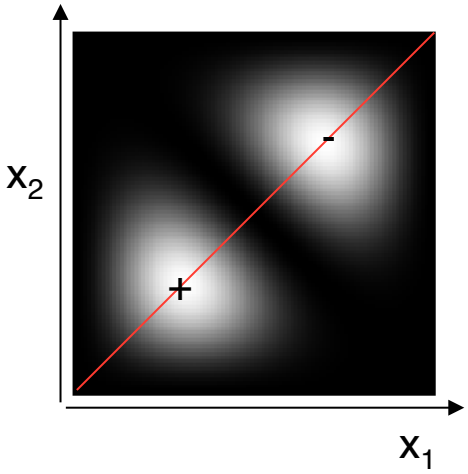
Singlet state: Ψ_s = ↑↑, Symmetric
Triplet state: $\Psi_r(r_1, r_2)$ is anti-symmetric



Particle-in-a-box example

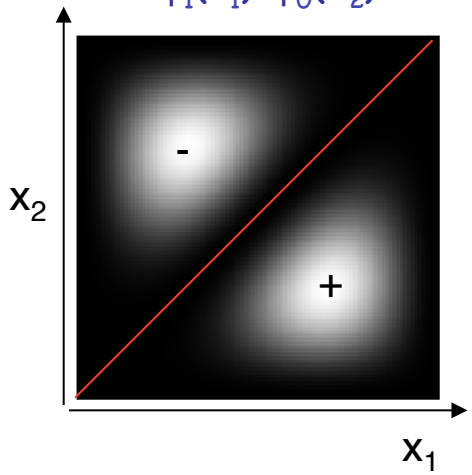


Symmetric
$\varphi_0(x_1)\ \varphi_1(x_2)$
$+\varphi_1(x_1)\ \varphi_0(x_2)$



High prob. of x1≈x2
High repulsion energy

Anti-symmetric
$\varphi_0(x_1)\ \varphi_1(x_2)$
$-\varphi_1(x_1)\ \varphi_0(x_2)$



Low prob. of x1≈x2
Low repulsion energy

TIMESCALES OF FLUORESCENCE

Transition	Process	Rate Constant	Timescale (Seconds)
S(0) => S(1) or S(n)	Absorption (Excitation)	Instantaneous	10^{-15}
S(n) => S(1)	Internal Conversion	k(ic)	10^{-14} to 10^{-10}
S(1) => S(1)	Vibrational Relaxation	k(vr)	10^{-12} to 10^{-10}
S(1) => S(0)	Fluorescence	k(f) or Γ	10^{-9} to 10^{-7}
S(1) => T(1)	Intersystem Crossing	k(pT)	10^{-10} to 10^{-8}
S(1) => S(0)	Non-Radiative Relaxation Quenching	k(nr), k(q)	10^{-7} to 10^{-5}
T(1) => S(0)	Phosphorescence	k(p)	10^{-3} to 100
T(1) => S(0)	Non-Radiative Relaxation Quenching	k(nr), k(qT)	10^{-3} to 100

Stocks shift

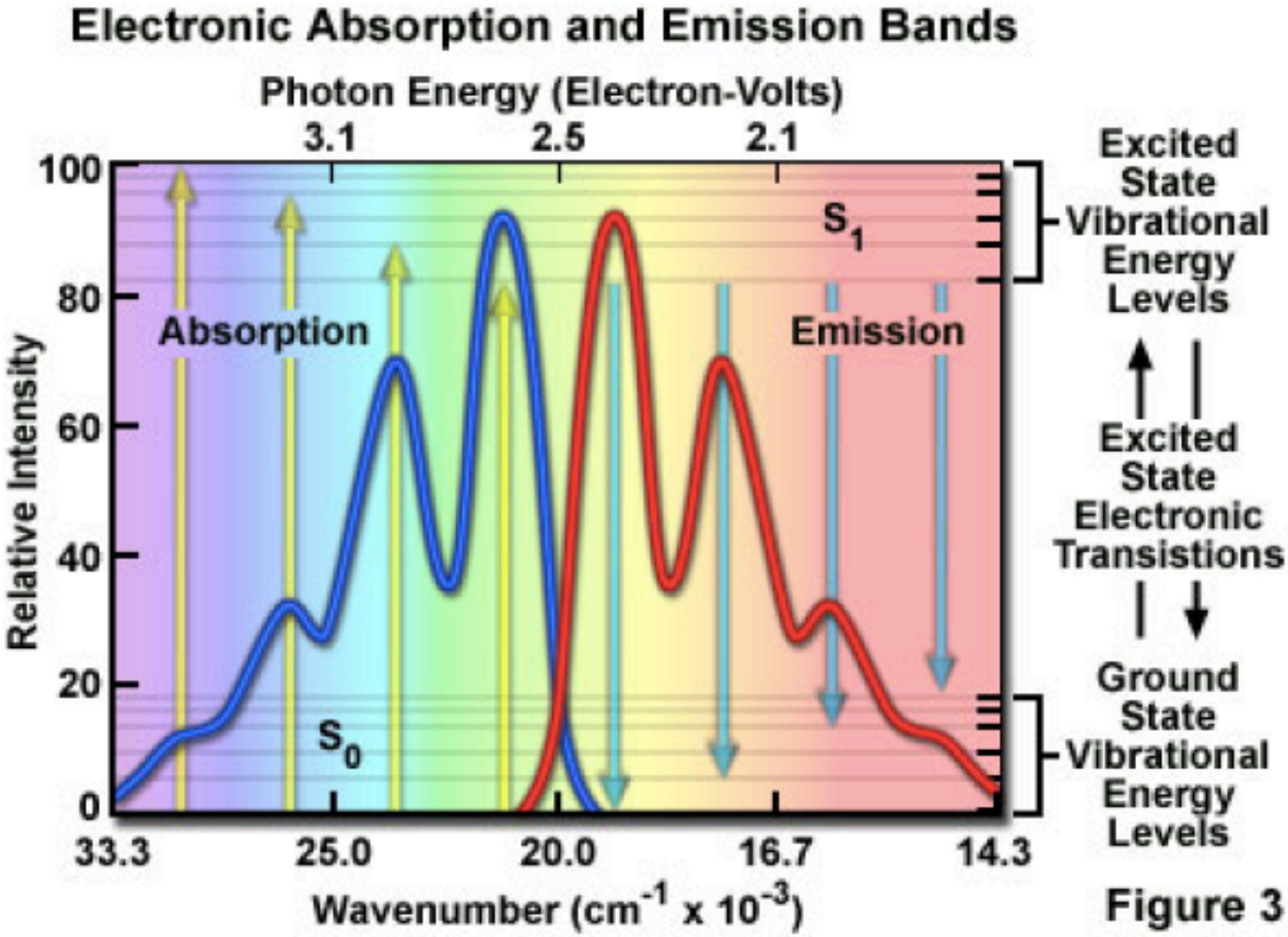


Figure 3

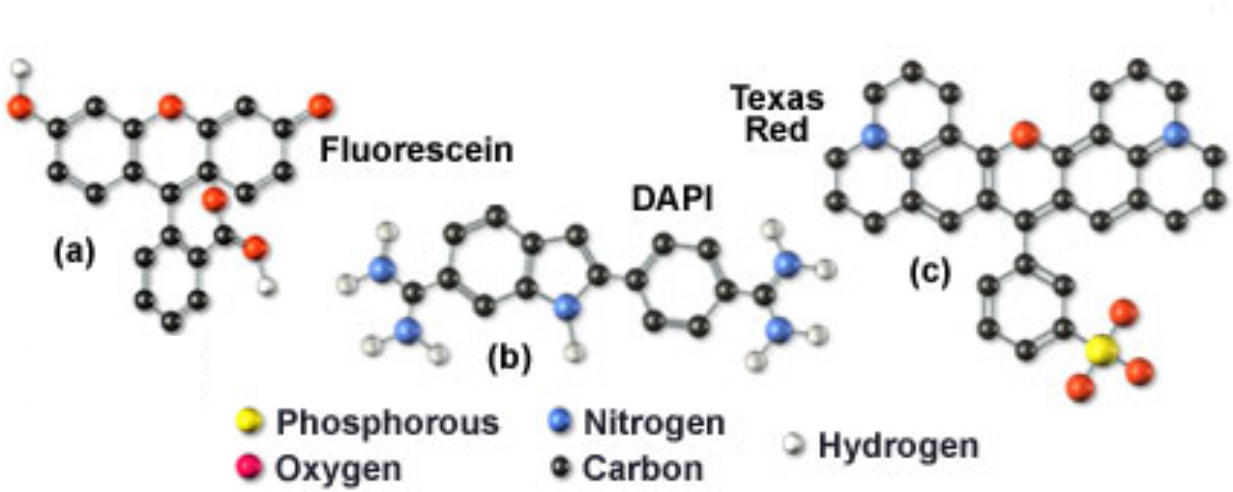
TWO IMPORTANT PROPERTIES

- Quantum efficiency:
photons emitted/photons absorbed

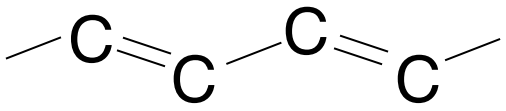
- Photobleaching efficiency:
probability of bleaching/photon absorbed

Fluorophores

Fluorescent molecules

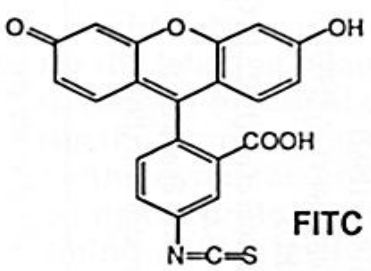


Systems of conjugated bonds
that share electrons

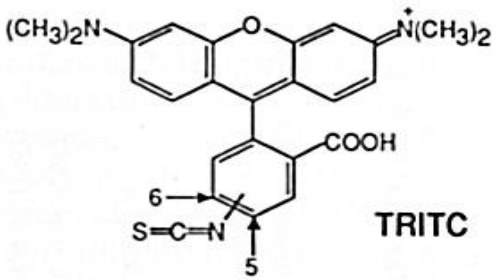


Larger system → longer wavelength

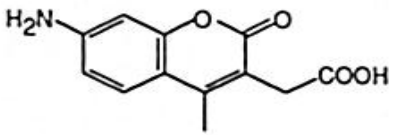
SOME COMMON FLUOROPHORES



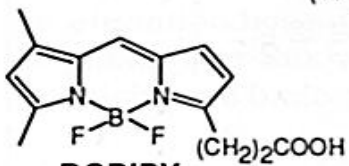
FITC



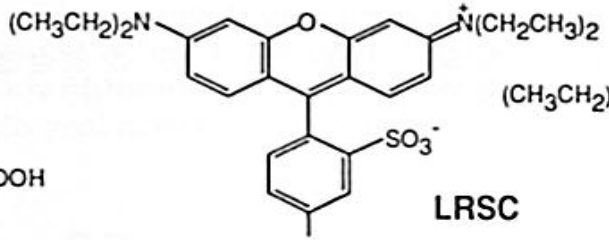
TRITC



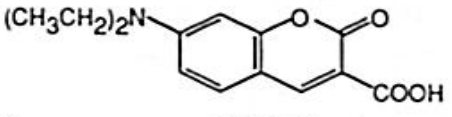
AMCA



BODIPY

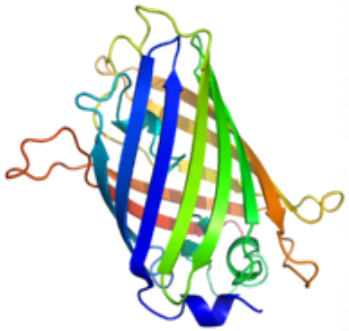


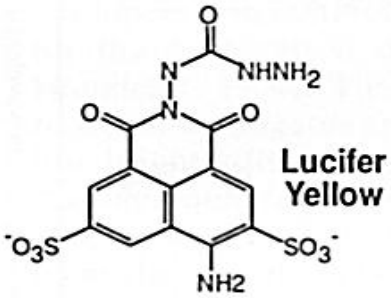
LRSC



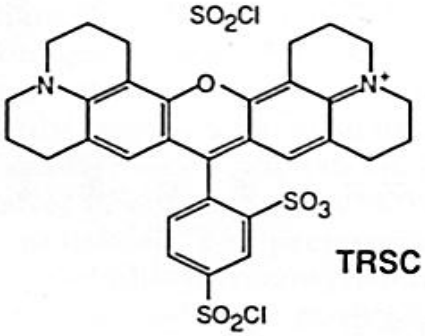
DAMC

~G/R FP



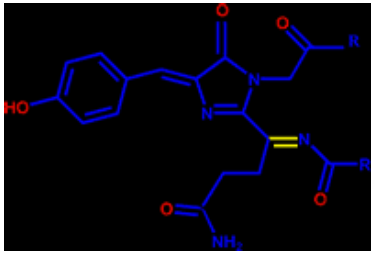


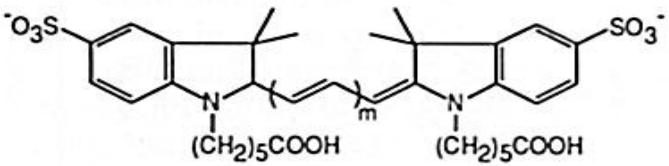
Lucifer Yellow



TRSC

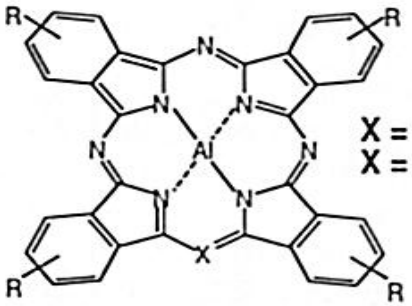
Cascade Blue





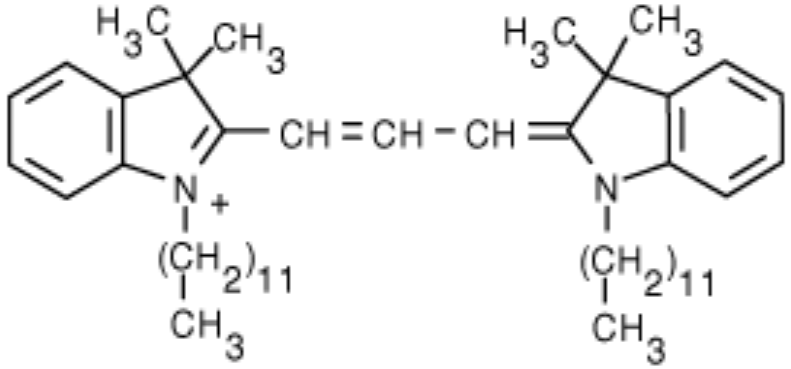
m = 1 CY3.18
m = 2 CY5.18

~Chlorophils

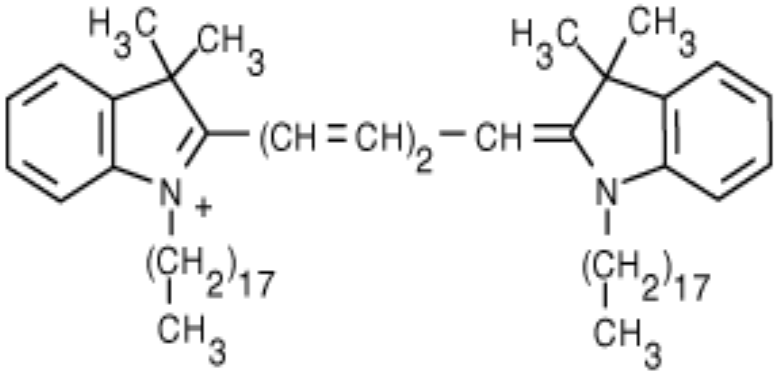


X = N Al-PC
X = C Al-TBTP

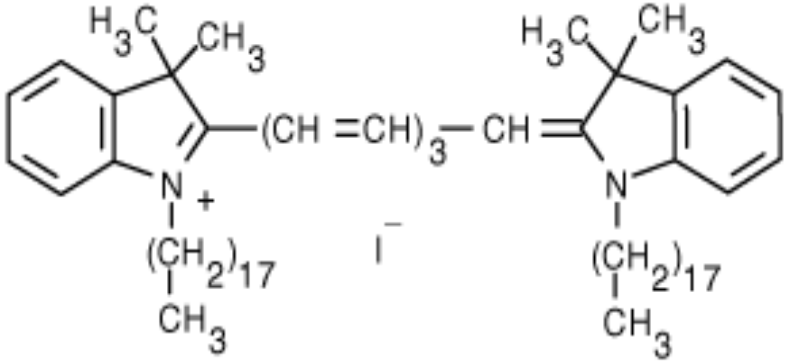
DYES COME IN FAMILIES



Abs = 549nm, Em = 565nm

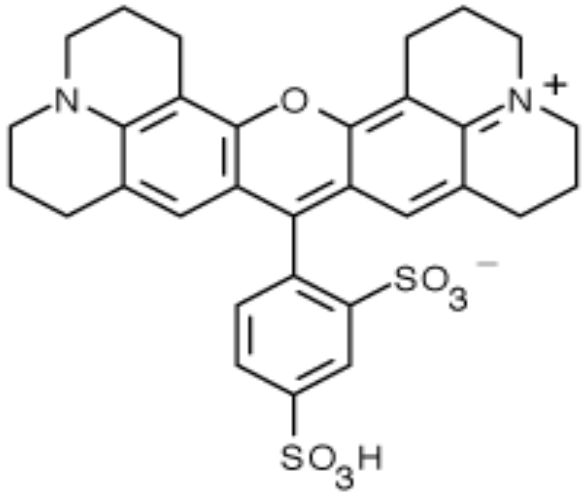


Abs = 650nm, Em = 670nm



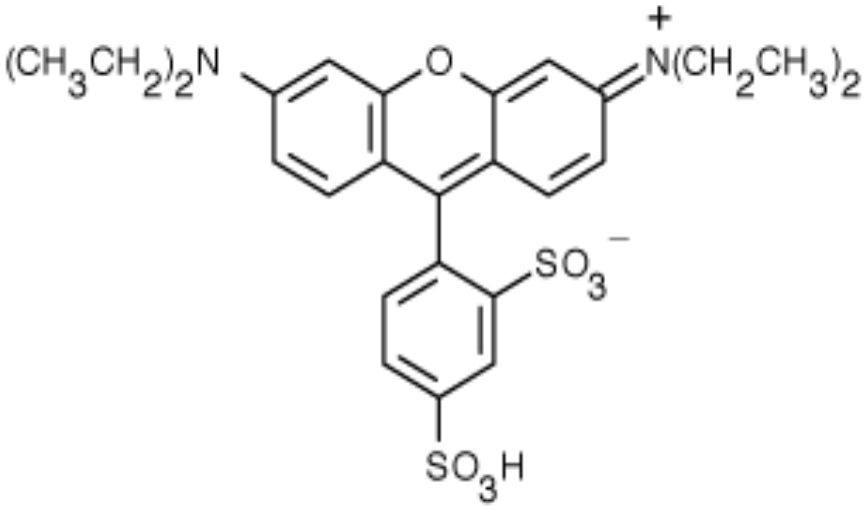
Abs = 748nm, Em = 780nm

XANTHENE DYES



Sulforhodamine 101
(precursor for Texas Red)

Abs = 586nm, Em = 605nm



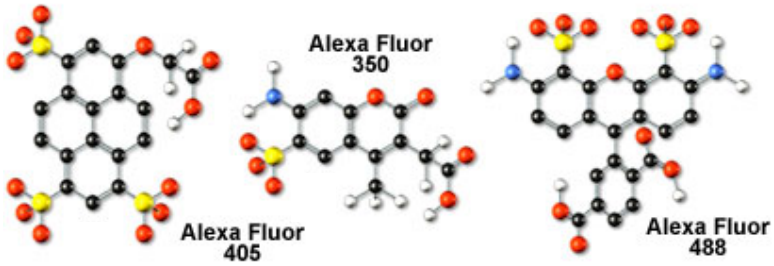
Sulforhodamine B
(precursor for Lissamine)

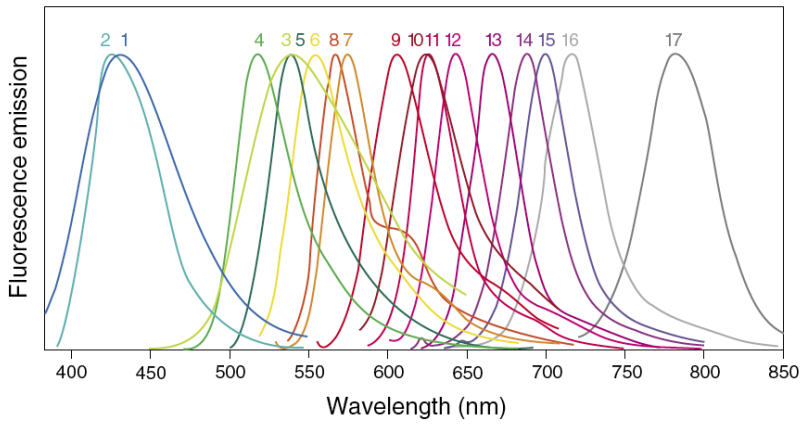
Abs = 565nm, Em = 586nm

General Fluorescent Molecules

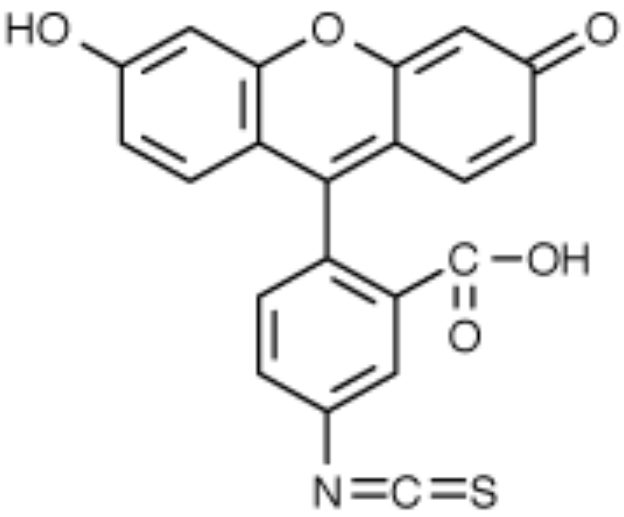
Hundreds to choose from

- **Fluorescein**
  - Bright but pH sensitive & bleaches fast
- **Rhodamine, Texas Red,..**
  - Red, more photostable
- **Bodipy, ...**
- **Cyanine dyes**
  - Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7
- **Alexa Fluor**
  - 405, 488, 532, 546, 633, 680, 750...
  - Bright, photostable

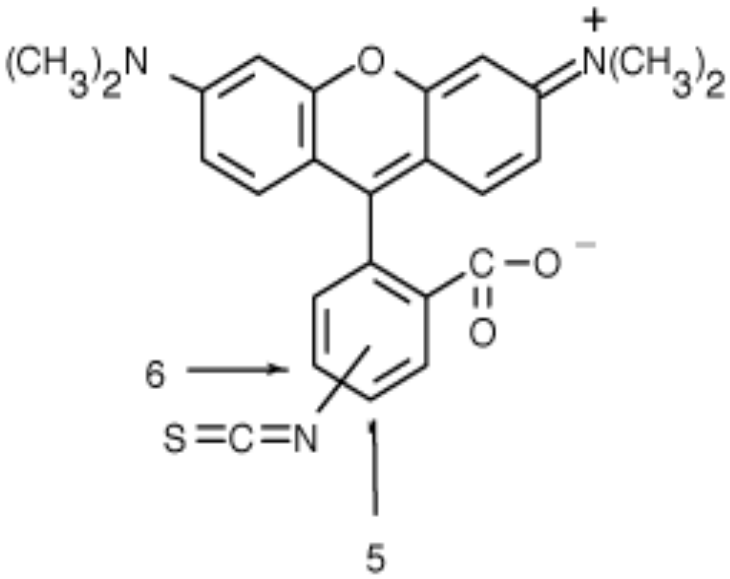




Alexa Fluor emission spectra

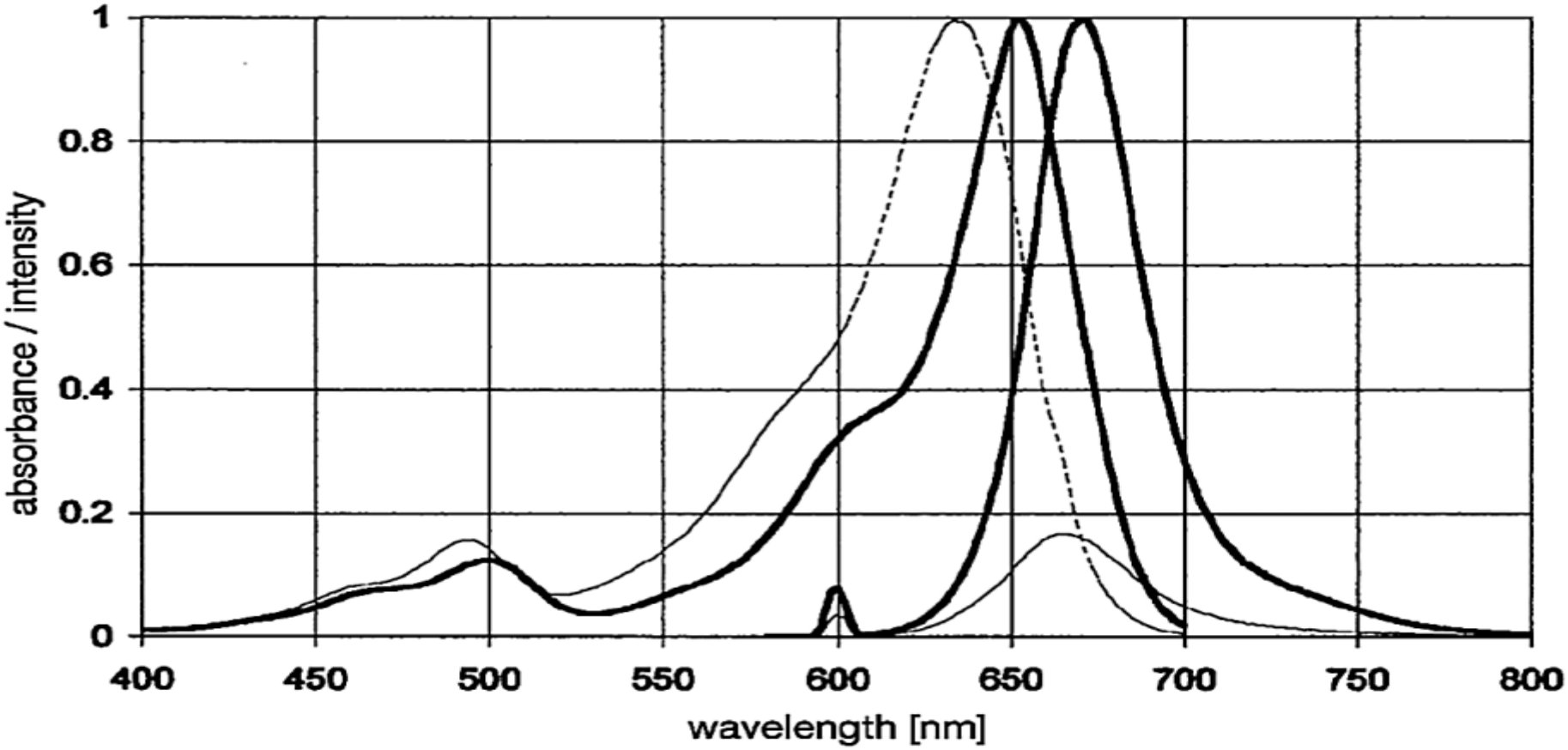


FITC: pH dependent



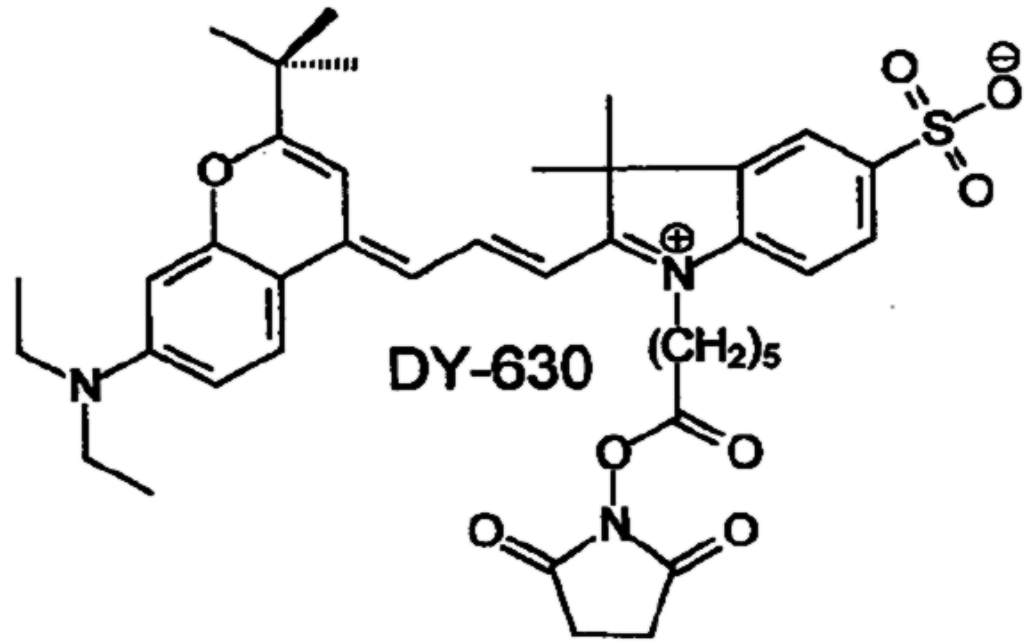
TRITC: pH independent
(almost...)

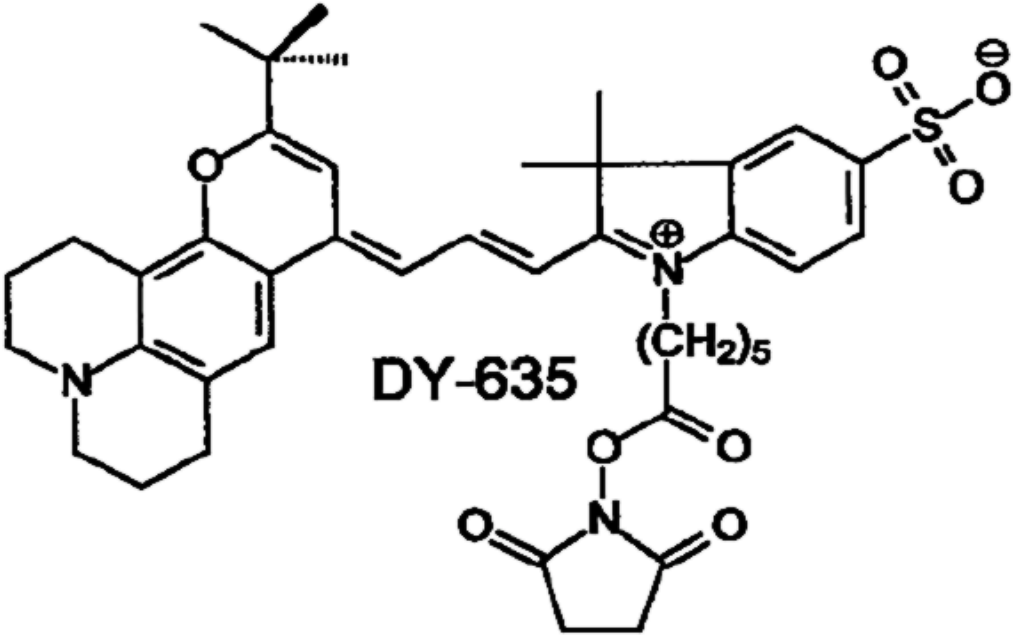
ENVIRONMENT MATTERS



Thin line: free dye in water

Thick line: dye bound to protein

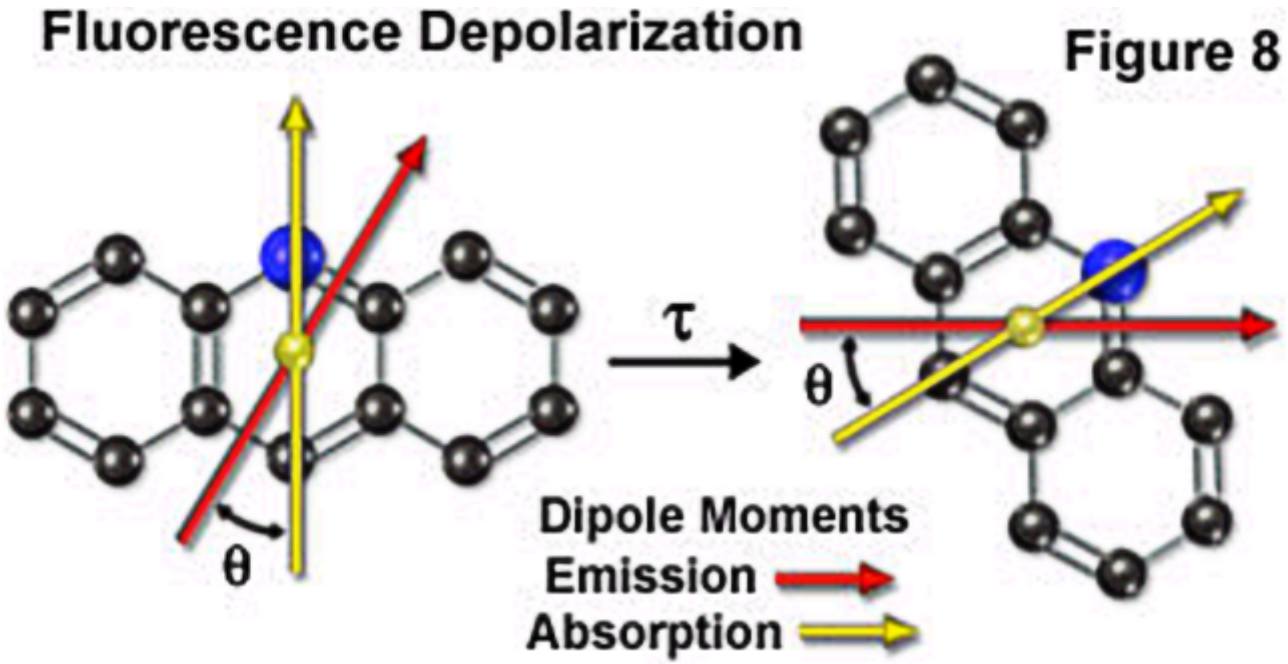




Tailor-Made Dyes for Fluorescence Correlation Spectroscopy (FCS)

Biol. Chem., Vol. 382, pp.495 – 498, March 2001

Dyomics GmbH,



$$p = \frac{(I_{par} - I_{perp})}{(I_{par} + I_{perp})}$$

polarization

$$r = \frac{(I_{par} - I_{perp})}{(I_{par} + 2I_{perp})}$$

anisotropy

THINGS DYES CAN SENSE

- Ca, Mg, Na, pH
- DNA, RNA, double-stranded or single
- Membrane Potential (fast and slow dyes)
- Lipid vs. aqueous surroundings
- Temperature
- Viscosity
- Each other

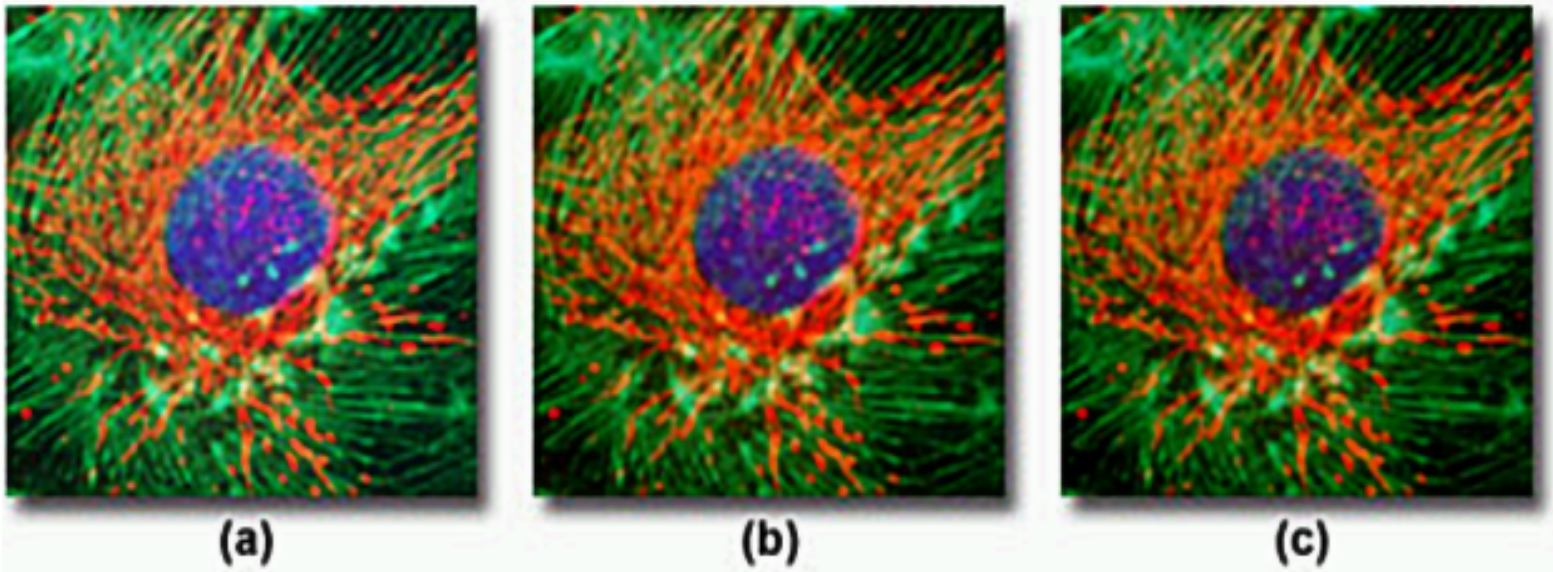
CHROMOPHORE HEADACHES

- Photobleaching
- Spectral shifts
- Broad spectra (especially emission)
- Toxicity

PHOTOBLEACHING

- Nominally an irreversible mono-exponential process
- Proportional to total exposure (but not always, depends on mechanism)
- Antifade agents can help (but not with live cells).
- Different rates for different labels can invalidate ratio measurements.

Differential Photobleaching in Multiply-Stained Cell Cultures



(a) (b) (c)

(d) (e) (f)

Figure 6

QUENCHING

- Usually refers to nondestructive reduction in fluorescence emission
- Causes: ions (eg oxygen). May be caused by triplet exchange
- Energy transfer (nonradiative, chemical, or other)
- Can be used to probe environment

SATURATION

- Fluorescence lifetime limits the rate at which a dye molecule can be excited.
- The saturation intensity is that which brings the dye to this limit.
- Further increases in excitation intensity will not produce increased emission.

TYPICAL SATURATION CALCULATION

- Assume incident power = 1mW @ 488nm
- Assume diff. Limited Gaussian spot (0.5μm dia)
- FITC cross section σ = 3.06×10^{-16} cm^2/molecule
- FITC QE = 0.9
- FITC fluorescence lifetime = 4.5nsec ($k_f = 2.2 \times 10^8$/sec)
- Photon flux at waist: I = 1.25×10^{24} photons/cm^2/sec

Q: in what microscope mode is saturation crucial?

EMISSION RATE

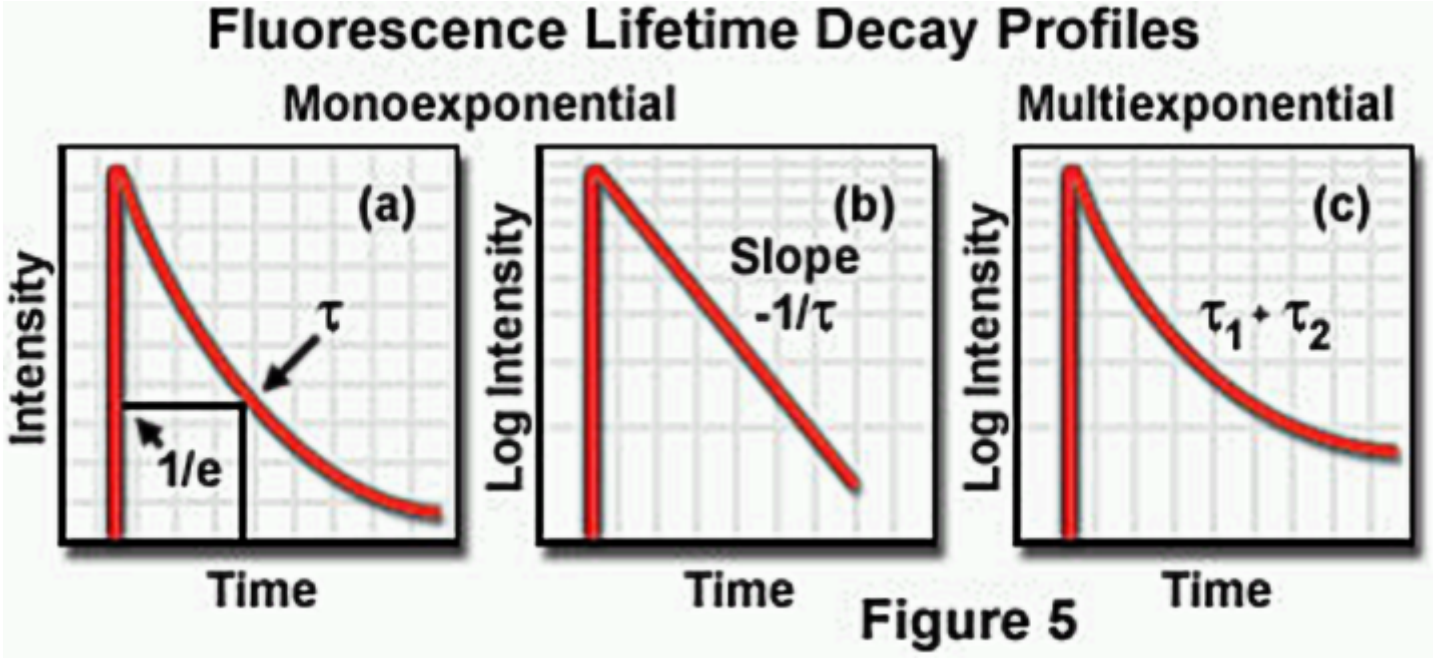
- Rate of de-excitation: $k_f = 2.2 \times 10^8$/sec
- Rate of optical excitation: $k_a = \sigma I = 3.8 \times 10^8$/sec
- Steady state:
  k_f[excited-fraction] = k_a(1 − [excited-fraction])
- [excited-fraction] = 63%
- Rate of emission per molecule = 1.3×10^8photons/sec.

  (Theoretical Max: 2.0×10^8photons/sec)

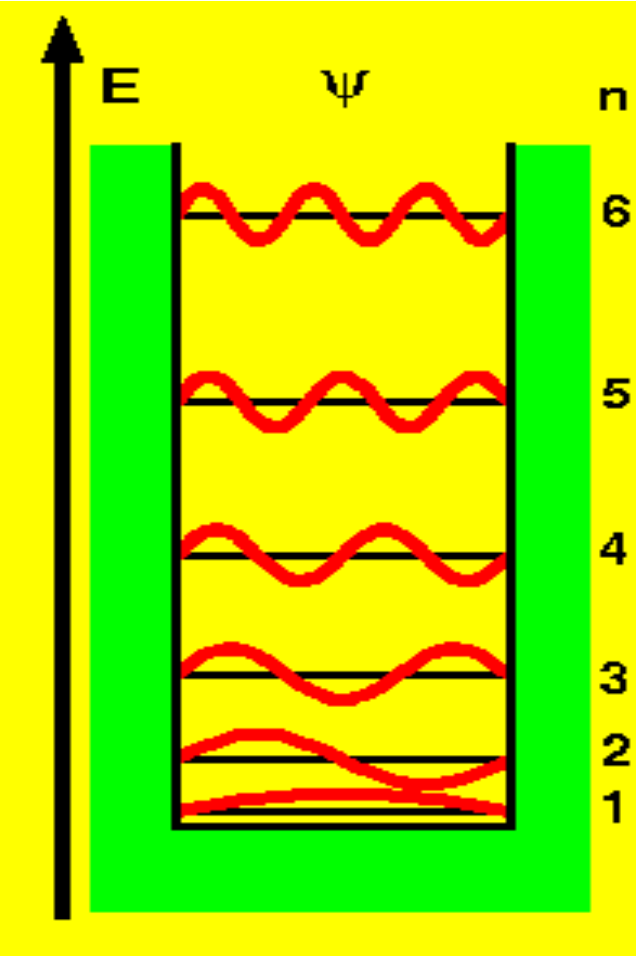
WHEN SHOULD WE WORRY?

- 0.5-1mW focused into a diffraction limited spot is enough to saturate a high QE sample.
- In laser scanning microscopy, this is easily achieved.
- In widefield microscopy, the field of view for high mag lens is about 400 microns diameter. So we have 150000-200000 difference in photon density: 200Watts, saturation is not likely.

LIFETIME: Mean Time in Excited State before Fluorescence Emission



PARTICLE IN A BOX
A MODEL FOR ORGANIC DYES



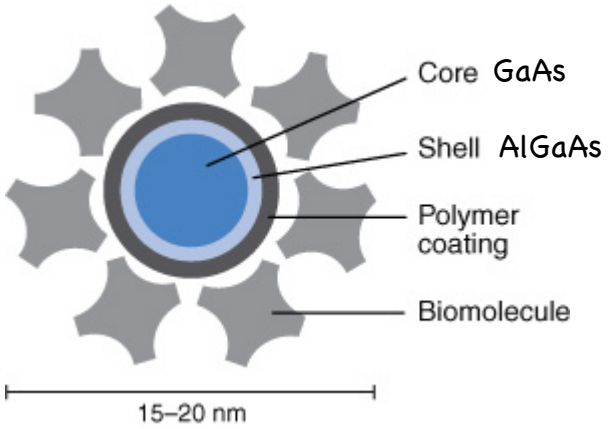
$$\Delta E = 2(n+1)h^2/8mL^2$$

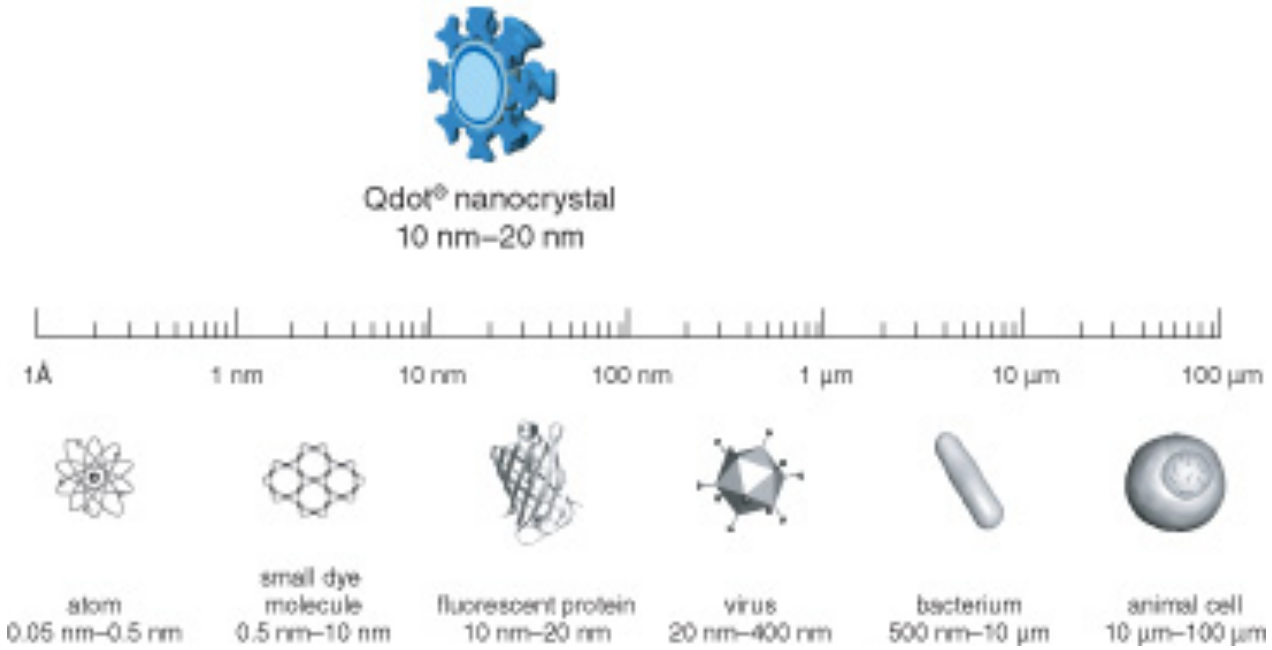
The key point: as the box gets wider, the energy difference decreases

http://itl.chem.ufl.edu/4412_aa/Gifs/box1_03.gif

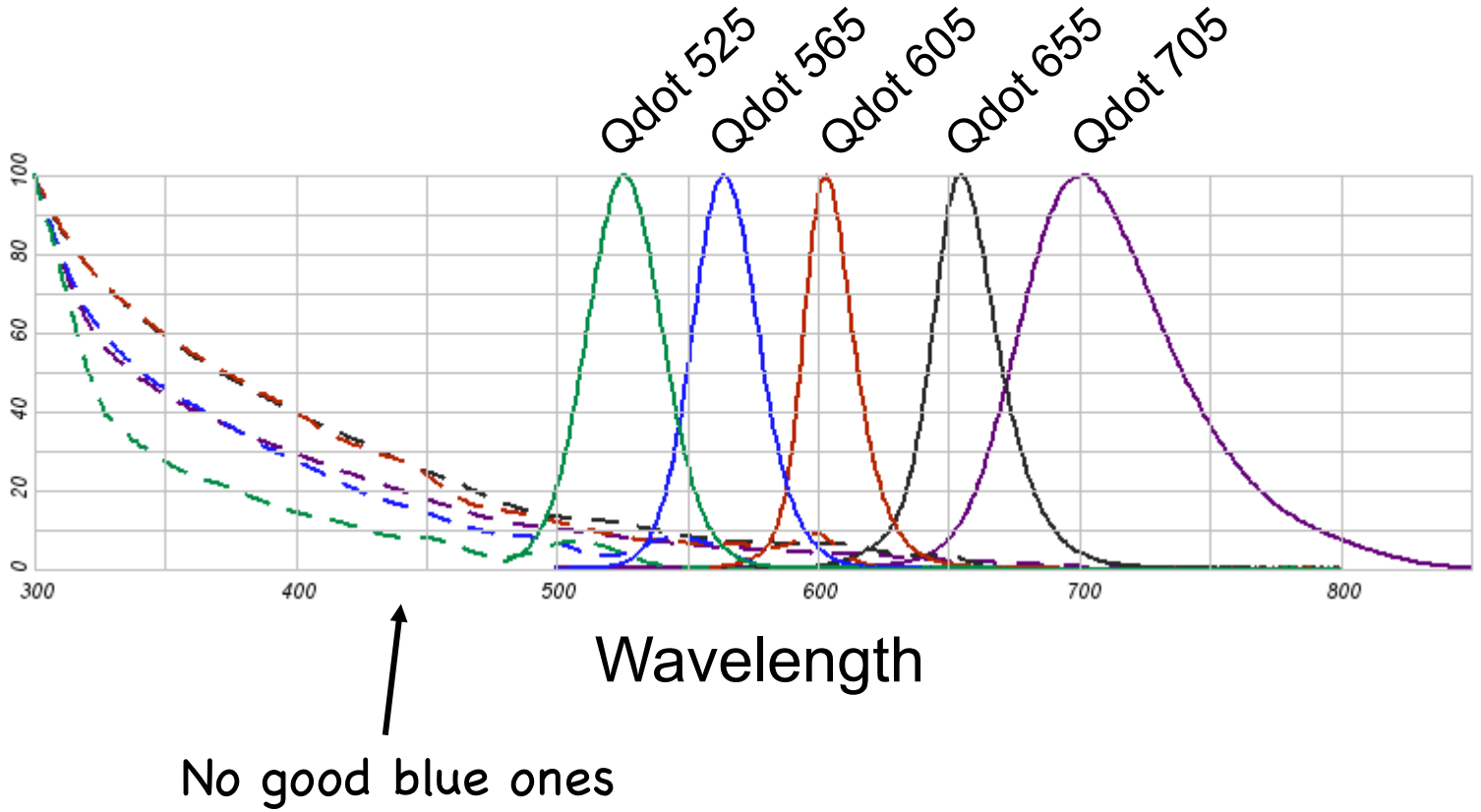
Quantum dots

- semiconductor nanocrystals
- Small size → Quantum confinement
- Size ↔ color [electron in a box]





Quantum dot spectra



Quantum dots - pros / cons

- Little photobleaching, very stable
- Very bright ? ? ?
- Can use single excitation wavelength for multiple colors
- Narrow emission spectra → can use many colors

- Large compared to small molecule dyes
  coating for bio-compatibility
  and independence on environment
- Hard to get into live cells
- Single qdots blink
- No good blue qdots available

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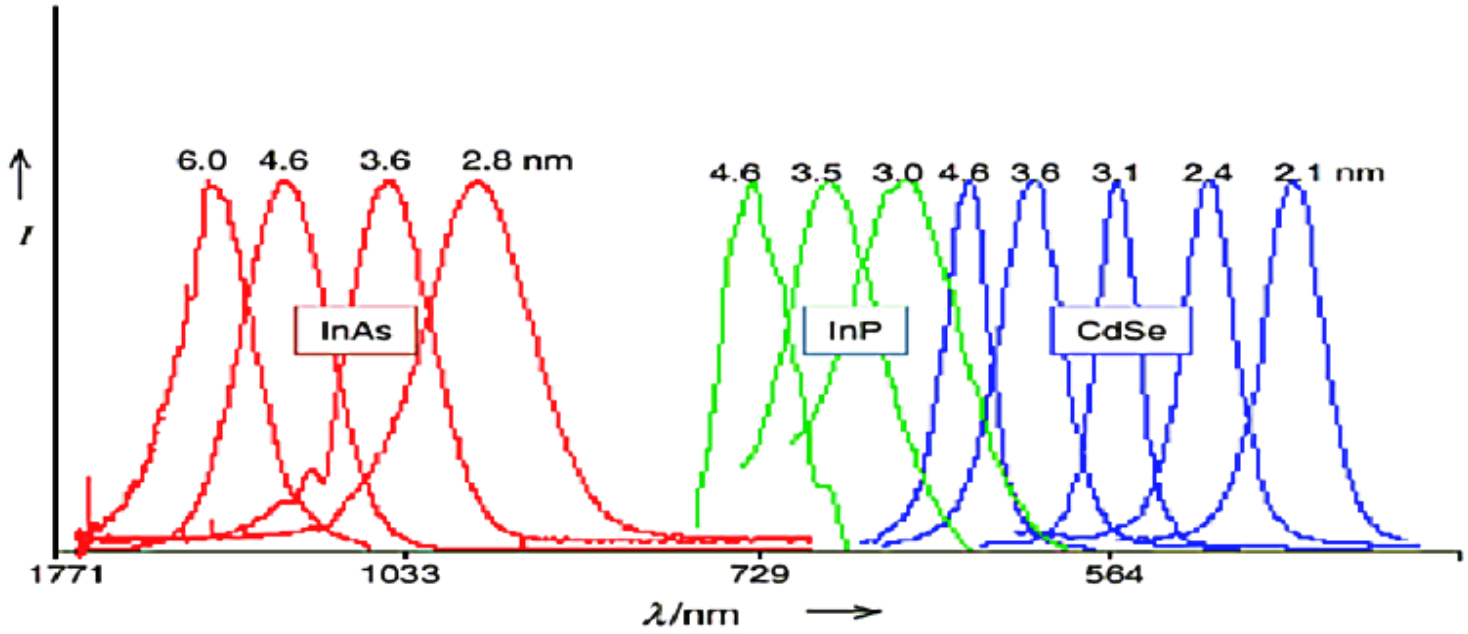
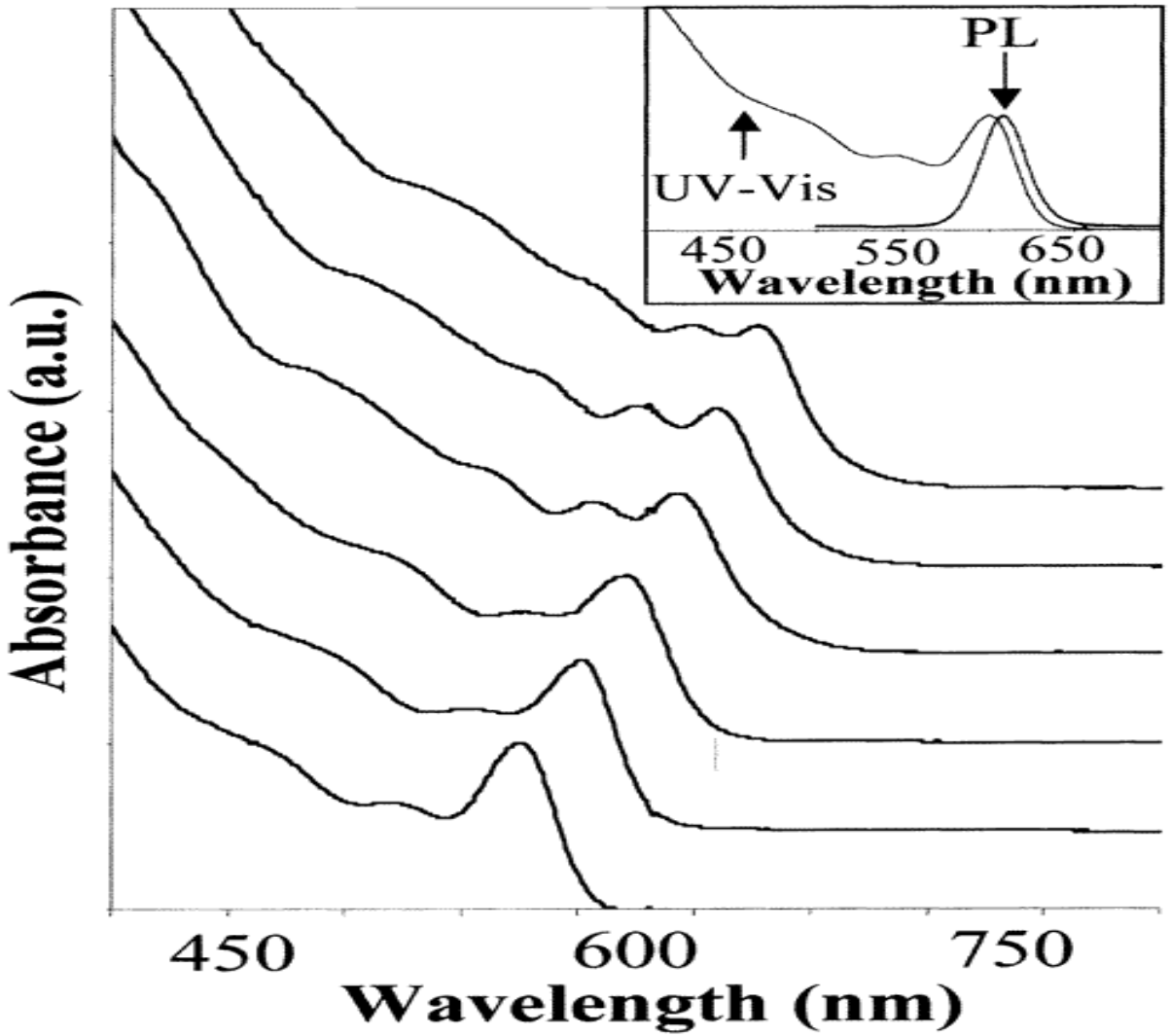
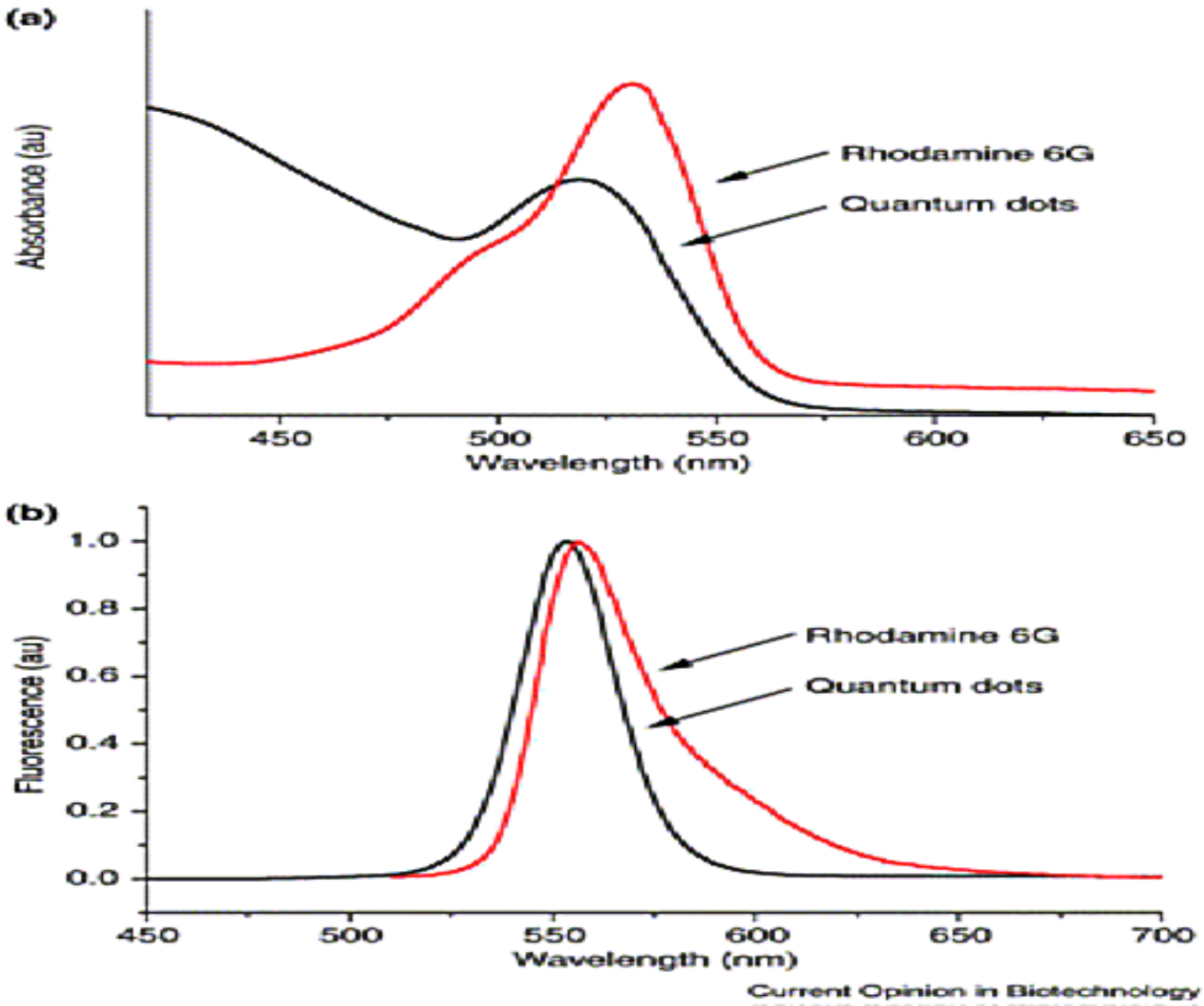


BROAD ABSORPTION SPECTRA



Q: Why is this of importance?

NARROW EMISSION SPECTRA



QDOT SUMMARY

- Qdots are very photostable
- Narrow, stable spectra
- Large (around 5nm diameter + bio-coat)
- Twinkeling

Filters

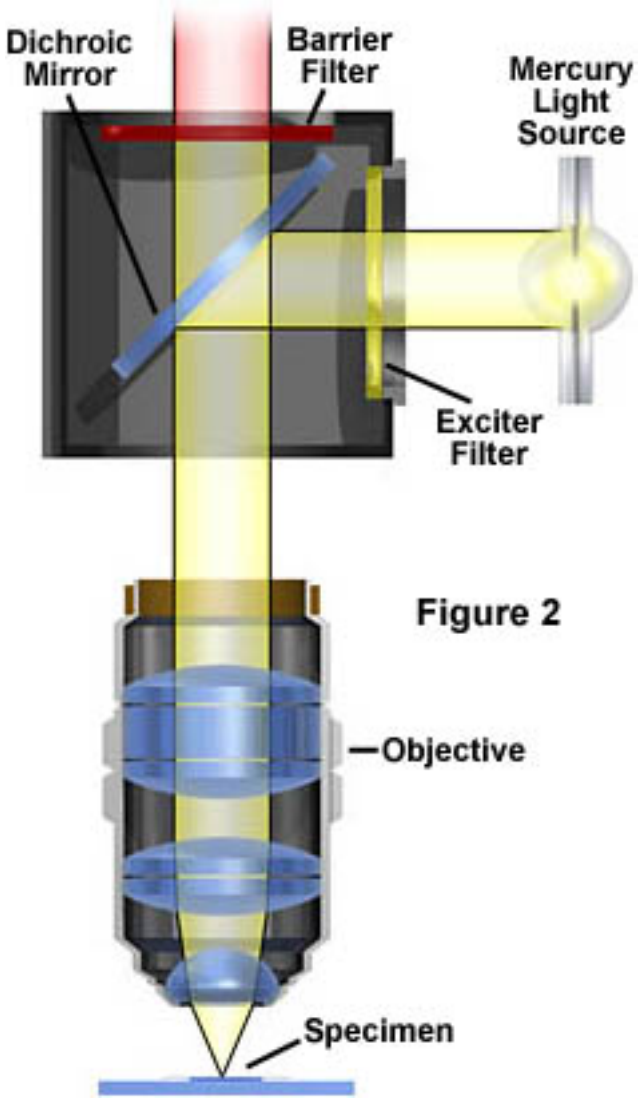
Filters are crucial

Since the ratio of the intensities of Excitation/Fluorescence = 10^6

Filter components

Excitation filter
Dichroic mirror
Emission filter

Each ~10^2 rejection
together ~10^6



FILTER CUBES

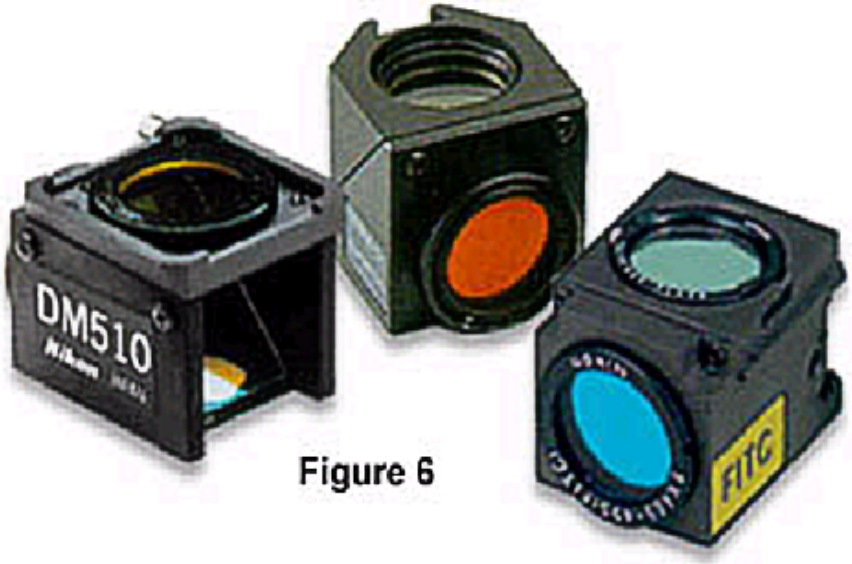


Figure 6

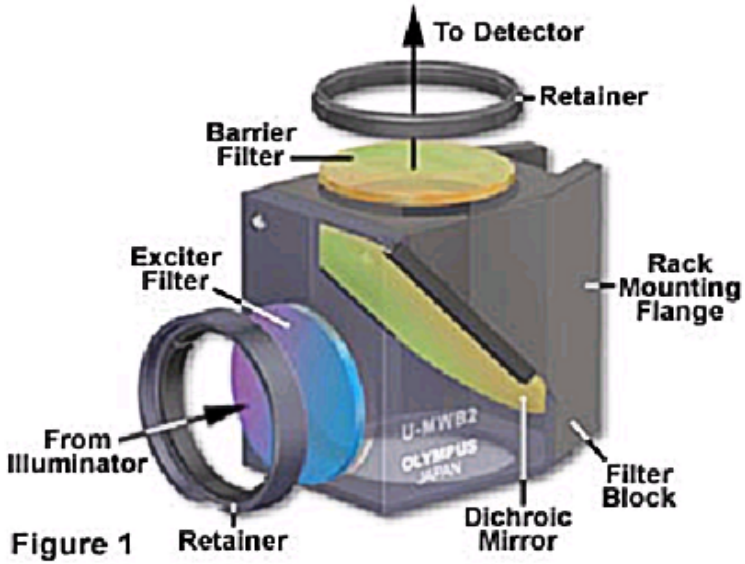


Figure 1

Interference filters:
Sharp transitions
Whigley

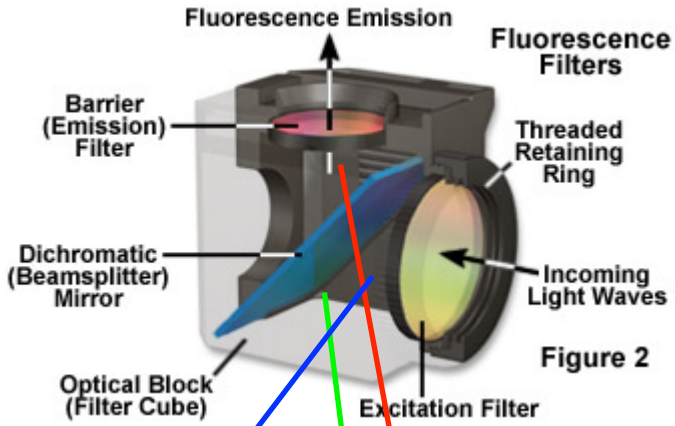
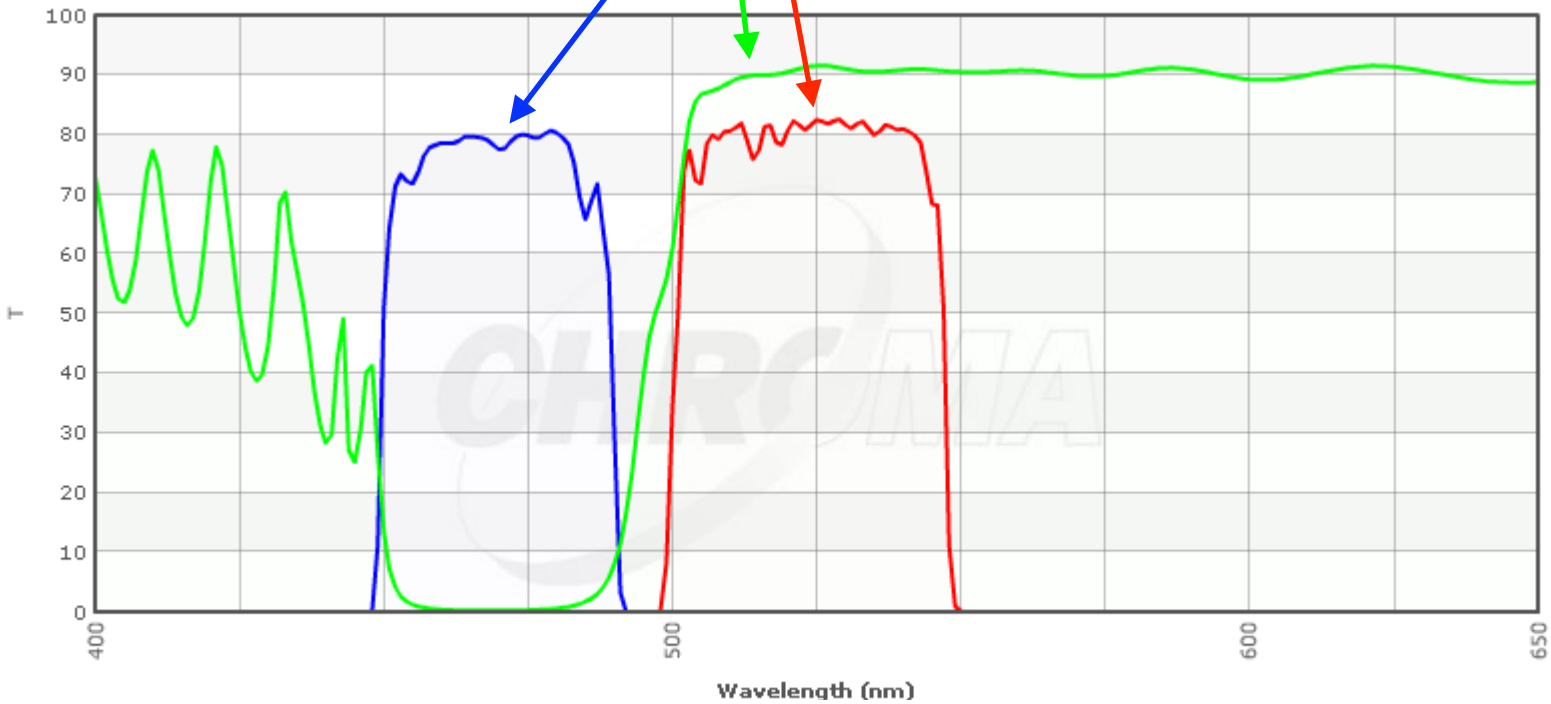


Figure 2



TYPES OF FILTERS

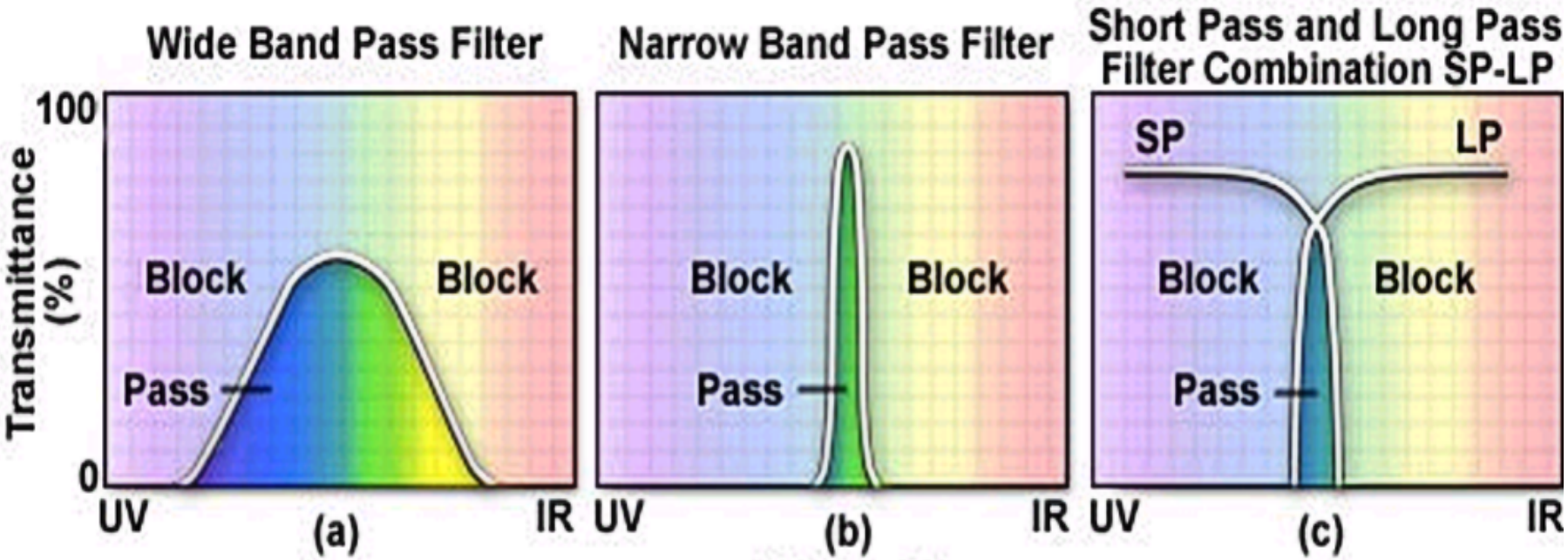
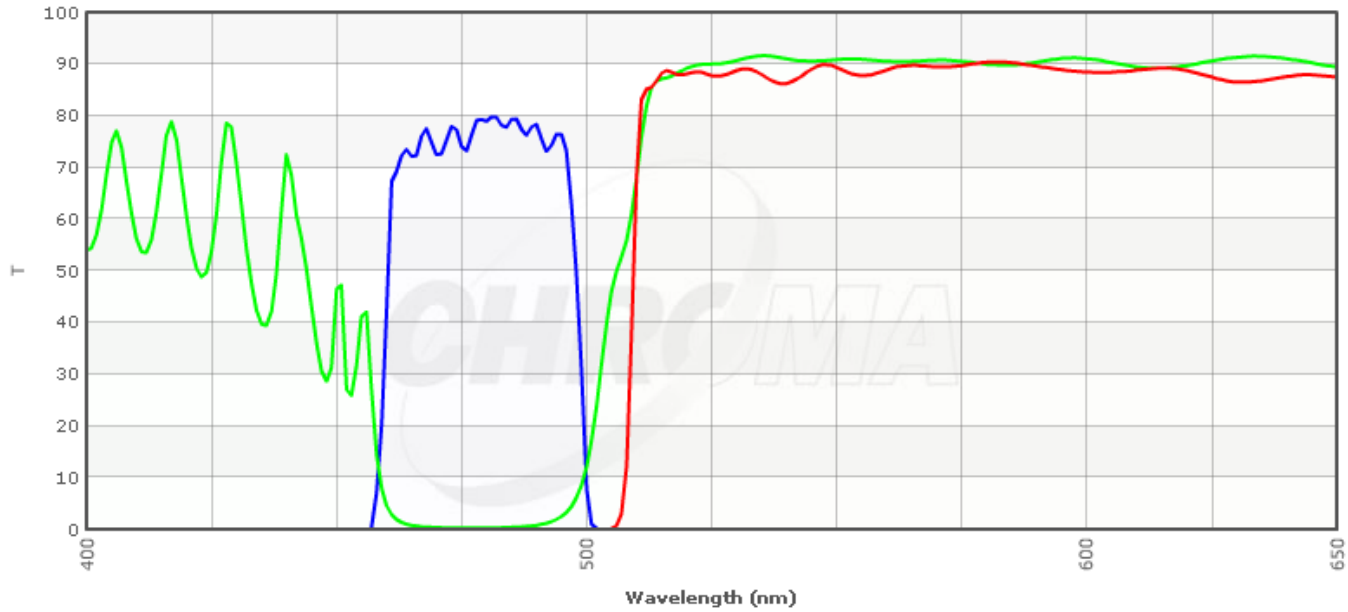
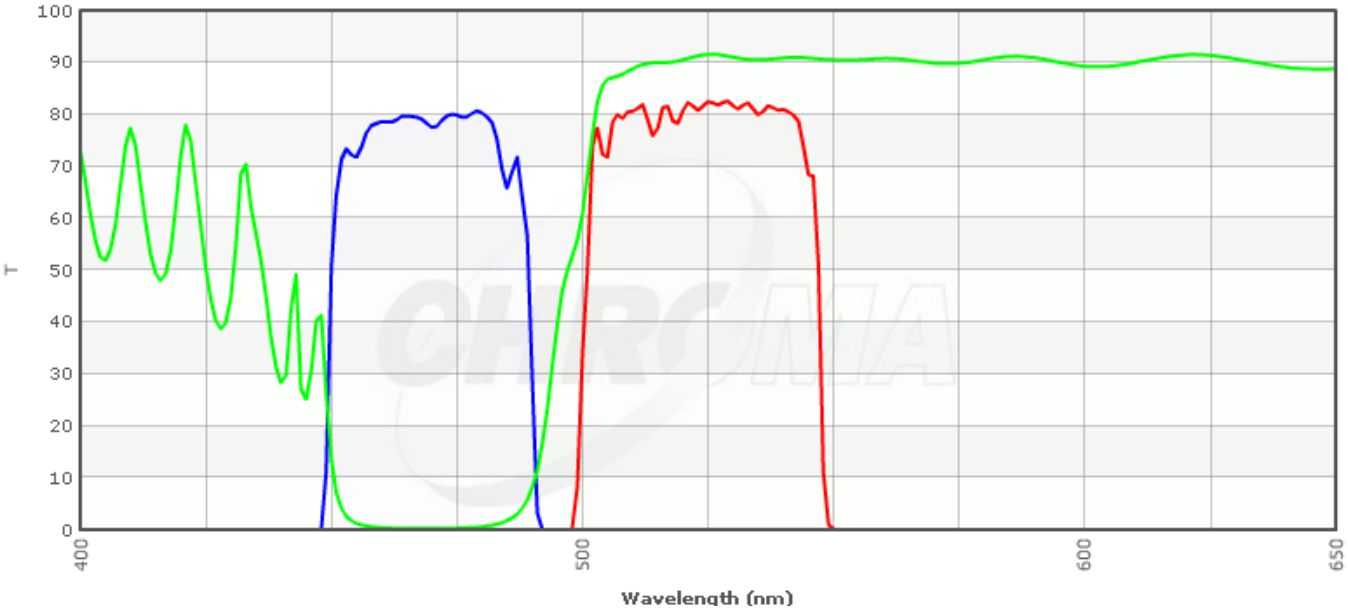


Figure 3

Longpass

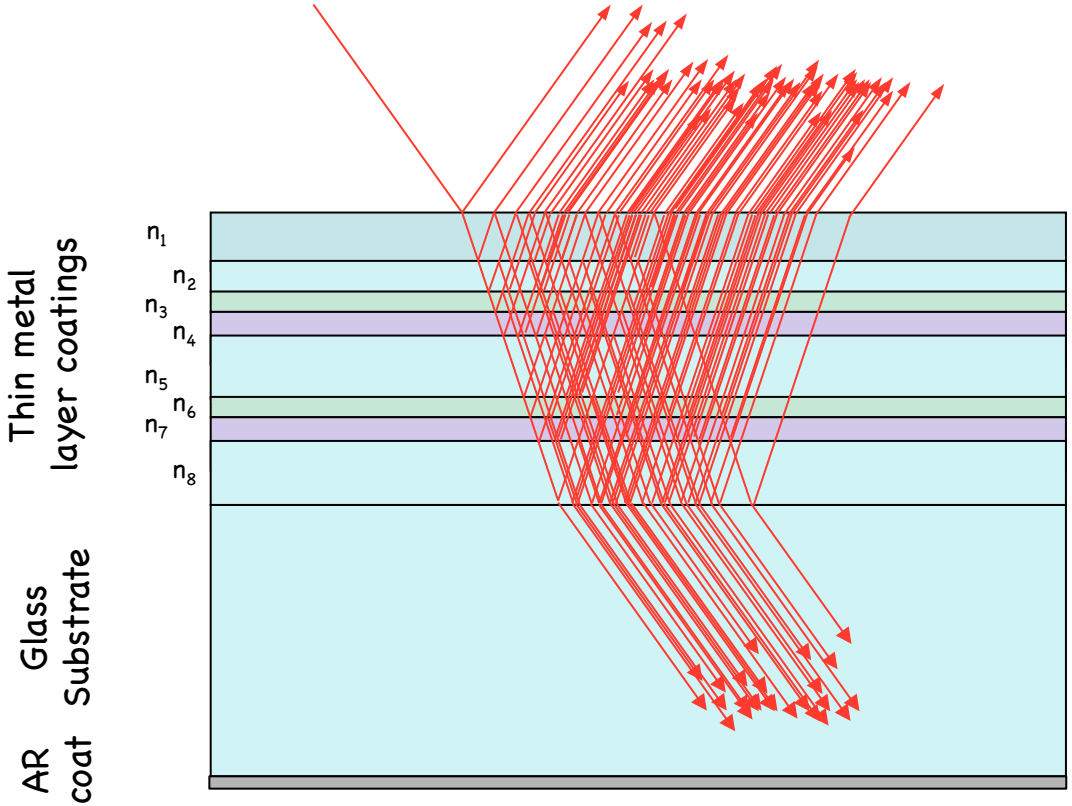


Bandpass



Q: when would you use band- and when long-pass filters

Interference filters



Interference

→ Wavelength-Dependent transmission & reflection

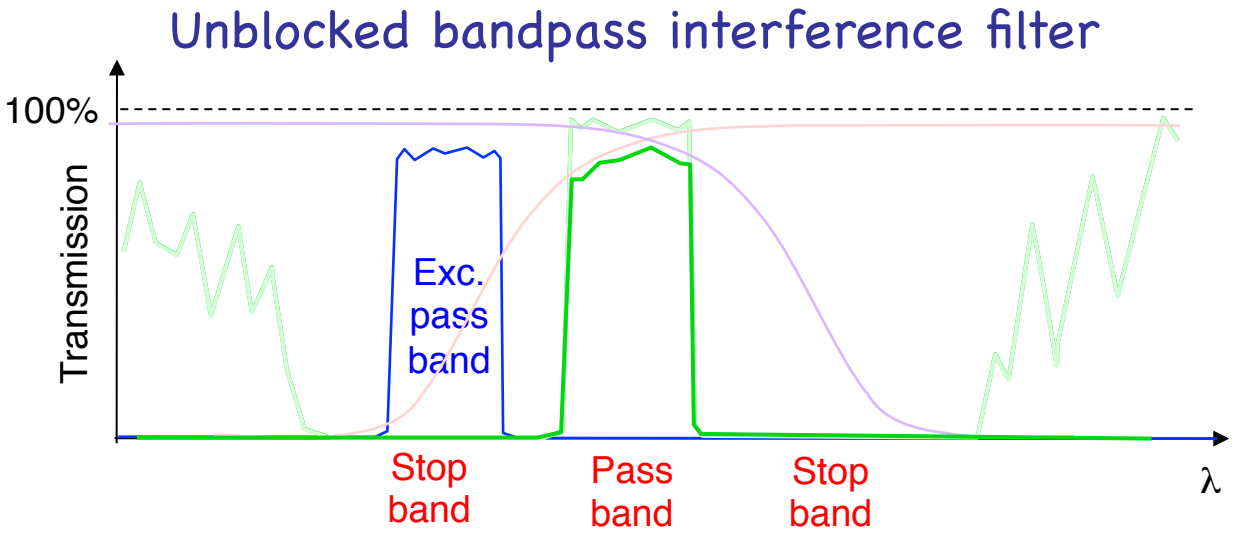
→ filter

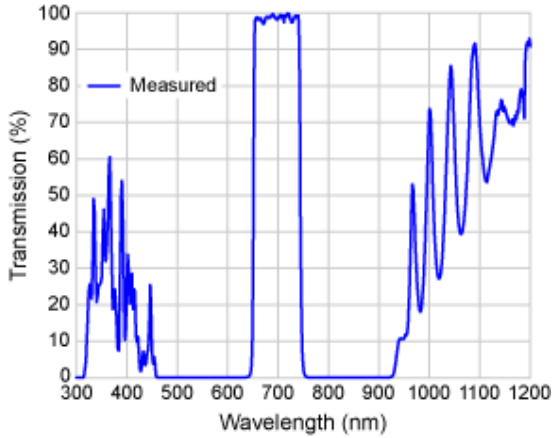
Filter makers : Chroma
Semrock
Omega

Transmitted color-band depends on angle

Blocking

Interference filters have finite stop bands

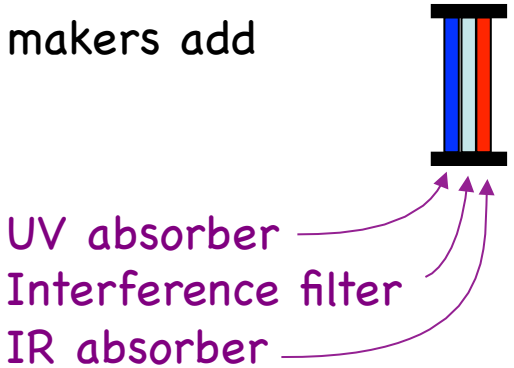




Semrock 697/75

To block unwanted transmission from UV to IR, filter makers add *absorption glass* to the filter.

Often excitation filters are blocked,
but emission filters *unblocked*.
→ Red autofluorescence or room light
may get through your blue emission filter

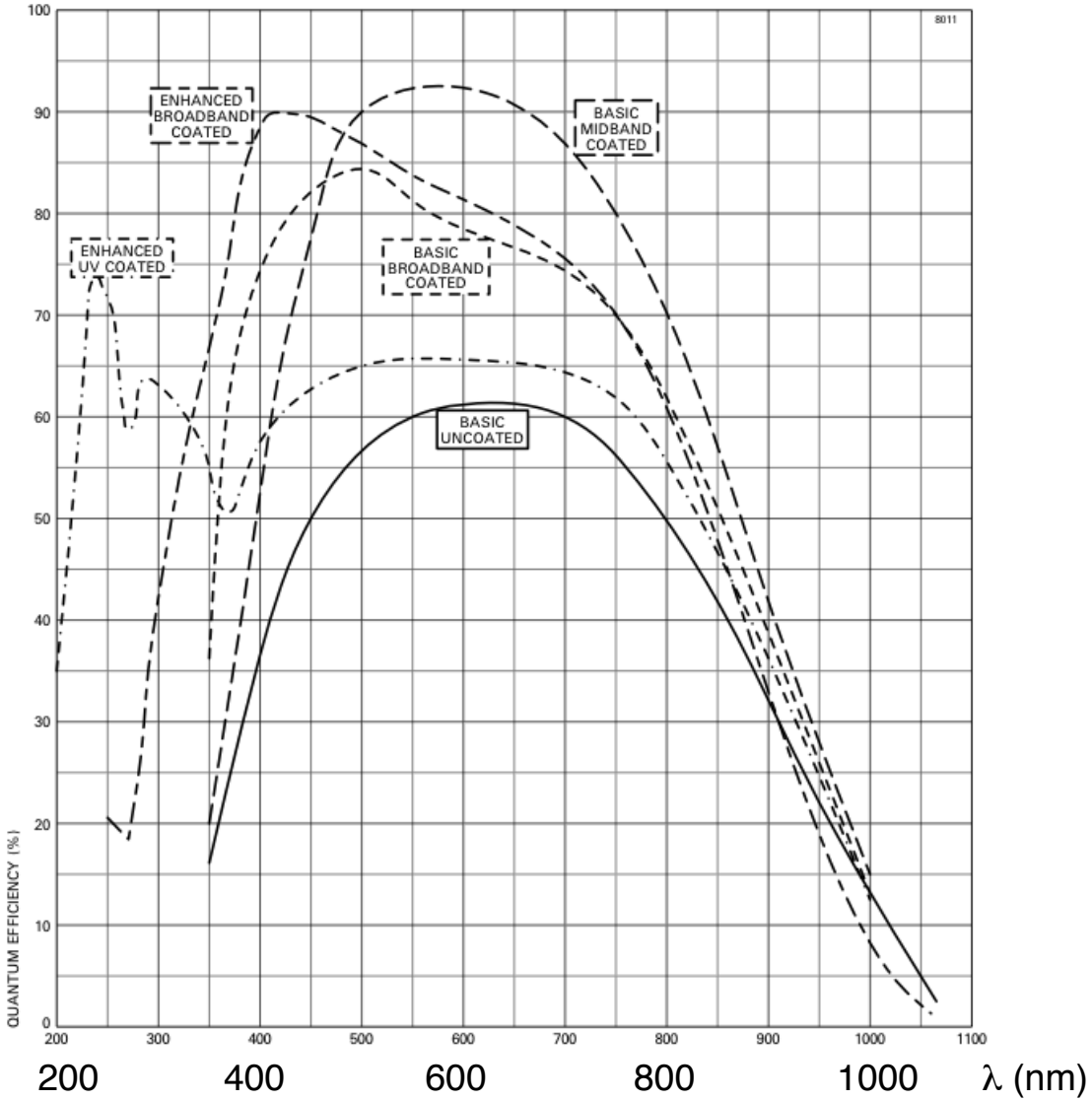


Blocking range

CCD cameras are sensitive from <300 nm to ≈1100 nm

Need to block this range

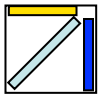
Can use separate IR filter in lamp

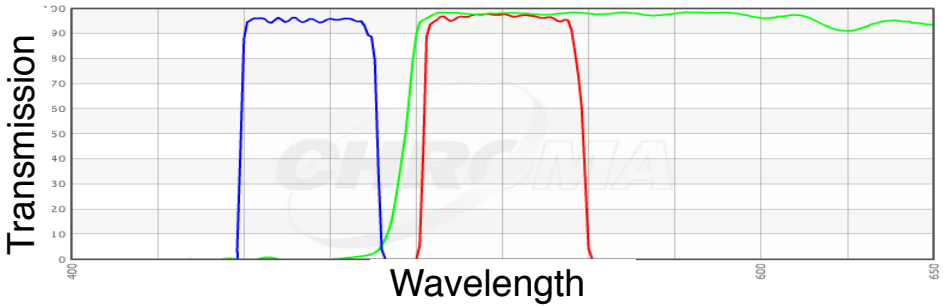


Filter schemes

Single wavelength sets

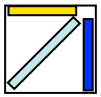
- Most efficient
- Best separation
- Very slow to change λ

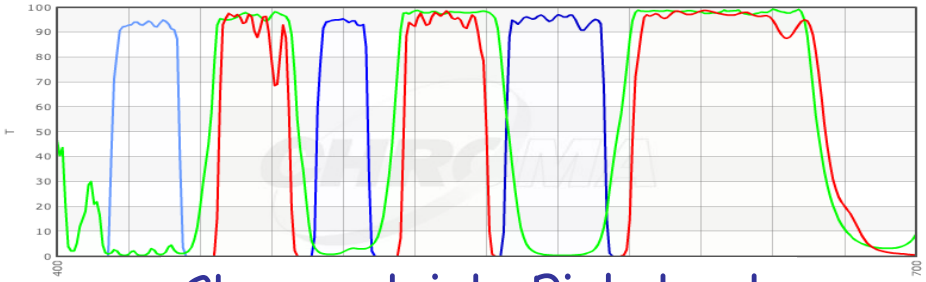




Multi-band filters

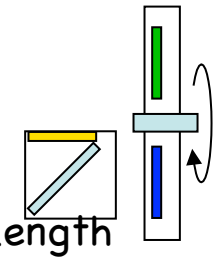
- Multi-band everything
  - See all colors at once
  - For color cameras
  - Bad crosstalk

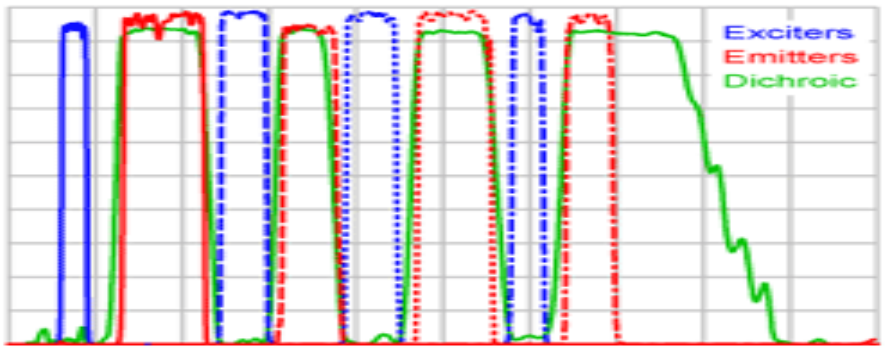




Chroma triple Pinkel set

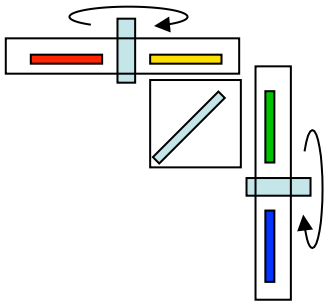
- "Pinkel" scheme
  Multi-band dichroic
  Multi-band emitter
  Single-λ exciters
  - Excitaton filter wheel
  - Separate image at each wavelength
  - Better separation



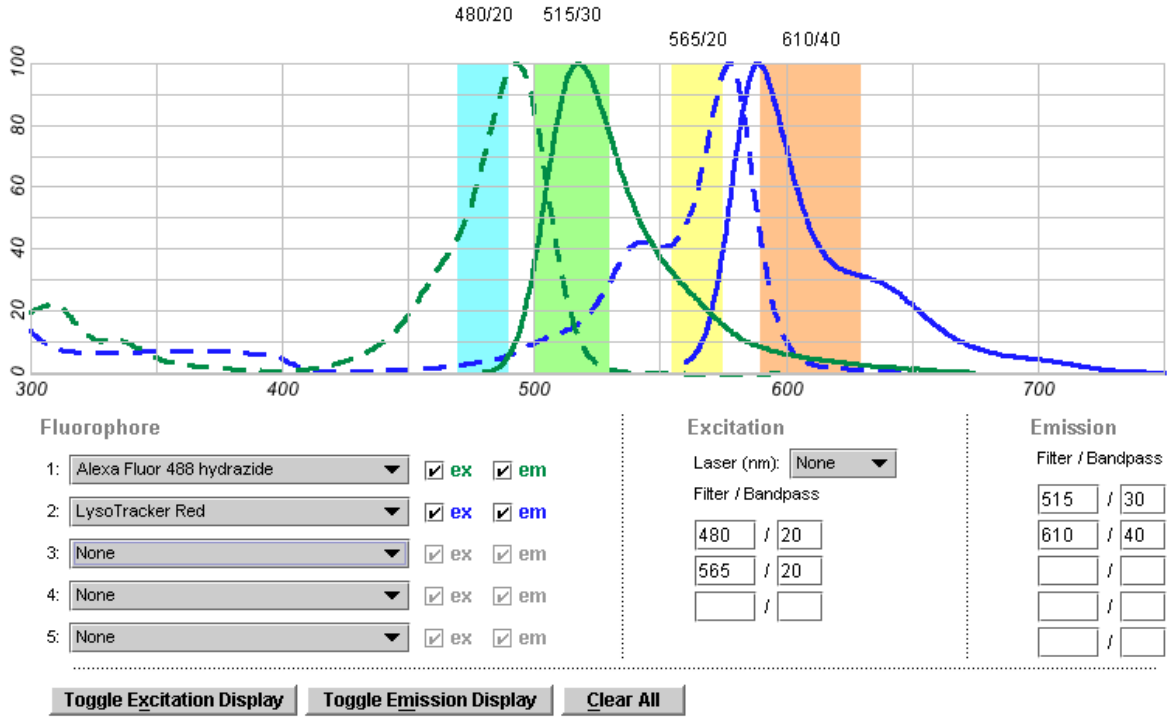


Semrock quad Sedat set

- "Sedat" scheme
  Multi-band dichroic
  single-band emitters
  Single-λ exciters
  - Two filter wheels
  - Even better separation



Matching Filters and Fluorophores



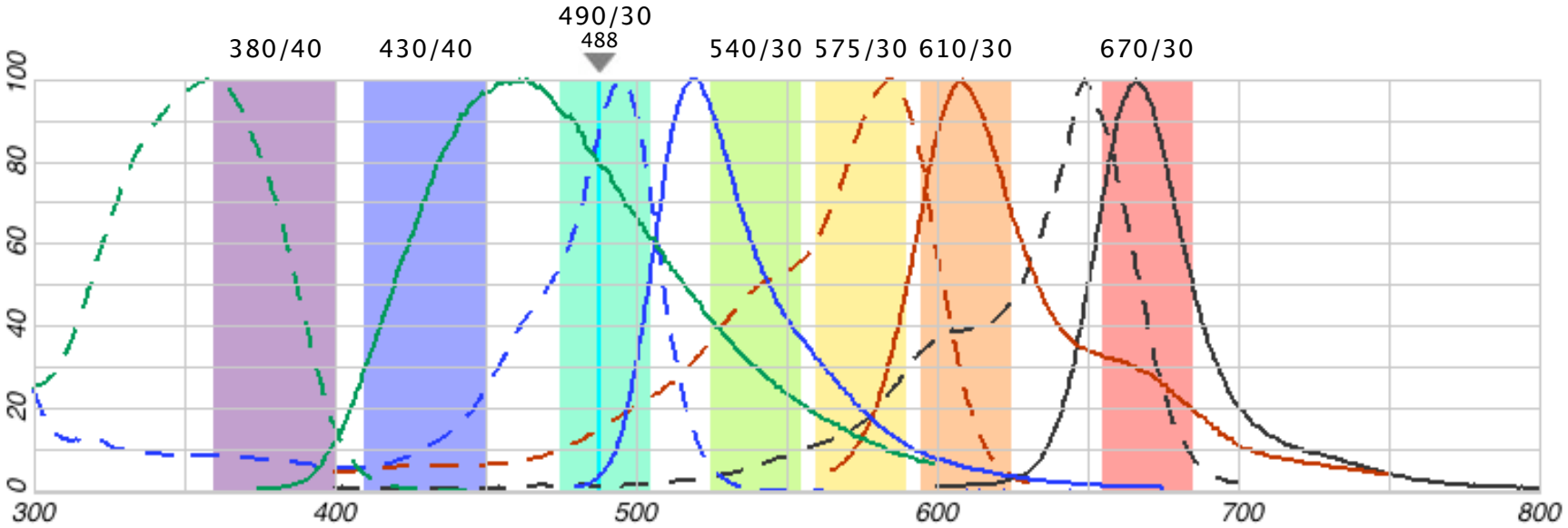
http://probes.invitrogen.com/resources/spectraviewer/

http://fluorescence.nexus-solutions.net/frames6.htm

https://www.omegafilters.com/curvo2/index.php

Fluorescence SpectraViewer

Now you can plot and compare spectra and check the spectral compatibility for many fluorophores offered by Molecular Probes. The Spectra Viewer ca
printed by capturing a screen-shot and printing the resulting image file. For printing instructions or to answer other questions you have, see our User Gu



Fluorophore

1:	DAPI	☑ ex	☑ em
2:	FITC (fluorescein, antibody conjugate)	☑ ex	☑ em
3:	Texas Red DHPE	☑ ex	☑ em
4:	Cy5 dye (antibody conjugate)	☑ ex	☑ em
5:	None	☑ ex	☑ em

Excitation

Laser (nm): 488

Filter / Bandpass

380	40
490	30
575	30

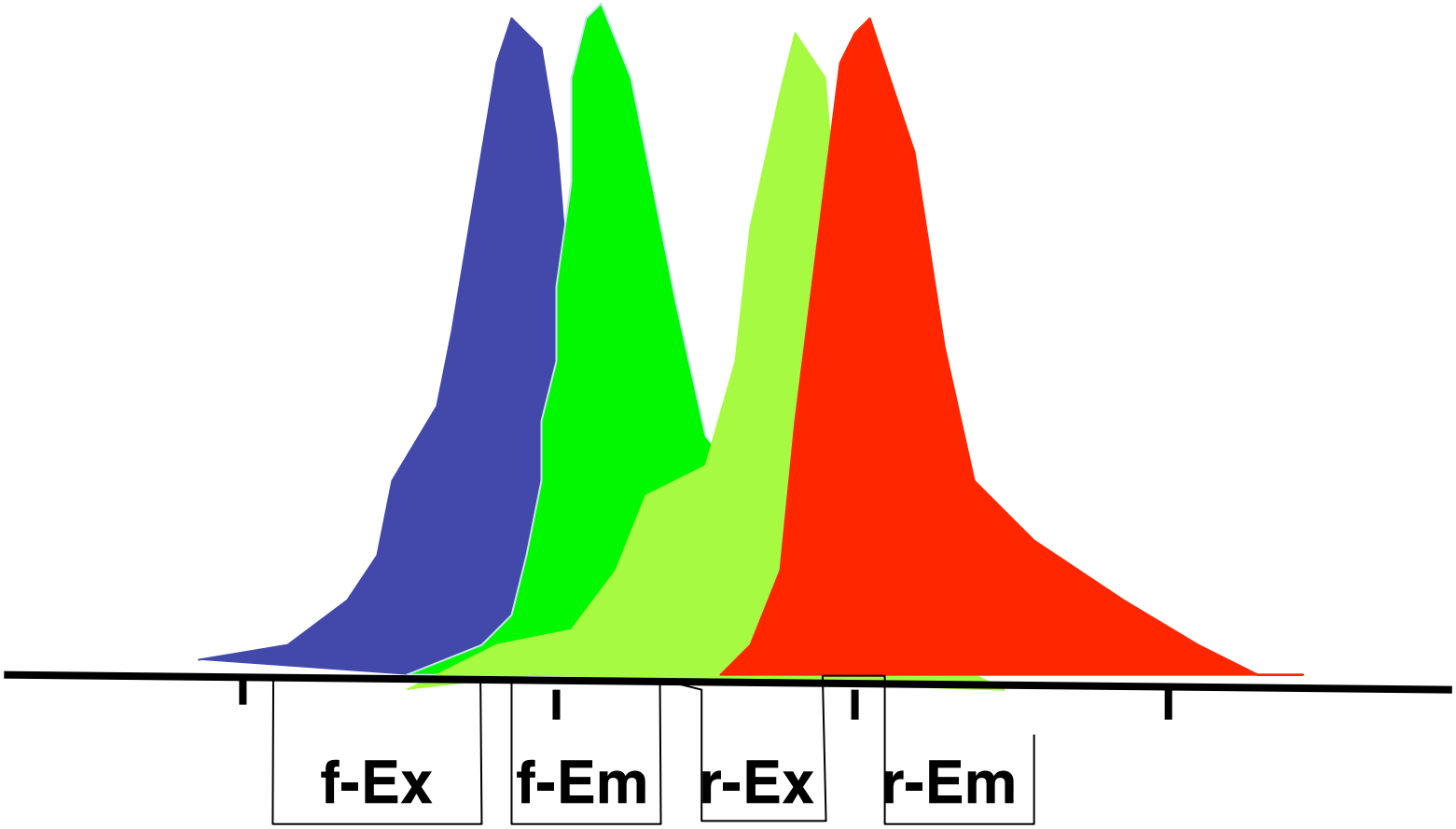
Emission

Filter / Bandpass

430	40
540	30
610	30
670	30

Toggle Excitation Display | Toggle Emission Display | Clear All

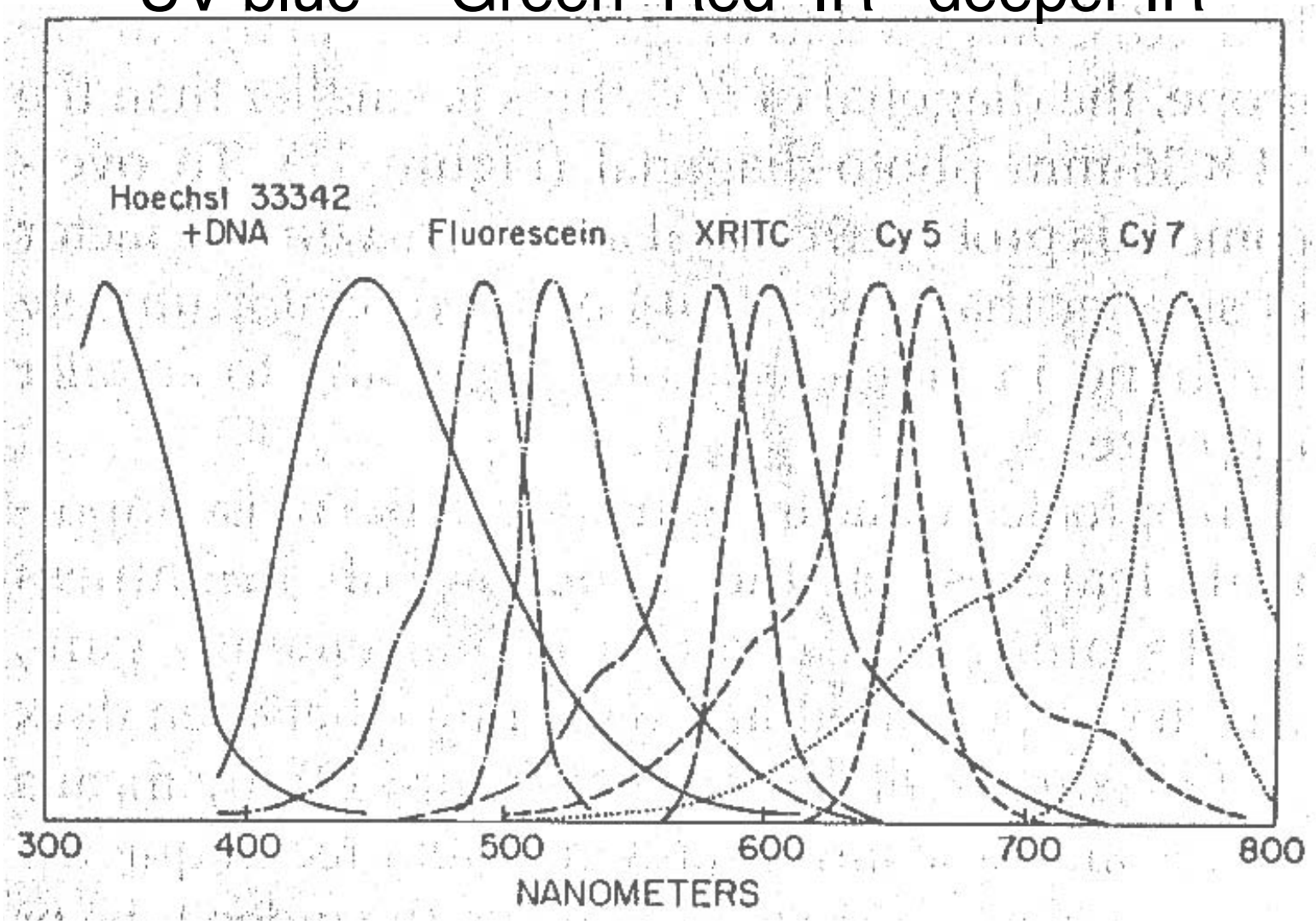
Multi-color labeling:
FITC & TRITC Exitation & Emission spectra



Q: What limits how close can the spectra be?

5 possible simultaneous fluorescent “channels”

UV-blue Green Red IR deeper IR



Light Sources

LIGHT SOURCES

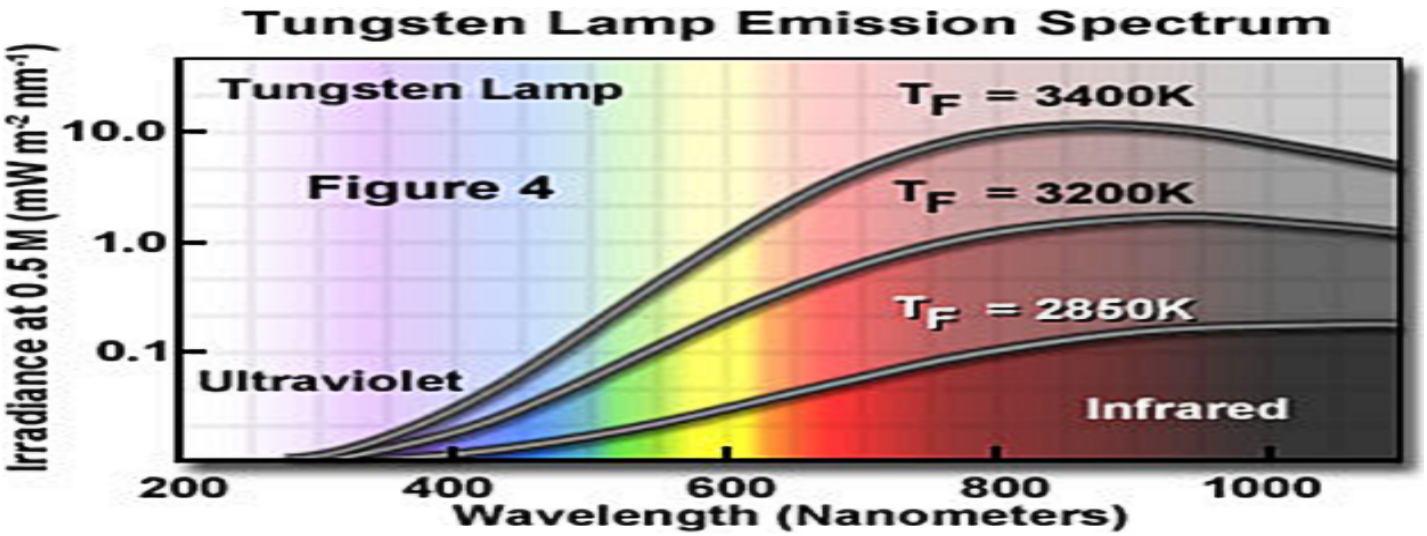
- Black body sources (halogen lamps)
  -spectrum is continuous
  -spectrum peak depends on temp.
  ("color temperature")

- Spectral sources (Hg, Xe, other arc lamps, lasers, LED)
  -spectrum has structure (peaks)
  -spectrum is a function of the
  electronic properties of the gas
  - VERY BRIGHT

Light sources: General considerations

- Wide field versus scanning system
  - Uniform (wide field)
  - Focused to a diffraction limited point (scanning systems)
- Brightness
  - Brightness of light source
  - Light source optics
- Spectra
  - Broad / Narrow
    - Range
  - Spectral uniformity
- Modulation
  - Depth
  - Rate
- Stability
  - Short term fluctuations
  - Long term stability

Halogen source spectra

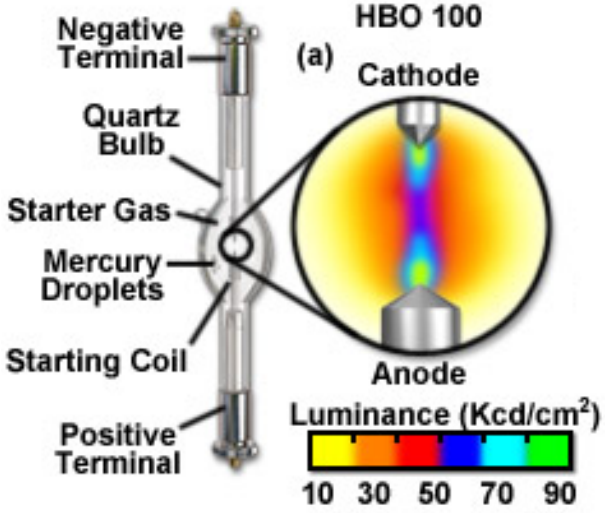


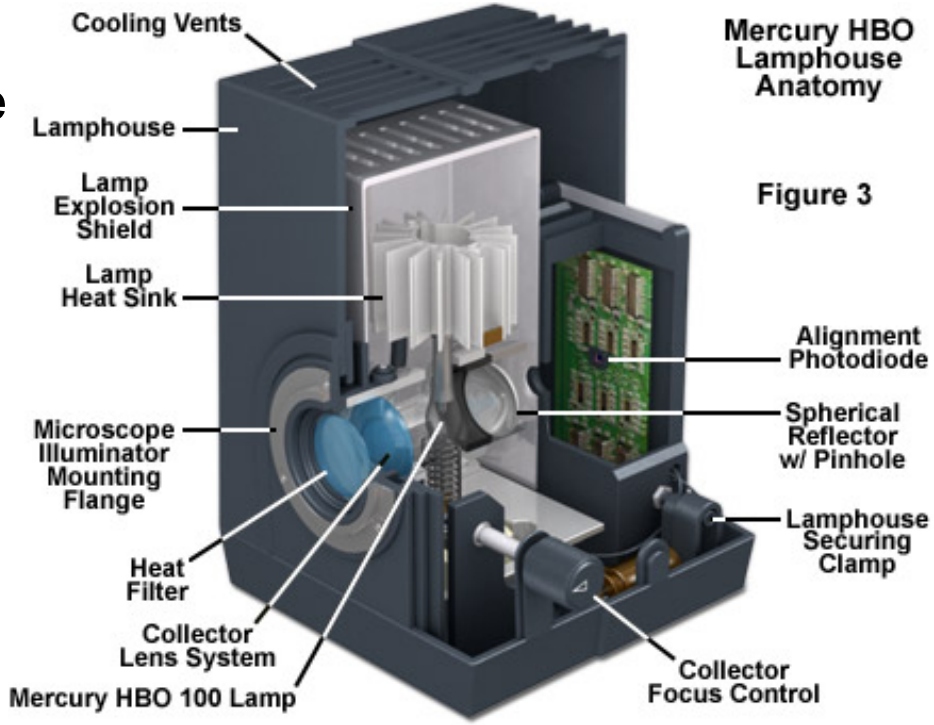
SOURCE SIZE = 5mm or more

Light sources: Mercury lamps

- Very bright light source
- Highly popular in fluorescence microscopes
- Classically referred to as HBO lamps
- Based on plasma arc-discharge lamp
- Spectrum based on mercury vapor
- Lamp enclosed in external housing built to withstand explosion, dissipate heat
- Housing has collector lens and alignment systems, as well as a reflector to collect additional light
- Driven by special power supply needed for ignition and constant current

SOURCE SIZE = 0.5-1 mm

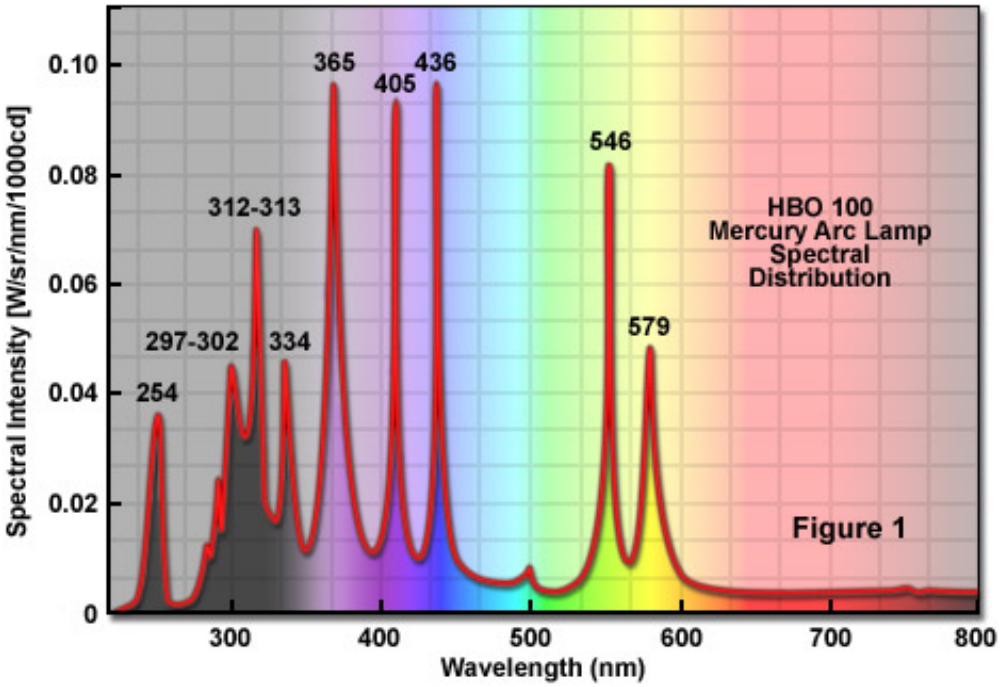


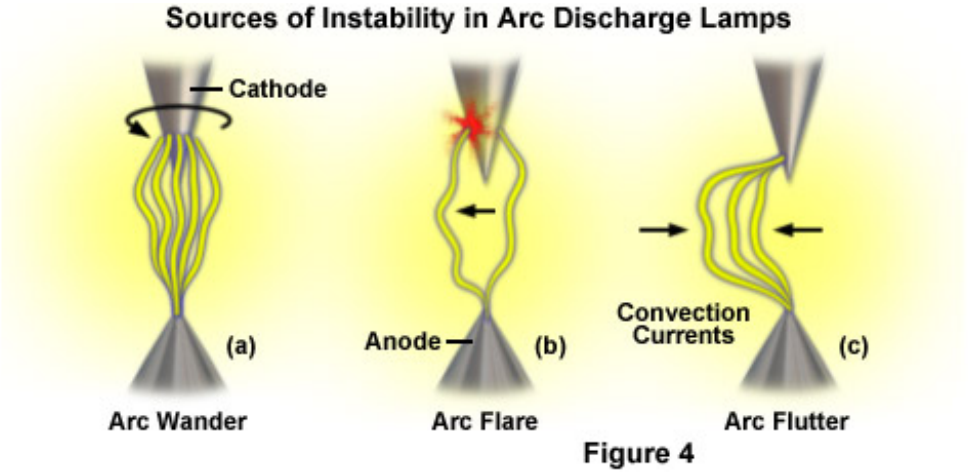


Light sources: Mercury lamps

Disadvantages:

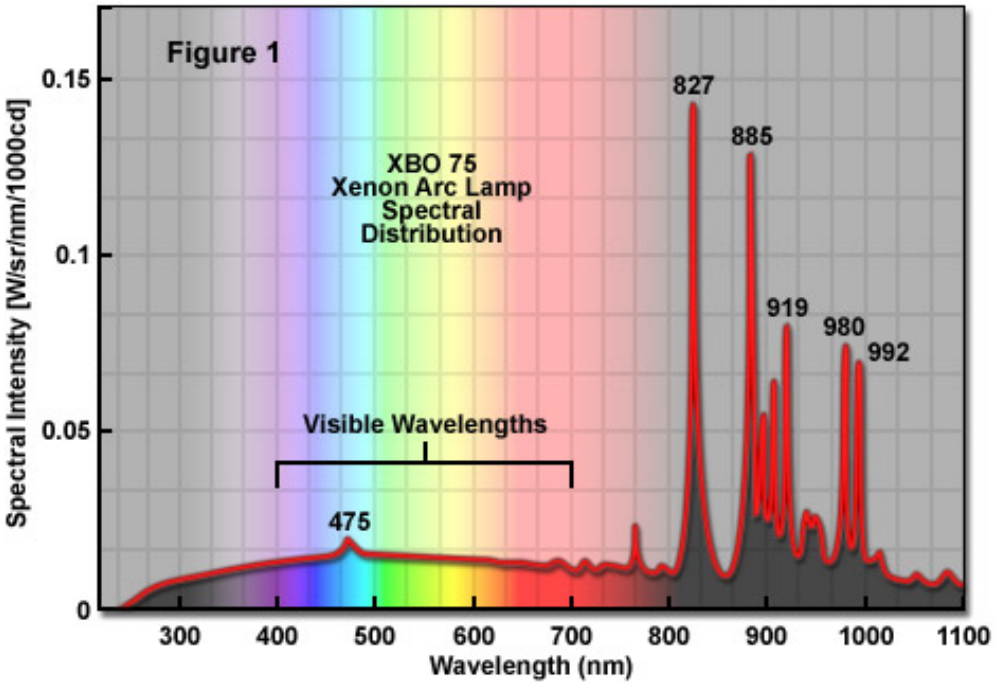
- Discontinuous spectrum
- Limited lifetime (~200 hours), further reduced by repeated on/off cycles
- Specialized lamphouse and power supply requirements (internal pressure > 75 Atms!)
- ~50% of energy in UV
- Fluctuations on short and long time scales

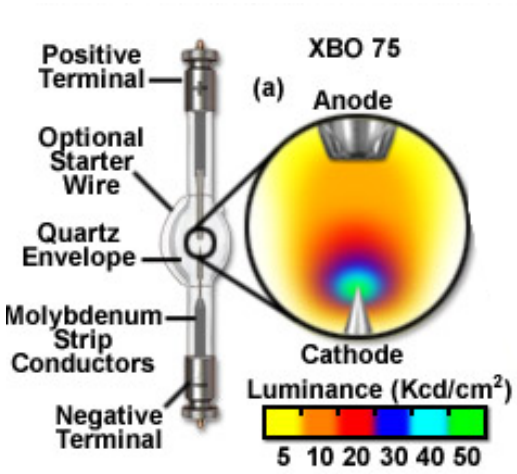




Light sources: Xenon lamp

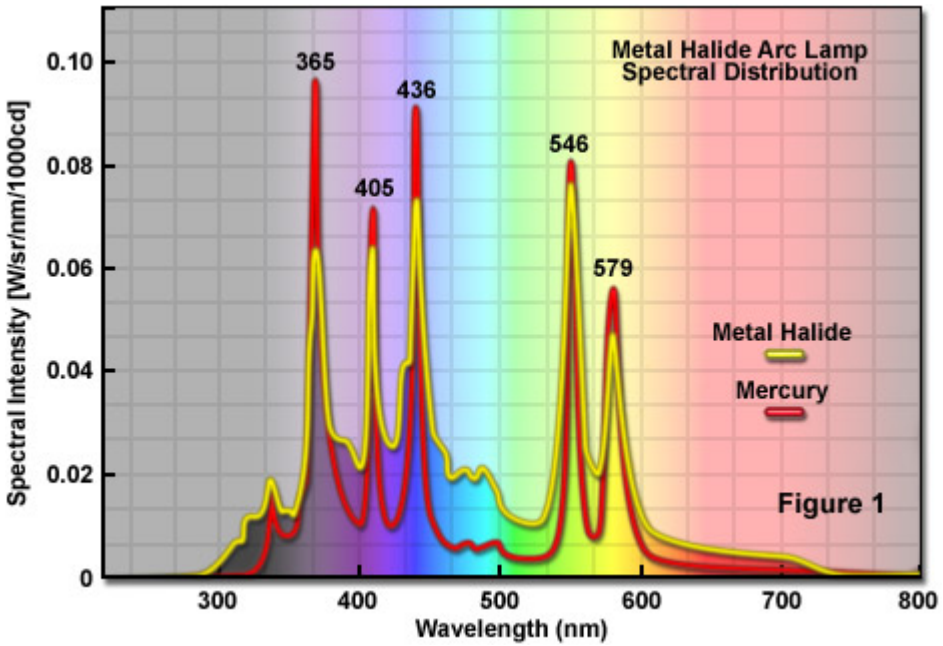
- Bright light source
- Classically referred to as XBO lamps
- Much more uniform spectrum (but less bright) as compared to HBO lamps
- Nearly ideal point source
- Based on plasma arc-discharge lamp
- Spectrum based on xenon gas
- Lamp enclosed in external housing built to withstand explosion, dissipate heat, similar to that of HBO lamp
- Excess energy in IR – use of IR blocking filter recommended to protect cells and imaging devices
- Short and long stability can be better than HBO lamps (i.e. “super quiet” lamps and special power supply units)
- Life time ~400 hours

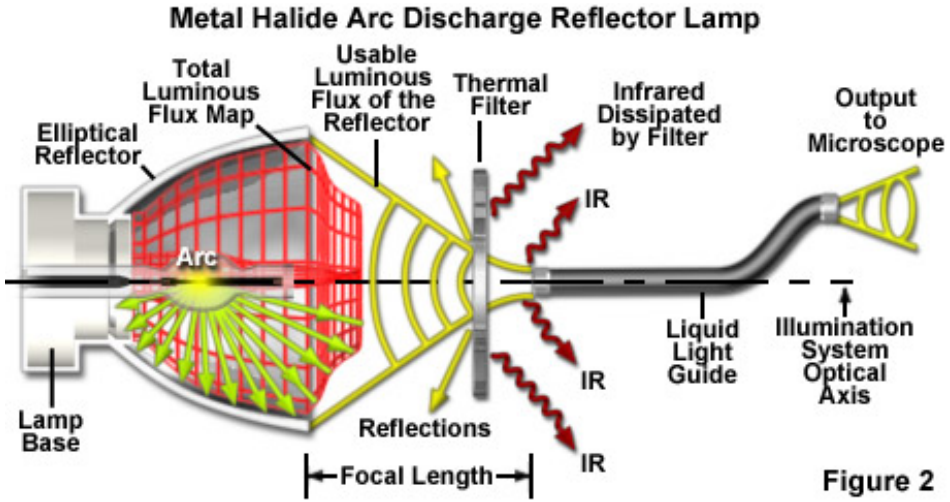




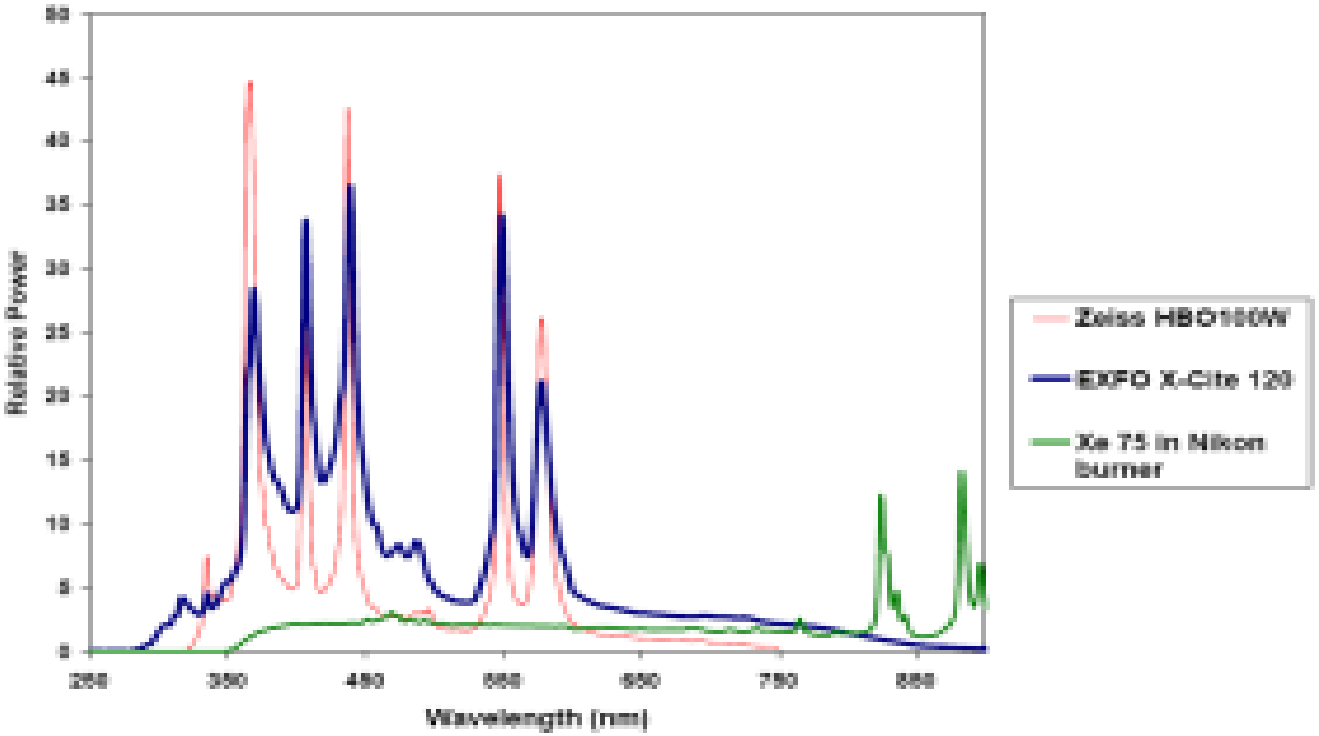
Light sources: Metal Halide

- Very bright light source, rapidly replacing HBO light sources
- Based on mercury lamp technology with enhancements (use of rare earth metal - halide salts)
- While spectra exhibits peaks, these are broader, with higher radiation levels in between
- Commercial systems typically include heat filters, a light guide, fixed alignment lamp mounting, ND filter wheels, a shutter and remote control via computer interfaces
- Lamp life time 500-2000 hours.
- Important not to shut off before lamp heat up is completed (~5min)





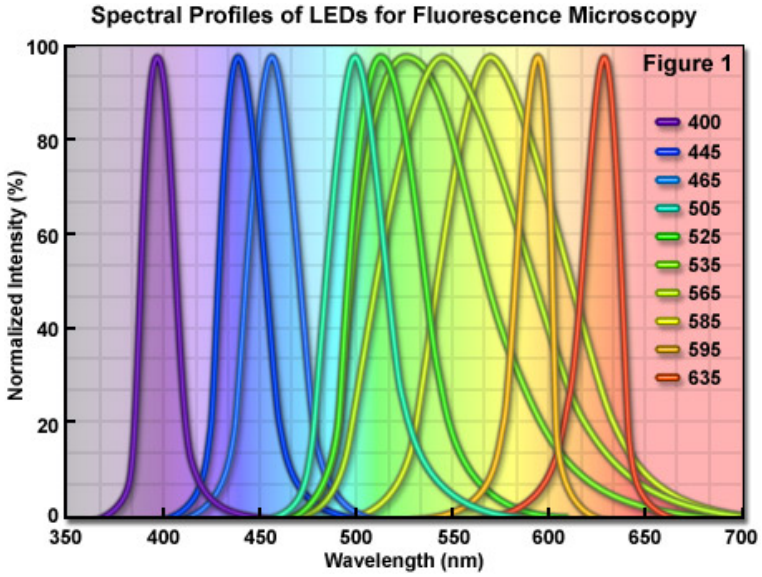
Meta-Halide

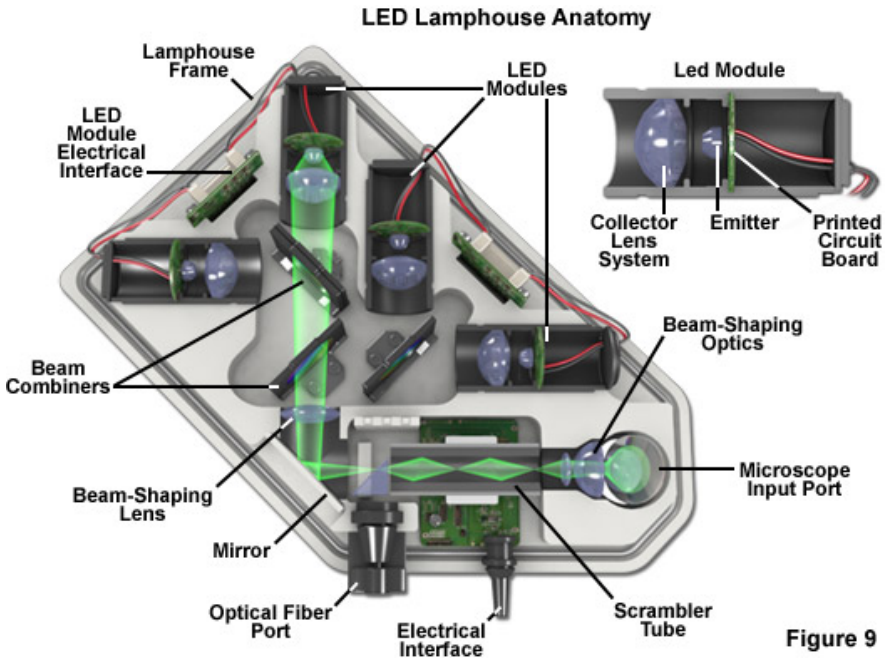


Fiber light-guide (scrambling)
Define NA and size

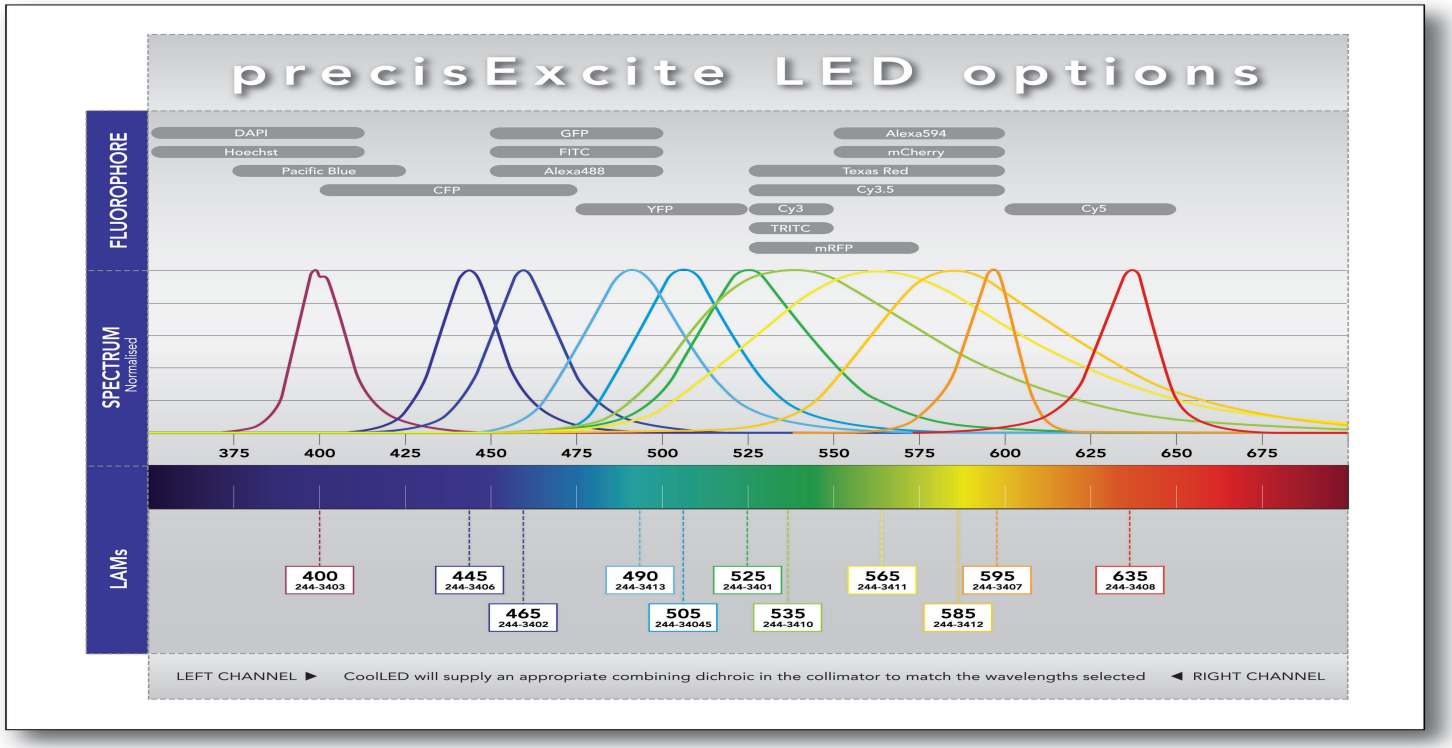
Light sources: LEDs

- LED (light emitting diode) a semiconductor light source
  - Becoming increasingly brighter every year, approaching XBO levels
    - ~Monochromatic light sources
  - Very efficient – low power consumption, little heat generation, not dangerous
- Extremely long life times (10,000-100,000 hours)
- Can be turned on and off repeatedly and modulated (<msec), by altering current levels, without damage
  - Commercial systems combine multiple modules to provide sets of desirable spectra.
    - No light outside these spectra





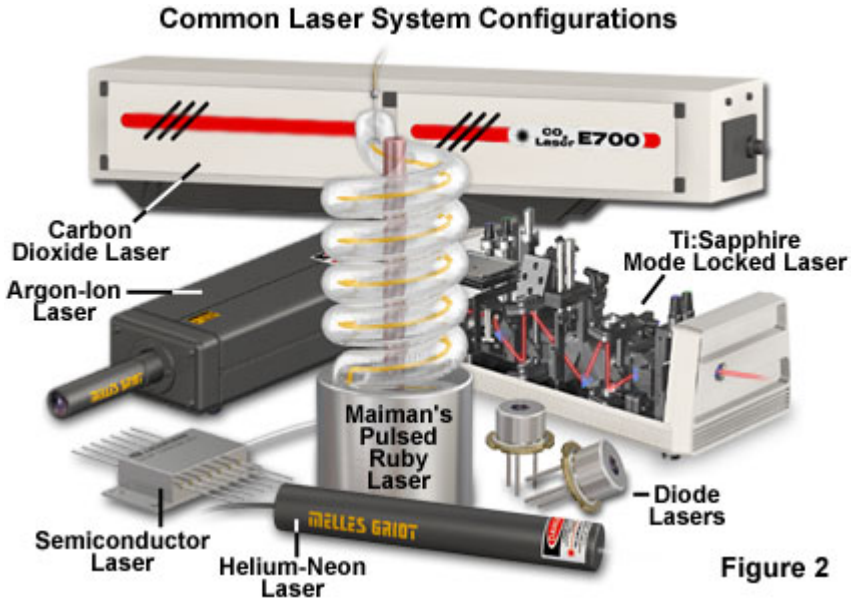
Light-emitting Diodes (LED)

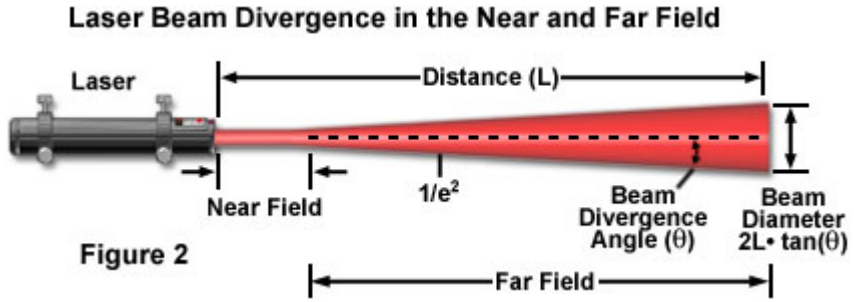


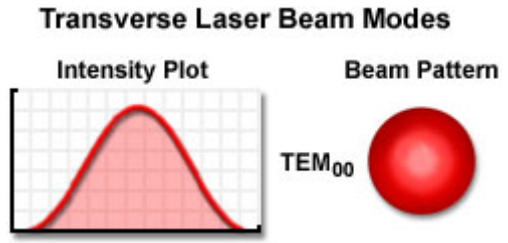
Green-gap -> phosphorescence
SIZE = 2-4mm
Flat illumination of field

Light sources: Lasers

- Laser = Light Amplification by the Stimulated Emission of Radiation
  - Very bright light source
- Emits light at one or more discrete wavelengths
  - Coherent, very narrow beam
- Usually Gaussian beam profile (TEM_{00})
- Mainly (but not exclusively) used for scanning systems
  - In this mode the specimen is illuminated *point by point*, by *scanning* a diffraction limited spot across the specimen (as opposed to uniform illumination in wide field microcopy)
  - Many laser technologies
    - Gas
    - Diode pumped solid state (DPSS)
    - Diode
    - …
- Typically expensive to *very* expensive







Light sources: Lasers

Common continuous wave (CW) lasers used in microscopy

Argon (gas) (457) 488 514 – air cooled, noisy, very useful

Argon Krypton 488 568 647 air cooled, noisy, limited life span

Helium Neon (gas) (543) 633 – simple, quiet, high beam quality, long lifetime

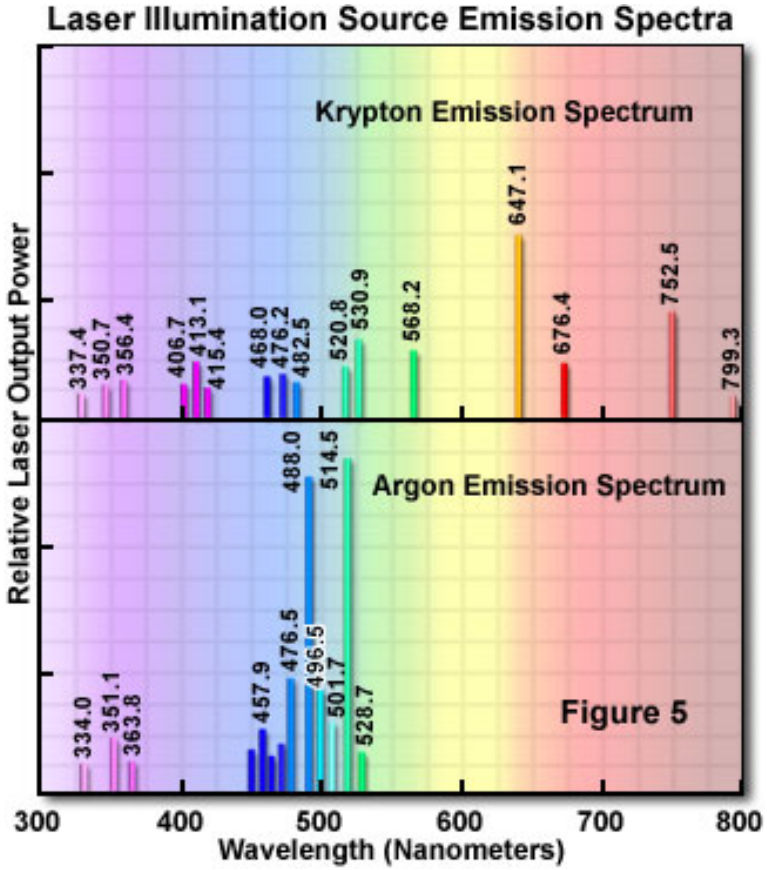
DPSS 457 473 491 505 515 532 542 561 594 – tiny, silent, cool, ~expensive

Diode Lasers 375 405 440 642 – tiny, silent, mediocre beam

Many solid state lasers can be modulated electronically (on/off, intensity)

Solid state lasers are often fiber coupled to improve beam quality

Lasers



EFFECTIVE SIZE = 0

Focusable to a diffraction spot

Flat illumination of field [speckle…]

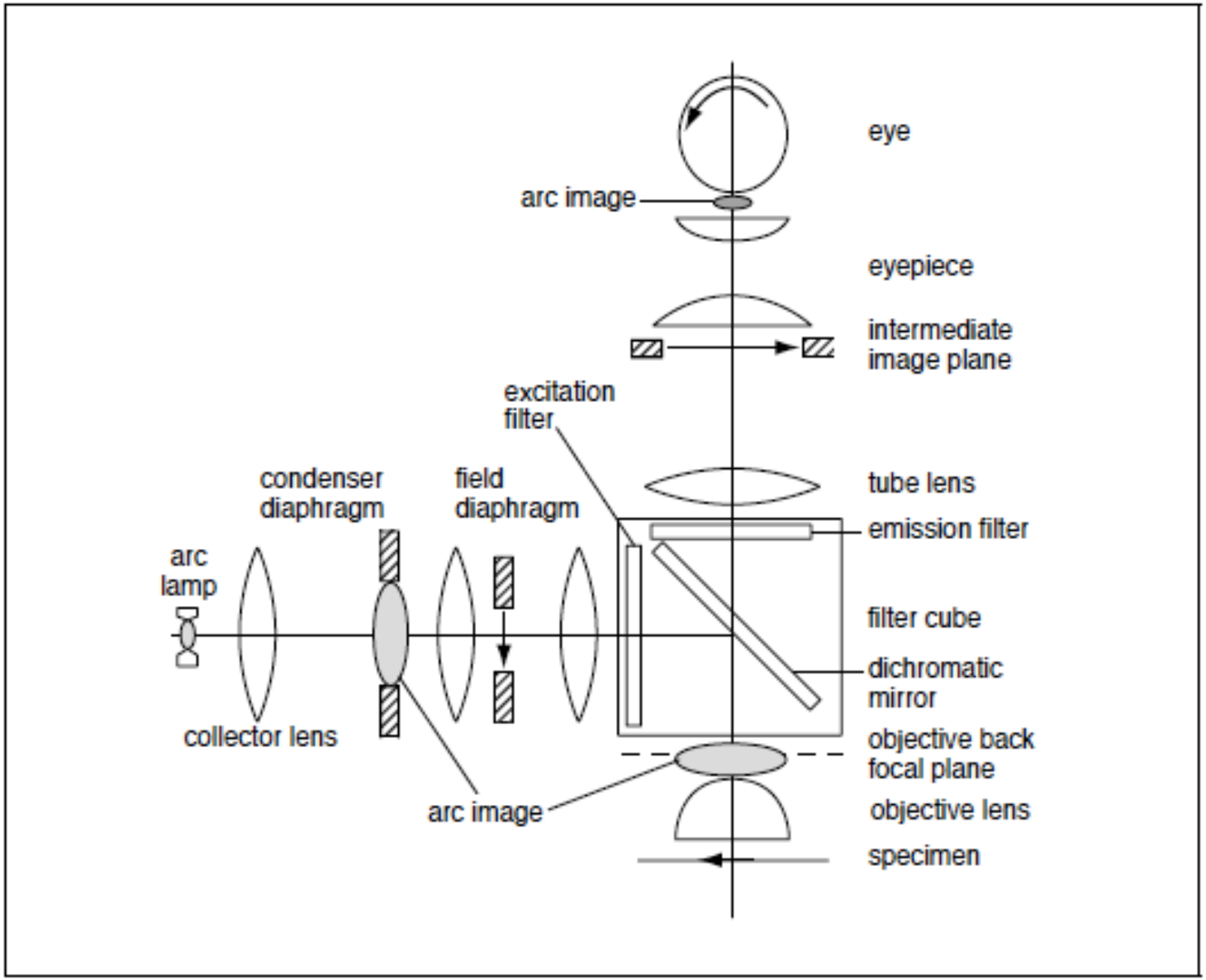
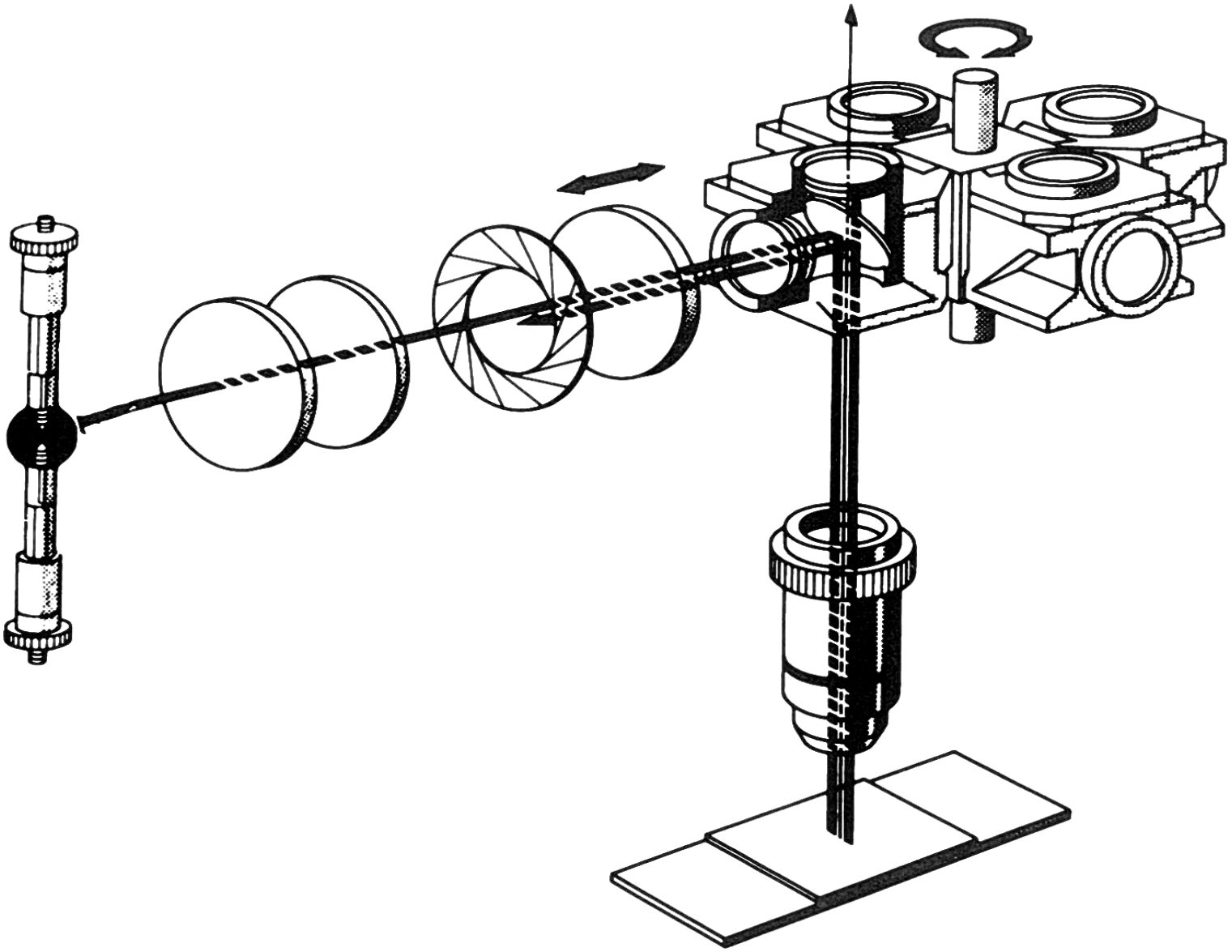


Figure 21.1.4 Microscope alignment for epifluorescence Köhler illumination.

EPIFLUORESCENCE LIGHT PATHS



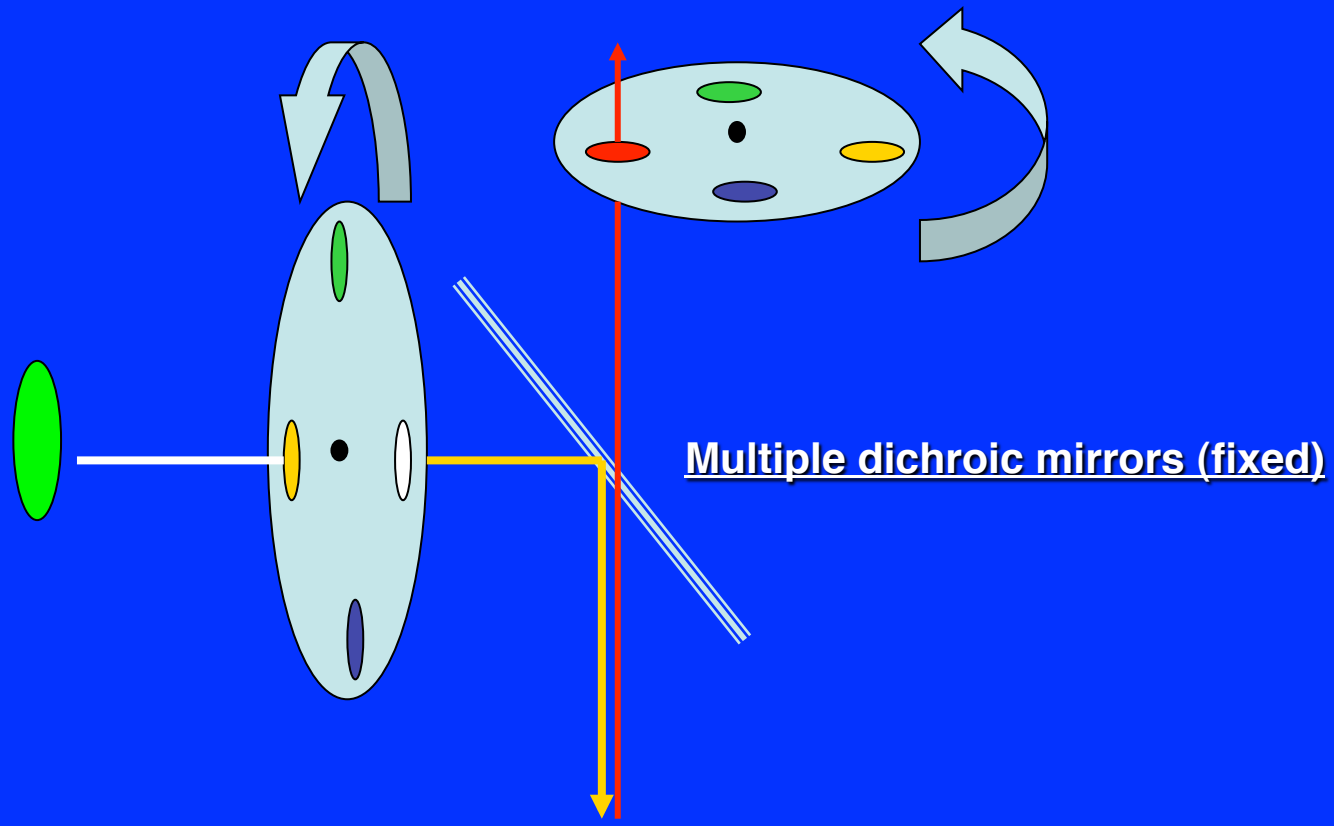
Filter wheels

- Simplest solution for modulating:
- Illumination power - by means of a series of neutral density filters
- Spectra - by means of a series of interference filters
- Typically motorized

FW1ADF

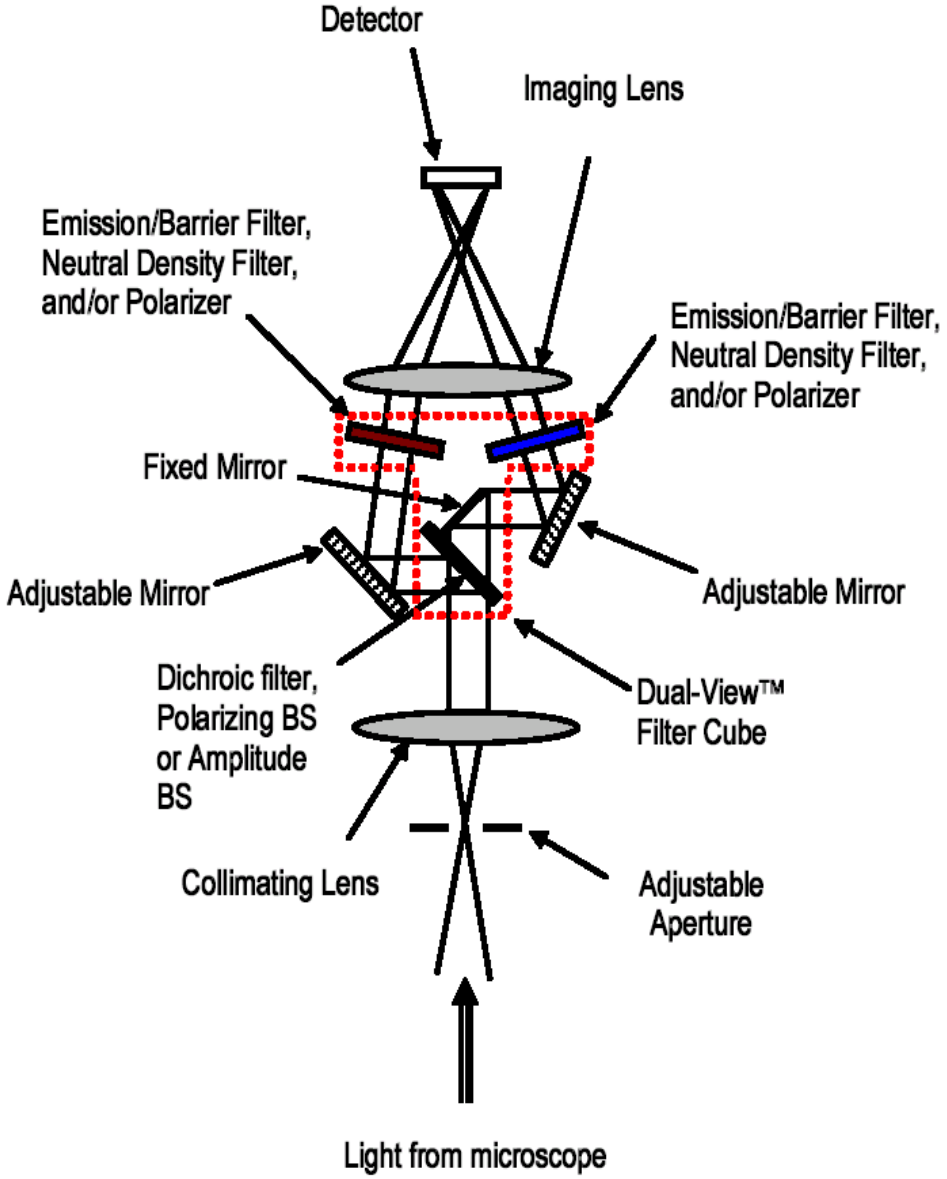
Multiple dichroic mirrors;

Enable automated and fast analysis of multiple (2-5) labeling.
Requirws one dichroic mirror with multiple excitation-emission "windows" and Ex. & Em. Filter wheels



Multiple dichroic mirrors (fixed)

ONE CAMERA, TWO CHANNELS



TWO CAMERAS, TWO CHANNELS

The Epifluorescence Microscope

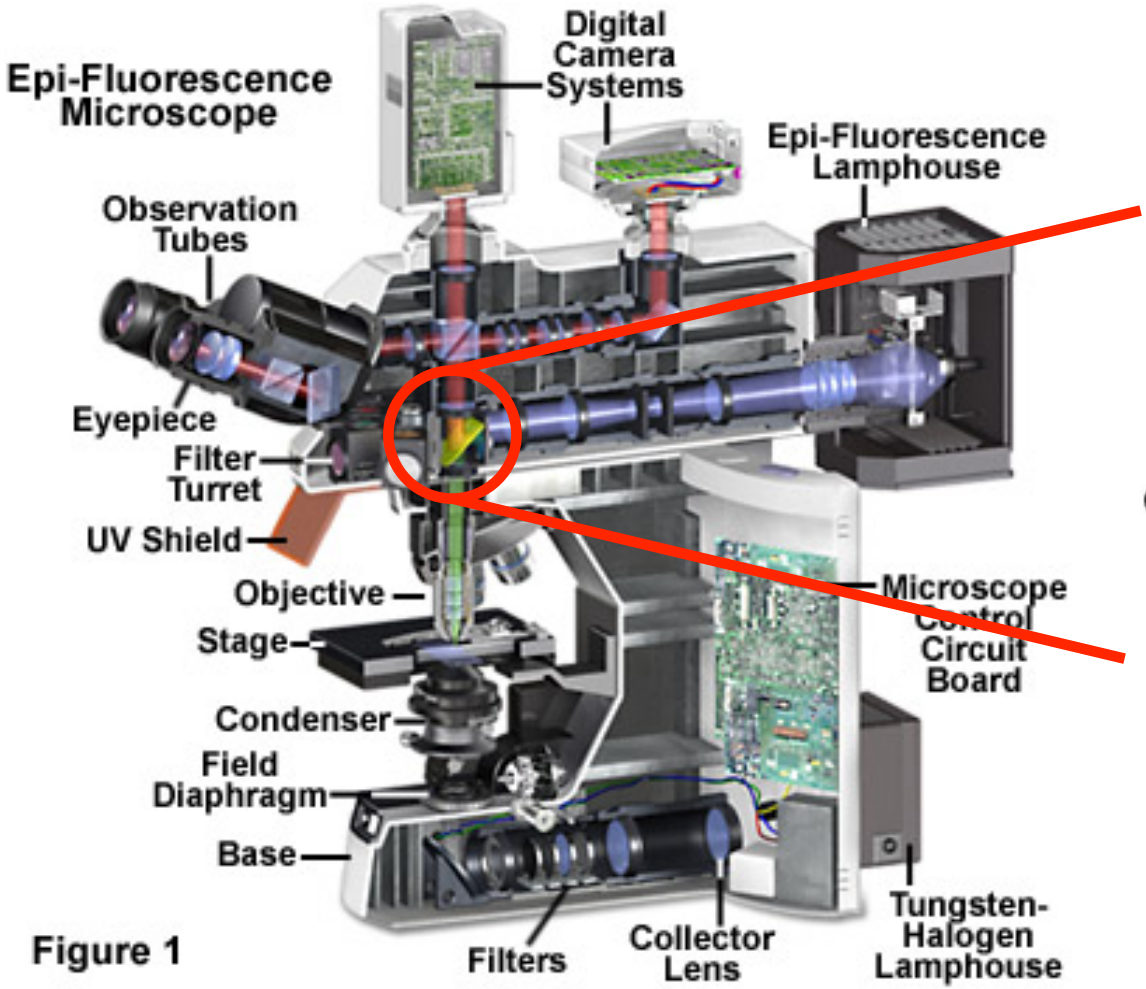


Figure 1

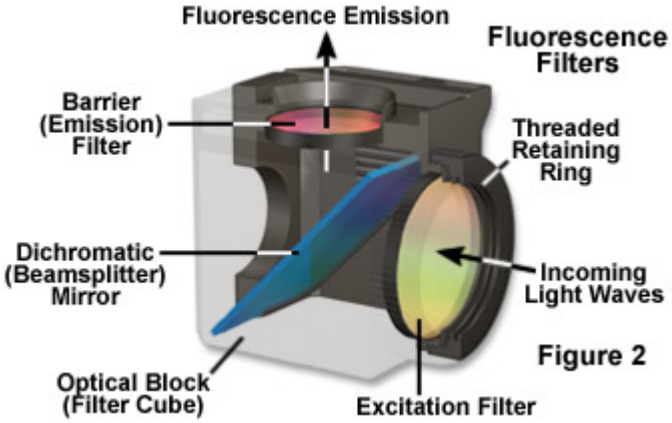


Figure 2

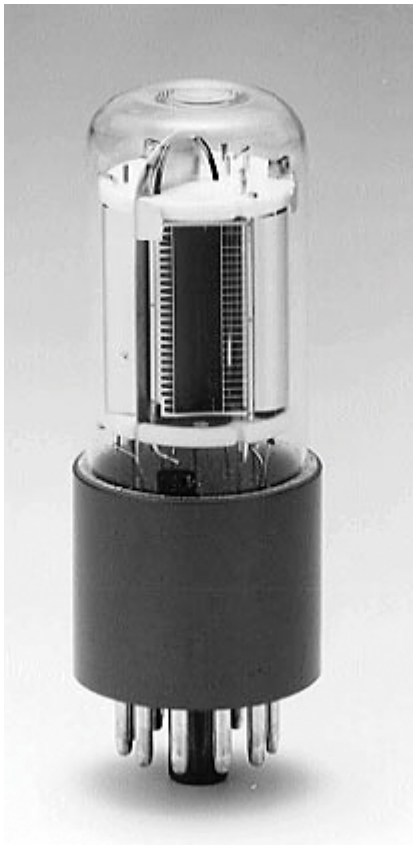
Detector/Imagers/Cameras

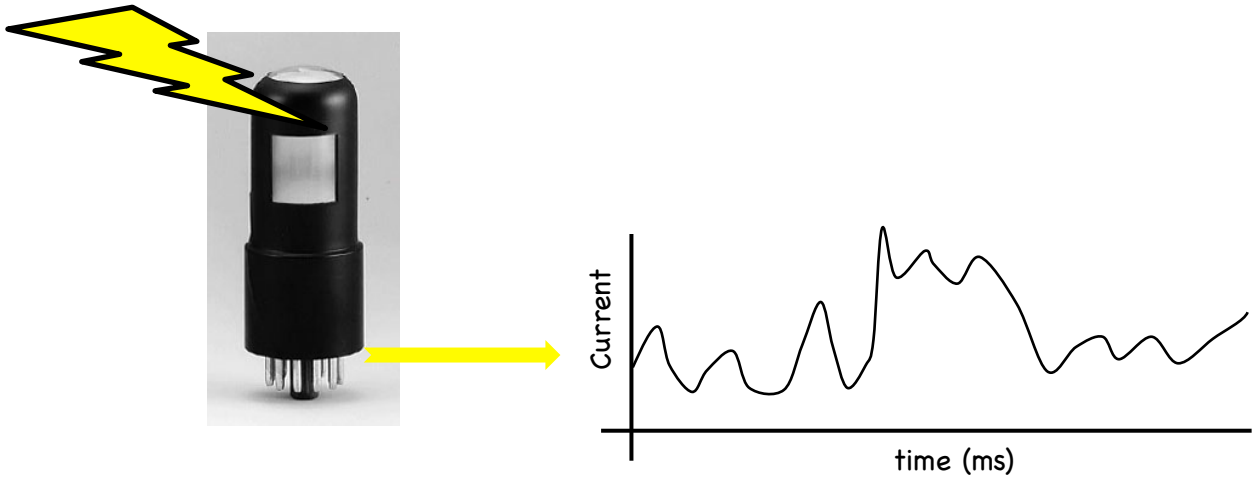
DETECTION ISSUES

- Fluorescence is weak
- Need to make best use of all of the available photons
- Use high QE detectors
- Use monochrome cameras and optimal filters

Detectors: Photomultipliers

- Sensitive light detectors that convert photons absorbance rates to current in a proportional manner
- Used mainly in scanning systems such as laser scanning confocal microscopes
- No spatial information – temporal only
- Relatively low quantum efficiency (typically <20%), that depends on wavelength.
- Shot noise is major noise source
- Many different types, differ in geometry, sensing material, amplification stages spectral range, QE,etc.





Detectors: Photomultipliers

- Photons hitting photocathode release electronswith some probability (typically < 20%)
- Released electrons are accelerated by electric fields
- Impact with next dynode leads to release of more electrons than originally hit target ("electron multiplication")
- Process repeated many times (>10) resulting in millions fold amplification
- Requires special high voltage power supplies (>1000V) and voltage divider
- Current is usually translated to voltage and thereafter digitized by A/D converter

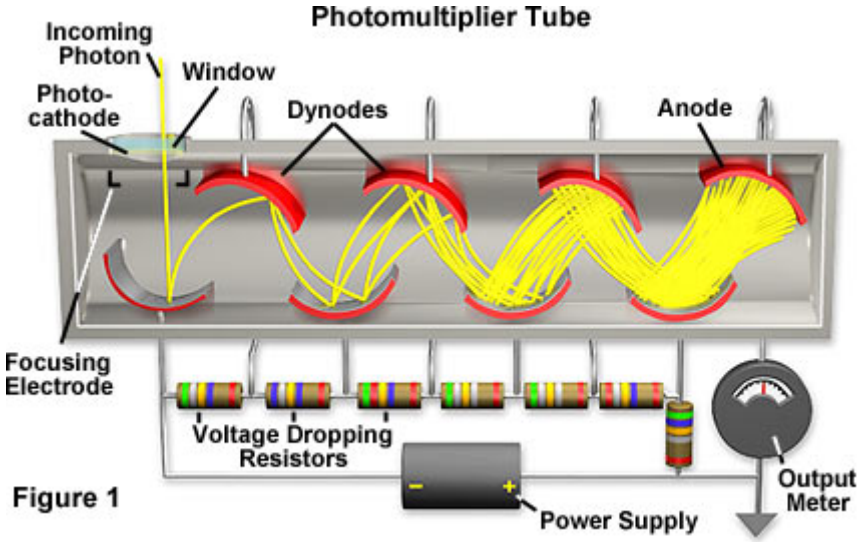
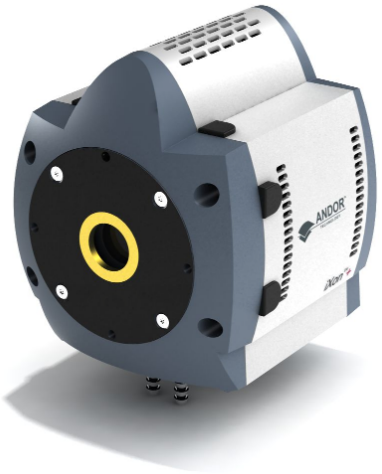


Figure 1

Detectors: CCDs



- Based on a CCD (charged coupled device) photon detector
- The CCD is a thin silicon wafer divided into a regular array of thousands or millions of light-sensitive regions (elements) equivalent to pixels
- Unlike PMTs, the CCD generates an entire 2 dimensional image (typically 512x512 or 1024x1024)
- Each element stores an electrical charge in a "potential well".
- Charge is proportional to the integrated amount of light that hit the element
- Output is typically sent to computer or converted to standard video signal
- Usually comes with software for controlling acquisition timing and options

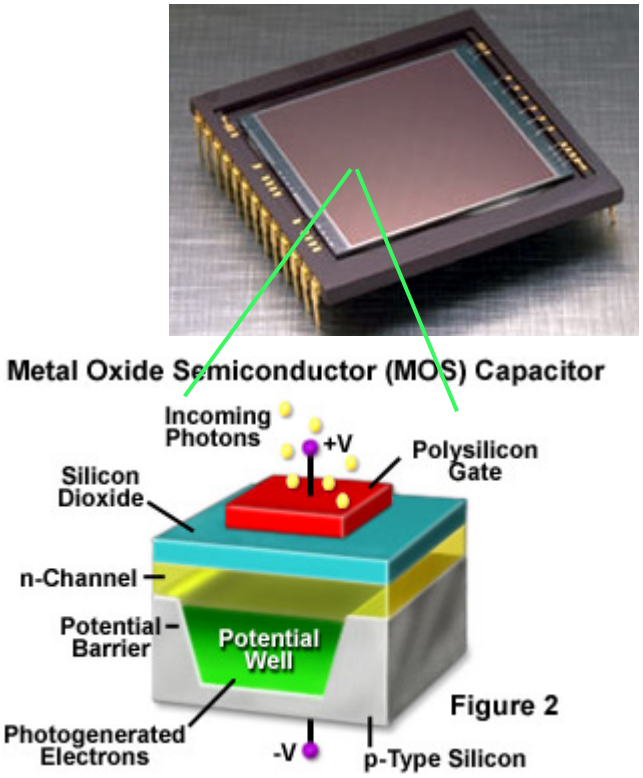
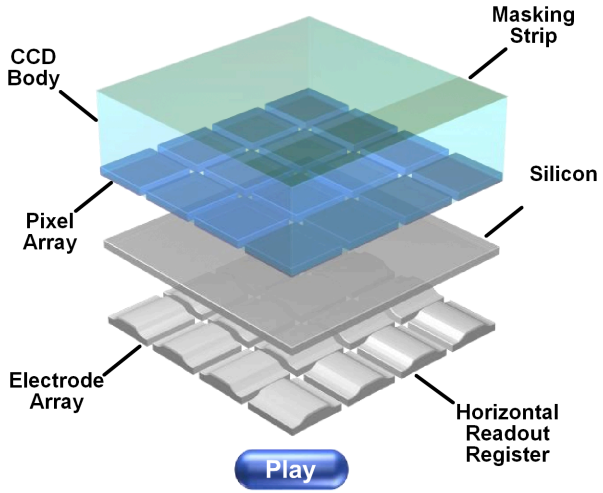


Figure 2

Detectors: CCDs

- Signal intensity depends on integration (exposure) time, typically 20 msec to several seconds.
- Although acquisition is done in parallel, readout is sequential and is often a limiting factor in determining frame rate (frames/sec)
- Spatial resolution depends on pixel size and number of pixels
- Dynamic range depends on "well" capacity: Too many photons ➔ too many electrons ➔ well overflow (saturation)

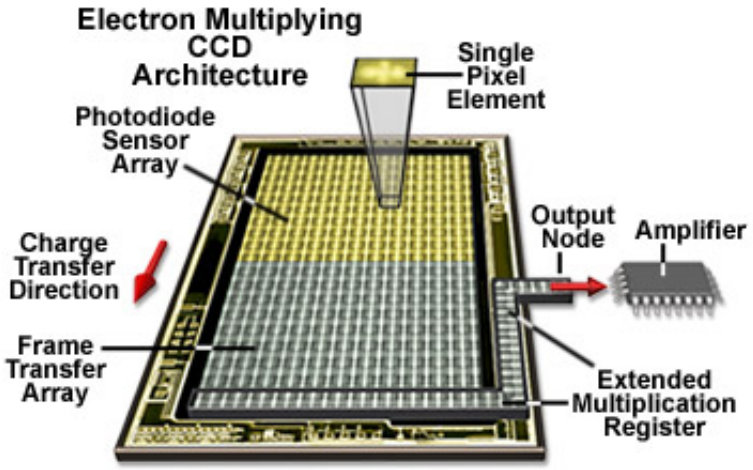


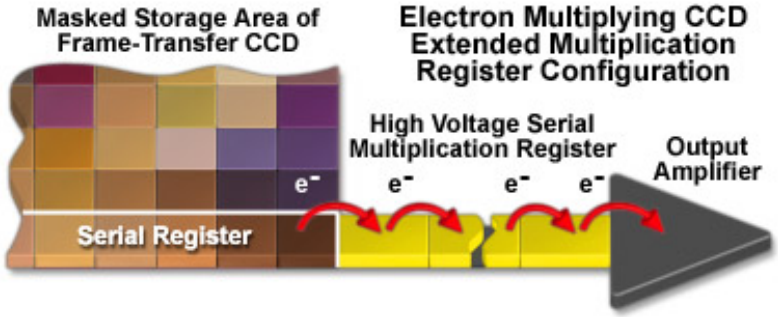
Detectors: EMCCDs

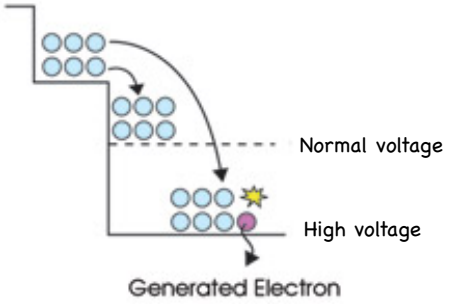
- The quantum efficiency of CCDs is typically much higher than that of PMT (>90% in certain CCDs!)
  - One problem is noise (spontaneous electron emission). This can be improved by cooling the CCD chip.
  - Another major noise problem, however occurs during charge measurement:

When each well contains only few electrons, the signal derived (real) electron numbers and spontaneously arising electron numbers in readout system ("noise") become comparable

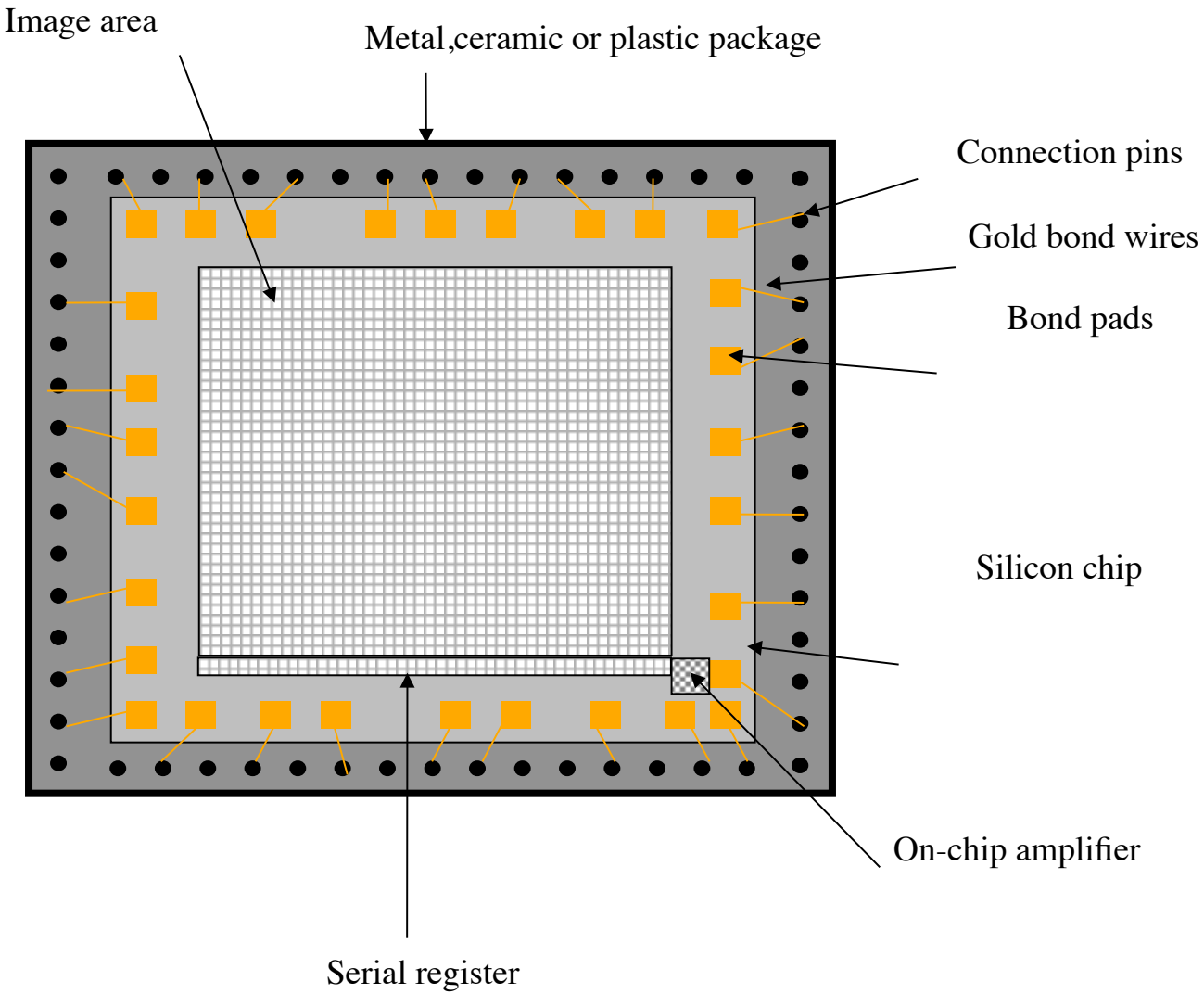
- This has been solved by adding a special shift register that multiplies the number of light-derived charges enormously before these are read out (➔ Electron Multiplication)
- This is done in a manner similar to that done in PMTs, in this case by impact ionization in silicon
- Probability of generating "impact" electrons is low (1.010 to 0.016) but with many shifts (~500) the gain is huge $(1.015^{500} \approx 1710)$







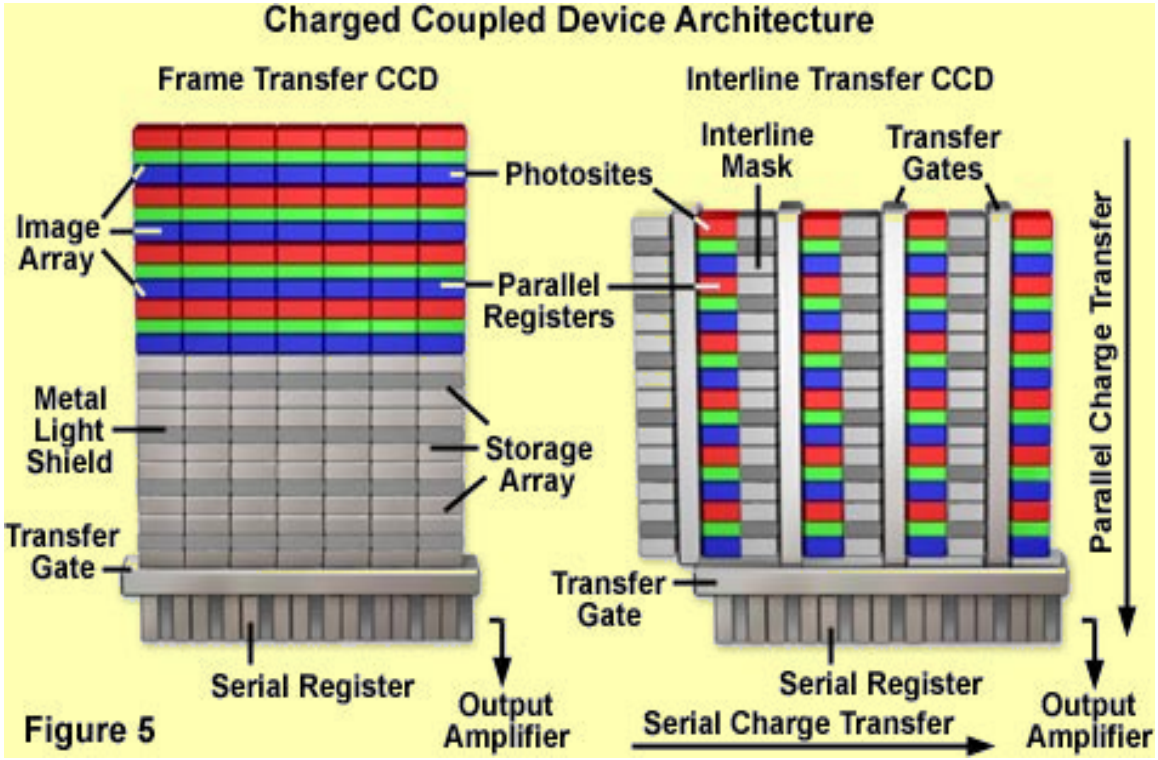
CCD (Charge Coupled Device)



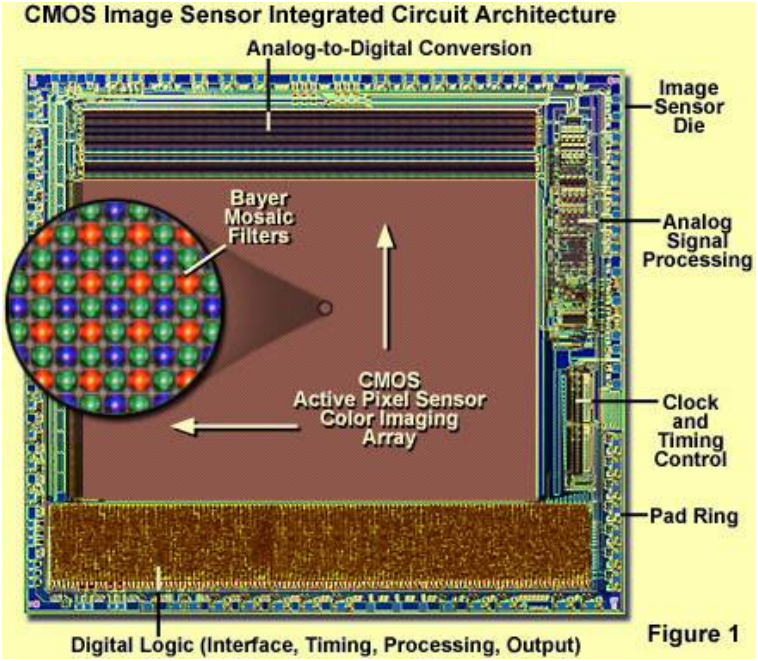
From: www.ing.iac.es/~smt/CCD_Primer/CCD_Primer.htm

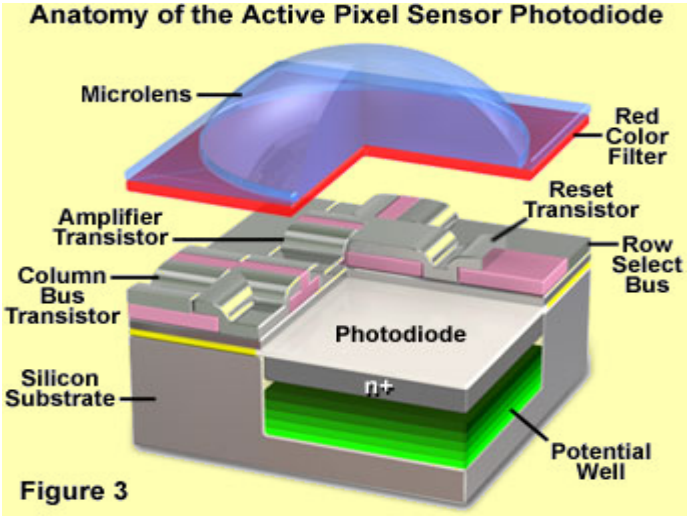
CCD (continued)

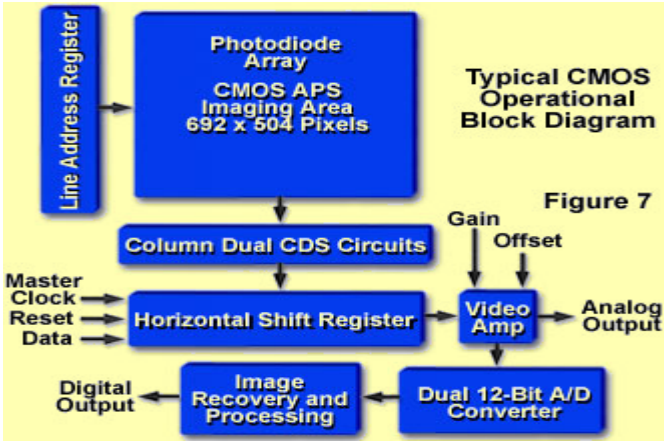
1. Frame transfer
2. Line transfer
3. Full frame



CMOS Imagers:



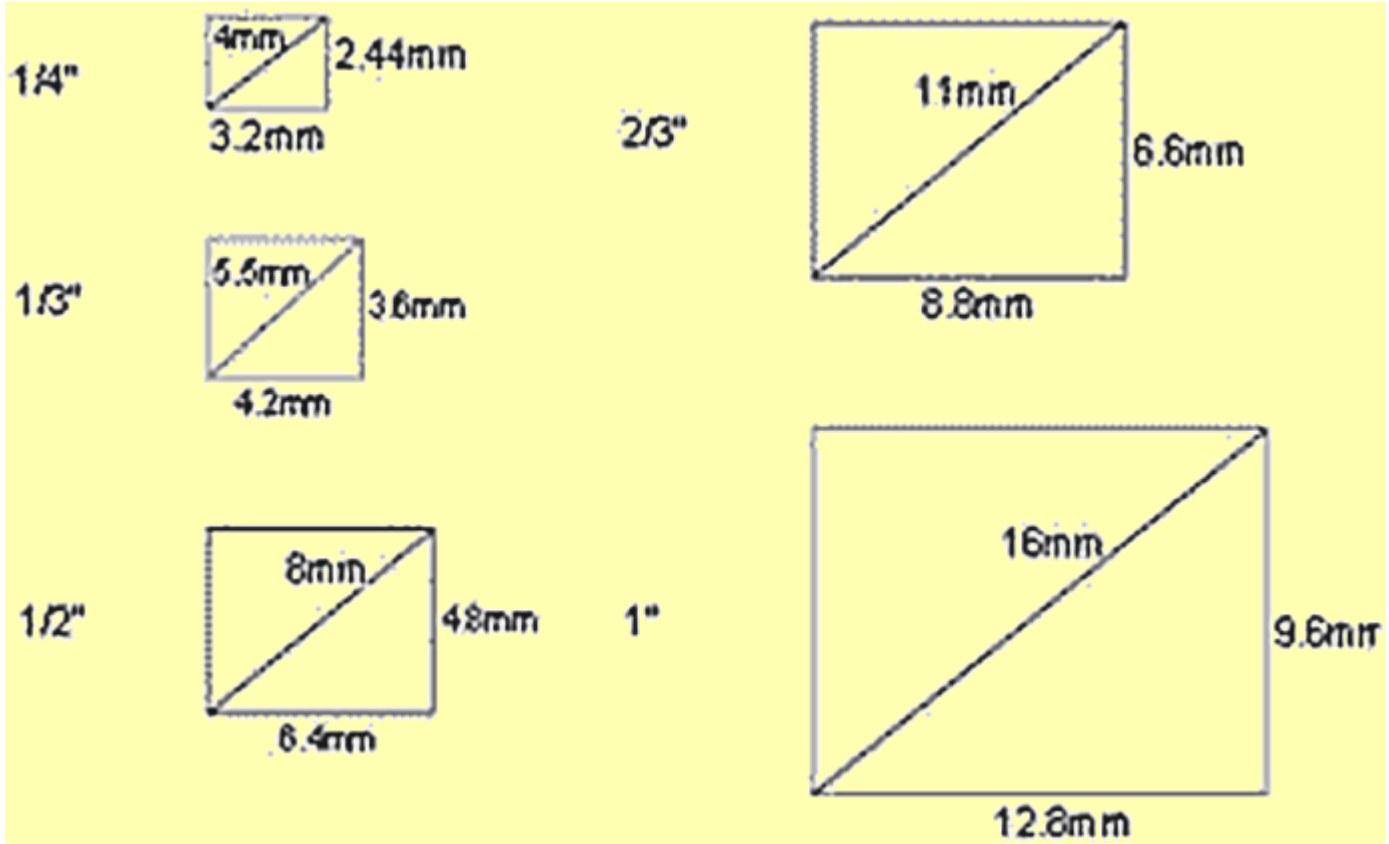




Solid State Imager Characteristics

- Spatial sampling
- Digitized intensity output ->EMCCD photon counting quality with low noise
- Subarray scanning (for fast dynamics)
- No geometric distortion
- Linear radiometric response
- High quantum Efficiency

Chip Format

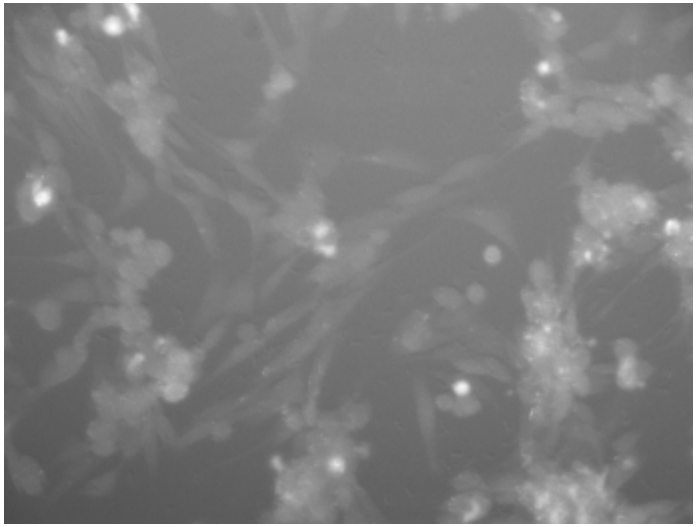

1/4"
4mm
2.44mm
3.2mm
1/3"
5.5mm
3.6mm
4.2mm
1/2"
8mm
4.8mm
6.4mm
2/3"
11mm
6.6mm
8.8mm
1"
16mm
9.6mm
12.8mm

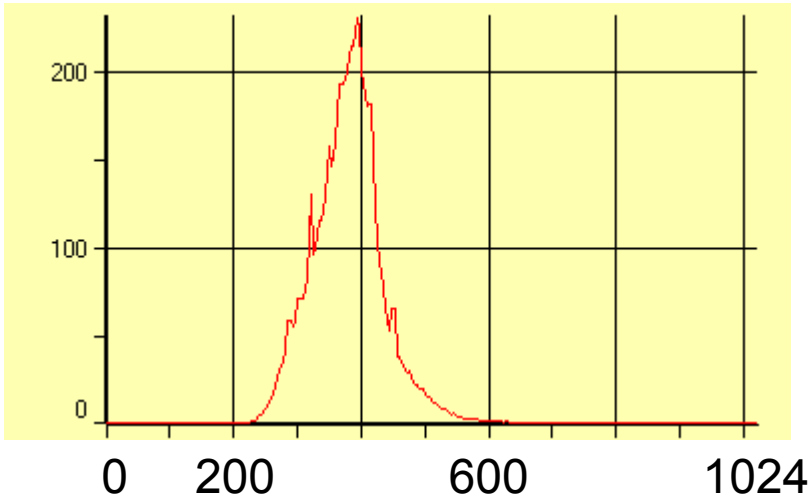
Well Capacity and Pixel Size

- Well capacity = # of electrons to fill the detector before image is “saturated”
- Well capacity proportional to pixel size.
- Dynamic range increase with well capacity
- Tradeoff: spatial resolution vs dynamic range
- Typical numbers: 15,000 – 250,000 electrons

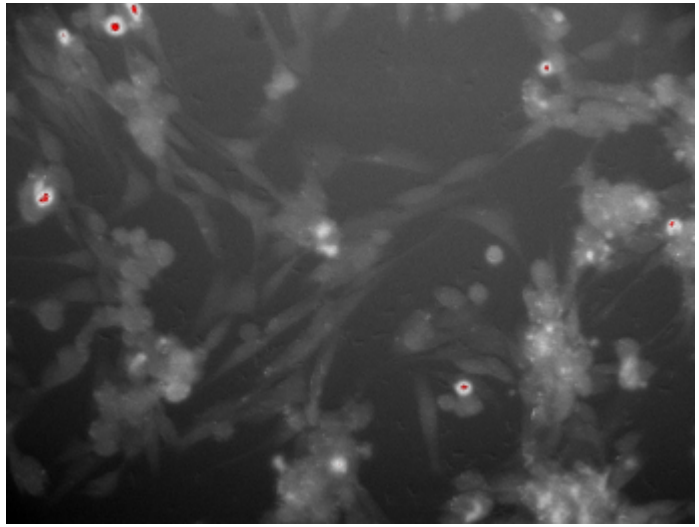
How Many Bits Do I Need?

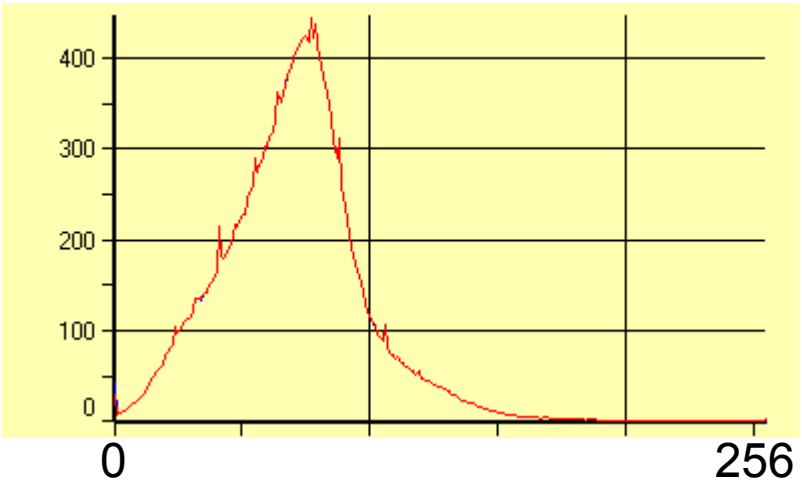
12 Bit Image

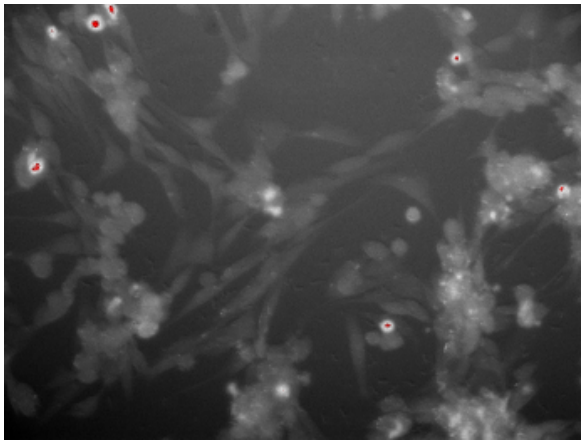


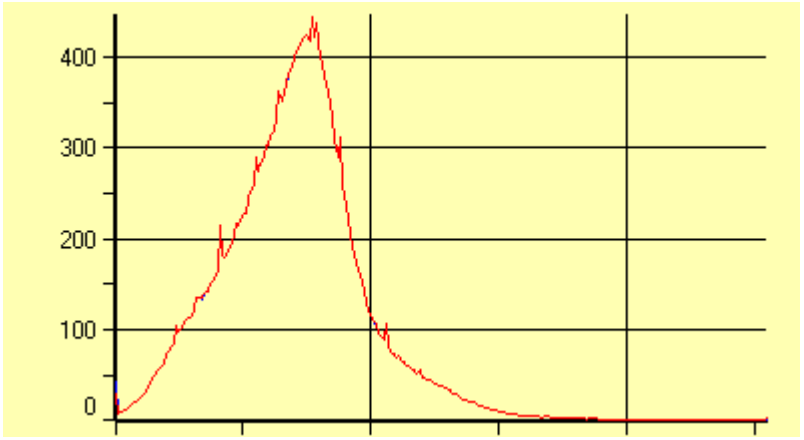


8 Bit Image

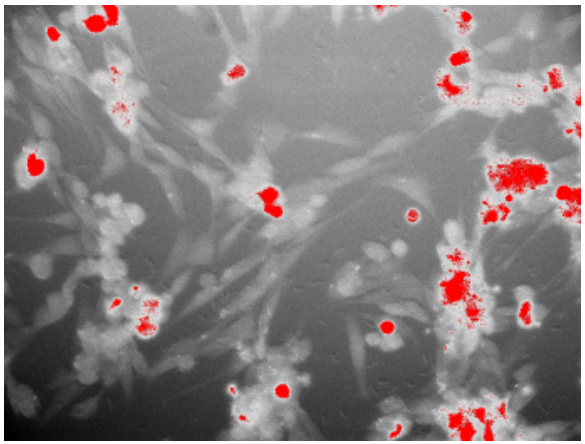


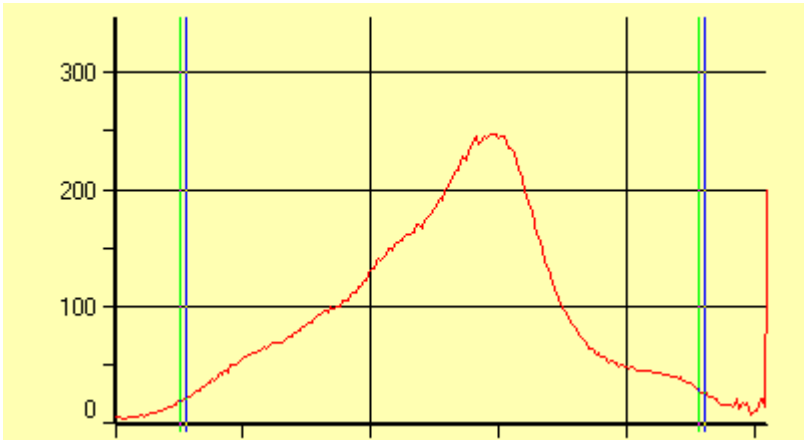




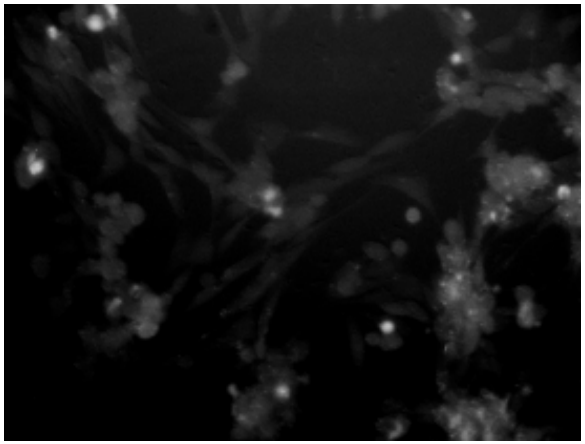


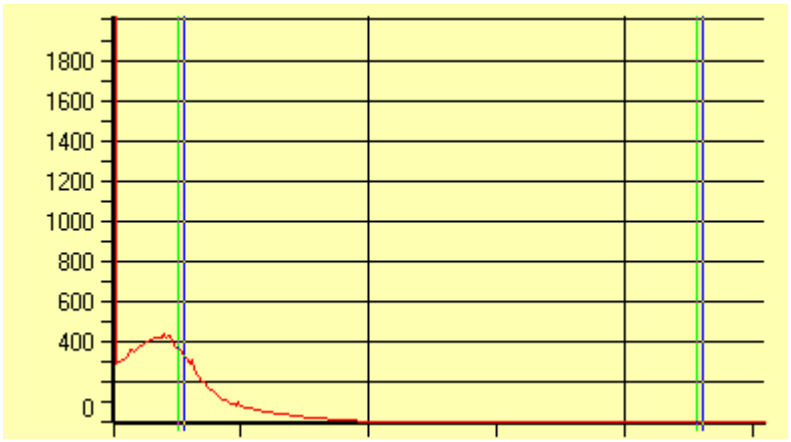
Best fit





Saturated





Under-exposed

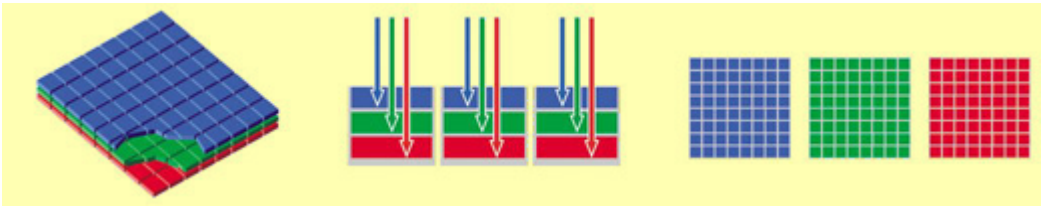
Color Image Acquisition

- 3-CCD cameras
- Single CCD, integrated color filters (rgb, cmy)
- Sequential color acquisition

  --filter wheel

  --liquid crystal tunable filter (LCTF)

  --acousto-optical tunable filter (AOTF)
- Depth color filter:



Monochrome or Color?

Monochrome

- Higher resolution
- Higher sensitivity
- Faster acquisition
- Color depends on filters

Color

- Convenience
- Faster color acquisition (3CCD and 1CCD)
- Low cost (consumer cameras)
- Colors are RGB

Digital Imaging Fundamentals

- Direct digitization at source
  (photon counts)
- Slow or fast readout
- High or low resolution (Binning)
- High or low sensitivity
- Dynamic range (bits/pixel, noise)
- Noise (photons/pixel, electrons/pixel, readout)

Noise

- **Photon noise** – square root of #photons
  -- acquire more photons, use high QE CCD
- **Thermal charge generation** in detector
  -- cool the detector
- **Electronic readout noise**
  -- Slow readout (fast electronics are noisy)
  -- Avalanche amplification register (EMCCD)

QE of CCDs

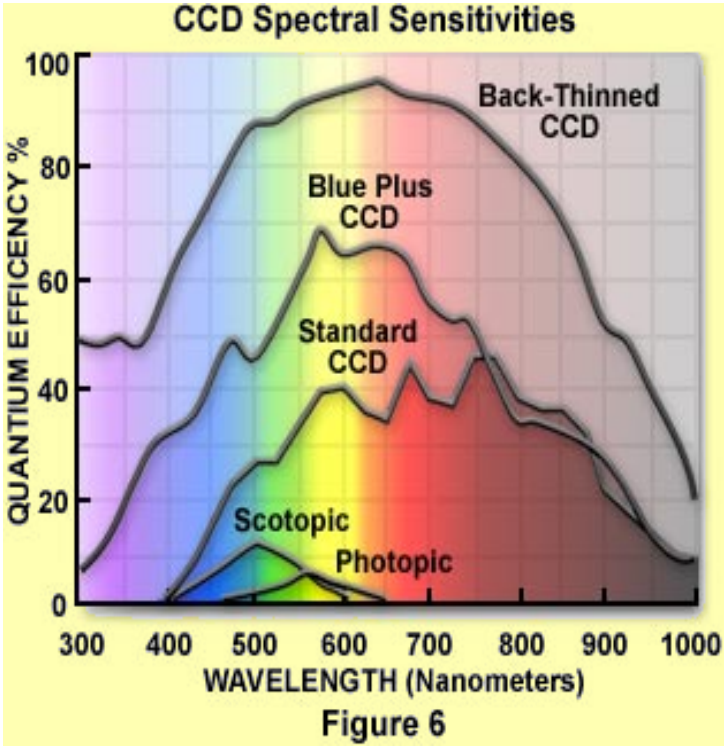
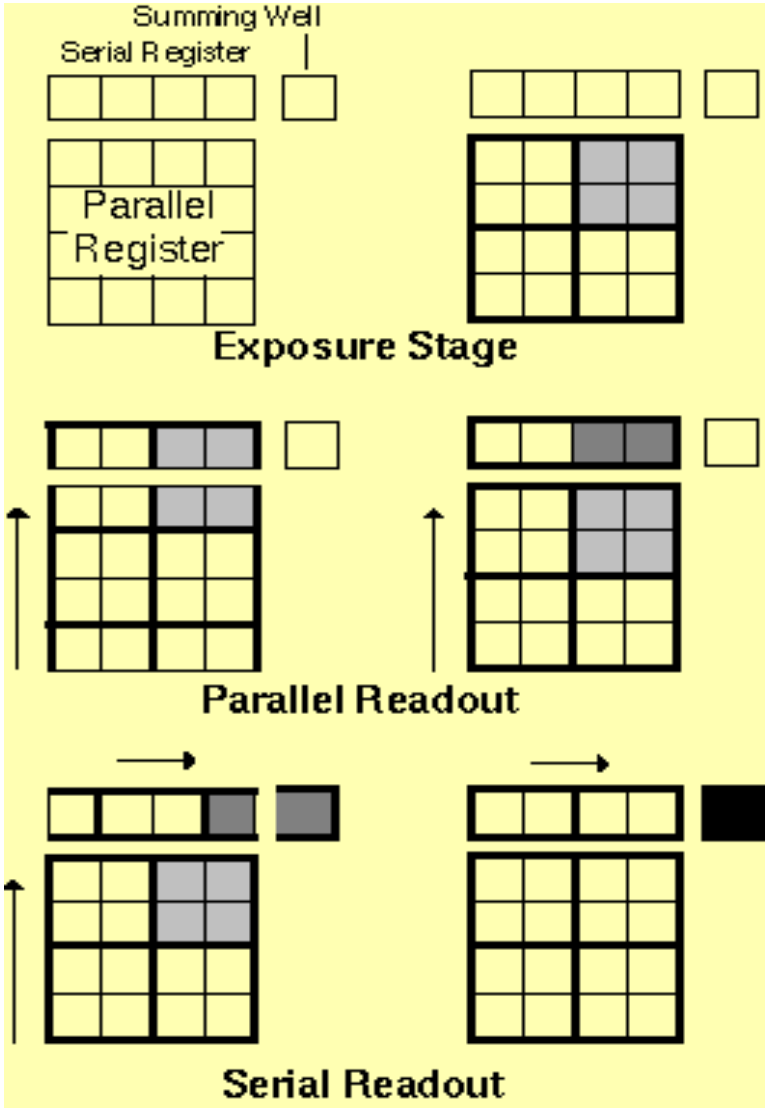


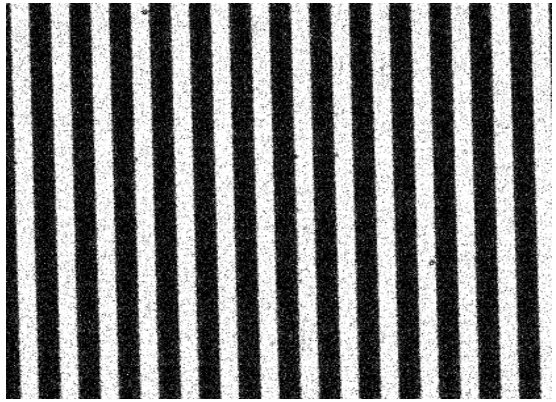
Figure 6

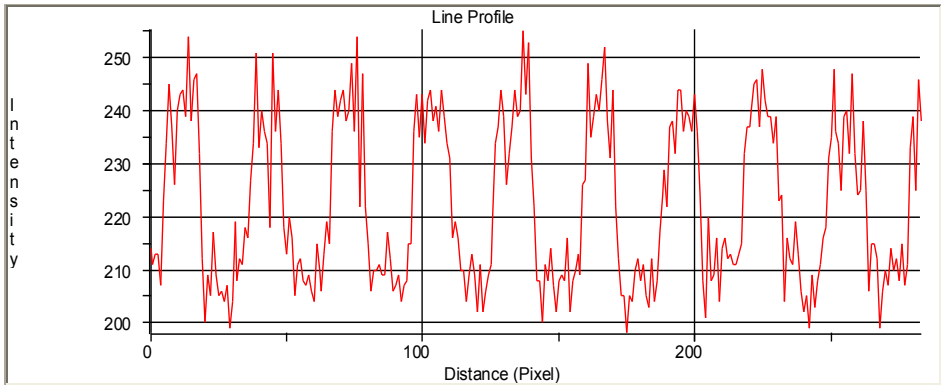
Resolution, Binning, Dynamic Range

- Binning -reduces spatial resolution
  - Increases dynamic range
    - Increases S/N



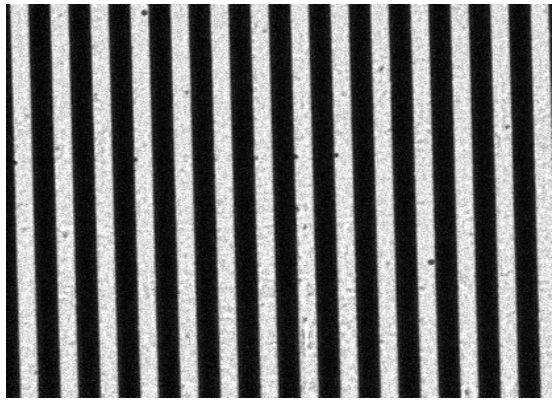
Autorange Display

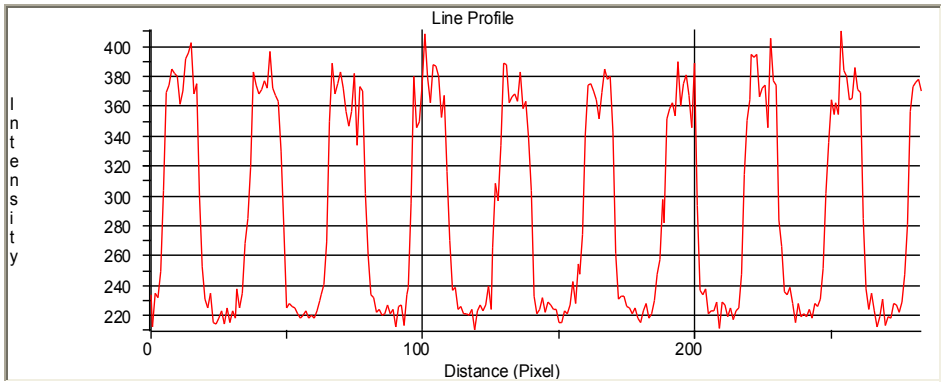




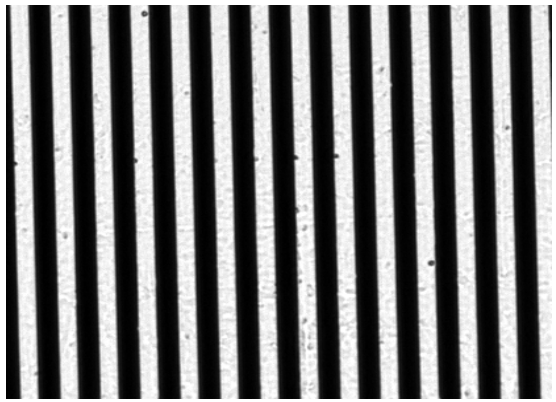
Exposure

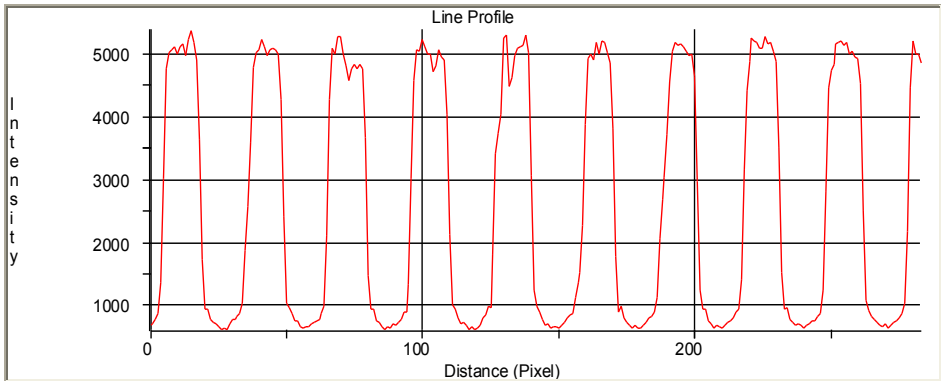
0.1ms





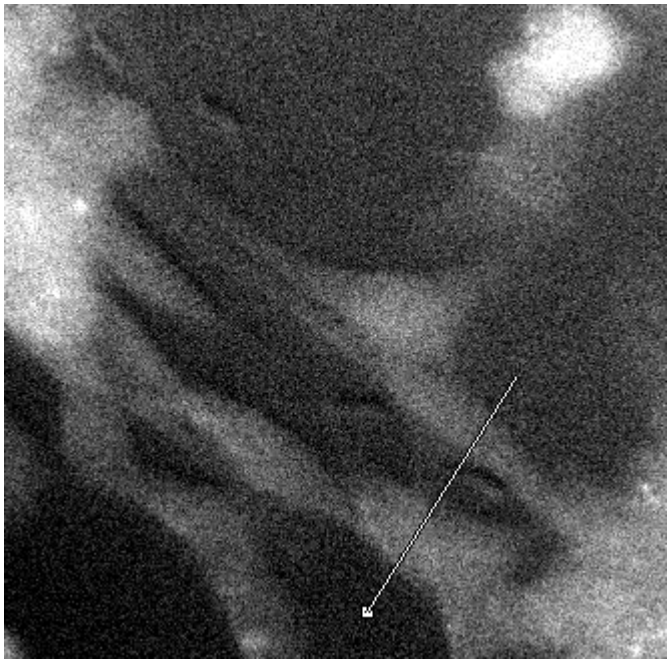
1ms

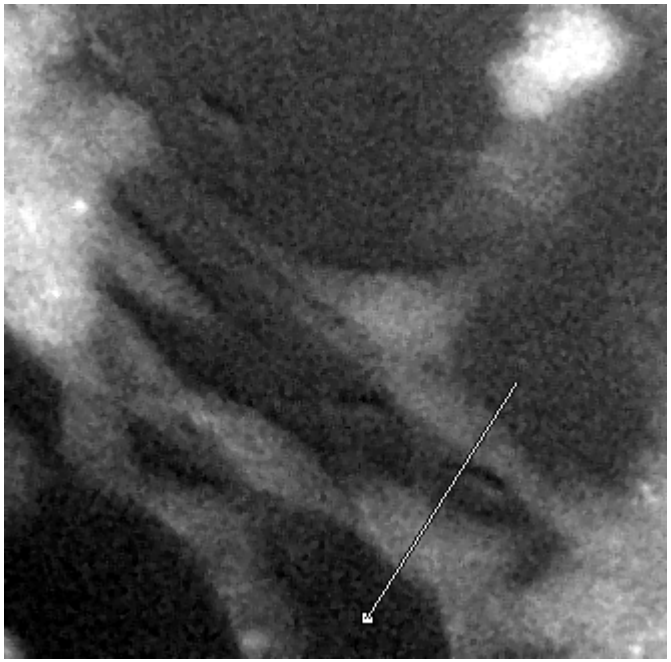


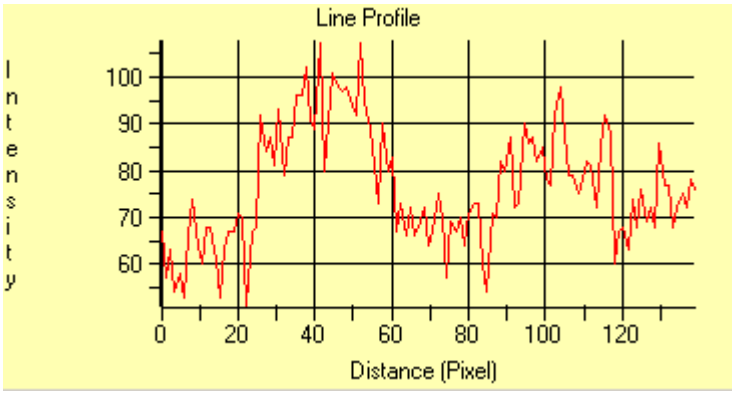


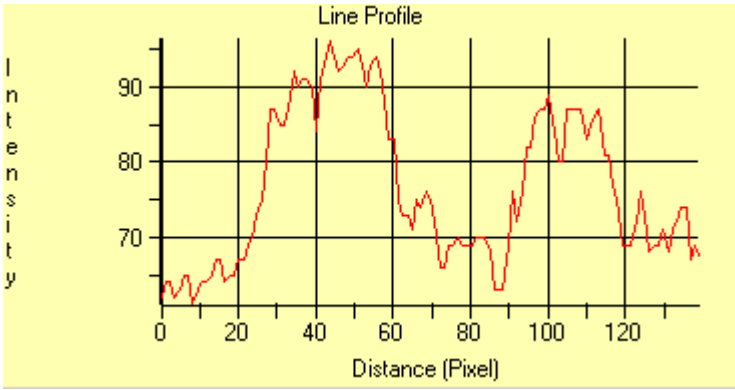
30ms

Photon counting noise



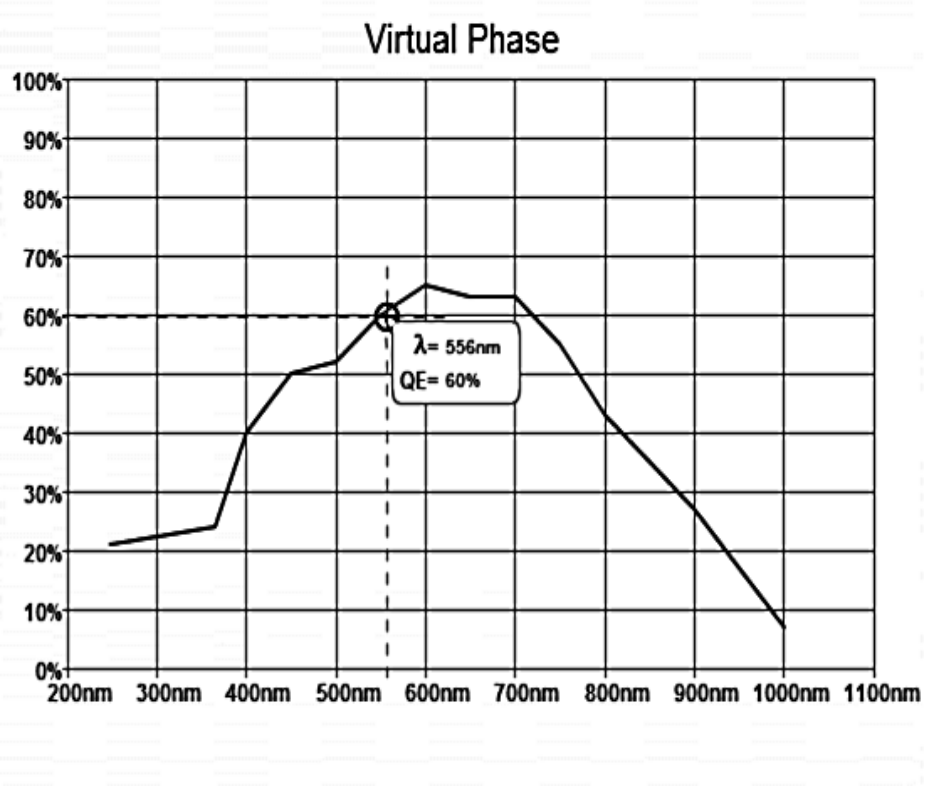




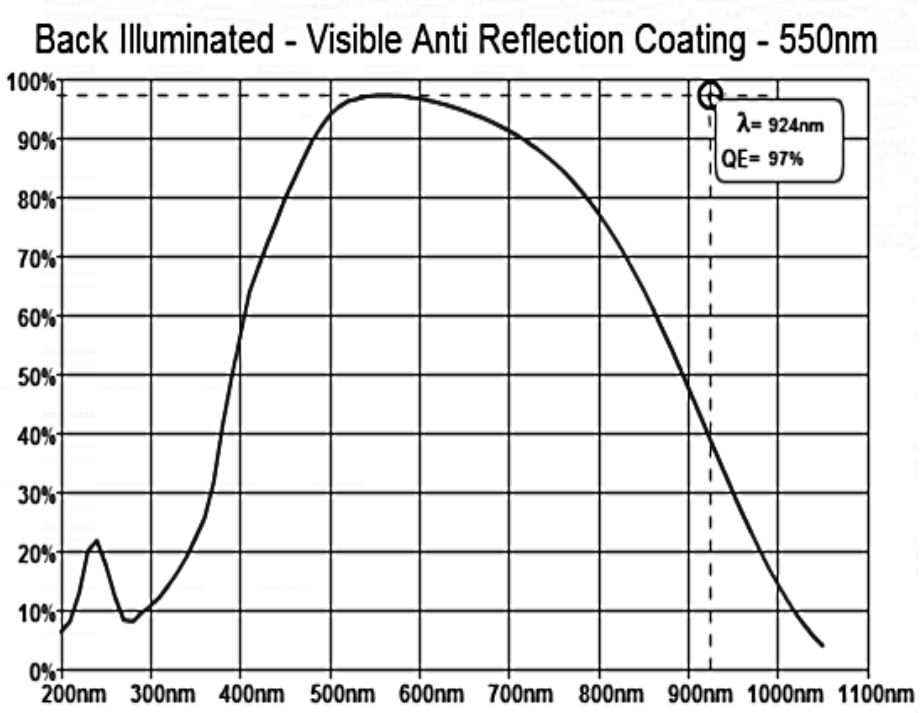


Front vs Back Illumination
(from Andor IXON cameras)

Front illuminated



Back illuminated



sCMOS Imager Highlights

Sensor format: 5.5 megapixels (2560(h) x 2160(v))

Read noise: < 2 e- rms @ 30 frames/s; < 3 e- rms @ 100 frames/s

Maximum frame rate: 100 frames/s

Pixel size: 6.5 μm

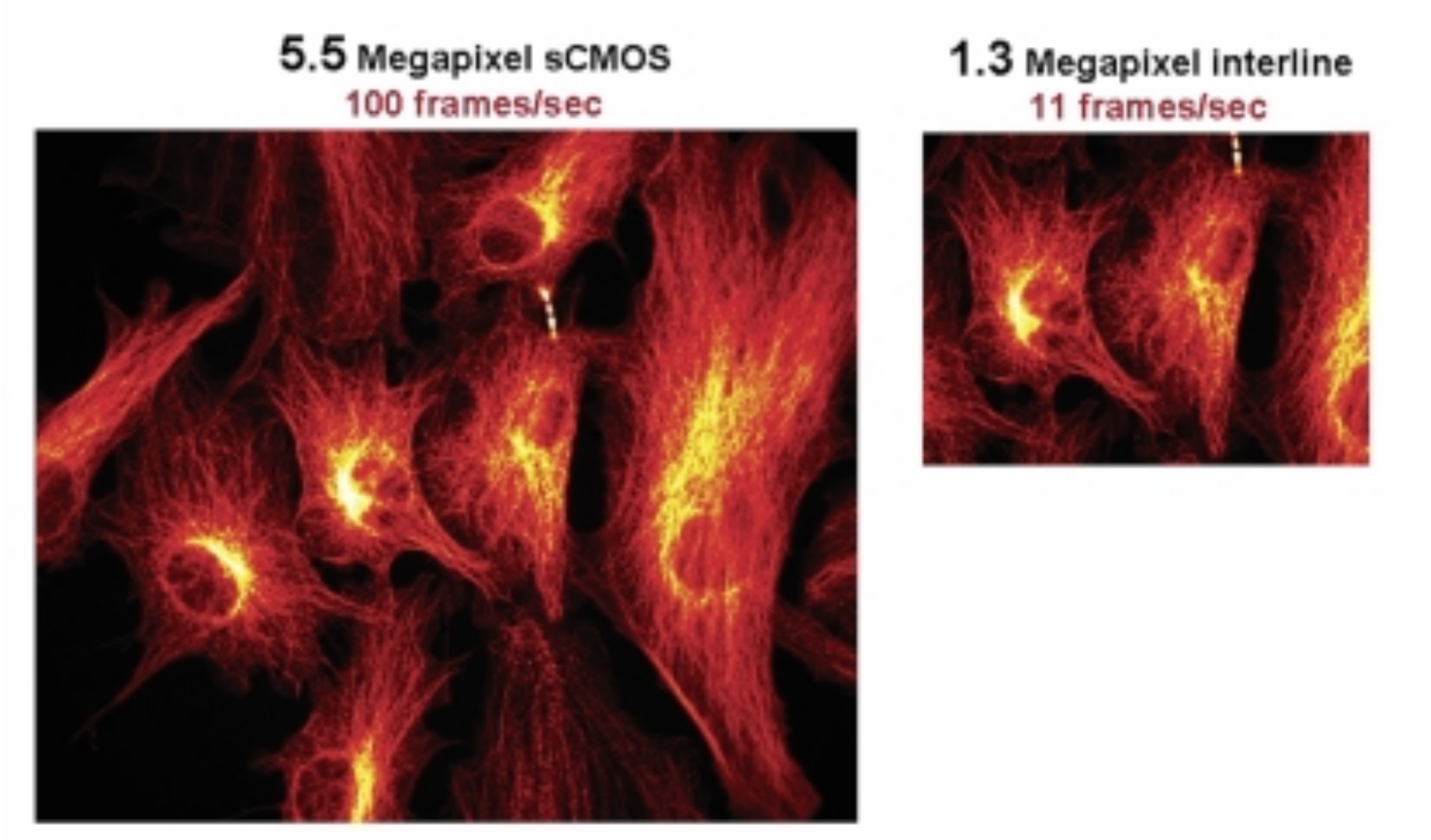
Dynamic range: > 16,000:1 (@ 30 frames/s)

QE_{max}: 60% (with excellent red/NIR response)

Read out modes: Rolling and Global shutter (user selectable)

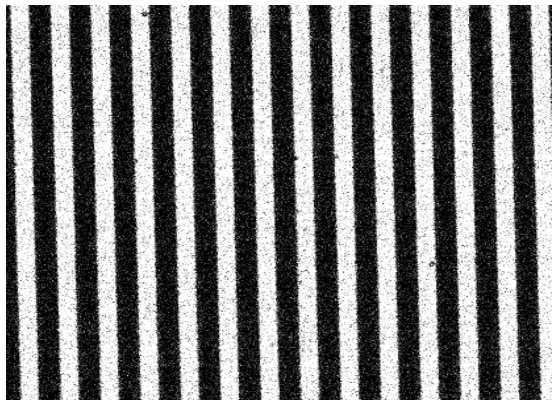
From: http://www.scmos.com

Comparison of field of view



From SCMOS white paper: http://www.scmos.com

Niquist sampling rule



The Biology

Labeling Technologies

	Pro	Con
Fixed Cells	low background	modify sample, artifacts
Antibodies	enhanced fluorescence surface epitopes (FACS) direct/indirect	cross reactivity
Live Cells	dynamics	high background
GFP	Gene clones->labeling	large pigibag (c/n terminus)
SNAP CLIP vital stains	permiable, microinject	

How to label your target?

- Dyes that bind to the target and get activated by it
  DNA/RNA by DAPI, Hoechst, ...
- Dyes that are accumulated into the target by the living cell
  Organelle compartments by mitotracker, ...
- Inject fluorescently labeled target molecule or substrate
- Direct or indirect immunofluorescence
  Label fixed cells with antibodies
- Fluorescent In-Situ Hybridization (FISH)
  Specific DNA sequences in fixed cells
- Genetically encoded fluorescent proteins
  GFP and allies

⋮

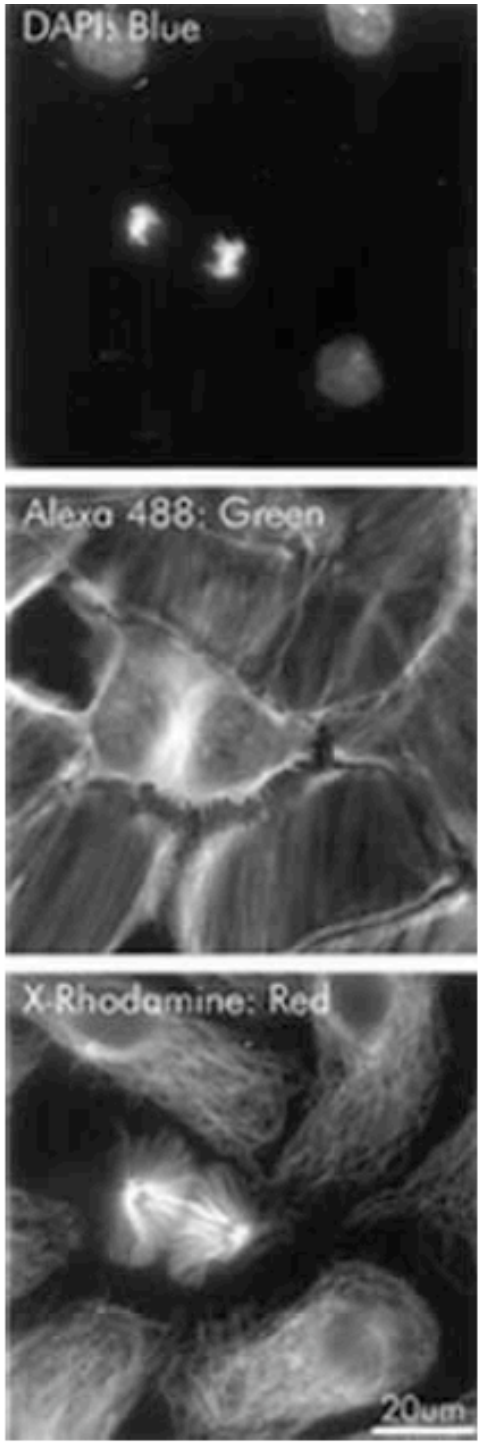
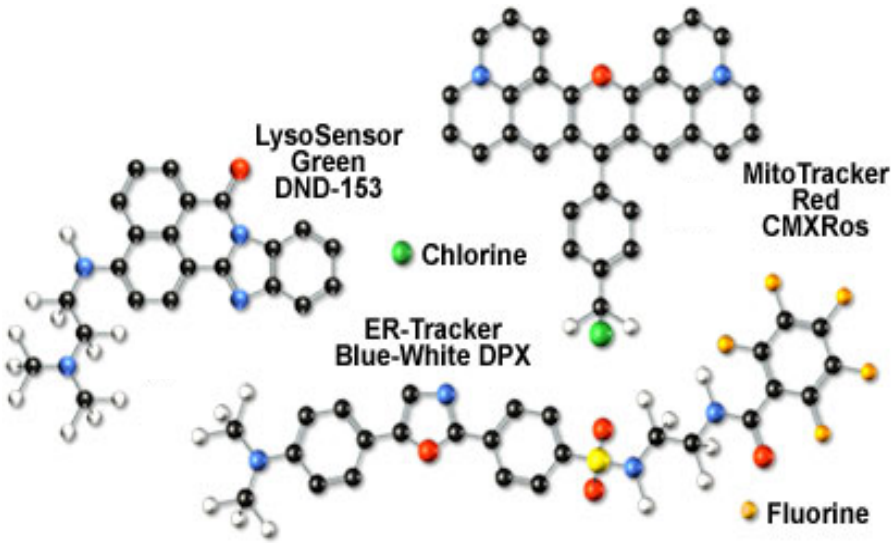


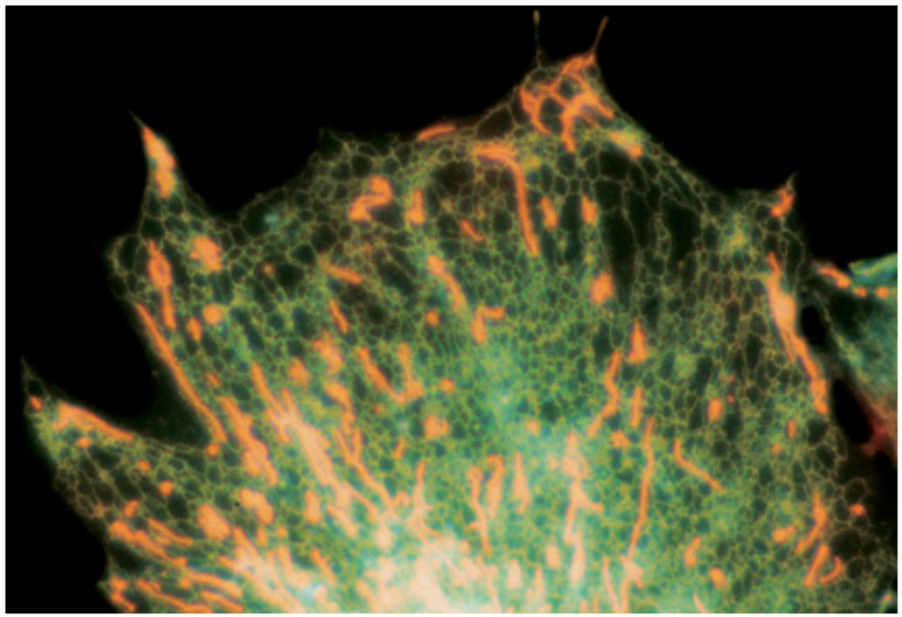
Figure 4.1.9 Epifluorescent images of fixed tissue culture cells stained with (**A**) DAPI, making DNA fluorescent blue; (**B**) Alexa 488 bound to phalloidin to label actin filaments fluorescent green; and (**C**) X-rhodamine labeled antibodies against tubulin to label microtubules fluorescent red. Bar = 20 μm. Images recorded with a 40×/(NA = 1.4) Plan Fluor objective, 1.5 magnification, to a cooled CCD camera and the multi-modem multiwavelength microscope described by Salmon et al. (1998).

Organelle-specific labels

Label specific organelles in live cells

- MitoTracker (various colors)
- LysoSensor
- ER-Tracker





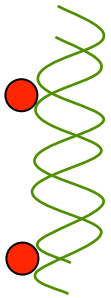
ER-Tracker Blue-White DPX and
MitoTracker Red CM-H2XRos

DNA / RNA-binding dyes

Fluorescence increases by large factor on binding

- DAPI
- Hoechst

Minor-groove binders



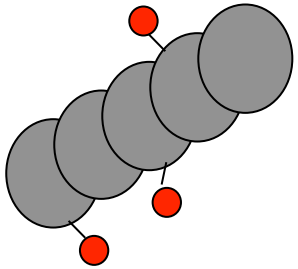
- Ethidium bromide
- PoPo, YoYo,...
- Oligreen
- ⋮

Intercalators

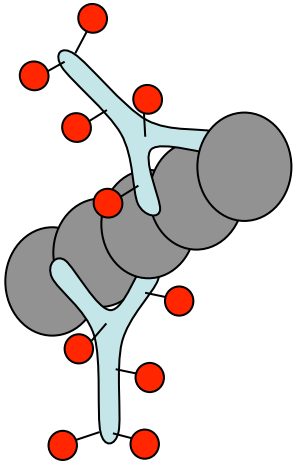
Risk disrupting structure

Fluorecent labeling

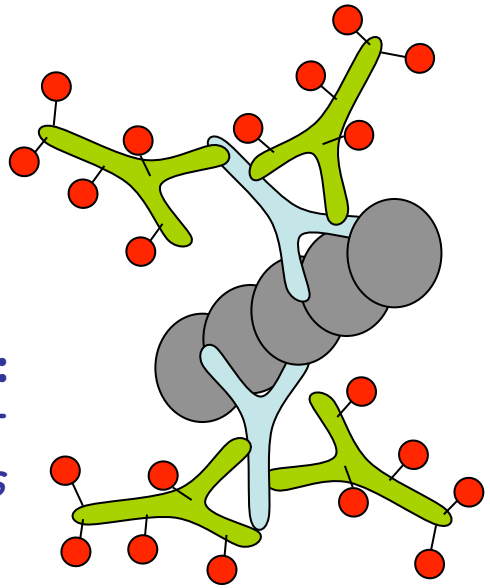
Direct labeling (& microinjection)
of target molecules



Direct immunofluorescence:
labeled antibodies against target

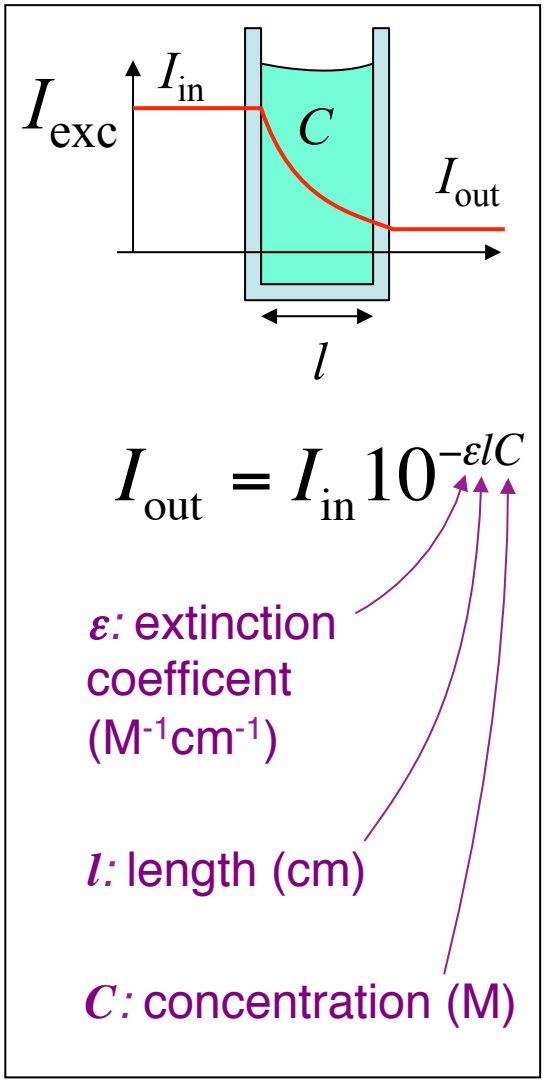


Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies *against those antibodies*



Parameters of fluorescent molecules

- Excitation & emission maxima
- Extinction coefficient ε
  - $\propto$ absorption cross section
  - $\varepsilon \approx 50{,}000$–$100{,}000$ $M^{-1}cm^{-1}$
- Fluorescence quantum yield Q_f
  - = # Photons emitted / # photons absorbed
  - $Q_f \approx 25$–90%
  - Brightness $\propto \varepsilon Q_f$
- Photo-bleaching quantum yield Q_b
  - = average # of photons emitted per molecule before bleaching.
  - Depends on environment.
  - $\propto Q_f / Q_b$



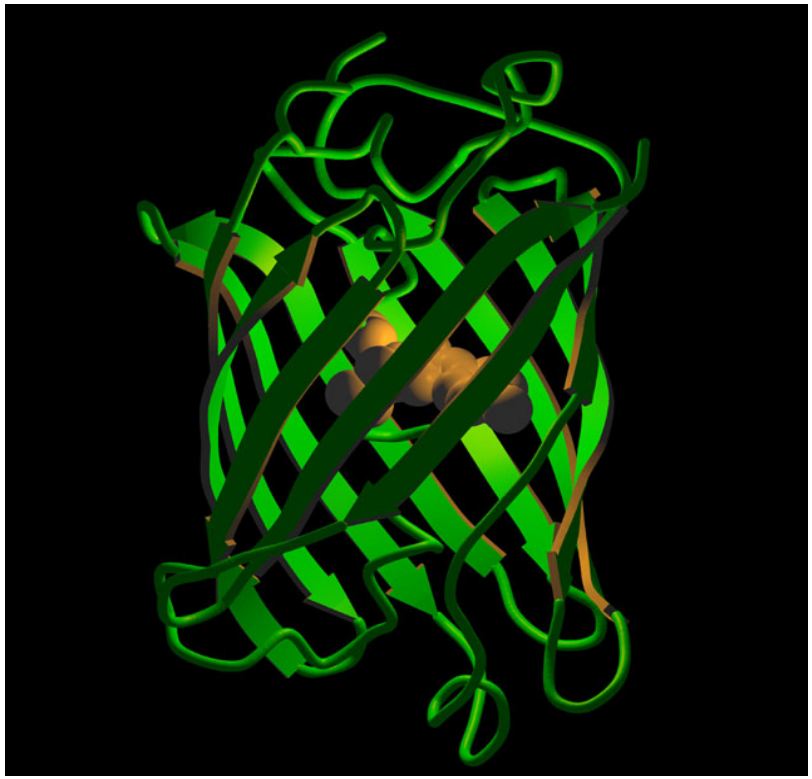
Parameters of some fluorophores

Dye	l_{ex}	l_{em}	ε	QY	brightness
DAPI	350	470	27000	0.58	15.7
Fluorescein	490	520	67000	0.71	47.6
Alexa 488	494	517	73000	0.6	43.8
Rhodamine	554	573	85000	0.28	23.8
Cy3	554	568	130000	0.14	18.2
Cy5	652	672	200000	0.18	36
GFP	488	507	56000	0.6	33.6
mCherry	587	610	72000	0.22	15.8
CFP	433	475	32500	0.4	13
YFP	516	529	77000	0.76	58.5

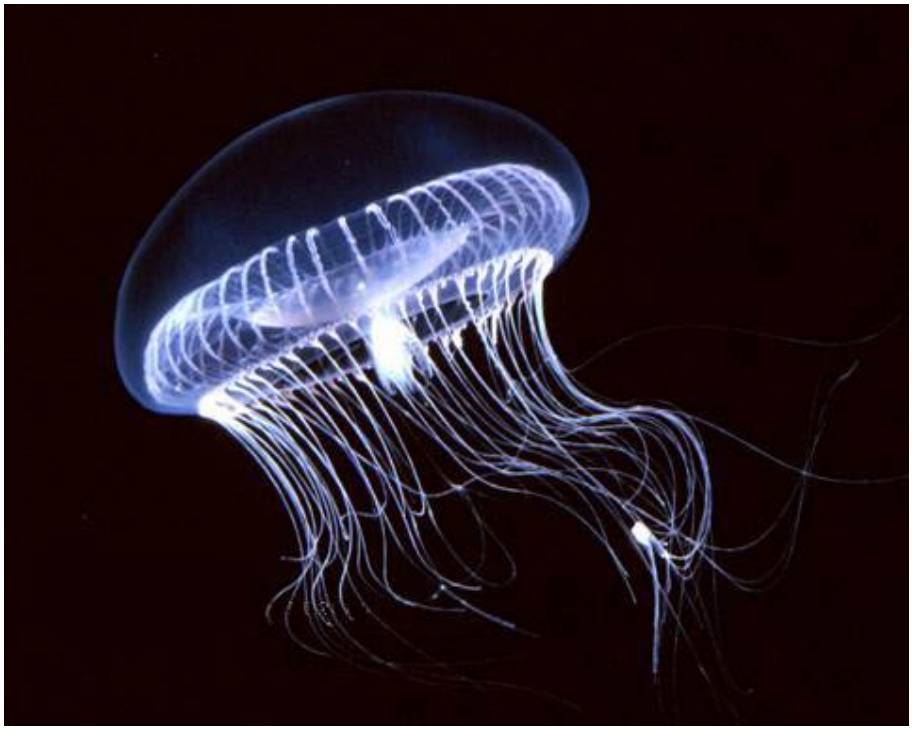
Small molecules - pros / cons

- 1000s available - huge spectral range
- Easy to acquire
- Precisely tailored properties, including environmental sensitivity
- Require fixing and staining, which can lead to artifacts
- Potential self-quenching and environmental sensitivity

Fluorescent Proteins



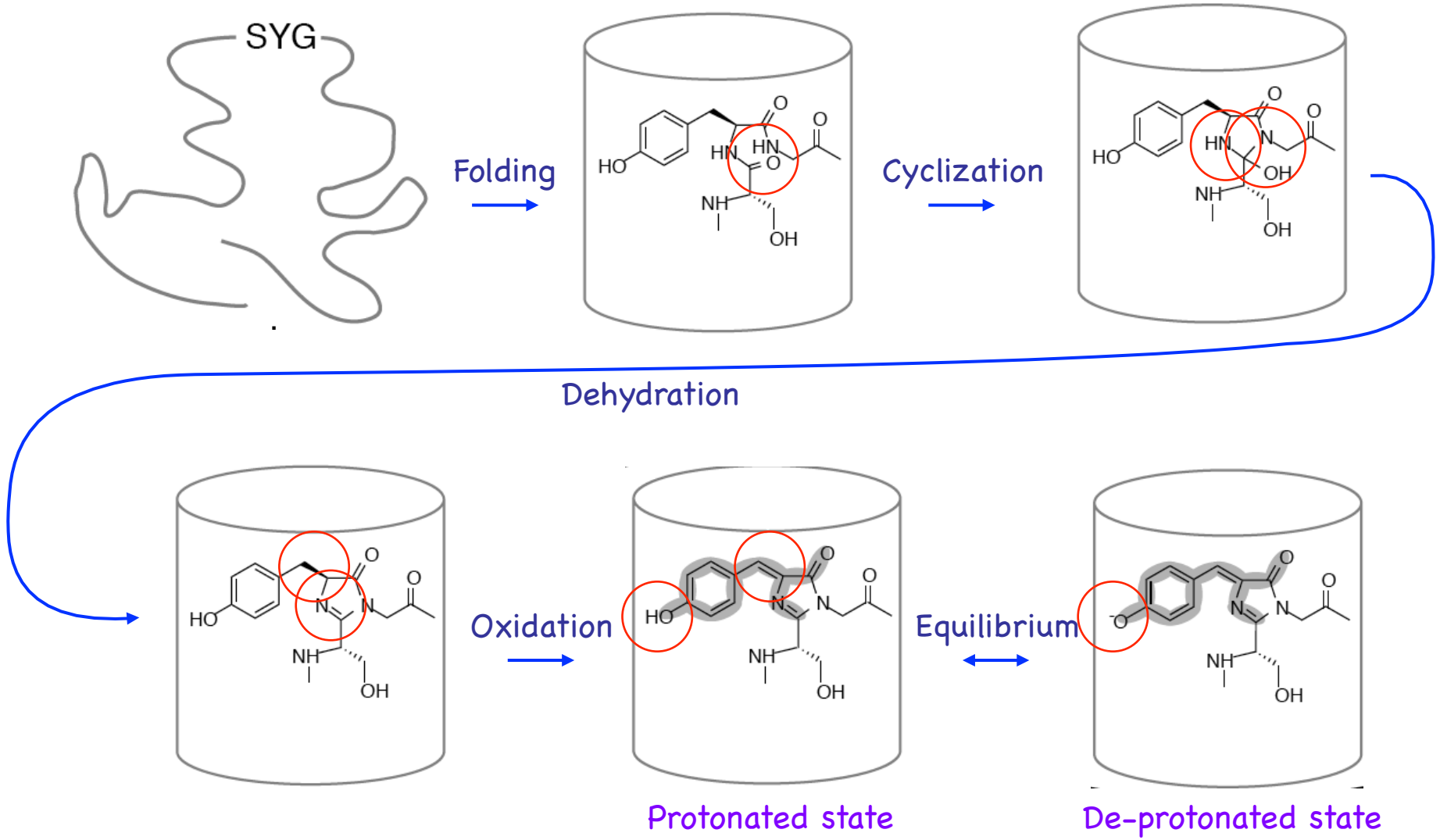
The Green Fluorescent Protein, GFP



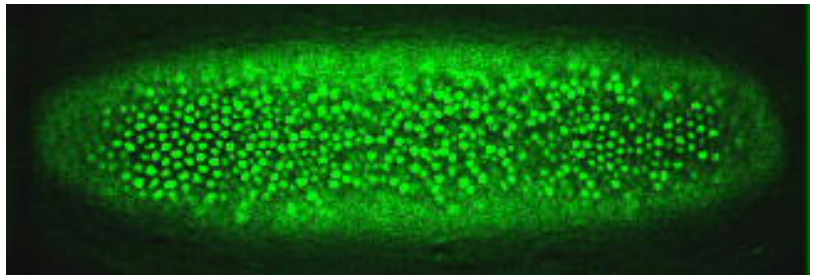
The hydromedusa Aequoria victoria

GFP Fluorophore Formation

Auto-catalysis

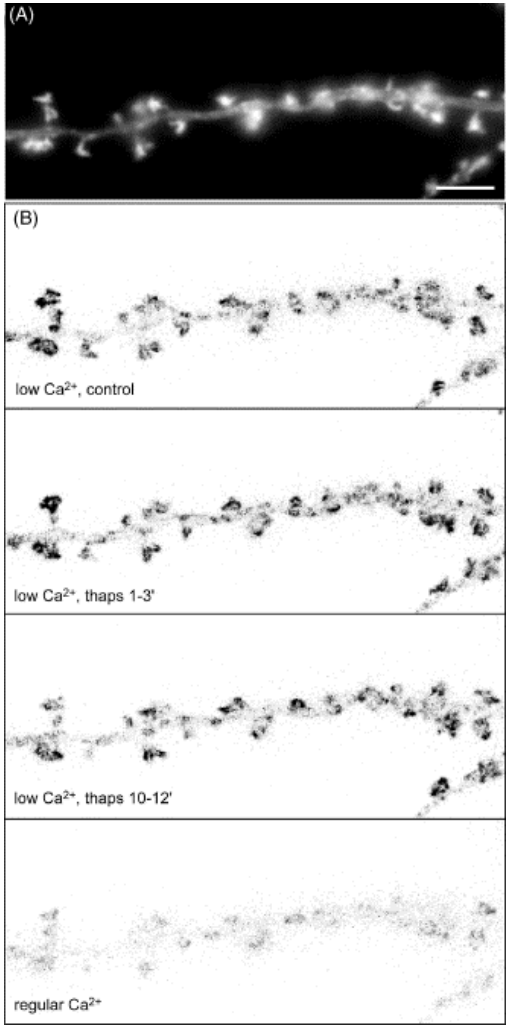


GFP-based live microscopy



Nuclear-targeted GFP
in Drosophila embryo
during gastrulation

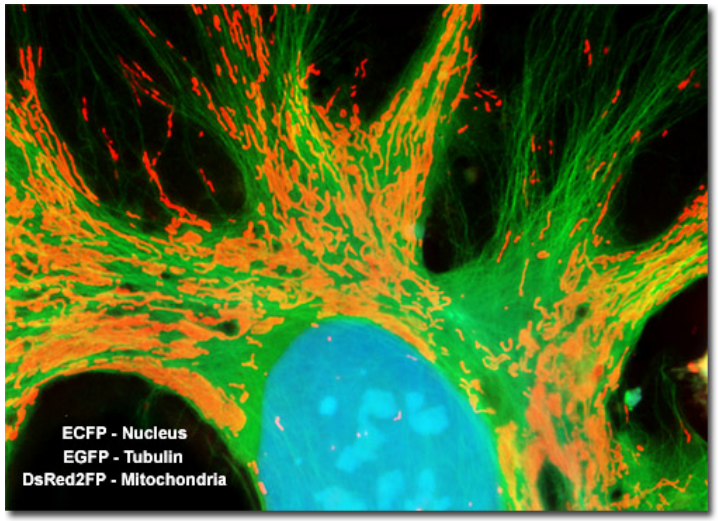
Ilan Davis
U. of Edinburgh, UK



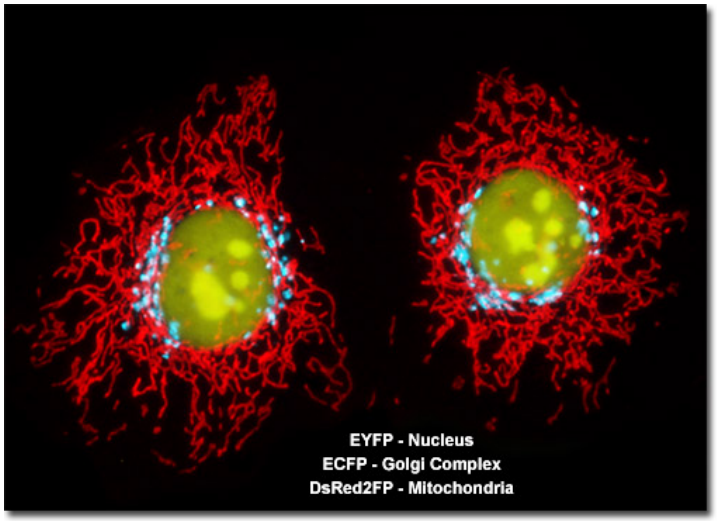
GFP-actin
In cultured
hippocampal
rat neurons

Stefanie Kaech, Heike Brinkhaus, and Andrew Matus
Neurobiology **96**, 10433-10437, 1999.
Volatile anesthetics block actin-based motility in dendritic spines

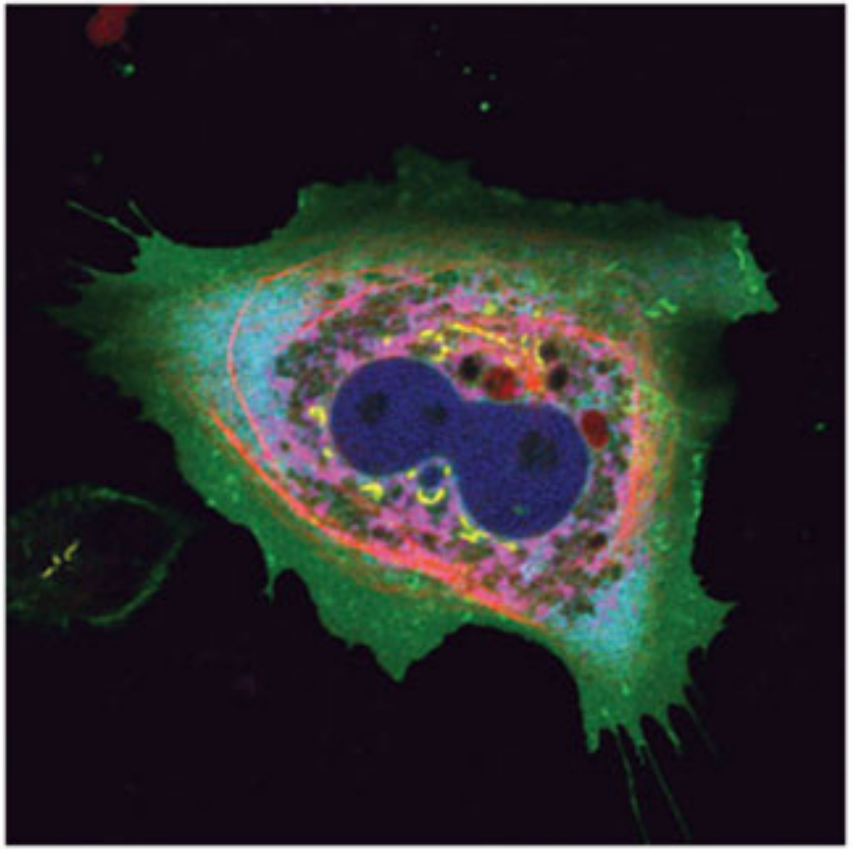
Multicolor microscopy with fluorescent proteins



Opossum Kidney Cortex Epithelial Cells (OK cells)



HeLa cells



African green monkey kidney (Vero) cell
labeled with six different fluorescent proteins
all excited at a single wavelength.
(Required linear unmixing)
(Atsushi Miyawaki)

Variants of *A.v.* GFP

GFP

↓

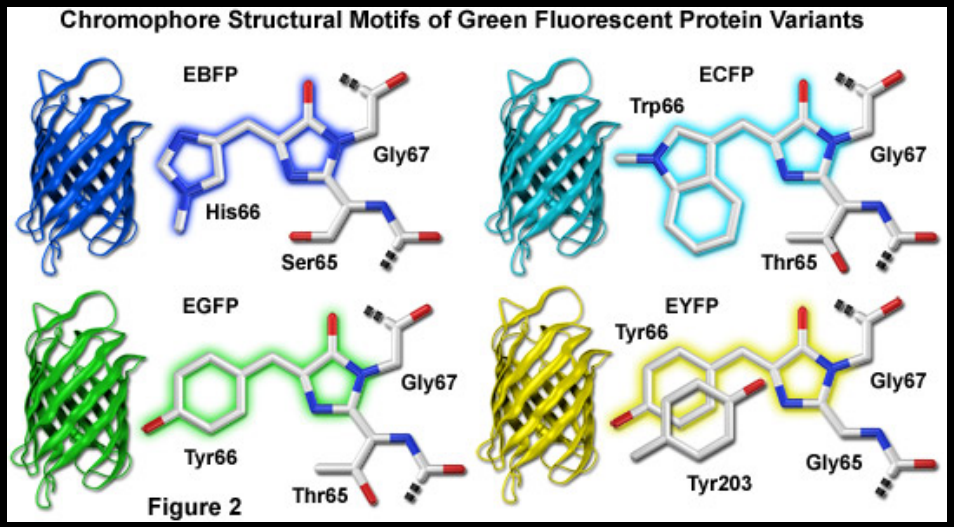
EGFP

↓

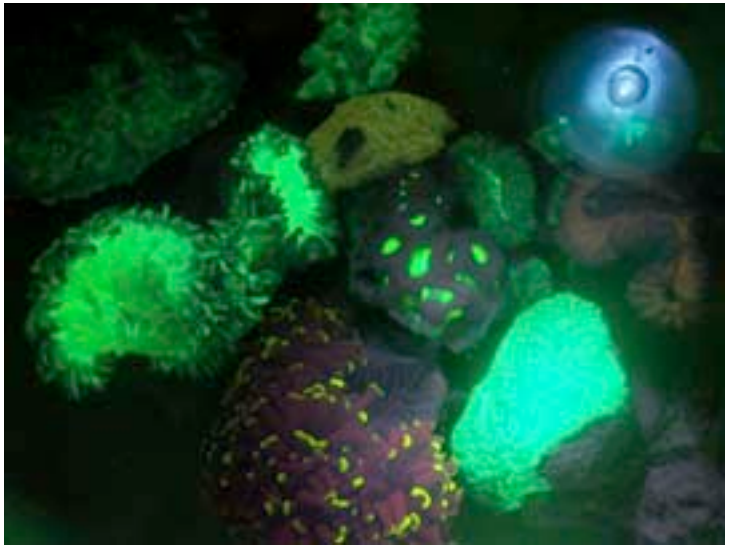
BFP, CFP, YFP

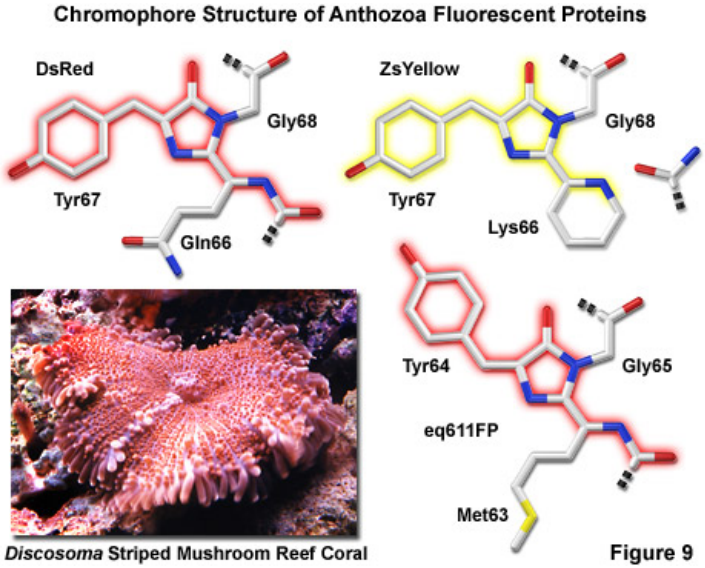
↓

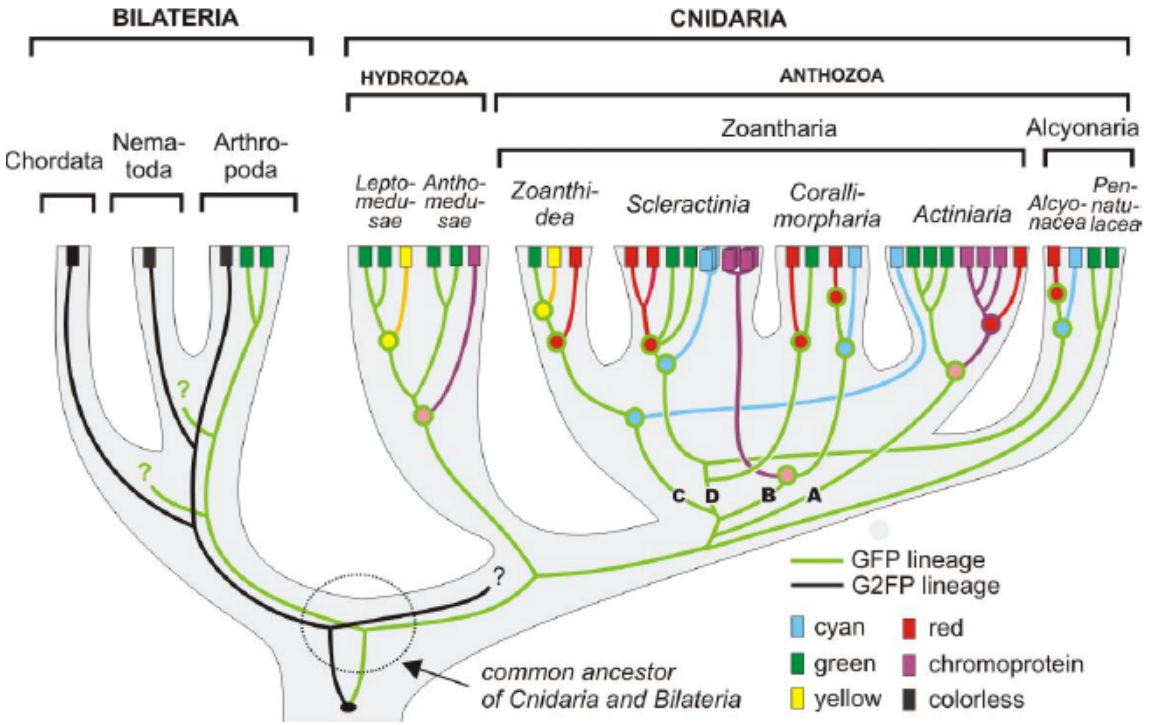
Cerulean , CyPet, Sapphire, Venus, Citrine , Ypet…



GFP-related proteins exist in many (most?) animals



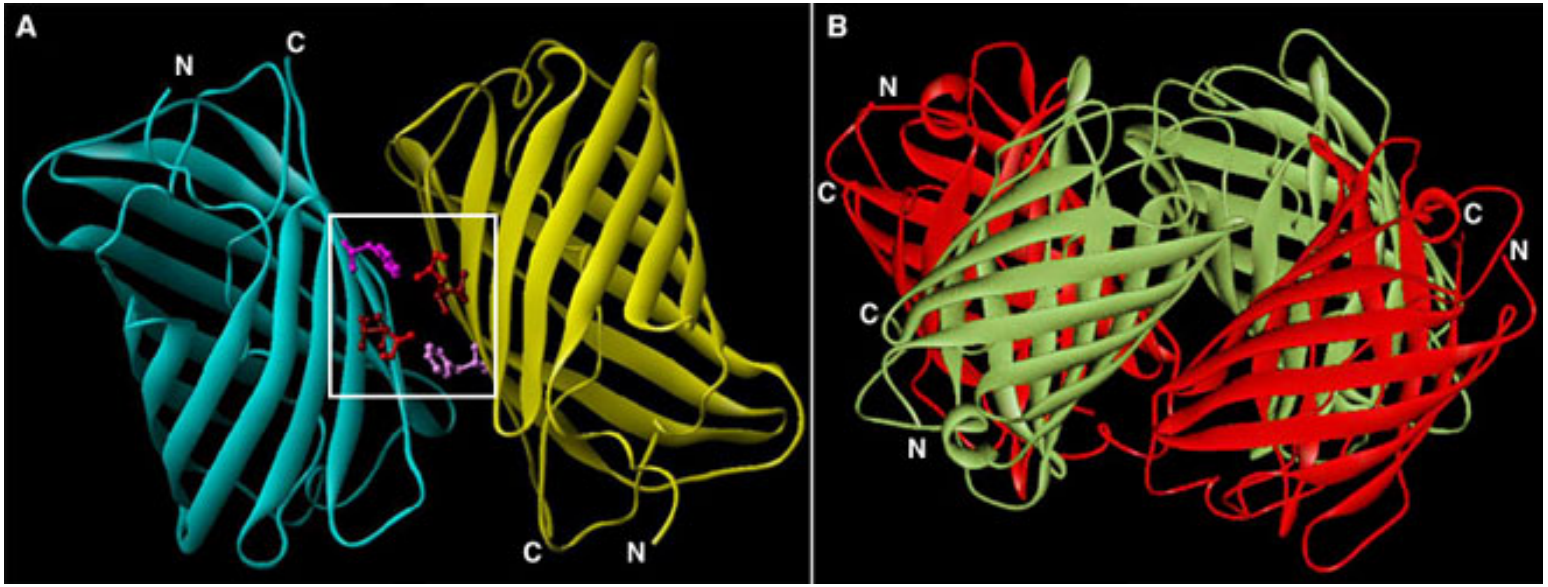




Oligomerization

GFP

dsRed

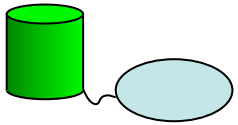


Monomer / weak dimer

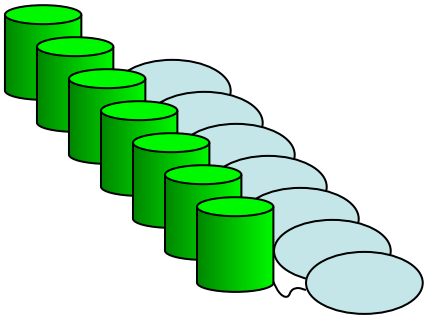
Tetramer

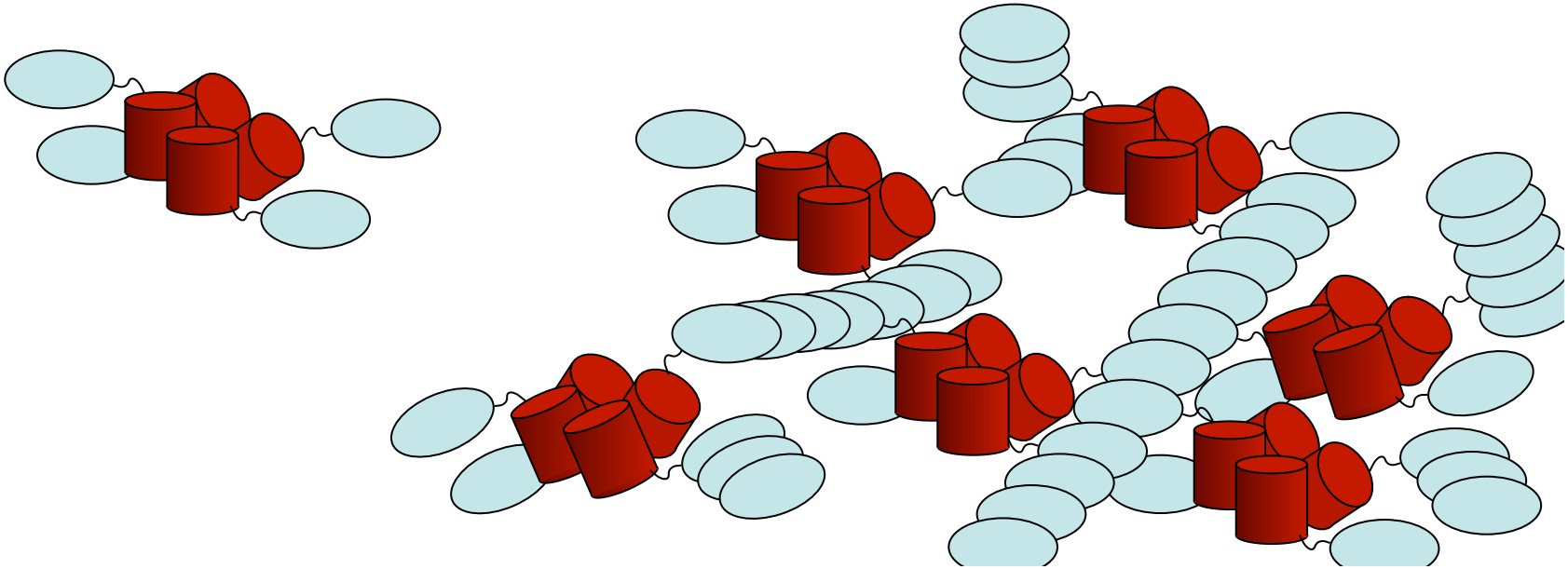
Why is oligomerization a problem?

Fusion to inert protein
or targeting sequence



Fused to interacting protein





Variants of dsRed

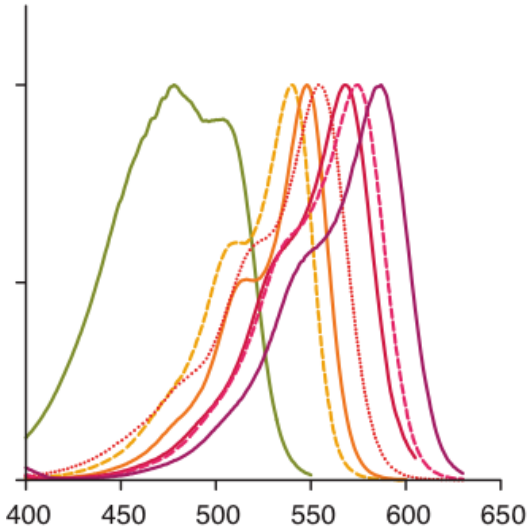
dsRed (tetramer)

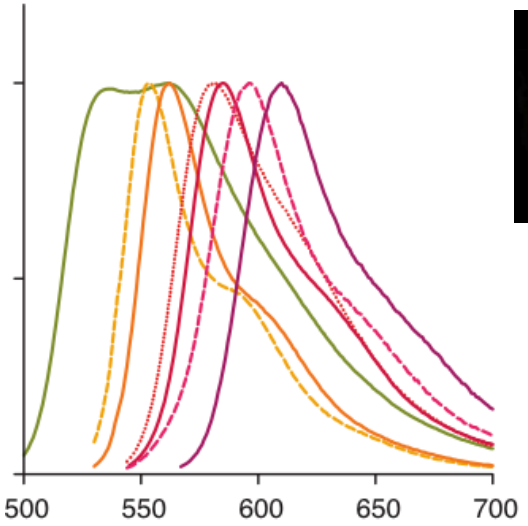
↓

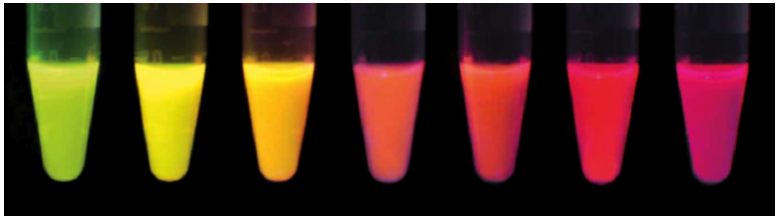
mRFP1 (monomer)

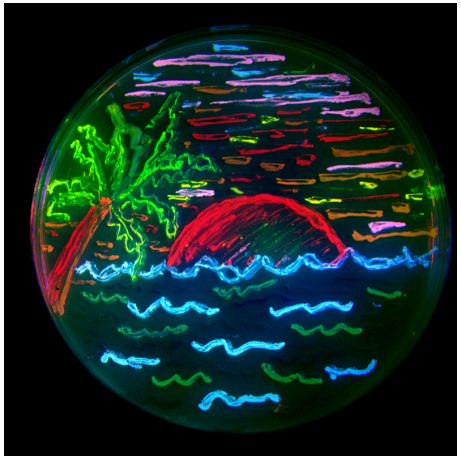
↓

mHoneydew, mBanana, mOrange, tdTomato,
mTangerine, mStrawberry, mCherry, mPlum,...





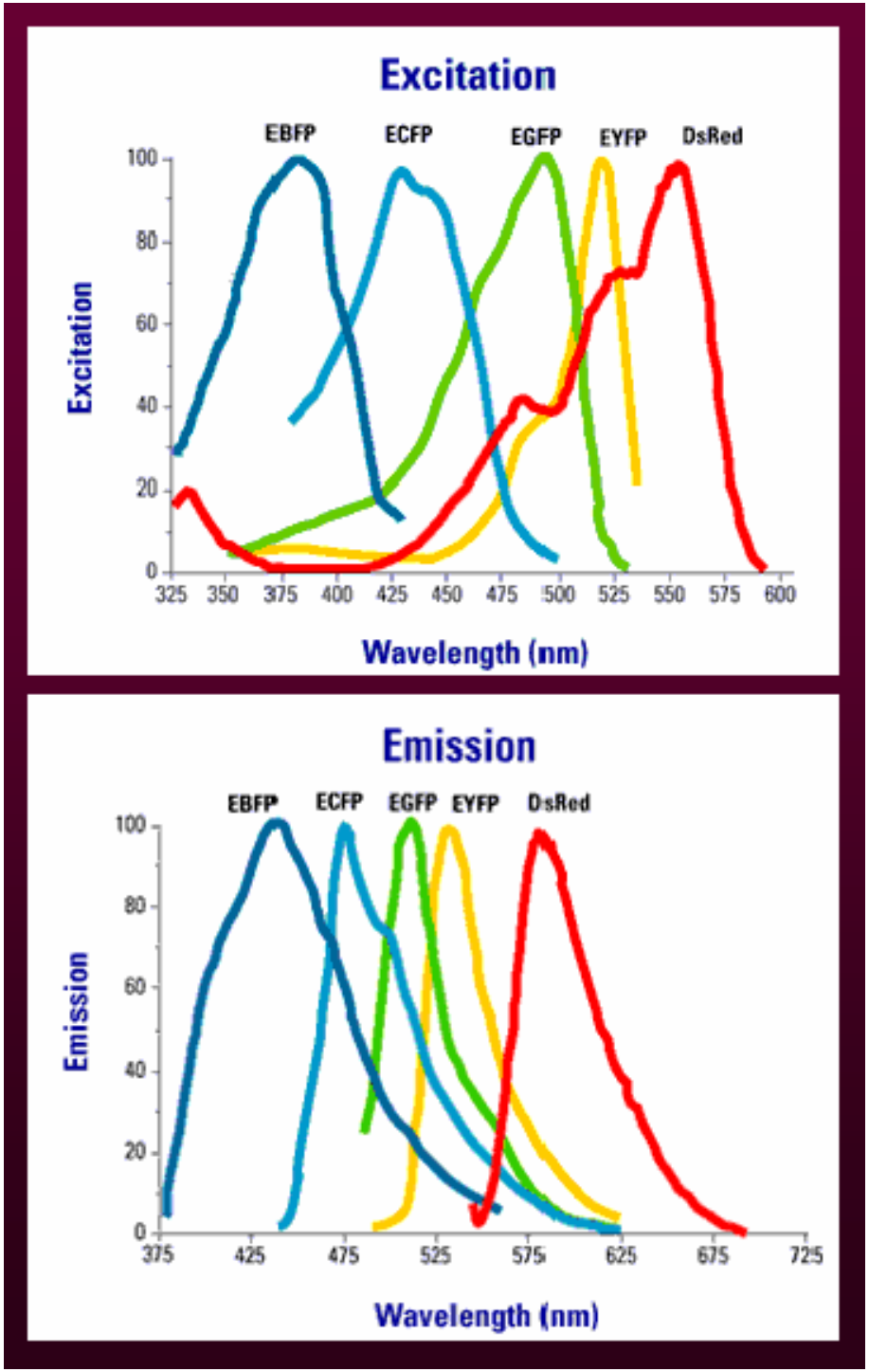




Tsien lab agar plate art

Fluorescent proteins

Protein	λex	λem	ε	QY	Brightness	Source
CFP	433	475	32500	0.4	13.0	Tsien
GFP	488	507	56000	0.6	33.6	Tsien
Citrine	516	529	77000	0.76	58.5	Tsien
PhiYFP	525	537	130000	0.4	52.0	Evrogen
MkOrange	548	559	51600	0.6	31.0	Miyawaki
tdimer2	552	579	120000	0.68	81.6	Tsien
tdtomato	554	581	138000	0.69	95.2	Tsien
DsRed-monomer	556	586				Clontech
mRFP1	584	607	44000	0.25	11.0	Tsien
mCherry	587	610	72000	0.22	15.8	Tsien
tHcRed	590	637	160000	0.04	6.4	Clontech



Multicolor labeling
Cross Talk between Channels

Properties of fluorescent proteins

*N.C. Shaner, P.A. Steinbach, & R.Y. Tsien, Nature Methods **2**:905 (2005)*

Wavelength Class	Protein	Source Lab	Organism	Ex (nm)	Em (nm)	Extinction coefficient per chain, $M^{-1}cm^{-1}$	Fluorescence quantum yield	Brightness (EC*QY) (mM*cm)^-1	Brightness of fully mature protein (% of fluorescein)	$t_{0.5}$ for bleach, sec	photostability (fold improvement over fluorescein)	pKa	$t_{0.5}$ for maturation at 37° C	Oligomerization	References
Far-red	mPlum	Tsien	*Discosoma sp.*	590	649	41,000	0.10	4.1	5.9	53	7.3	<4.5	100 min	monomer	5
Red	mCherry	Tsien	*Discosoma sp.*	587	610	72,000	0.22	16	23	96	13.1	<4.5	15 min	monomer	4
	tdTomato	Tsien	*Discosoma sp.*	554	581	138,000	0.69	95	138	98	13.5	4.7	1 hr	tandem dimer	4
	mStrawberry	Tsien	*Discosoma sp.*	574	596	90,000	0.29	26	38	15	2.1	<4.5	50 min	monomer	4
	J-Red	Evrogen	Unidentified Anthomedusa	584	610	44,000	0.20	8.8	13	13	1.8	5	ND	*dimer*	*x*
	DsRed-Monomer	Clontech	*Discosoma sp.*	556	586	35,000	0.10	3.5	5.1	16	2.2	4.5	ND	*monomer*	*y*
Orange	mOrange	Tsien	*Discosoma sp.*	548	562	71,000	0.69	49	71	9.0	1.2	6.5	4.5 hr	monomer	4
	mKO	MBL Intl.	*Fungia concinna*	548	559	51,600	0.60	31	45	122	16.7	5	2.5 hr	monomer	10
Yellow	mCitrine	Tsien	*Aequorea victoria*	516	529	77,000	0.76	59	85	49	6.7	5.7	ND	monomer	16, 23
	Venus	Miyawaki	*Aequorea victoria*	515	528	92,200	0.57	53	76	15	2.0	6	ND	weak dimer	1
	YPet	Daugherty	*Aequorea victoria*	517	530	104,000	0.77	80	116	49	6.7	5.6	ND	weak dimer	2
	EYFP	Invitrogen	*Aequorea victoria*	514	527	83,400	0.61	51	74	60	8.3	6.9	ND	weak dimer	18
Green	Emerald	Invitrogen	*Aequorea victoria*	487	509	57,500	0.68	39	57	0.69	0.1	6	ND	weak dimer	18
	EGFP	Clontech*	*Aequorea victoria*	488	507	56,000	0.60	34	49	174	23.9	6	ND	weak dimer	*y*
Cyan	CyPet	Daugherty	*Aequorea victoria*	435	477	35,000	0.51	18	26	59	8.1	5	ND	weak dimer	2
	mCFP	Tsien	*Aequorea victoria*	433	475	32,500	0.40	13	19	64	8.8	4.7	ND	monomer	23
	Cerulean	Piston	*Aequorea victoria*	433	475	43,000	0.62	27	39	36	5.0	4.7	ND	weak dimer	3
UV-excitable green	T-Sapphire	Griesbeck	*Aequorea victoria*	399	511	44,000	0.60	26	38	25	3.5	4.9	ND	weak dimer	6
Reference	fluorescein pH 8.4			495	519	75,000	0.92	69	100	7.3	1.0	6.4			

* No longer commercially available
x www.evrogen.com
y www.clontech.com
ND = not determined

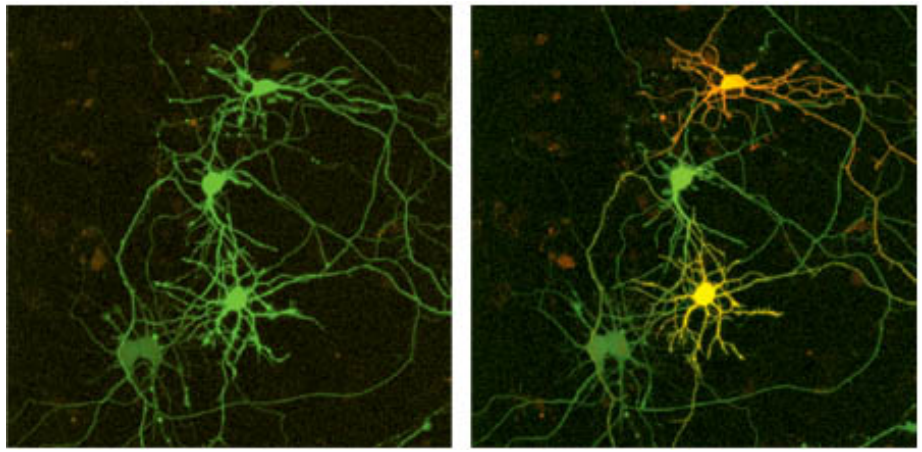
*Other, **less** recommended FP:s*

Protein	Source	Comments
AceGFP	Evrogen	no clear advantage over well-validated Aequorea GFPs
AcGFP1	Clontech	no clear advantage over well-validated Aequorea GFPs
AmCyan1	Clontech	tetrameric
AQ143	Lukyanov	tetrameric
AsRed2	Clontech	tetrameric
Azami-Green/mAG	MBL Intl.	no clear advantage over well-validated Aequorea GFPs
cOFP	Stratagene	tetrameric
CopGFP	Evrogen	no clear advantage over well-validated Aequorea GFPs
dimer2, tdimer2(12)	Tsien	slower maturation than dTomato/tdTomato
DsRed/DsRed2/DsRed-Express	Clontech	tetrameric
EBFP	Clontech	Fast bleaching, dim, no longer commercially available
eqFP611	Weidenmann	poor folding at 37C, tetrameric
HcRed1	Clontech	dimeric, dim
HcRed-tandem	Evrogen	fast bleaching, dim
Kaede	MBL Intl.	dimmer and less efficient at photoconversion than KikGR
mBanana	Tsien	dim, fast photobleaching
mHoneydew	Tsien	dim, fast photobleaching
MiCy	MBL Intl.	dimeric, less spectral separation from YFPs than Aequorea GFP-derived CFPs
mRaspberry	Tsien	faster bleaching than mPlum
mRFP1	Tsien	dimmer and less photostable than mCherry
mTangerine	Tsien	fast bleaching, dimmer than mStrawberry
mYFP	Tsien	Chloride sensitivity
PhiYFP	Evrogen	suspected aggregation, faster bleaching than other YFPs, potential problems with fusion constructs
Renilla GFPs	various	dimeric, no clear advantages over well-validated Aequorea GFPs
TurboGFP	Evrogen	no clear advantage over well-validated Aequorea GFPs
ZsYellow1	Clontech	tetrameric

Switchable fluorescent proteins

Fluorescence that can be activated
or altered by light

- Activatable
  - PA-GFP, ...
- Color-changing
  - Green-red:
    - Kaede, EosFP, KikGR,...
  - Cyan-green:
    - PS-CFP
- Reversibly switchable
  - KFP, Dronpa



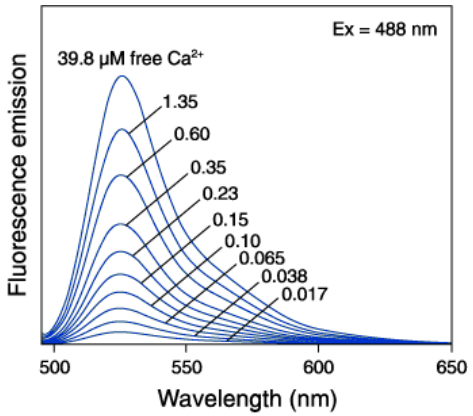
Fluorescent proteins – pros / cons

- Can be easily introduced into live cells
- Minimally perturbative
- Photoactivatible/photoconvertible versions exist
- Avoids fixing / staining

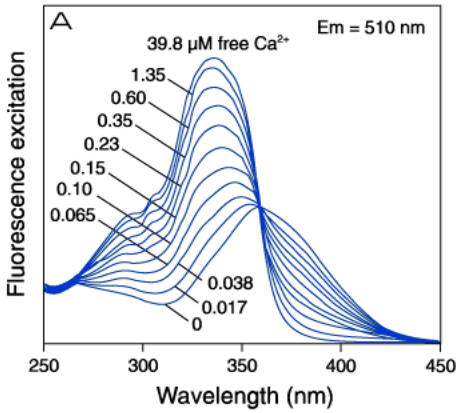
- Require genetically tractable system
- Folding and maturation can be slow
- Some are pH and Cl^- sensitive
- Some have very complicated photophysics (strange photoactivation / photobleaching behavior)

Ca^{2+} imaging

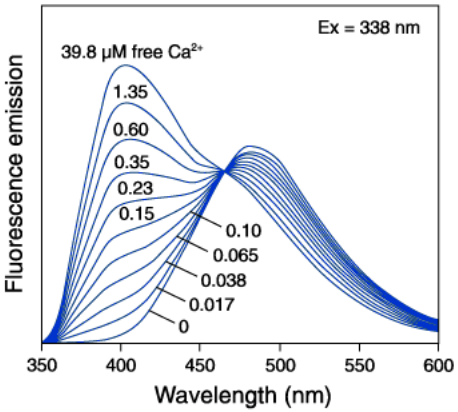
Intensity
Fluo-3



Exc. ratioing
FURA-2

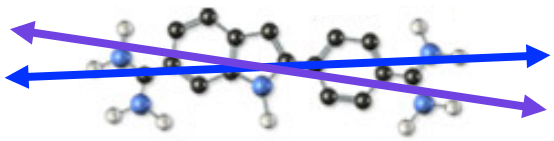


Em. ratioing
Indo-1



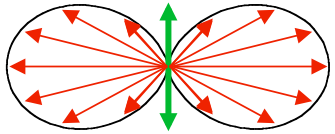
Fluorescence Polarization

Fluorophores have dipole directions



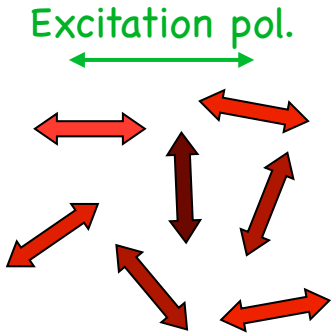
Emission dipole direction

Excitation dipole direction

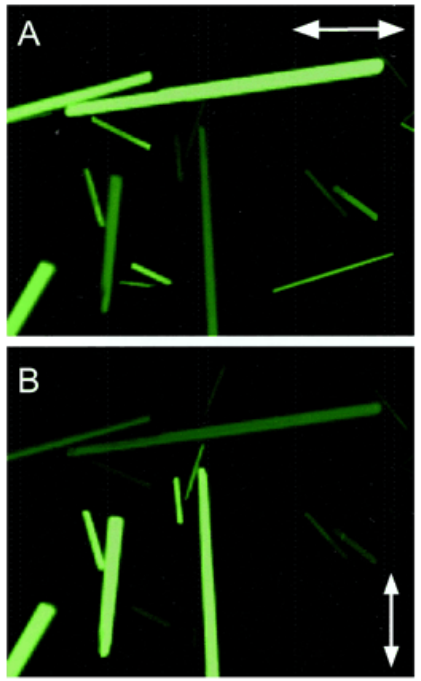


Angle dependence of exc. or em

- Is emission *polarized* under polarized excitation?
  - (Em-exc dipole angle)
  - Rotation rate

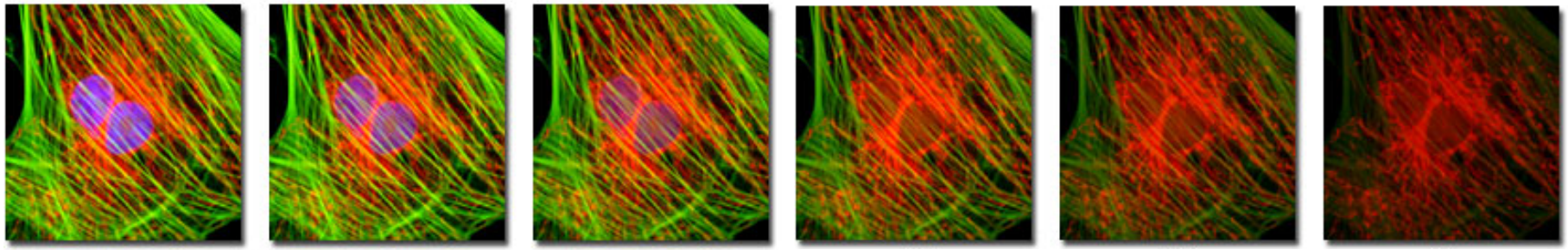


- Does emission *intensity* depend on excitation polarization?
  - Molecular orientation



GFP crystals (Shinya Inoué)

The Enemy:
Photo-bleaching



Decrease in emission intensity after exposure

Exciting a molecule once has a probability Q_b of killing it

Each molecule will emit only a finite number of photons

Photo-bleaching

Photostability varies between dues

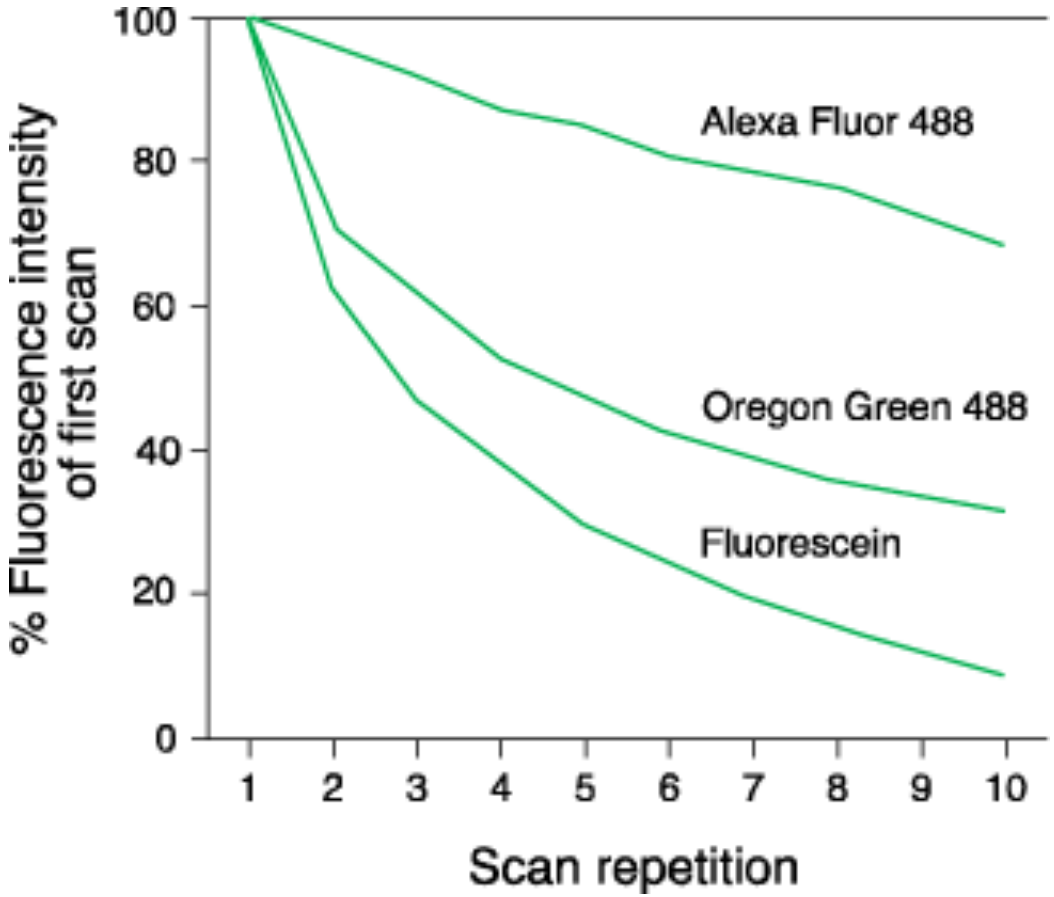
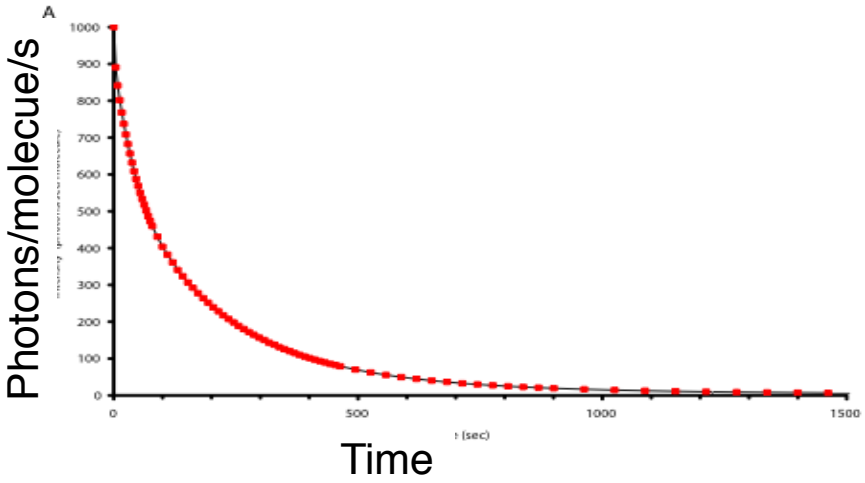
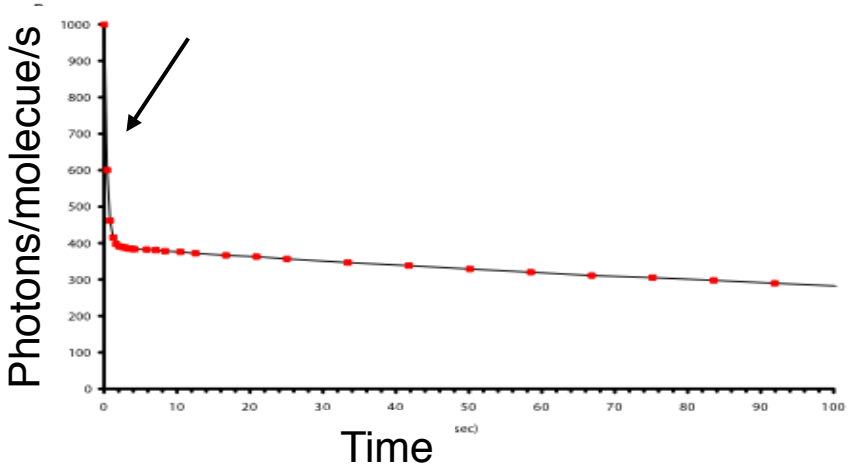


Photo-bleaching of fluorescent proteins



mCherry
Single-exponential bleaching



Emerald
Double-exponential bleaching
Fast- and slow-bleaching populations?

What to do about photo-bleaching?

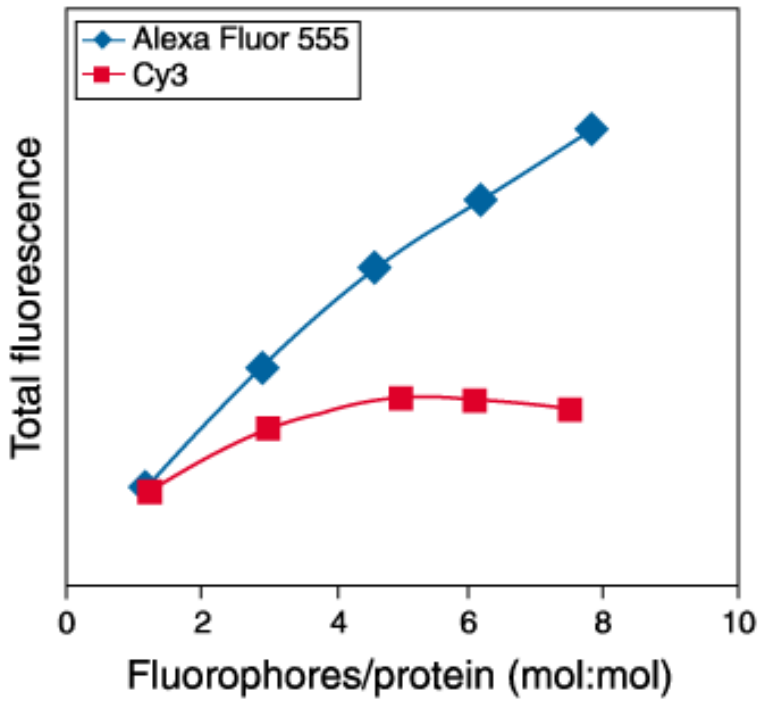
- Select fade-resistant dyes
- Label densely
- Decrease bleaching by *anti-fade mounting media*
  - Glycerol
  - Oxygen scavengers
  - Free-radical scavengers
  - Triplet state quenchers

  Note: some anti-fade agents quench some dyes.
- Budget the photons you have
  - Only expose when observing
  - Minimize exposure time & excitation power
  - Use efficient filter combinations
  - Use highly QE, low noise camera
  - Use simple light path

Self-Quenching

Dye molecules can ***self-quench*** if too close together

→ Label densely but not *too* densely



OPTIMIZATION

- Lamp alignment – do it right and double the light.
- Filter selection – more is not always better.
- Camera selection – a little effort can pay back very well.
- Objective selection

Resolution Properties of Fluorescence Microscpy

Same as brightfield with full aperture

$d_{lateral} \approx 200$ nm, $d_{axial} \approx 600$ nm