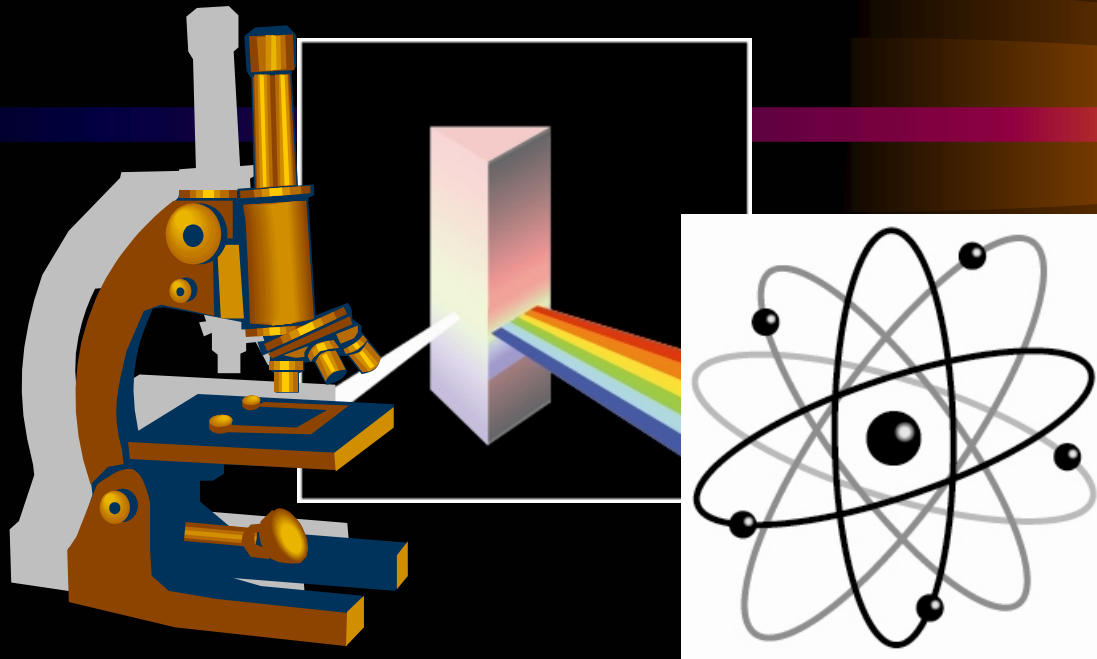# *Fundamentals of Fluorescence*

Sam Wells, 9/15/03

What is fluorescence?

Optical and Physical Properties

Detection and Measurement

Chemical properties and Biological applications to be discussed later

Definition of Fluorescence

Light emitted by singlet excited states of molecules following absorption of photons from an external source

## Requirements for Fluorescence

Fluorescent Dye (Fluorophore). A molecule with a rigid conjugated structure (usually a polyaromatic hydrocarbon or heterocycle).

**Excitation.** Creation of dye excited state by absorption of a photon.

Molecular Structure Features and Fluorescence

High ring density of π electrons increase fluorescence. Aromatic hydrocarbons (π to π * absorption) are frequently fluorescent.

Protonation (affected by e^- withdrawing/donating groups).   OH produces blue shift and decreased Q_f in fluorescein.   Halogens (e^- withdrawing) lower pK_a and alkyl groups (e^- donating) raise the pK_a.
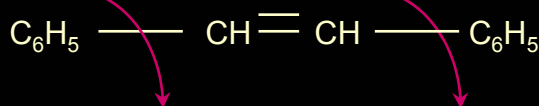
BCECF Acid
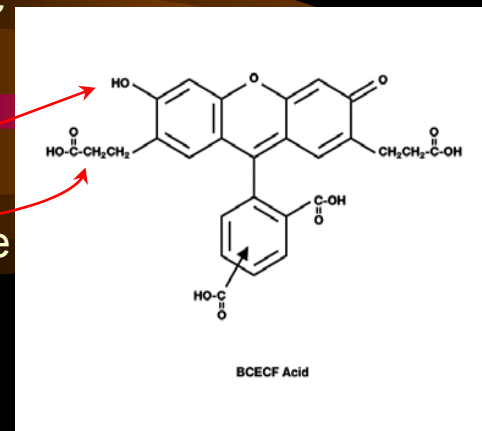
Increased hydrocarbon conjugation shifts π το π^* absorption to the red and increases the probability of fluorescence.
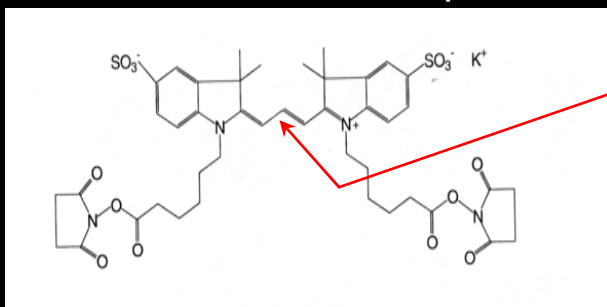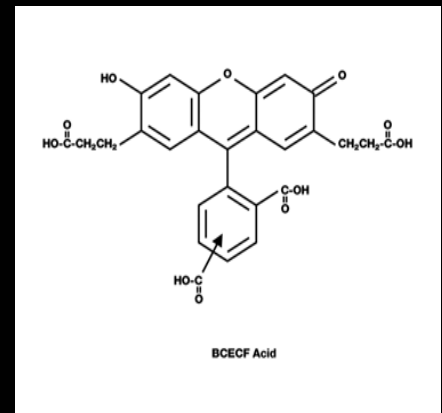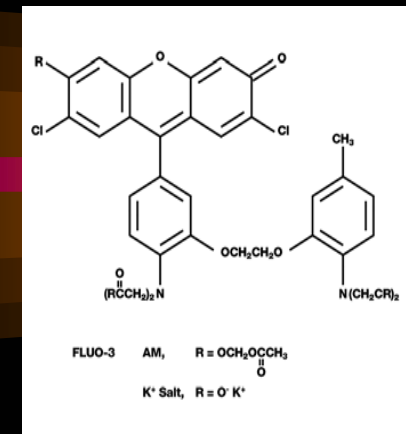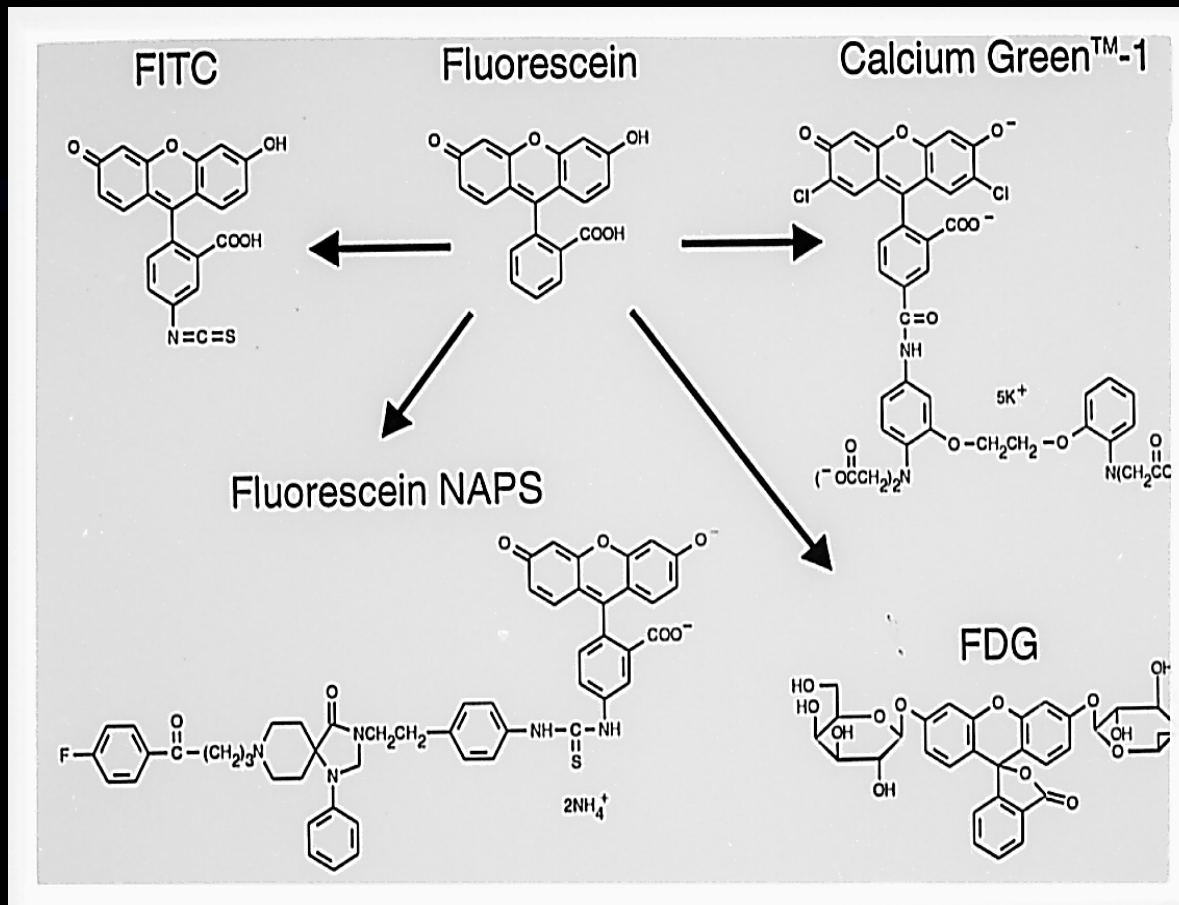
$(C_3H_3)_n$, n = 3,5,7 $\longrightarrow$ Cy3, Cy5, Cy7

Planarity and rigidity affect fluorescence. Increased viscosity may slow free internal rotations and increase fluorescence.

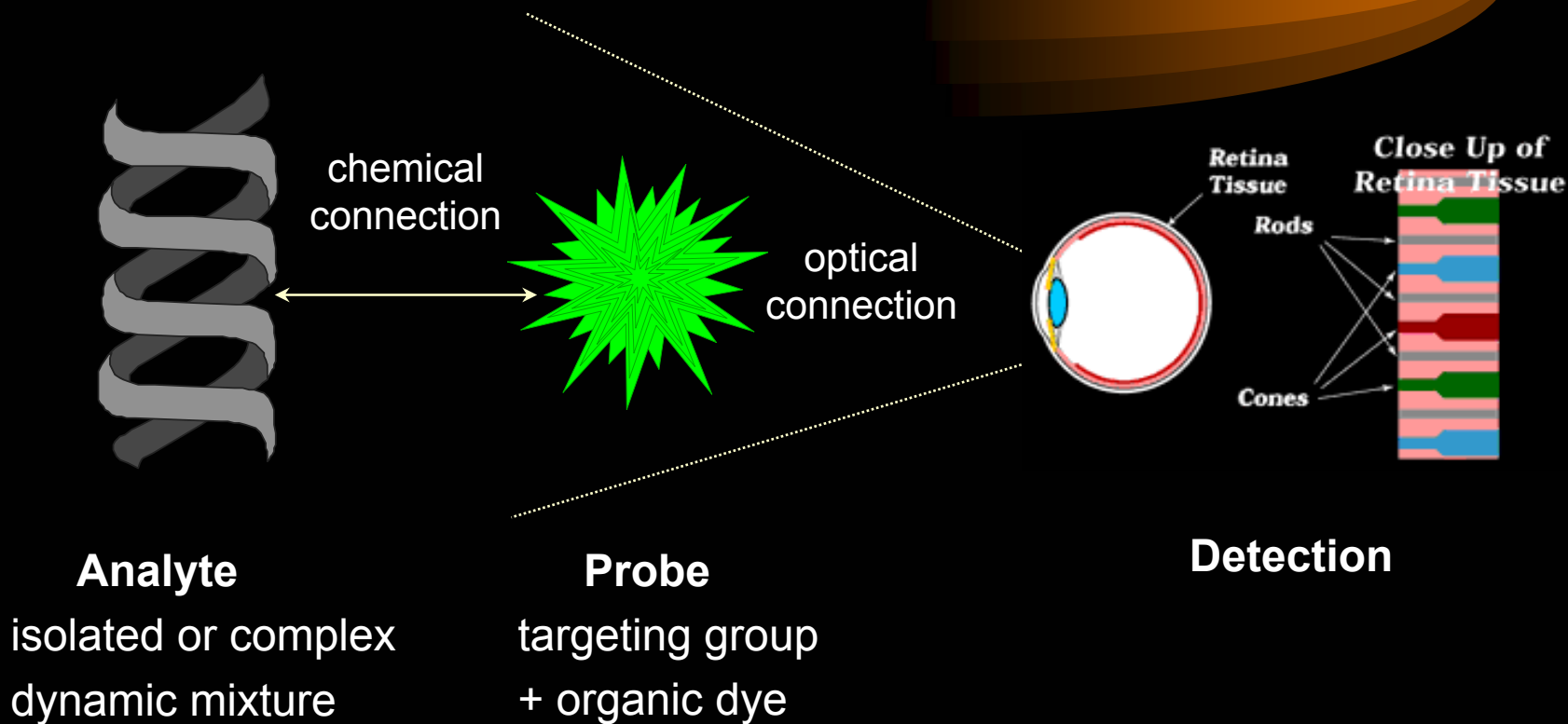C_6H_5 —— CH = CH —— C_6H_5

# Fluorescent PROBES: Tags, Tracers, Sensors, etc.

Fluorescent **tags** generate contrast between an analyte and background, e.g., to identify receptors, or decorate cellular anatomy.

Fluorescent **sensors or indicators** change spectral properties in response to dynamic equilibria of chemical reactions, intracellular ions e.g., Ca^{2+}, H^+, and membrane potential.

Fluorescence Combines Chemical and Optical Recognition

chemical connection

optical connection

Retina Tissue

Close Up of Retina Tissue

Rods

Cones

Analyte
isolated or complex
dynamic mixture

Probe
targeting group
+ organic dye

Detection

Wells, 9/15/03

ANALYTES can evaluated by fluorescence in a wide range of environments, including single molecules in solutions, gels or solids, cultured cells, thick tissue sections, and live animals.
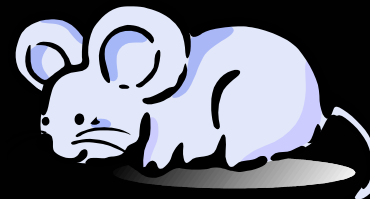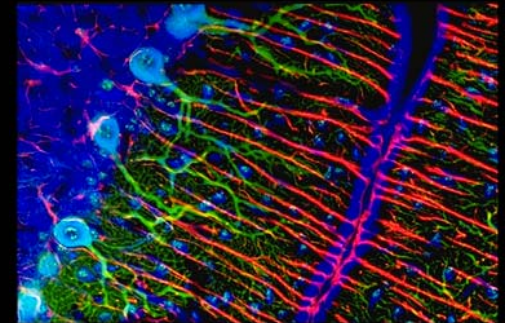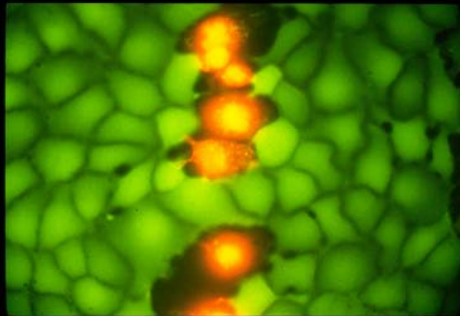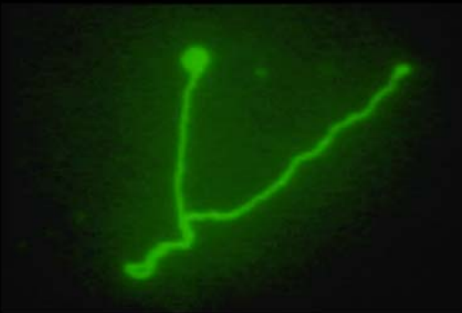
Optical Properties

Spectral properties of fluorescent probes define their utility in biological applications.

Fluorescence spectra convey information regarding molecular identity.

Variations in fluorescence spectra characterize chemical and physical behavior.

1 micron spheres viewed under transmitted incandescent "white" light
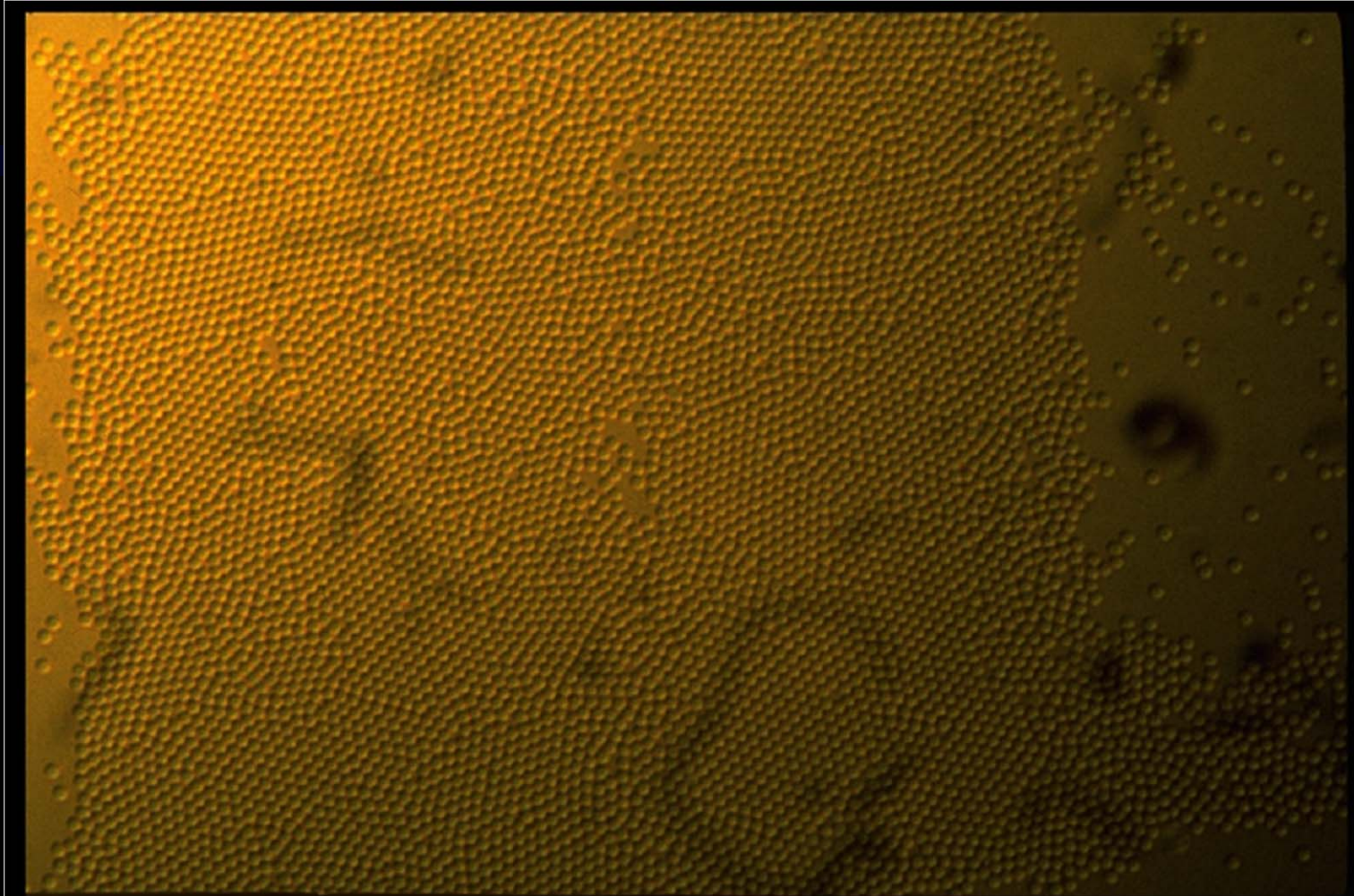
The light passing through the sample is diffracted, partially absorbed and transmitted to the detector.

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1 micron spheres emitting fluorescence

The light passing through the sample is diffracted and absorbed, then **FILTERED** to detect light originating from molecules within the sample.

Fluorescence-related Terminology

Excitation – <u>process of absorbing</u> optical energy.
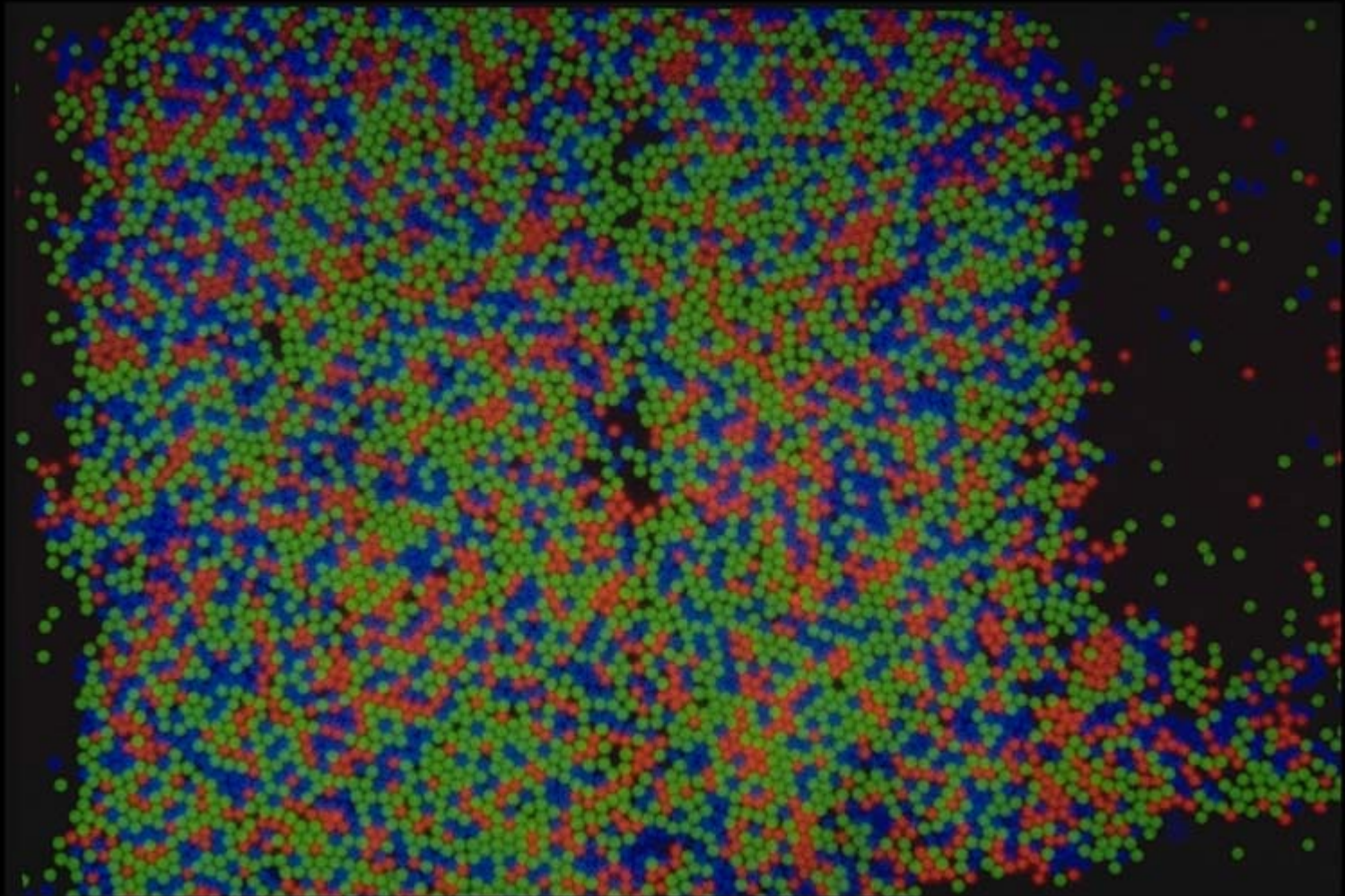
Extinction coefficient – <u>efficiency of absorbing</u> optical energy; this value is wavelength dependent.
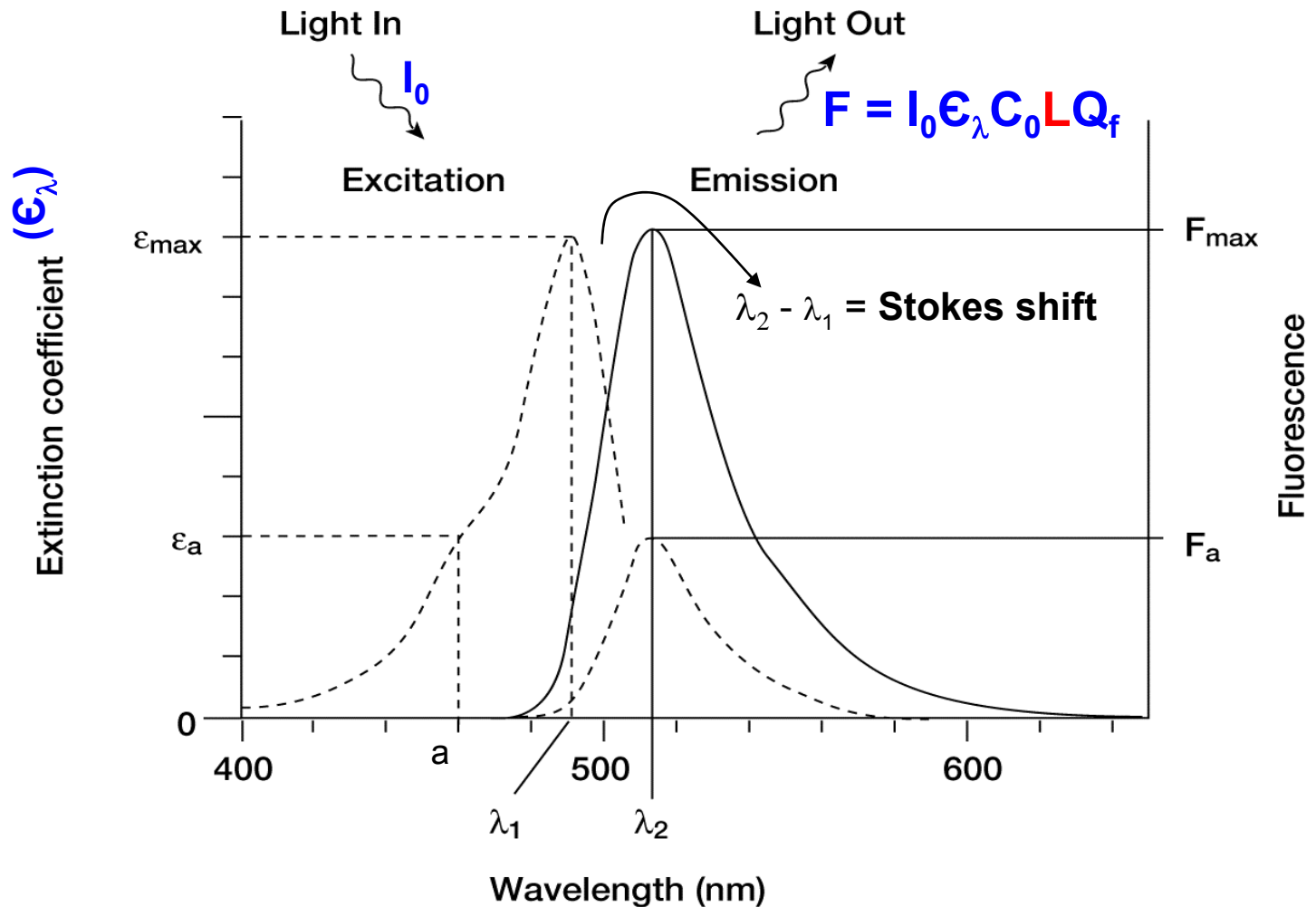
Emission – <u>process of releasing</u> optical energy.

Quantum yield – <u>efficiency of releasing</u> energy (0-1), generally in the form of fluorescent light; not wavelength dependent.

Stokes shift – <u>difference in energy</u> between excitation and emission wavelength maxima – the key to contrast generation.

Spectrum – distribution of excitation and emission wavelengths

Spectral Features of Fluorescence

Light In

I_0

Light Out

$F = I_0 \epsilon_\lambda C_0 L Q_f$

Excitation

Emission

Extinction coefficient (ϵ_λ)

ϵ_{max}

ϵ_a

0

$\lambda_2 - \lambda_1$ = Stokes shift

F_{max}

F_a

Fluorescence

400

a

500

λ_1

λ_2

600

Wavelength (nm)

Absorption and Fluorescence Excitation Spectra: Same or Different?

Incident intensity (I_o)   Transmitted intensity (I_t)

Fluorescence intensity (I_F)
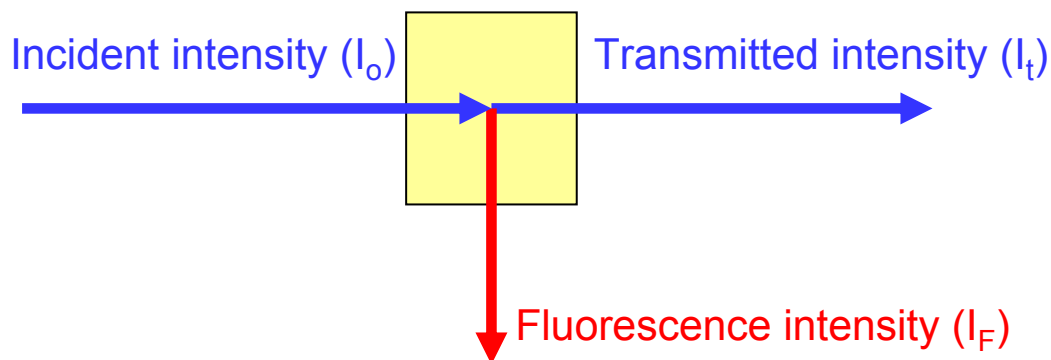
Absorption spectrum: Scan $I_o(\lambda)$, detect I_t
Fluorescence excitation spectrum: Scan $I_o(\lambda)$, detect I_F

For pure dye molecules in solution, the absorption and fluorescence excitation spectra are usually identical and can be used interchangeably. When more than one absorbing or fluorescent species is present in the sample, they are typically different, as in the case of protein-conjugated tetramethylrhodamine dyes, shown right.

Fluorescence excitation

Absorption

500   550   600

Wavelength (nm)
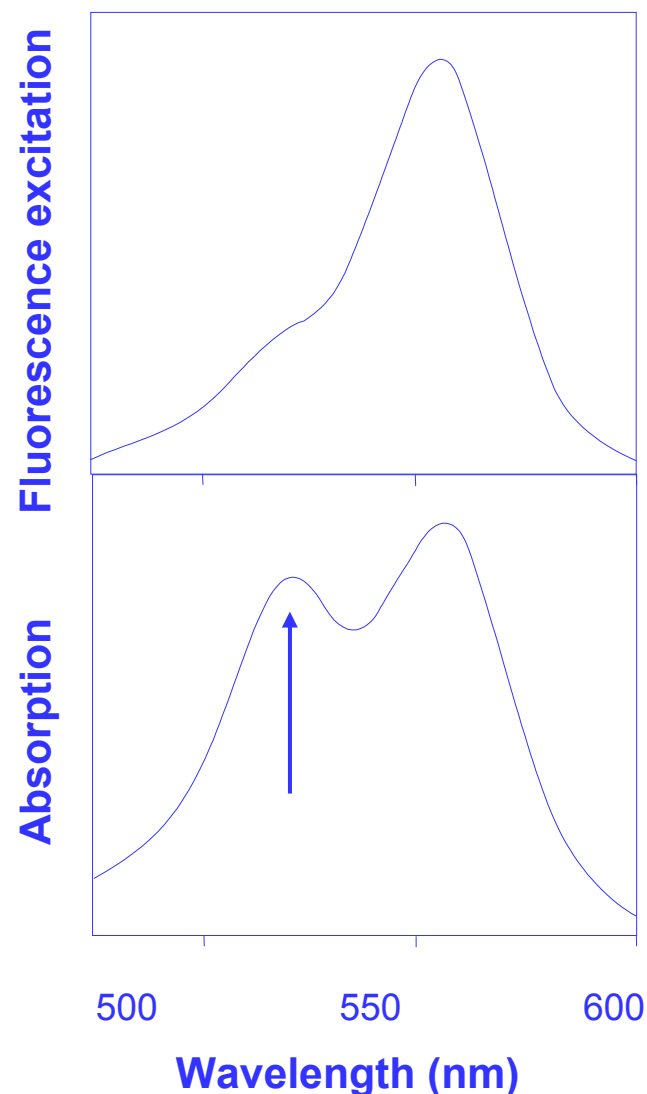
DETECTION involves only a single observable parameter = INTENSITY.

The spectral properties [COLOR] are discriminated by optical filtering and sometimes temporal filtering prior to measuring the intensity.

Therefore, it is extremely important to configure the filtering and optical collection conditions properly to avoid artifacts and faulty observations!

Contrast by Epi-illumination (reflection mode) and Optical Filtering
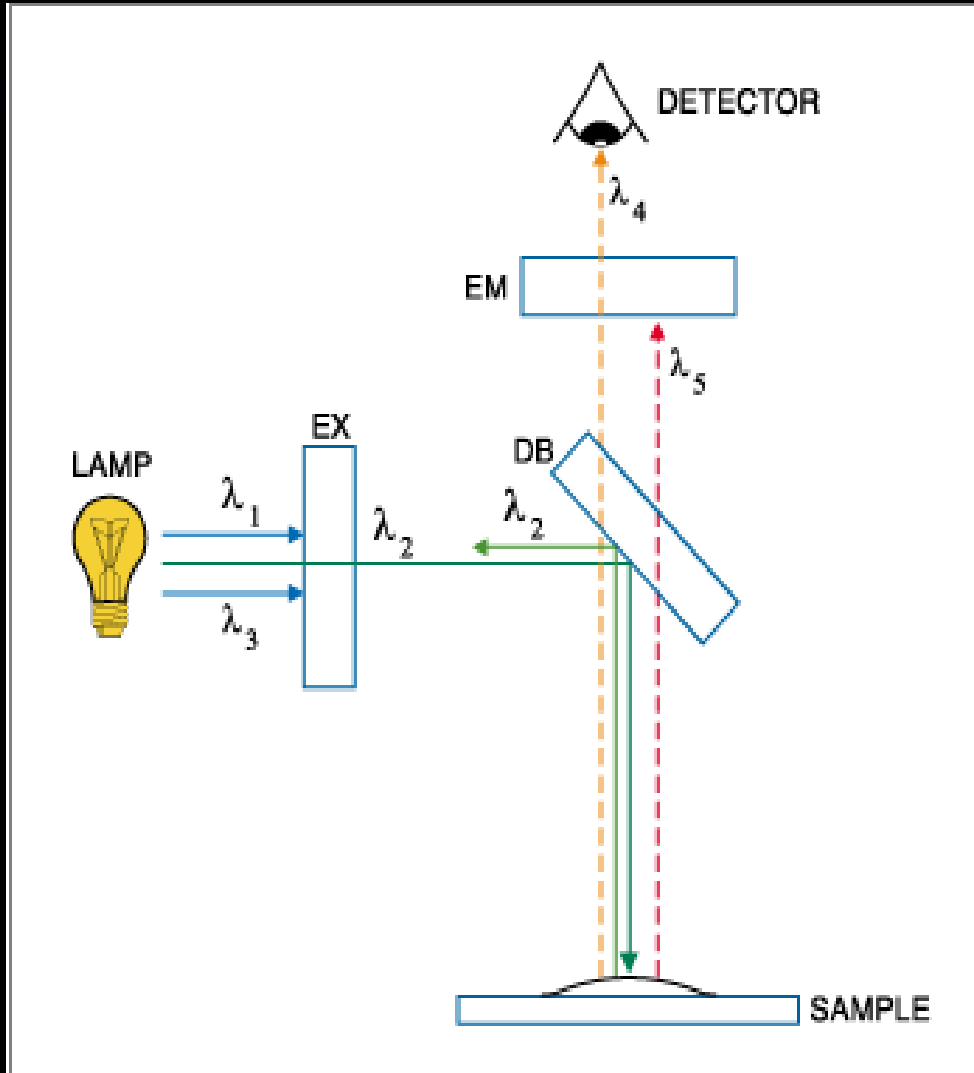
EX = excitation filter
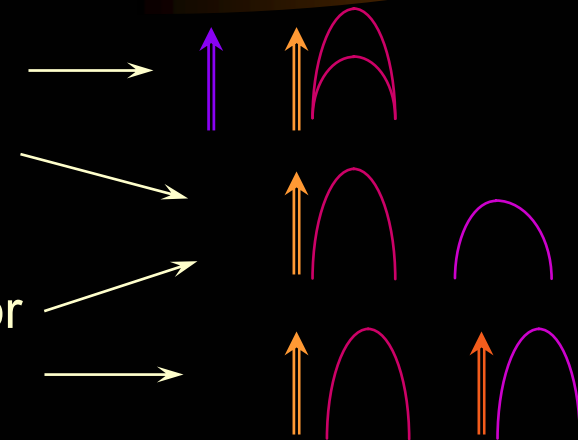DB = dichroic beamsplitter
EM = emission filter

Chemical Identification and Physical Properties by Excitation and Emission

(A)   1 dye:1 excitation, 1 emission
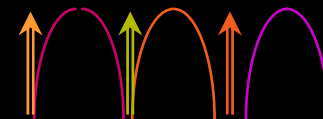
(B)   1 dye:2 excitation, 1 emission or
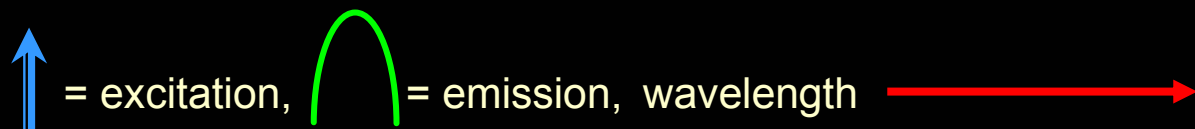(C)   1 excitation, 2 emission

(D)   2 dyes:1 excitation, 2 emission or
(E)   2 excitation, 2 emission

(F)   >1 dye: >1 excitation, >1 emission

= excitation, = emission, wavelength

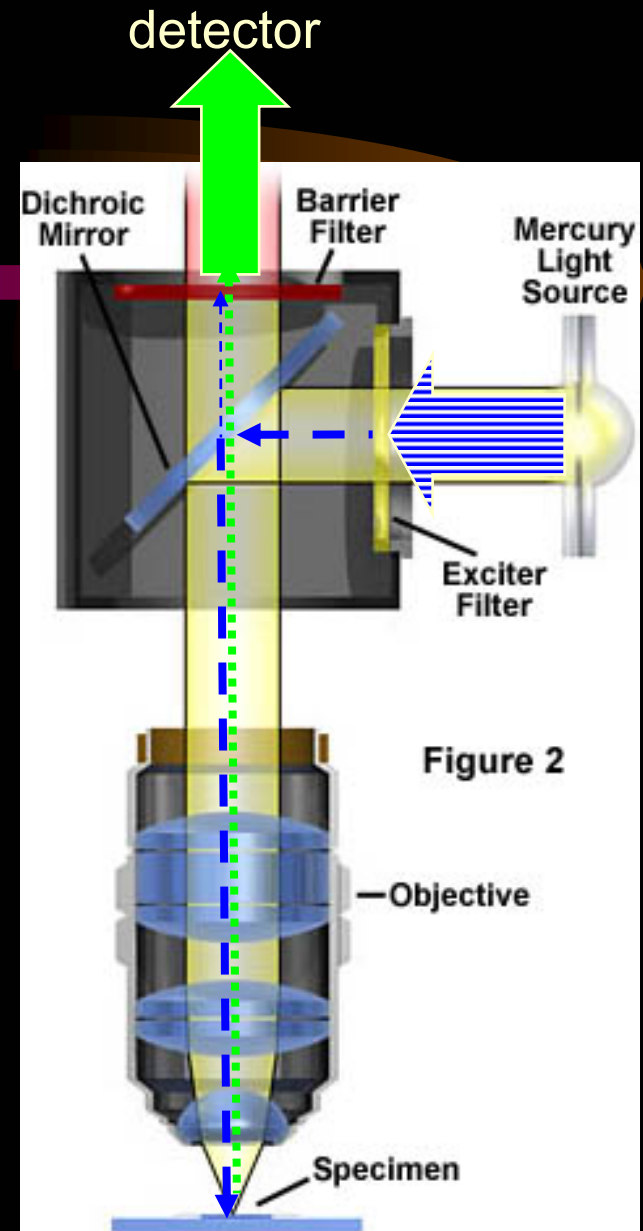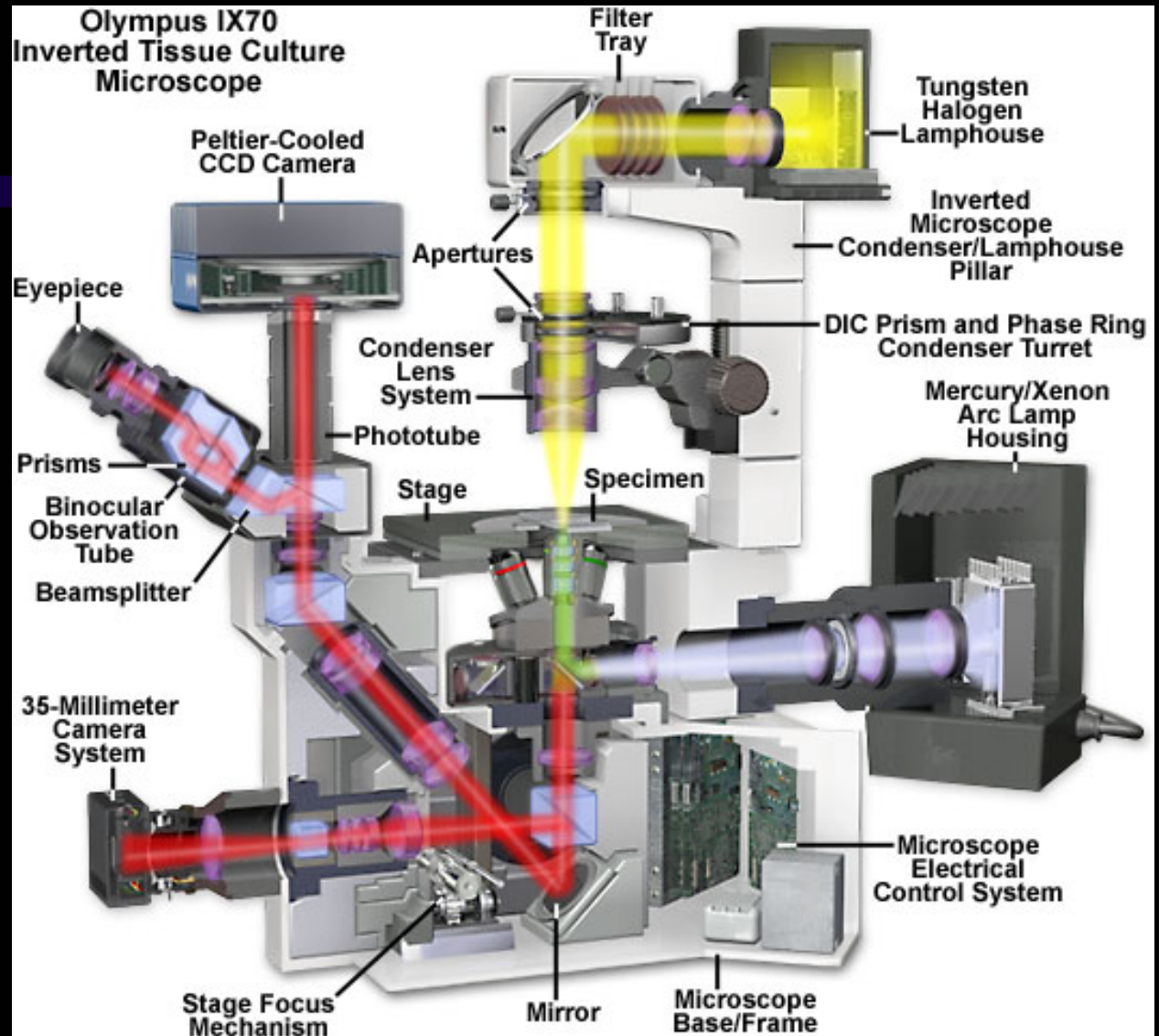## DETECTION / Microscopy, Imaging

Basic components for reflected light fluorescence microscopy, a.k.a. incident light fluorescence, or epi-fluorescence.

1. Excitation light source
2. Filter set
3. Objective lens (the microscope)
4. Fluorescent specimen (analyte + probe)
5. Detector

detector

Dichroic Mirror

Barrier Filter

Mercury Light Source

Exciter Filter

Figure 2

Objective

Specimen

Wide-field, inverted fluorescence microscope

Olympus IX70 Inverted Tissue Culture Microscope

Filter Tray

Tungsten Halogen Lamphouse

Peltier-Cooled CCD Camera

Inverted Microscope Condenser/Lamphouse Pillar

Apertures

Eyepiece

DIC Prism and Phase Ring Condenser Turret

Condenser Lens System

Mercury/Xenon Arc Lamp Housing

Phototube

Prisms

Specimen

Binocular Observation Tube

Stage

Beamsplitter

35-Millimeter Camera System

Microscope Electrical Control System

Stage Focus Mechanism

Mirror

Microscope Base/Frame

Spectral Optimization

- **Absorption wavelength** Optimize excitation by matching dye absorption to source output (Note: total excitation is the product of the source power and dye absorption)

- **Emission wavelength** Impacts the ability to discriminate fluorescence signals from probe A versus probe B and background autofluorescence

- Emission bandwidth   Narrow bandwidths minimize overspill in multicolor label detection

Limitations to Sensitivity (S/N)

Noise Sources

Instrument

Stray light, detector noise

Sample

Sample–related autofluorescence, scattered excitation light (particle size and wavelength dependent)
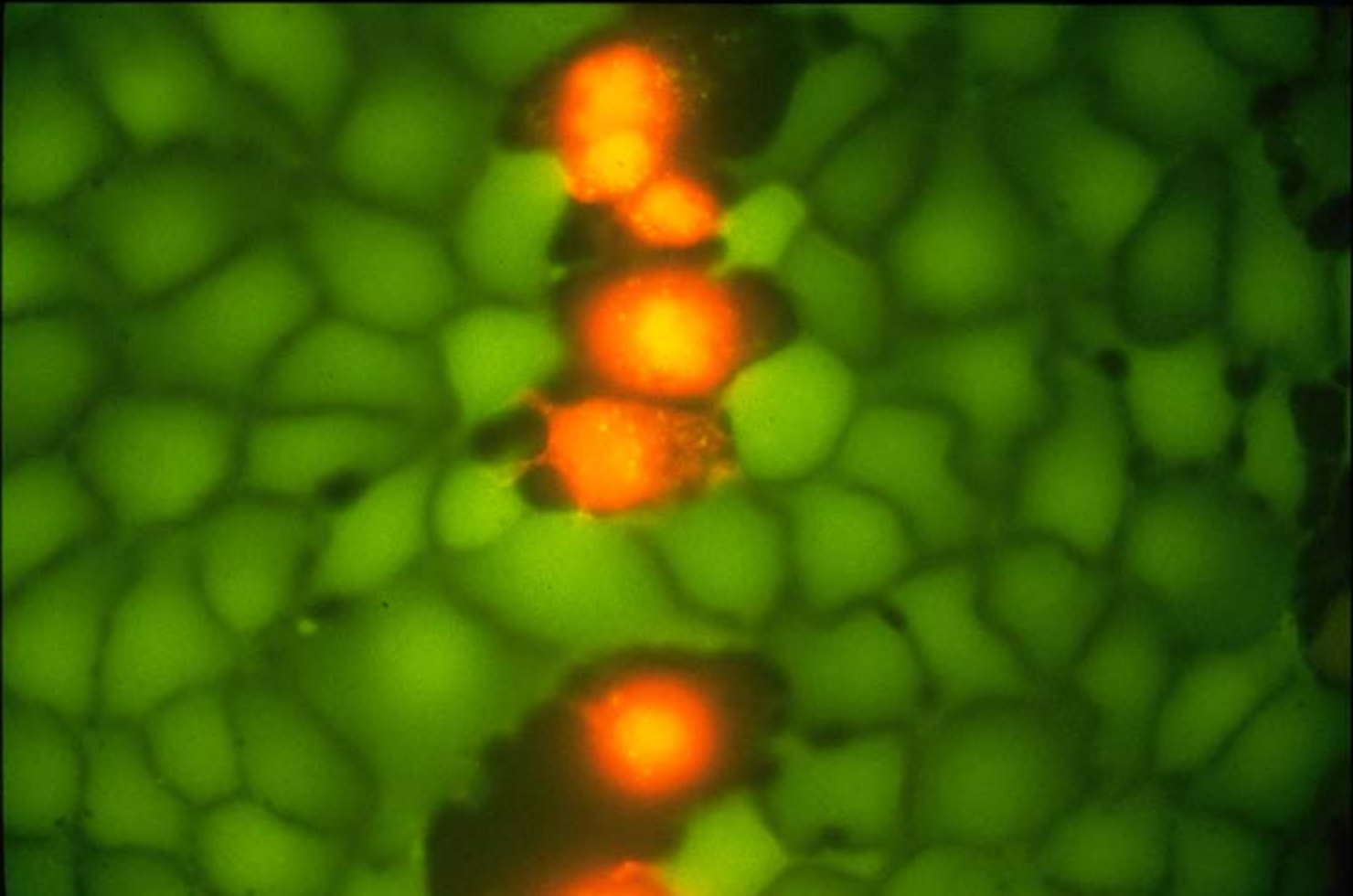
Reagent

Unbound or nonspecifically bound probes

Signal Losses

Inner filter effects, Emission scattering, *Photobleaching*

Single T2 genomic DNA macromolecule stained with YOYO-1 (10 nM loading in very low concentration DNA in agarose). Wide-field image using a 1.4NA objective and CCD camera.
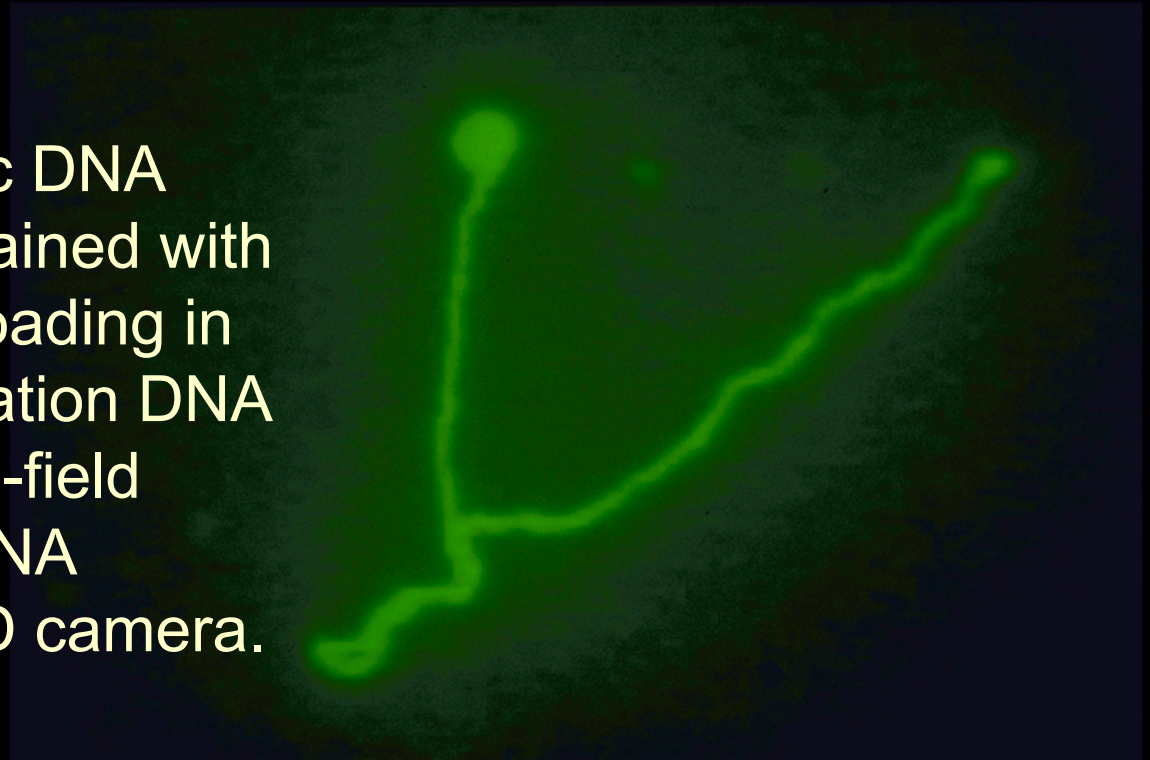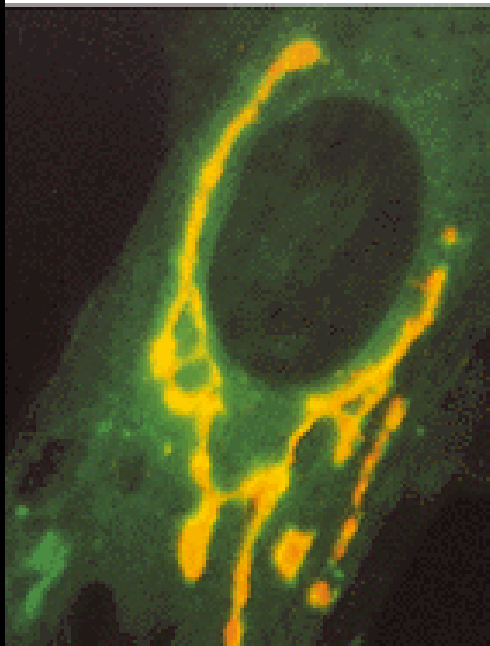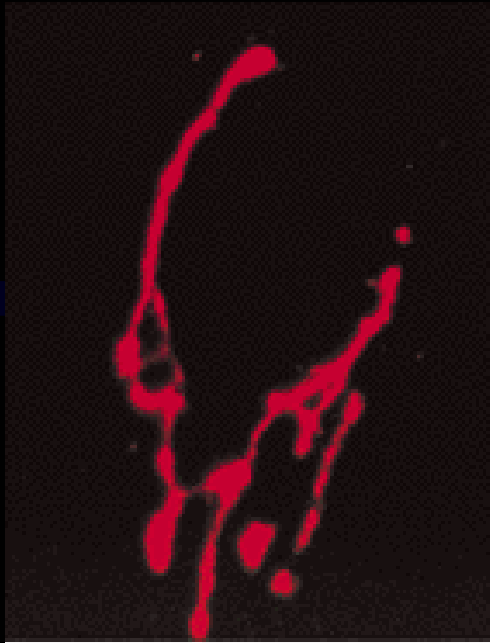
Unfortunately, YOYO-1 bleaches very fast.

Viability

2 dyes, 1excitation, 2 emissions, 1 LP filter set

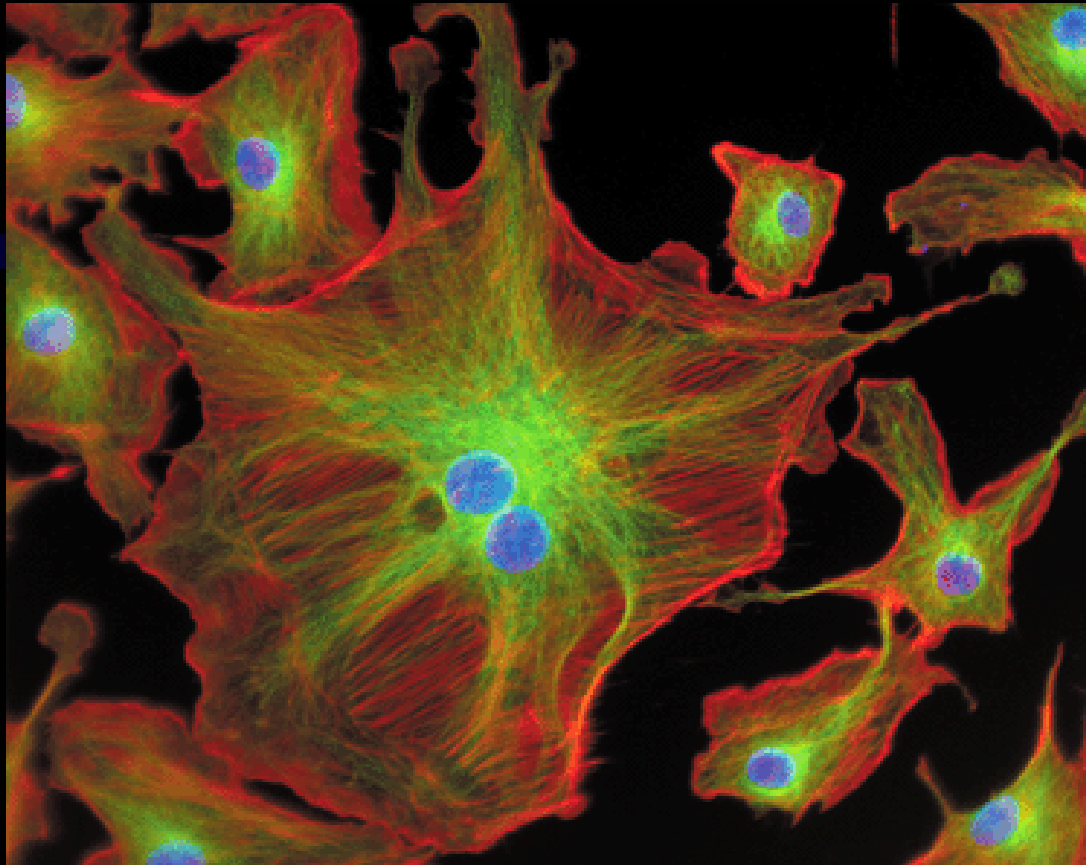calcien AM (green, live cells), ethidium homodimer (red, dead cells)

Organelles

1 dye, 2 excitation, 2 emissions,
switch between 2 filter sets

BODIPY FL C5-ceramide accumulation in
the trans-Golgi is sufficient for excimer
formation (top panel).

Images by Richard Pagano, Mayo Foundation.

Wells, 9/15/03

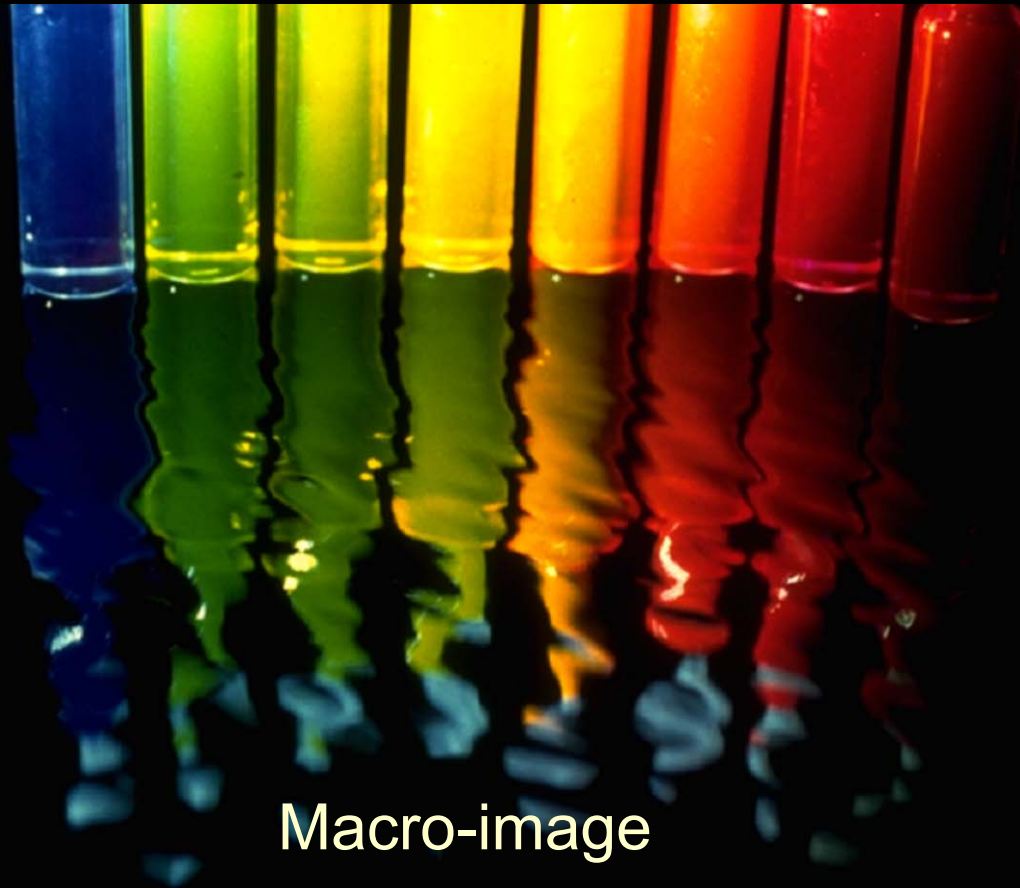**Cytoskeleton**

3 dyes, 3 excitation, 3 emissions

BPAE cells: microtubules (green, Bodipy-antibody), F-actin (red, Texas Red-X phalloidin) and nuclei (blue, DAPI). Triple exposure on 35mm film. This can be done with a triple-band filter set or by 3 filter sets.

1 (UV) excitation "band," 8 (visible) emission "bands" detected simultaneously on 35mm color film

Macro-image

Physical Origins of Fluorescence

1 Dye excited state created by absorption of a photon from an external light source

2 Fluorescence photon emitted has lower energy (longer wavelength) than excitation photon. Ratio of emitted photons/absorbed photons (fluorescence quantum yield) is usually less than 1.0

3 Unexcited dye is regenerated for repeat absorption/emission cycles. Cycle can be broken by photobleaching (irreversible photochemical destruction of excited dye)

Energy Flow: Photons→Electrons→Photons

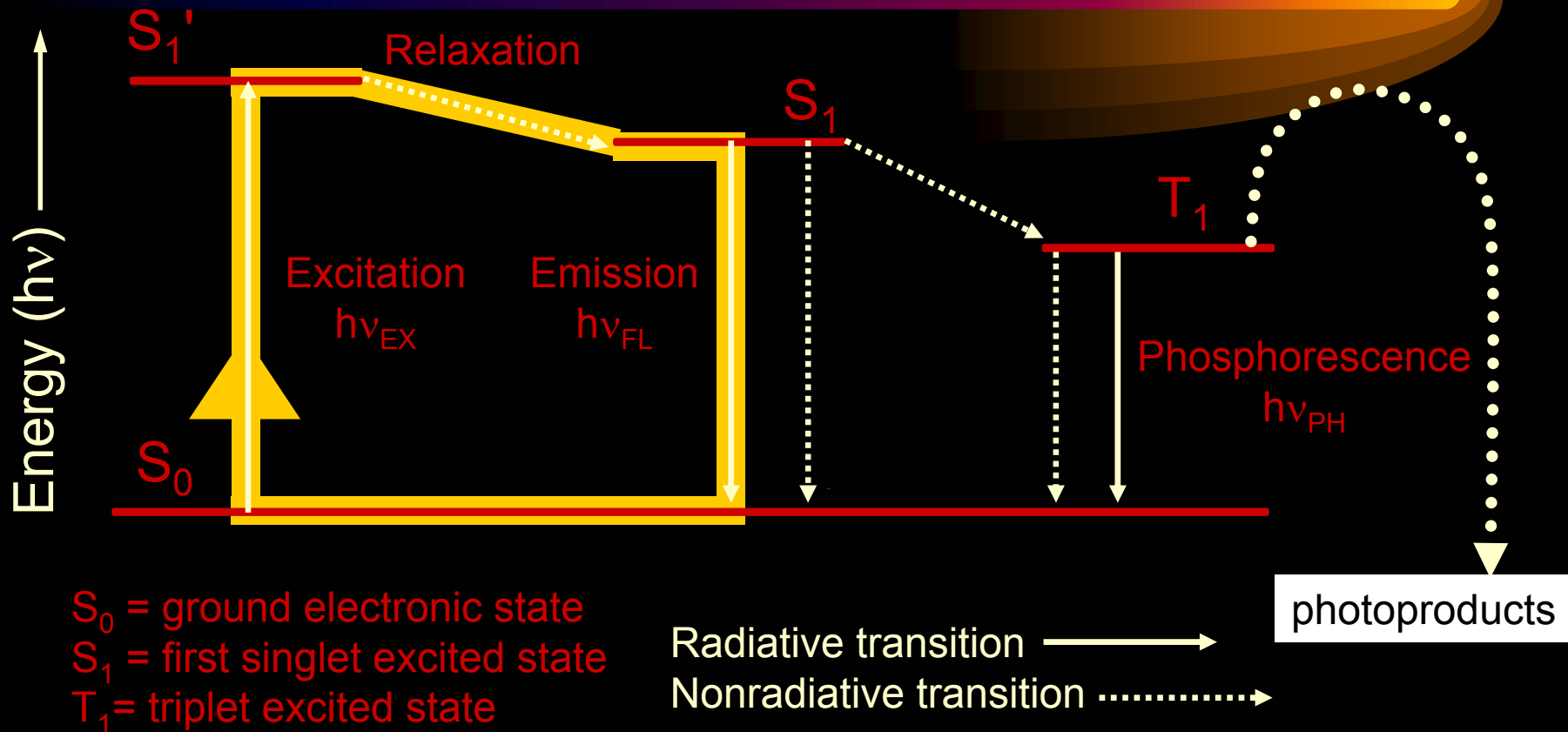Different dye molecules exhibit different excitation and emission "profiles."

BCECF Acid

Energy is proportional to $1/\lambda$
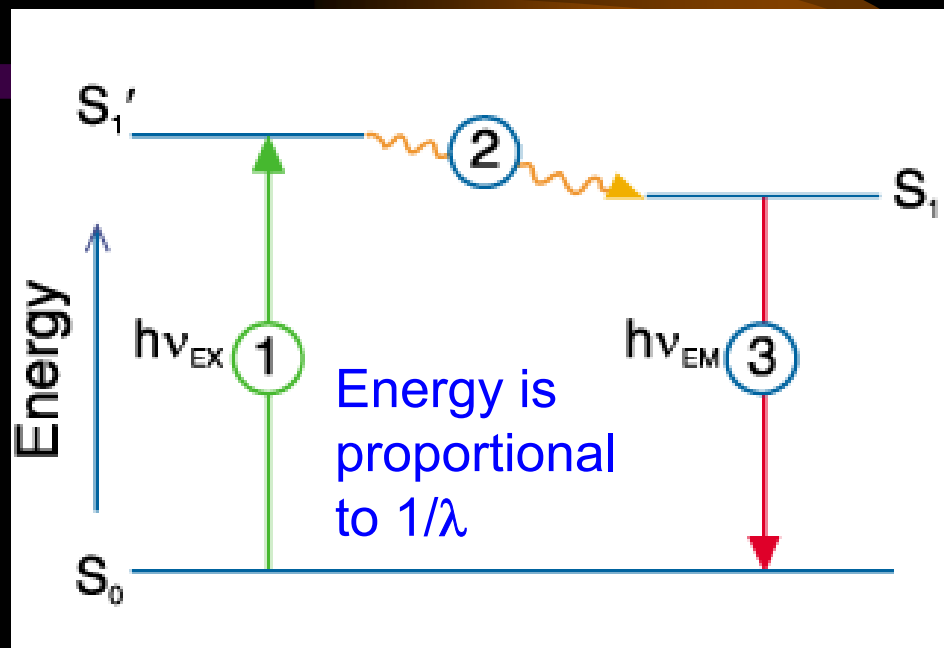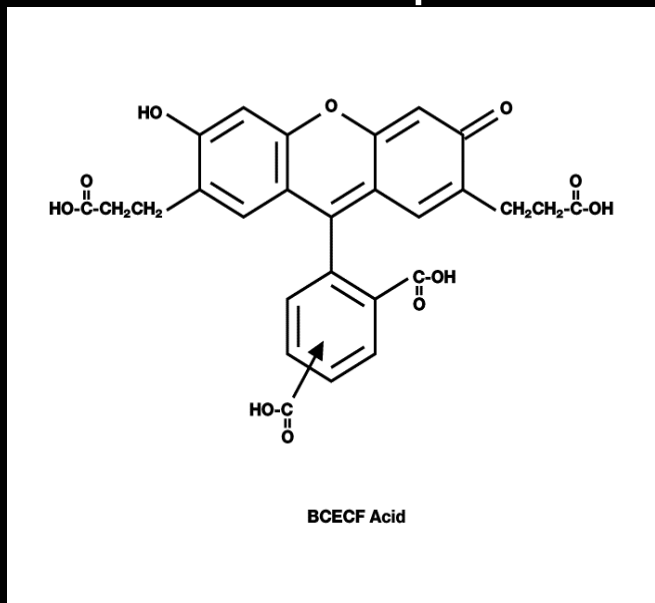
1 = absorbance of light (excitation)
2 = vibronic relaxation, (Stokes shift)
3 = emission of light

Fluorescence Excitation-Emission Cycle

Energy ($h\nu$)

S_1'

Relaxation

S_1

T_1

Excitation $h\nu_{EX}$

Emission $h\nu_{FL}$

Phosphorescence $h\nu_{PH}$

S_0

photoproducts

S_0 = ground electronic state
S_1 = first singlet excited state
T_1 = triplet excited state

Radiative transition ⟶
Nonradiative transition ┈┈▶

Iain Johnson [Molecular Probes]

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Molecular Electronic State Transitions
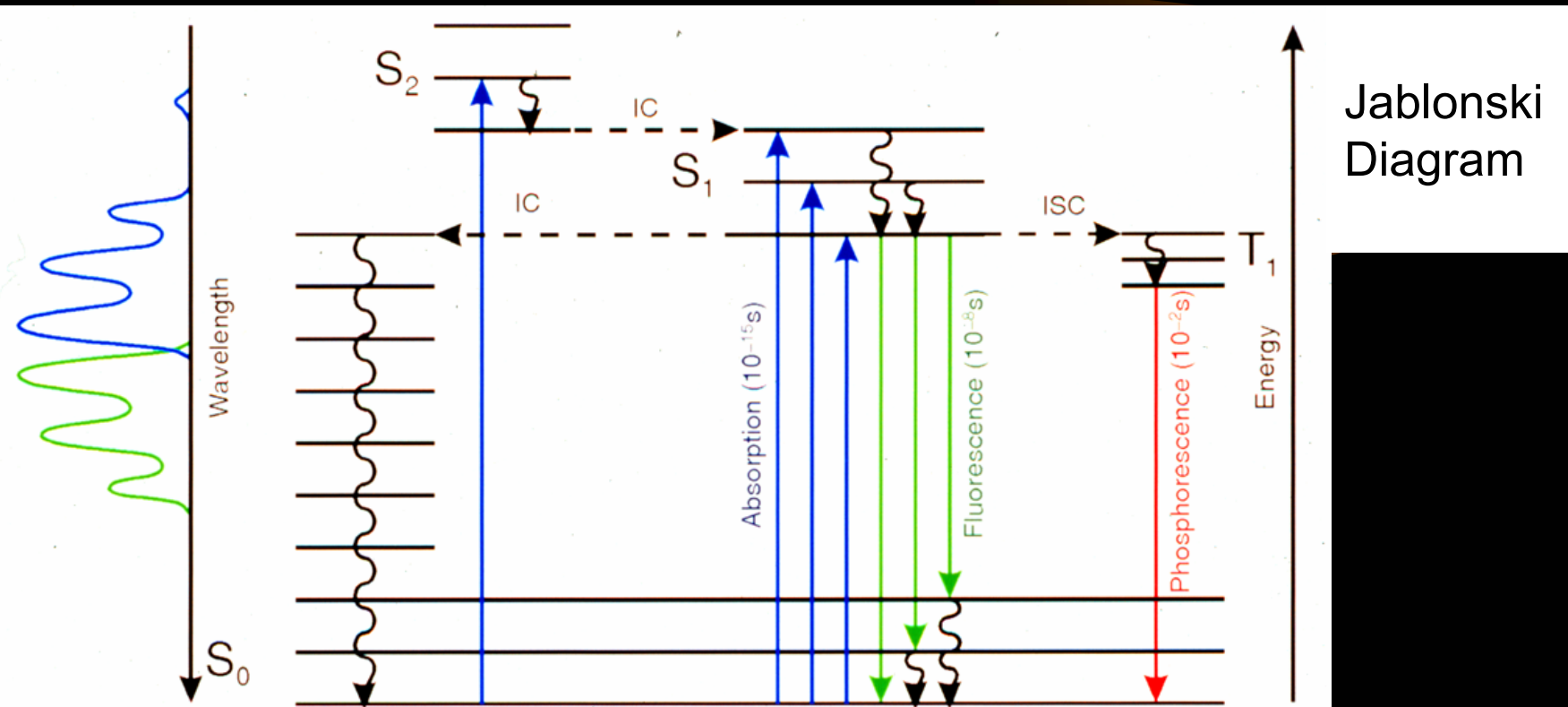
Jablonski Diagram

IC = Internal Conversion ($S_2 \rightarrow S_1$ 10^{-12}s; $S_1 \rightarrow S_0$ 10^{-8}s)

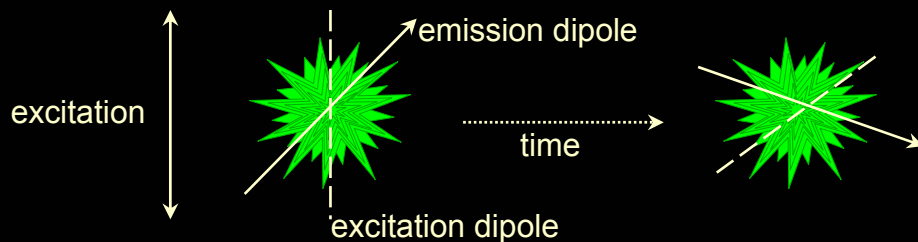ISC = Intersystem Crossing ($T_1 \rightarrow S_0$ 10^{-8}s)

$\sim\sim\sim\rightarrow$ = Vibrational Relaxation < 10^{-12}s

Iain Johnson [Molecular Probes]
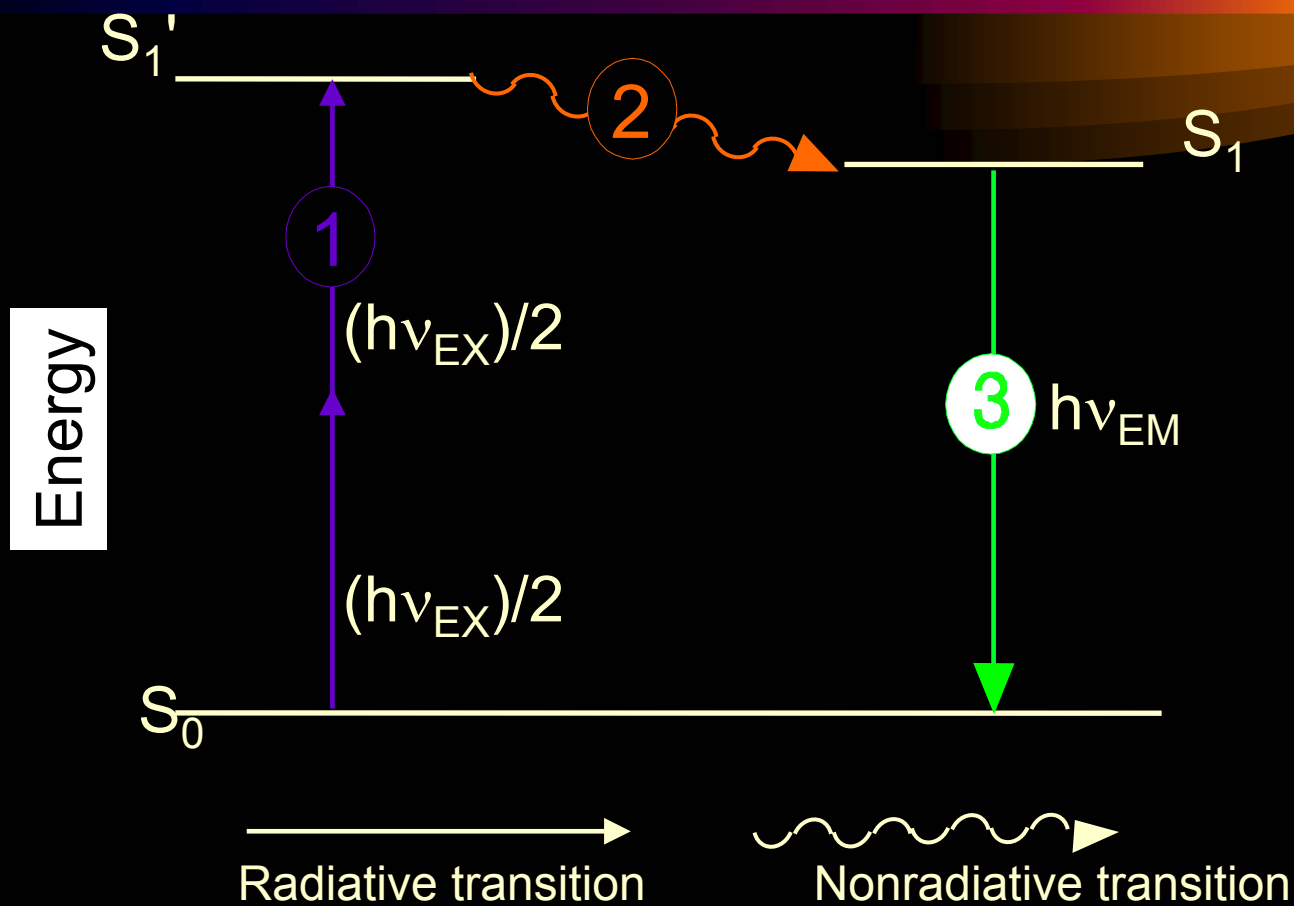
Molecular Interactions and Fluorescence

• Intermolecular Energy Transfer - characterized by donor-acceptor pairing
    short range exchange interactions within interatomic collision diameter
    long range interactions by sequential short range exchange
    optical interactions between transition dipoles - decays as $1/r^6$
    exciton migration = electron-hole pair migration - decays as $1/r^3$
    eximer = complex between excited and ground state of same type molecules
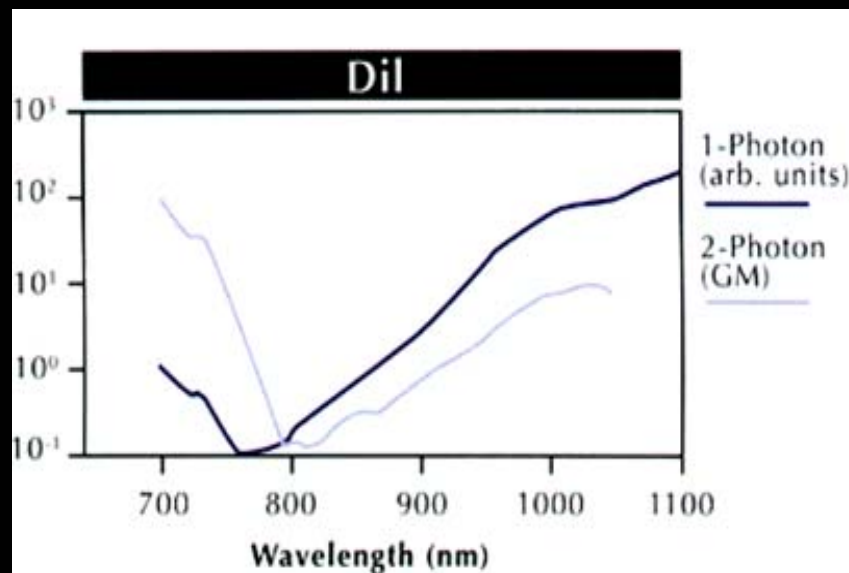   exiplex = complex between excited and ground state of different type molecules

excitation

D < 100 A
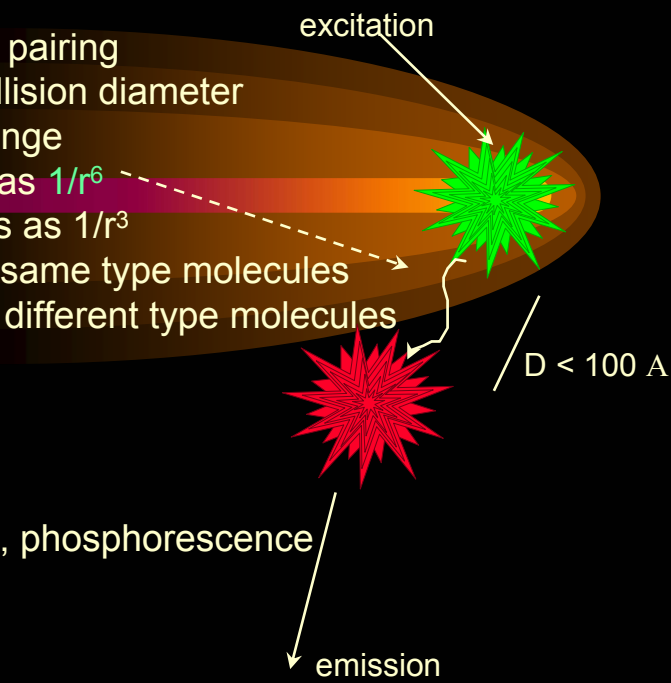
• Intramolecular Energy Transfer
    intersystem crossing, e.g., E-type delayed fluorescence, phosphorescence
    internal conversion, e.g., vibronic transitions

emission

• Polarized Transitions and Rotational Diffusion
    angular orientation between excitation and emitted light polarization changes
    when a fluorophore rotates during the excited-state lifetime (τ_f)

excitation

emission dipole

time

excitation dipole

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Two Photon Excitation

S_1'

② (circle 2, orange)

S_1

① (circle 1)

$(h\nu_{EX})/2$

③ $h\nu_{EM}$

Energy

$(h\nu_{EX})/2$

S_0

→ Radiative transition

∿→ Nonradiative transition

One-photon ≠ Two-photon Excitation Spectra
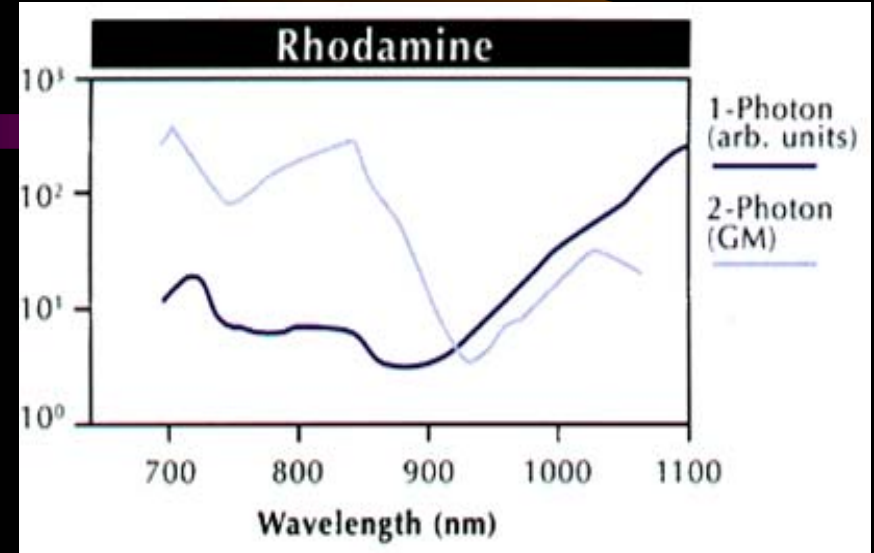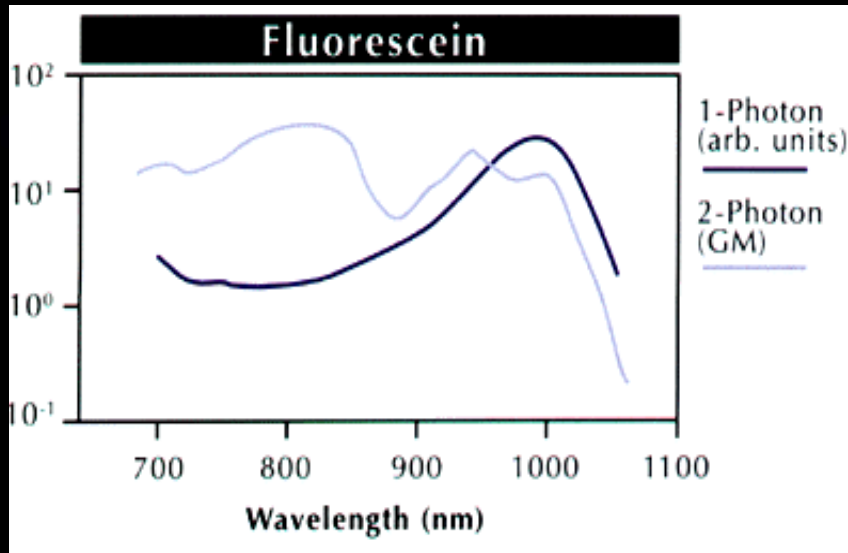
Xu, Zipfel, Shear, Williams, Webb. Proc Natl Acad Sci USA 93:10763 (1996)

Quantitative Fluorometry

• Measured fluorescence intensities are products of **dye-dependent** (ε, QY), **sample-dependent** (c, L) and **instrument-dependent** (I_0, k) factors

• Sample-to-sample and instrument-to-instrument comparisons should ideally be made with reference to standard materials such as fluorescent microspheres

• Attempts to increase signal levels by using higher concentrations of dye may have the opposite effect due to dye–dye interactions (self-quenching) or optical artifacts (e.g. inner filter effects, self-absorption of fluorescence)

Photometric Output Factors: Absorption

- Molar extinction coefficient (aka molar absorptivity). Symbol: ε Units: $cm^{-1}\ M^{-1}$
Defined by the Beer-Lambert law $\log I_0/I_t = \varepsilon \cdot c \cdot l$

- Quantifies efficiency of light absorption at a specific wavelength (ε_{max} = peak value)

- Typically 10,000 – 200,000 $cm^{-1}\ M^{-1}$

- Not strongly environment dependent

Photometric Output Factors: Emission

- Fluorescence quantum yield
  - Symbol: QY or ϕ_F or Q_F Units: none

- Number of fluorescence photons emitted per photon absorbed

- Typically 0.05 – 1.0

- Strongly environment dependent

- Other emission parameters: lifetime, polarization

Quantifying Fluorescence Intensity

$$I_F = I_0 \cdot QY \cdot (1 - e^{-2.303\,\varepsilon \cdot c \cdot L}) \cdot k$$

For $(\varepsilon \cdot c \cdot L) < 0.05$

$$I_F = I_0 \cdot QY \cdot (2.303 \cdot \varepsilon \cdot c \cdot L) \cdot k = \text{Fluorescence emission intensity at } \lambda_{EM}$$

QY = Fluorophore quantum yield

ε = Molar extinction coefficient of fluorophore at λ_{EX}

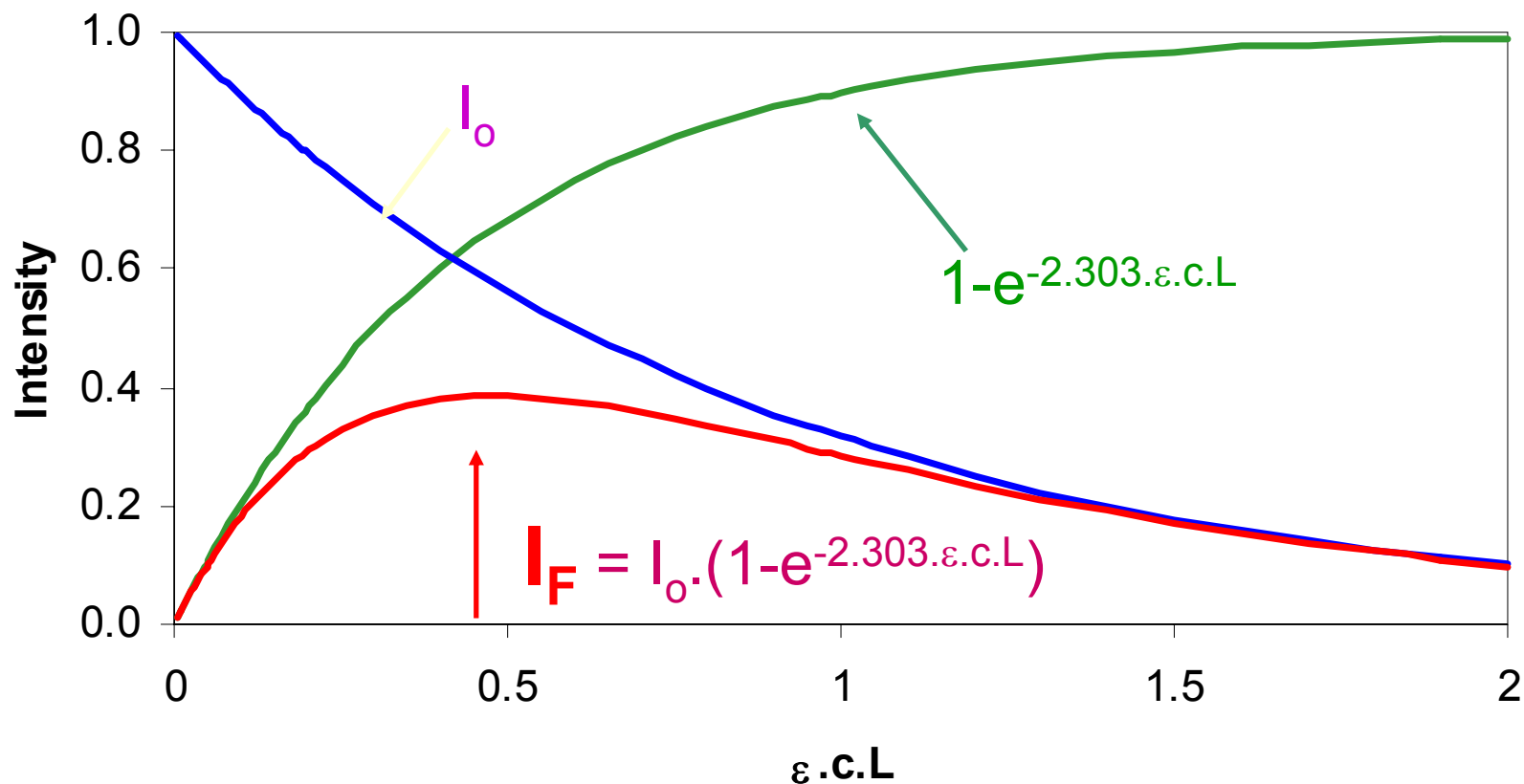c = Fluorophore concentration

L = Optical pathlength for excitation

I_0 = Excitation source intensity at λ_{EX}

k = Fluorescence collection efficiency*

*In a typical epifluorescence microscope, about 3% of the total available emitted photons are collected (k =0.03) [Webb et al, PNAS 94, 11753 (1997)]

I_F will decrease when $(\varepsilon \cdot c \cdot L) > 0.05$
Inner Filter Effect Simulation

I_o

$1-e^{-2.303 \cdot \varepsilon \cdot c \cdot L}$

$I_F = I_o \cdot (1-e^{-2.303 \cdot \varepsilon \cdot c \cdot L})$

Intensity

$\varepsilon.c.L$

Iain Johnson [Molecular Probes]

Total Output Limit: Photobleaching

- Irreversible destruction of excited fluorophore

- Proportional to time-integrated excitation intensity

- Avoidance: minimize excitation, maximize detection efficiency, antifade reagents

- Q_B (photobleaching quantum yield). Q_F/Q_B = number of fluorescence cycles before bleaching. About 30,000 for fluorescein.

Photobleaching Reactions

$$^3Dye^* + {}^3O_2 \longrightarrow {}^1Dye + {}^1O_2$$

Photosensitized generation of singlet oxygen

$$^1O_2$$
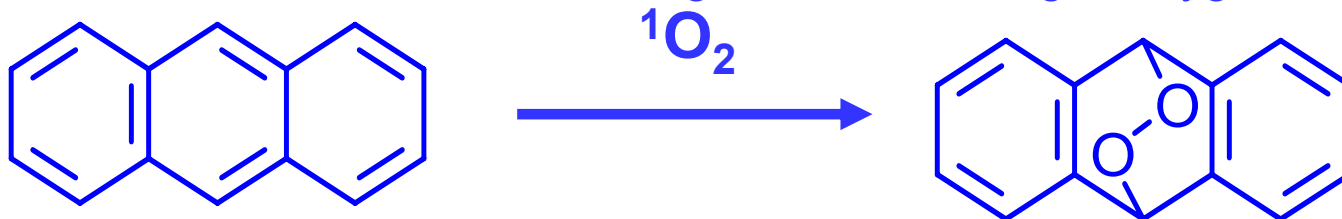
Anthracene → Nonfluorescent endoperoxide

The lifetime of $^1O_2$ in cells is <0.5 μs versus 4 μs in H_2O. Why?

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Dynamic Intensity Parameters

- Electronic Excitation-Relaxation Rate $\quad$ $R = I_0\ OD$

- Excitation-Relaxation Rate Limit $\quad$ $R_{max}\ \alpha\ (\varepsilon_\lambda\ \tau_f)^{-1}$

- Fluorescence Rate (zero bleaching) $\quad$ $K_f = Q_f\ R$

- Bleaching Rate $\quad$ $K_B = Q_B\ R$

- Total Photons Emitted / Molecule $\quad$ Q_f / Q_B

- Bleaching reduces the dye concentration $\quad$ $c(t) = c_0 exp(-K_B t)$

Fluorescence Rate (+ bleaching)

$$F(t) = K_f\ exp(-K_B t)$$

<u>Definitions</u>
I_0 = incident excitation intensity (W/cm^2)
c_0 = dye concentration prior to bleaching (mole/L)
ε_λ = molar extinction coefficient (M^{-1} -cm^{-1})
OD = optical density at <u>fixed λ and pathlength</u> = $\varepsilon_\lambda c_0$
$K_{f,B}$ = fluorescence (f) or bleaching (B) rate (seconds^{-1})
$Q_{f,B}$ = fluorescence (f) or bleaching (B) quantum yield (environmentally dependent)
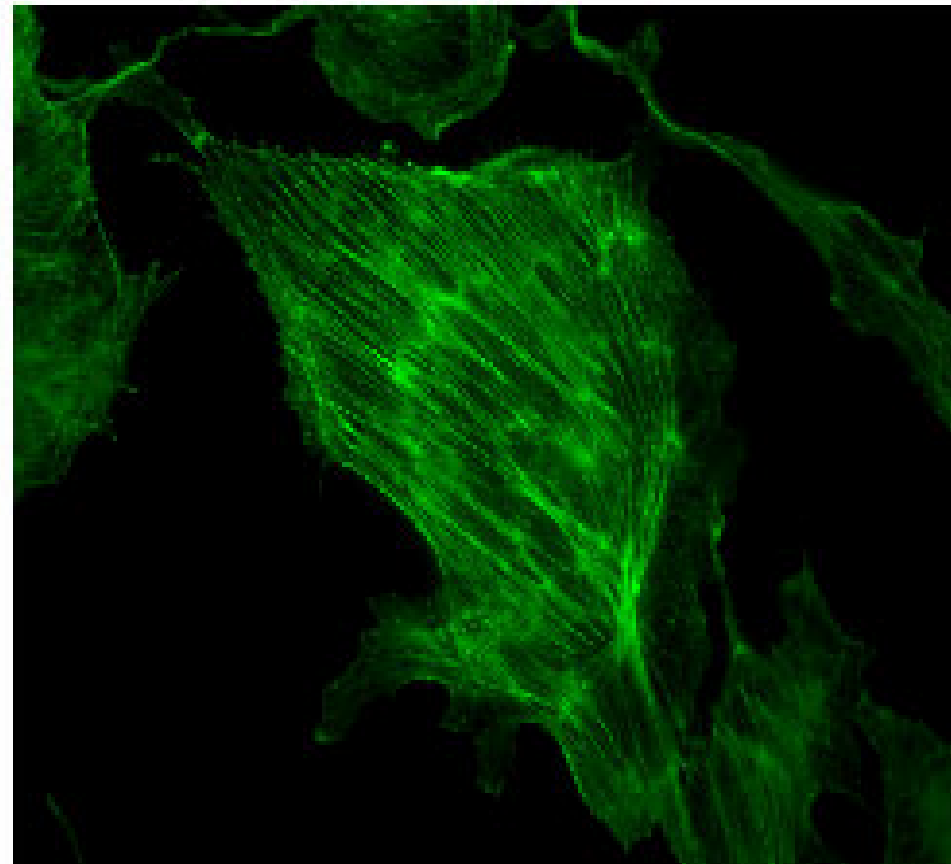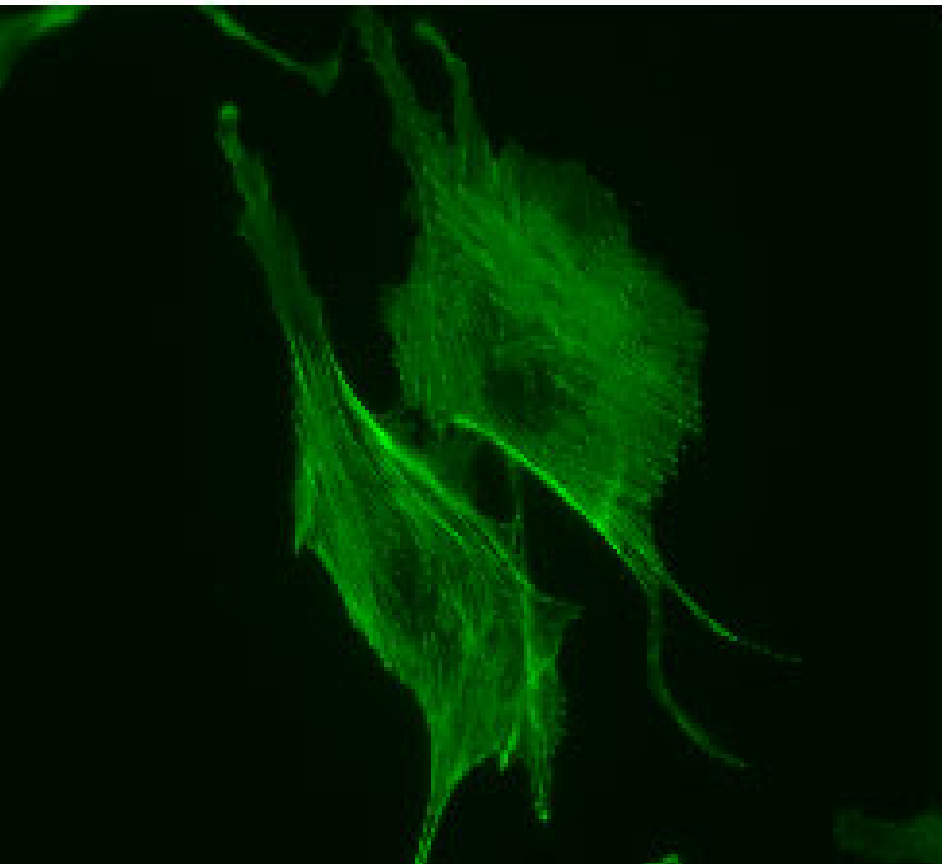τ_f = fluorescence lifetime (seconds)

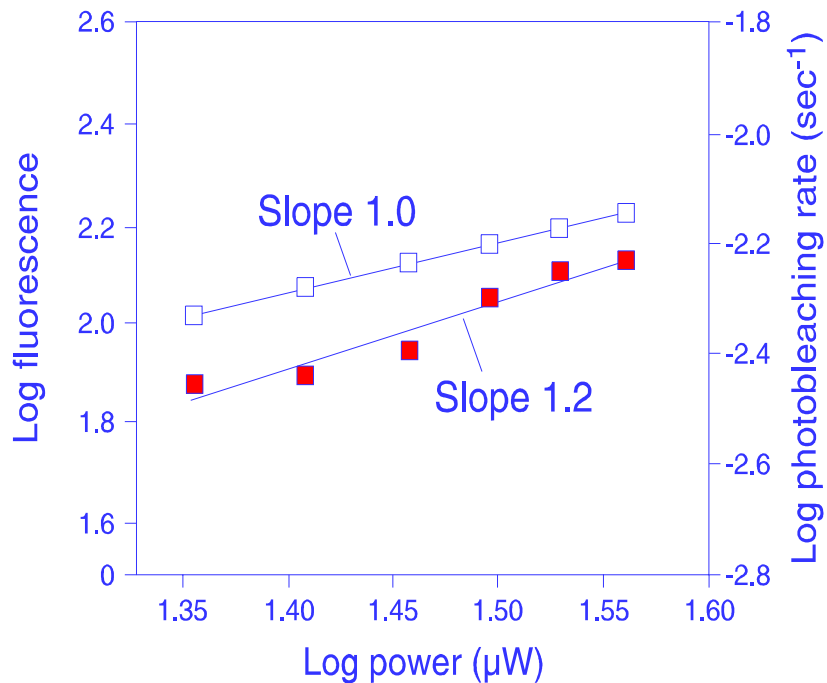# Photobleaching [Q_B] Varies between dyes and chemical environments

Fluorescein

Alexa Fluor 488

00:00
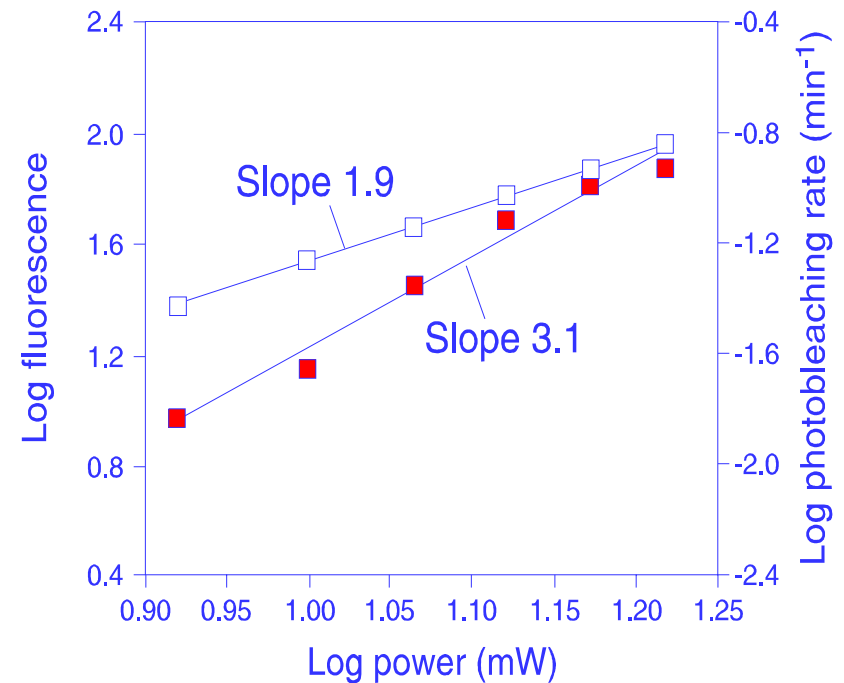
Fluorescein dextran photobleaching under one- and two-photon excitation
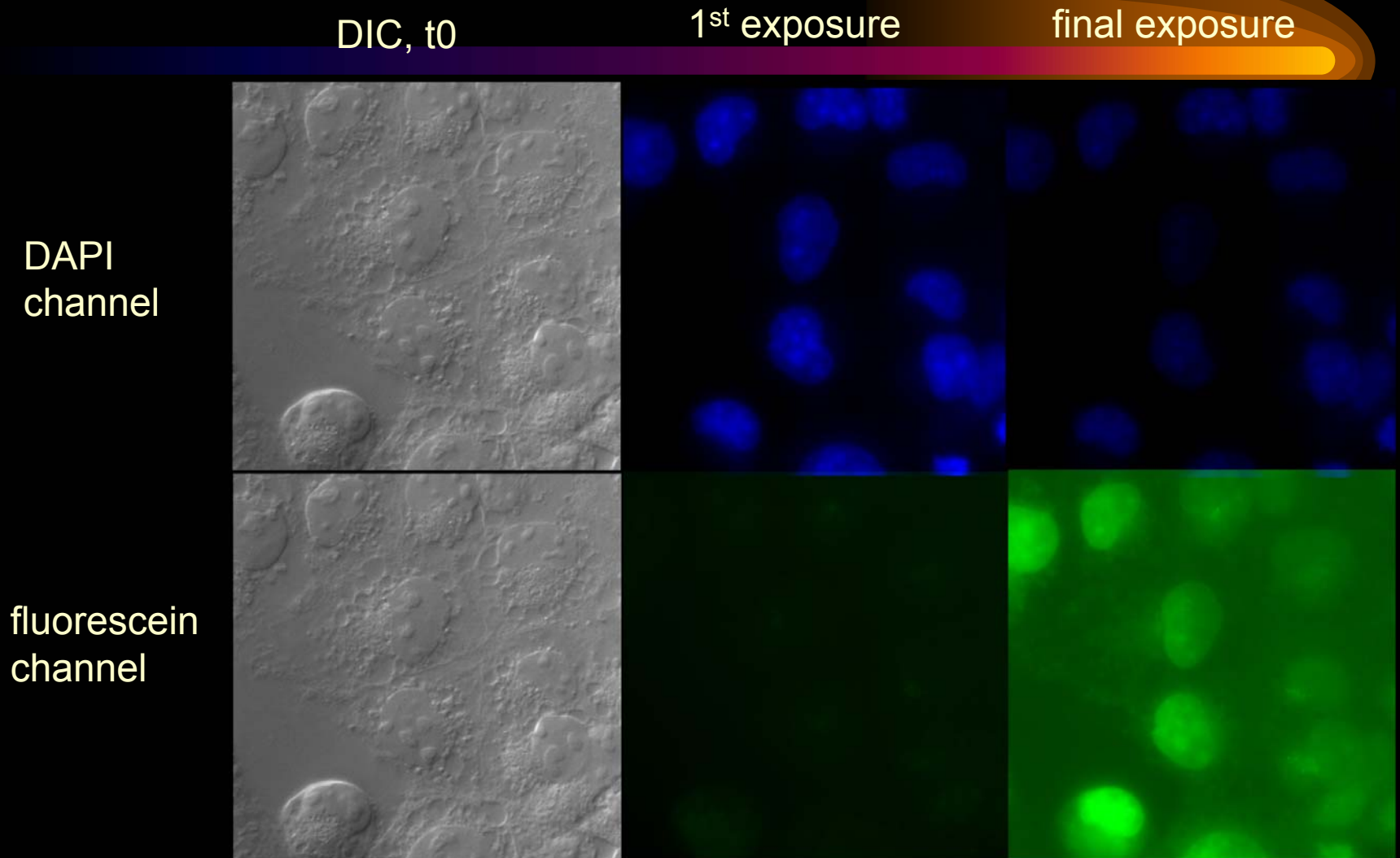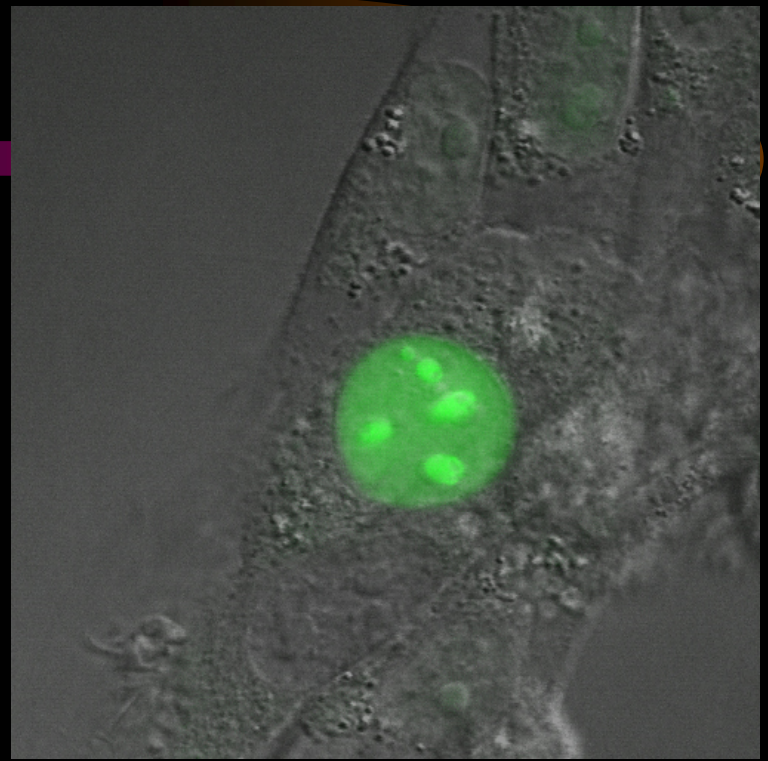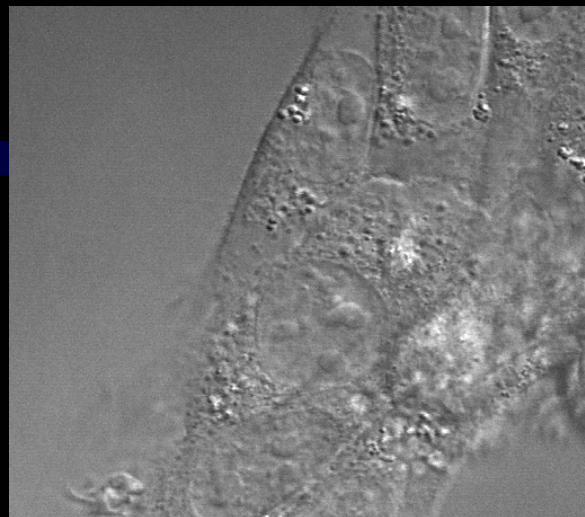
One-photon

Two-photon

☐ Fluorescence intensity   ■ Photobleaching rate

Photochemistry: everything you didn't want to know. Cells containing ONLY DAPI may turn green!
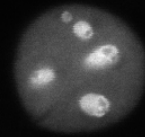

Wells, 9/15/03

Avoid dyes when there not essential to the experiment

DIC

fluorescence

DIC + fluorescence

Antifade Reagents

Fixed Specimens

P-phenylene diamine

N-propyl gallate

DABCO

Hydroquinone

Live Specimens

Mitochondrial particles

Bacterial membrane "Oxyrase"

Vit. C, E

Trolox (water soluble E analogue)

Trolox® is a registered trademark of Hoffman – La Roche

Guiding Principles for Imaging Fluorescence

Select the proper dye for the job – not always obvious

Optimize light collection conditions – use the proper optics, filters, detectors, etc.

Minimize excitation exposure – minimize bleaching, reduce sample perturbations, artifacts

Maximize the dynamic range – contrast depends on this!

Minimize background signal – maximize sensitivity