Principles of Fluorescence

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Outline for Fluorescence

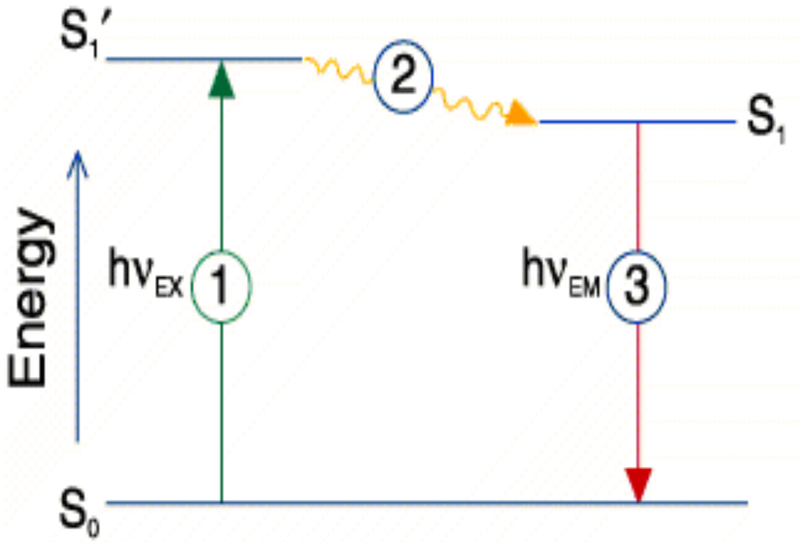
I. Principles of Fluorescence
II. Quantum Yield and Lifetime
III. Fluorescence Intensities
IV. Fluorophores
V. Detecting Fluorophores
VI. Fluorescence Measurements
VII. Applications

I. Principles of Fluorescence

- Fluorophores, native or man made
- Excite with one colour (wavelength A)
- Emits with a different colour (wavelength B)
- Different fluorophores have different colour properties
- Use specialised filters to split colours to see specific fluorescent probes

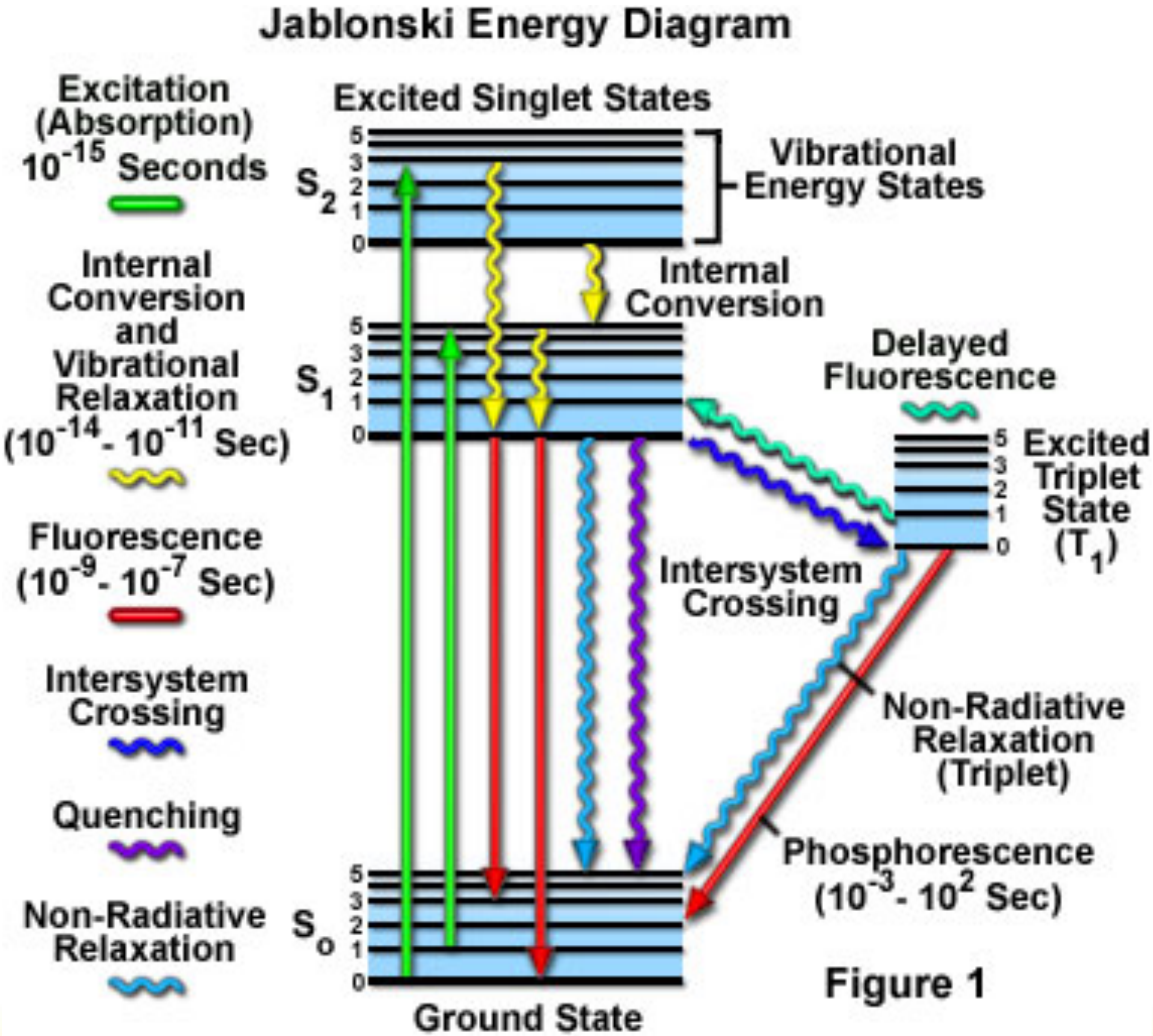
I. Principles of fluorescence

Fluorescence - Photon Release



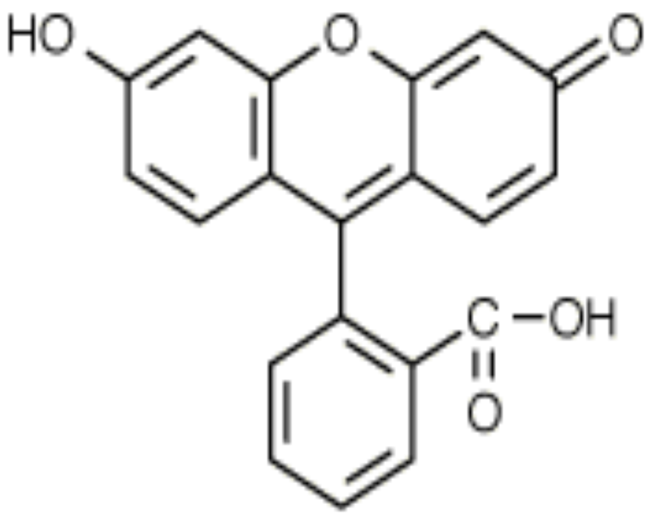
- Electron excited form ground state by absorption of light
- Fluorescence observed as electron decays - photon release
- Energy lost so light emitted at a longer wavelength

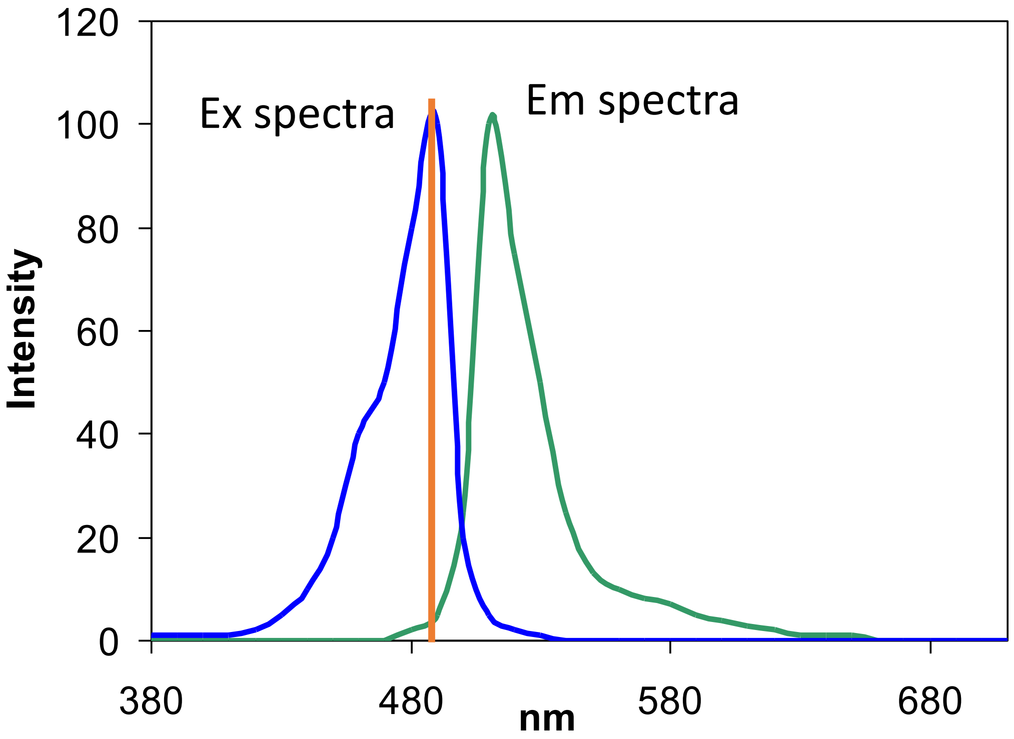
I. Principles of fluorescence



I. Principles of fluorescence

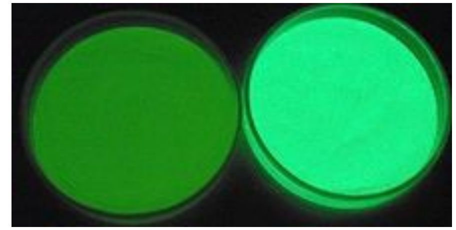
Fluorescein – A Typical Fluorescent Probe





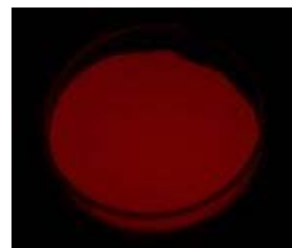
I. Principles of fluorescence

Typical Phosphorescent Probes

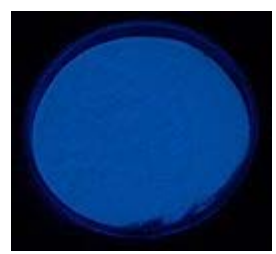


ZnS $Sr_4Al_{14}O_{25}$ ZnS $Sr_4Al_{14}O_{25}$

CaS



Alkaline earth metal silicates e.g. Calcium Silicate



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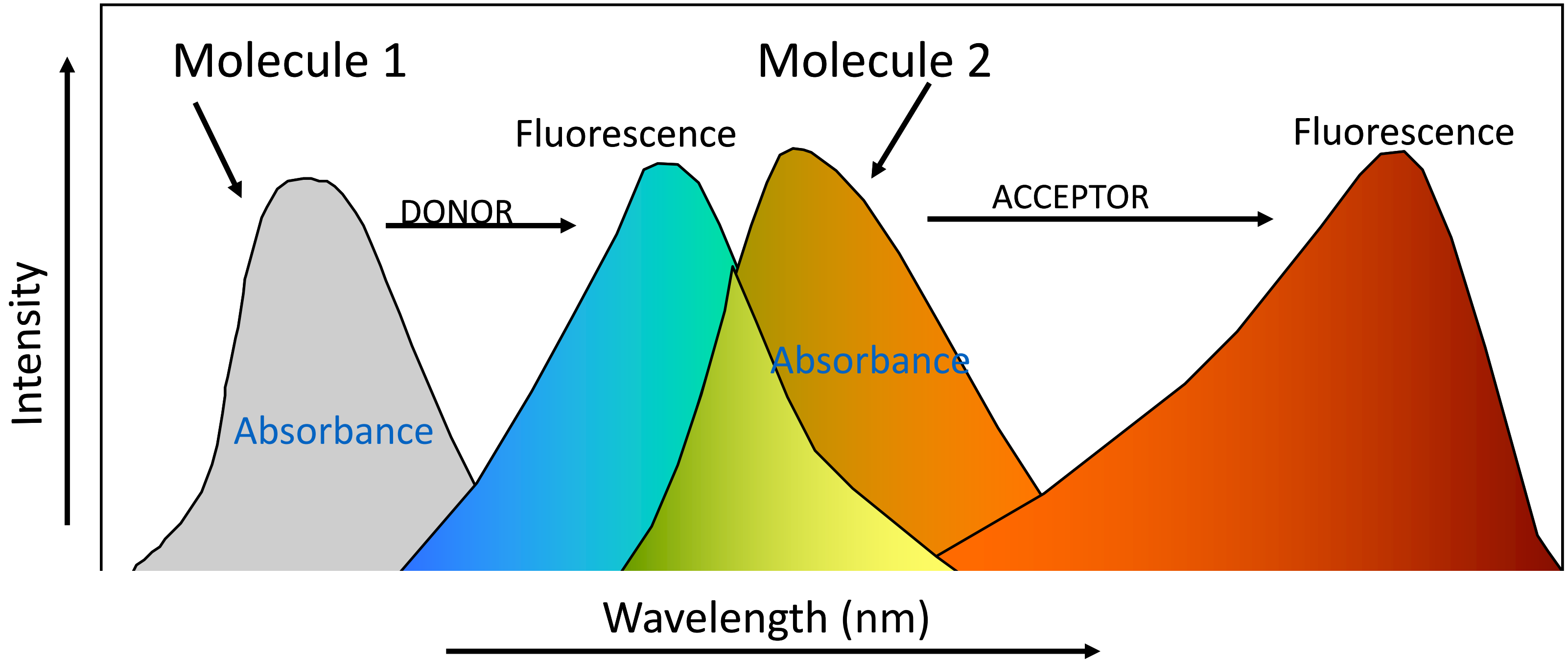
I. Principles of fluorescence

- Fluorescence energy transfer (FRET)



Non radiative energy transfer – a quantum mechanical process of resonance between transition dipoles

Effective between 10-100 Å only

Emission and excitation spectrum must significantly overlap

Donor transfers non-radiatively to the acceptor

II. Quantum yield

Quantum yield of fluorescence

- Quantum yield of fluorescence, Φ_f, is defined as:

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

- In practice, is measured by comparative measurements with reference compound for which has been determined with high degree of accuracy.

- Ideally, reference compound should have
  - the same absorbance as the compound of interest at given excitation wavelength
  - similar excitation-emission characteristics to compound of interest (otherwise, instrument wavelength response should be taken into account)
  - Same solvent, because intensity of emitted light is dependent on refractive index (otherwise, apply correction

$$\frac{\Phi_f^u}{\Phi_f^s} = \frac{I_f^u}{I_f^s} \times \frac{n^2(u)}{n^2(s)}$$

  - Yields similar fluorescence intensity to ensure measurements are taken within the range of linear instrument response

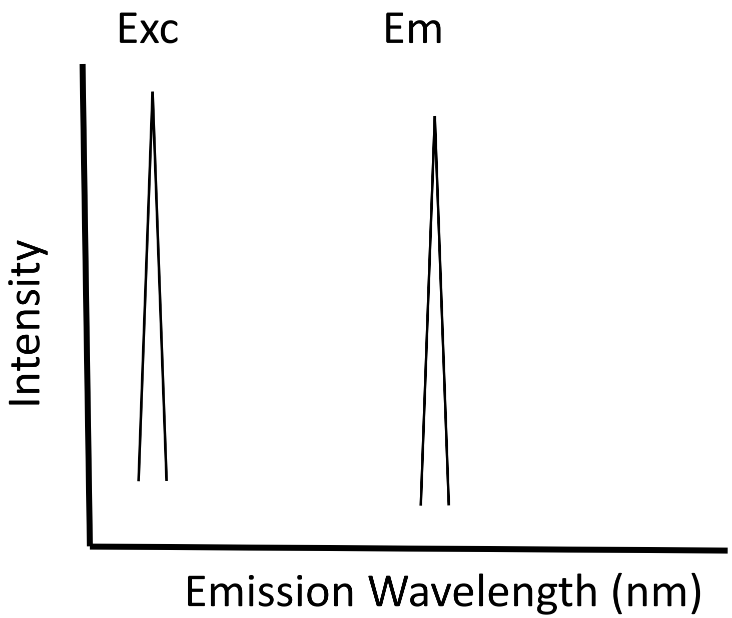
II. Fluorescence Lifetime

Effect on fluorescence emission

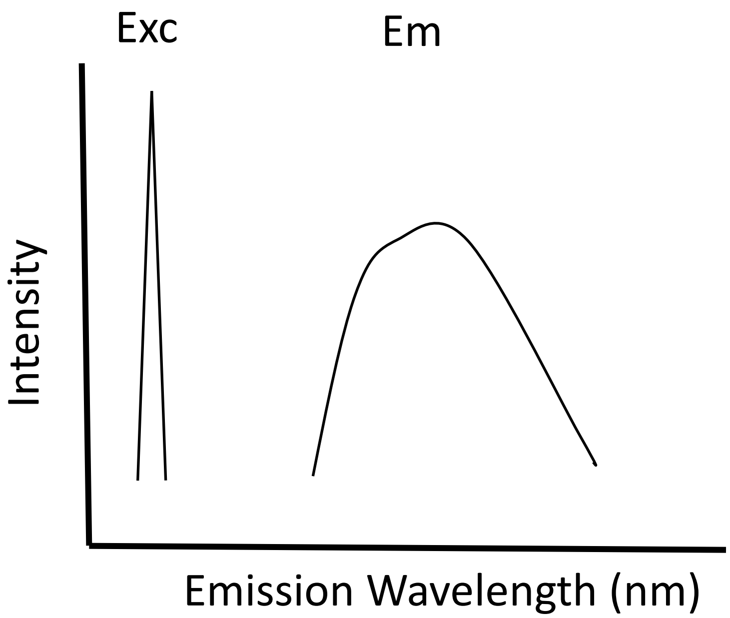
- Suppose an excited molecule emits fluorescence in relaxing back to the ground state
- If the excited state lifetime, τ is long, then emission will be monochromatic (single line)
- If the excited state lifetime, τ is short, then emission will have a wider range of frequencies (multiple lines from multiple vibrational states)

II. Fluorescence Lifetime

Large $\Delta\tau$ – small ΔE



Small $\Delta\tau$ – large ΔE



III. Fluorescence Intensities

1a. Fluorescence intensity

The fluorescence intensity (F) at a particular excitation (λ_x) and emission wavelength (λ_m) will depend on the absorption and the quantum yield:

$$\mathbf{F}(\lambda_{\mathbf{x}}, \lambda_{\mathbf{m}}) = \mathbf{I}_{\mathbf{A}}(\lambda_{\mathbf{x}})\phi(\lambda_{\mathbf{m}})$$

where,

I_A – light absorbed to promote electronic transition

ϕ – quantum yield

III. Fluorescence Intensities

1b. From the Beer-Lambert law, the absorbed intensity for a dilute solution (very small absorbance)

$$I_A(\lambda_x) = 2.303 I_o \varepsilon(\lambda_x) CL$$

$$\text{for } \varepsilon(\lambda_x) CL << 1$$

where,

I_o – Initial intensity

ε – molar extinction coefficient

C – concentration

L – path length

III. Fluorescence Intensities

1c. Fluorescence intensity expression

The fluorescence intensity (F) at a particular excitation (λ_x) and emission wavelength (λ_m) for a dilute solution containing a fluorophore is:

$$\mathbf{F}(\lambda_{\mathbf{x}}, \lambda_{\mathbf{m}}) = \mathbf{I_o}\, 2.303\, \varepsilon(\lambda_{\mathbf{x}})\mathbf{CL}\, \phi(\lambda_{\mathbf{m}})$$

where,

I_o – incident light intensity

C – concentration

L – path length

ϕ – quantum yield

ε – molar extinction coefficient

IV. Fluorophores

1. Native biological molecules
2. Organic Fluorophores
3. Quantum Dots
4. Up-conversion nanoparticles
5. Luminescent nanoparticles
6. Fluorescent nanoparticles

IV. Biological Fluorophores

–Endogenous Fluorophores

- amino acids
- structural proteins
- enzymes and co-enzymes
- vitamins
- lipids
- porphyrins

–Exogenous Fluorophores

- Cyanine dyes
- Photosensitizers
- Molecular markers – GFP, etc.

Endogenous fluorophores	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural proteins		
Collagen	325	400, 405
Elastin	290, 325	340, 400
Enzymes and coenzymes		
FAD, flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B_6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5′-phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340–395	540, 430–460
Ceroid	340–395	430–460, 540
Porphyrins	400–450	630, 690

FAD, flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; AND(P)H, reduced nicotinamide adenine dinucleotide phosphate.

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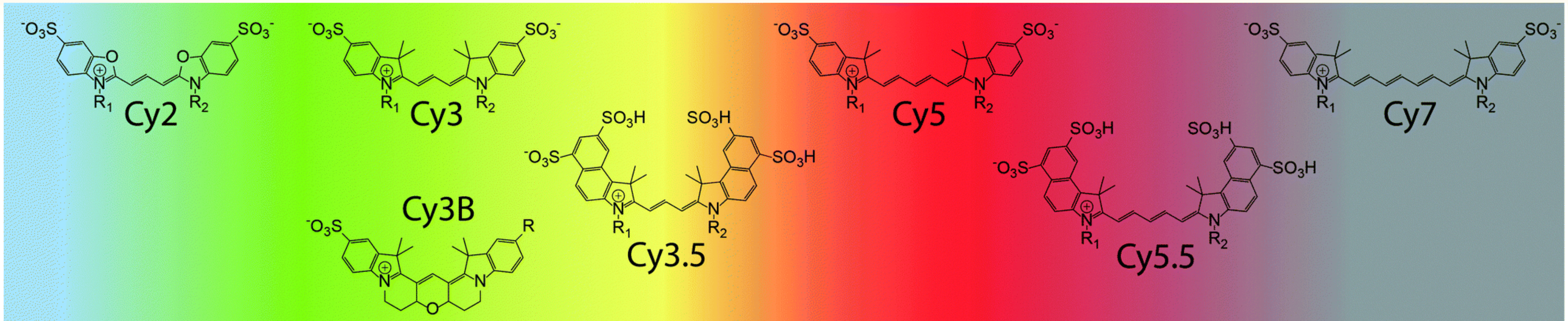
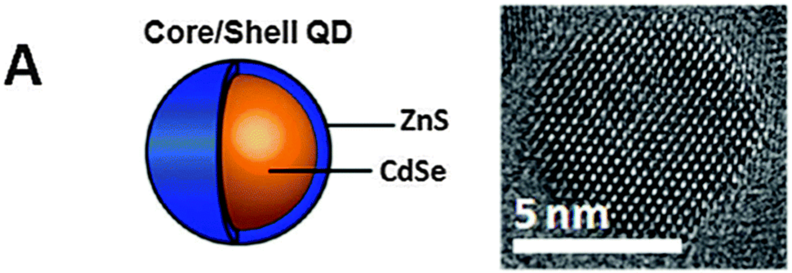
IV. Organic Fluorophores

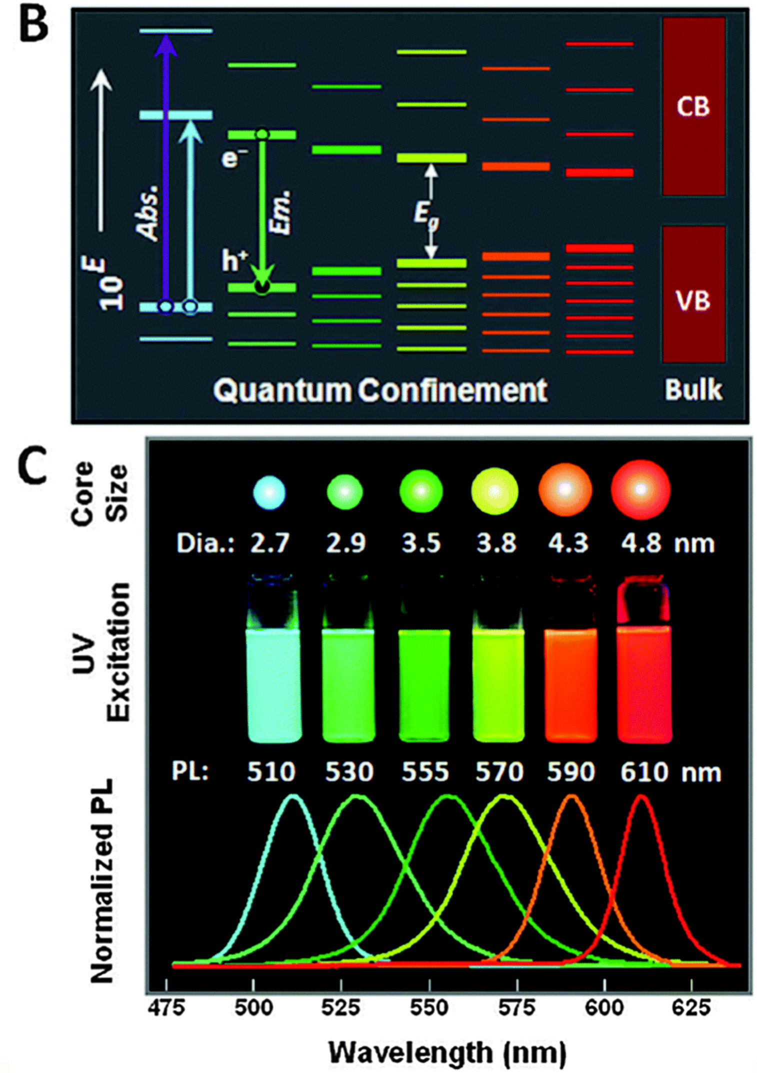


	Cy2	Cy3	Cy3B	Cy3.5	Cy5	Cy5.5	Cy7
λ_{abs} (nm)	489 nm [i,a]	550 nm [i,a]	559 nm [i,a]	581 [i,a]	649 nm [i,a]	675 nm [i,a]	747 nm [i,a]
λ_F (nm)	506 nm [i,a]	570 nm [i,a]	570 nm [i,a]	596 [i,a]	670 nm [i,a]	694 nm [i,a]	776 nm [i,a]
ε_{max} (10^5 $M^{-1}cm^{-1}$)	1.3 [ii,b,*]	1.5 [iii,a] 1.5 [v,a/b]	1.3 [iii,a]	1.2 [xiv,a] 1.25 [xiv,c]	2.2 [iv,b,*] 2.5 [v,a/b]	1.9 [xiv,a] 1.95 [xiv,c]	2.5 [vi,b,*] 2.0 [v,a/b]
τ_F (ns)	0.2 [vii,a]	0.2 [vii,a] < 0.3 [iii,a] 0.18 [xiii,a]	2.8 [iii,a] 2.7 [xiii,a]	0.6 [xvi,a]	0.9 [vii,a] 0.98 [iv,b,*]	0.83 [xvii,a]	0.4 [vii,a]
Φ_F	0.05 [viii,b,*] 0.04 [xi,e,*] 0.053 [xii,c,*]	0.04 [iii,a] 0.09 [xiii,a] 0.04 [v,a] 0.09[v,b]	0.67 [iii,a] 0.85 [xiii,a]	0.14 [xiv,a] 0.28 [xiv,c]	0.21 [iv,b,*] 0.27 [v,a] 0.4 [v,b]	0.23 [xiv,a] 0.24 [xiv,c]	0.28 [vi,b,*]
τ_T (μs)	[–]	520 [xv,b,*] 3.9 [ix,c]	[–]	[–]	60 [iv,b,*] 63 [x,d]	[–]	[–]
Φ_{ISC}	< 0.001 [xi,e,*]	< 0.001 [iv,e,*] 0.03 [ix,c]	[–]	[–]	< 0.003 [iv,b,*]	[–]	[–]

http://pubs.rsc.org/en/content/articlehtml/2014/cs/c3cs60237k

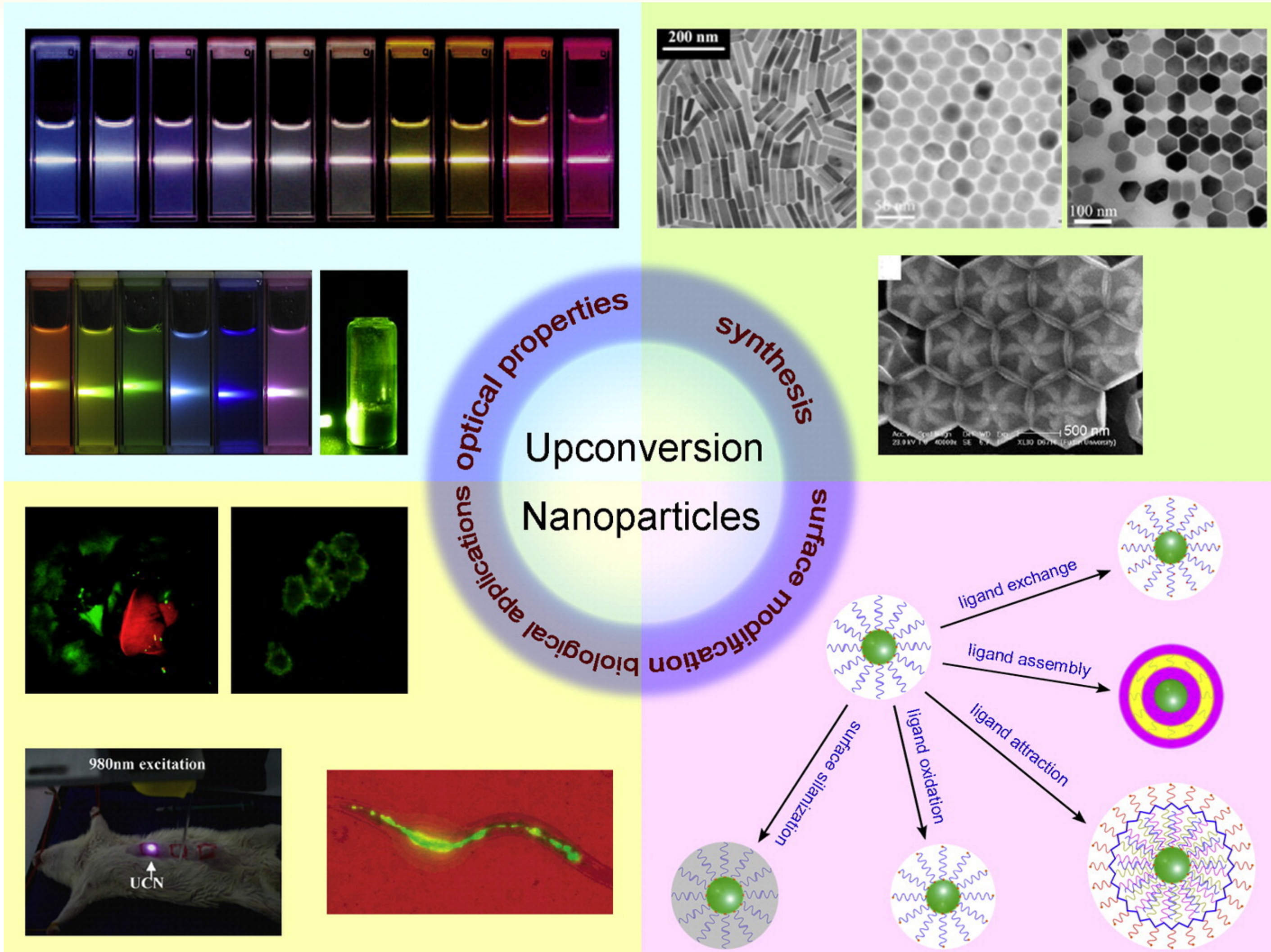
IV. Quantum Dots





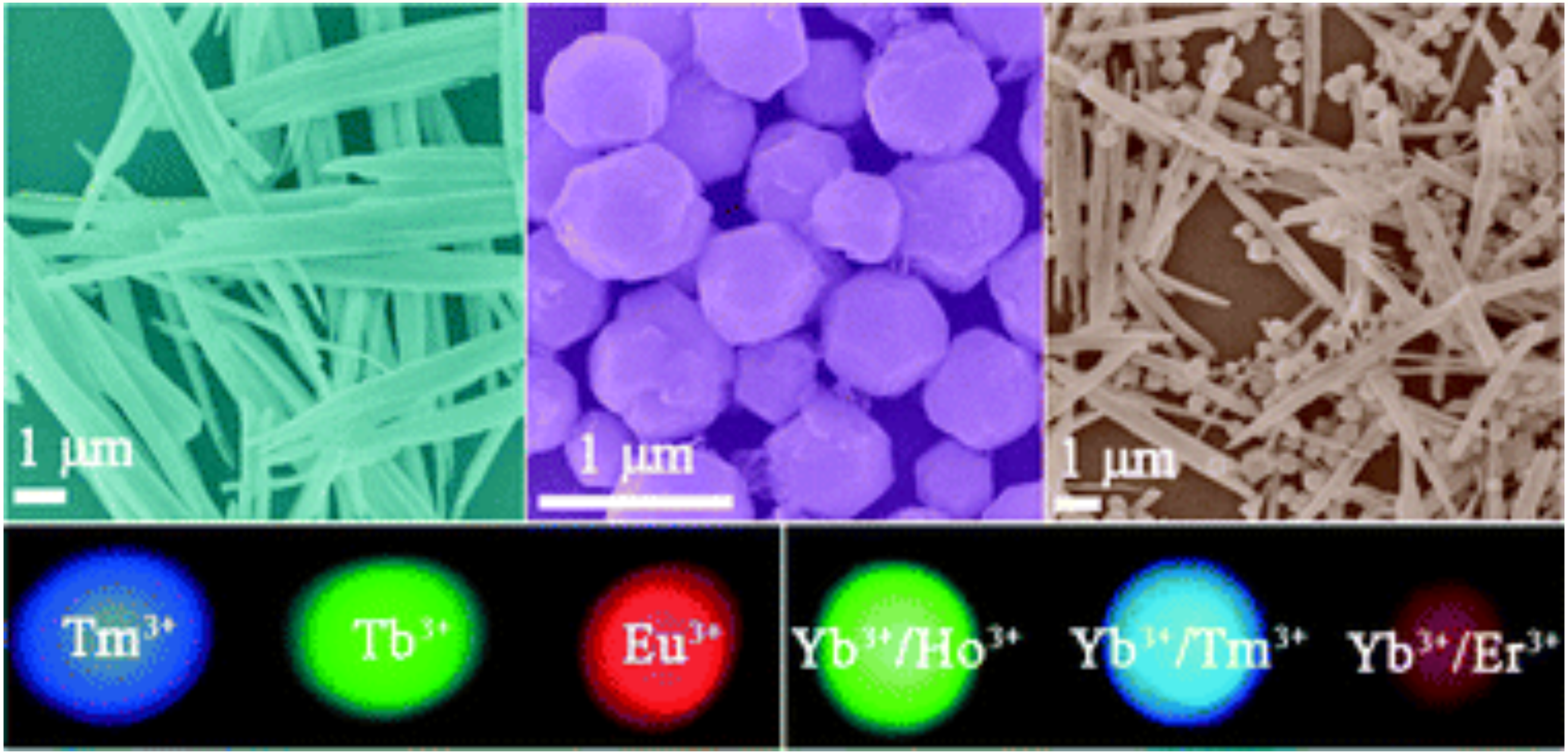
http://pubs.rsc.org/en/Content/ArticleHtml/2015/CS/c4cs00532e

IV. Up-conversion Nanoparticles



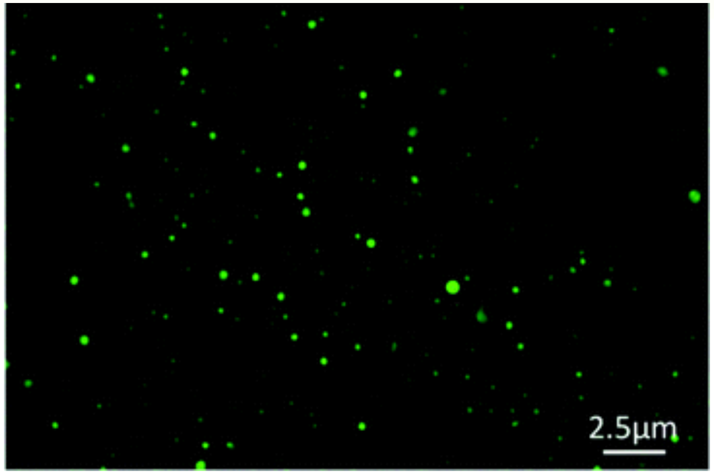
http://www.sciencedirect.com/science/article/pii/S1549963411000979#f0025

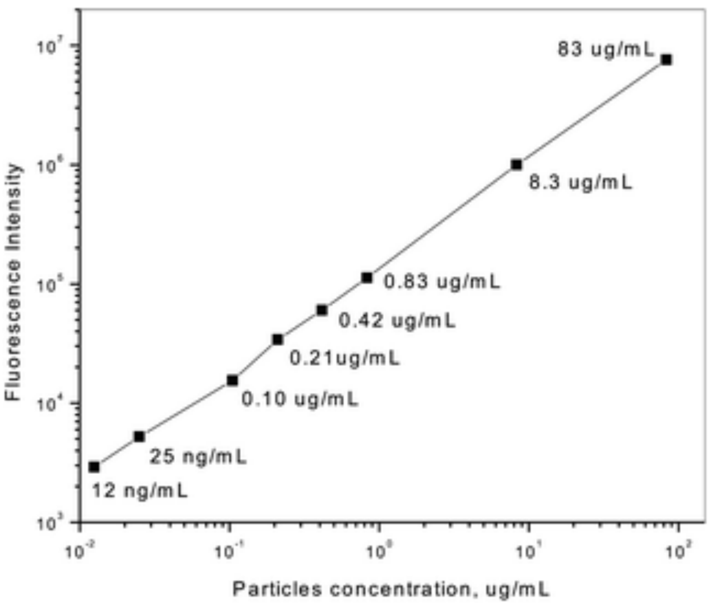
IV. Luminescent Nanoparticles

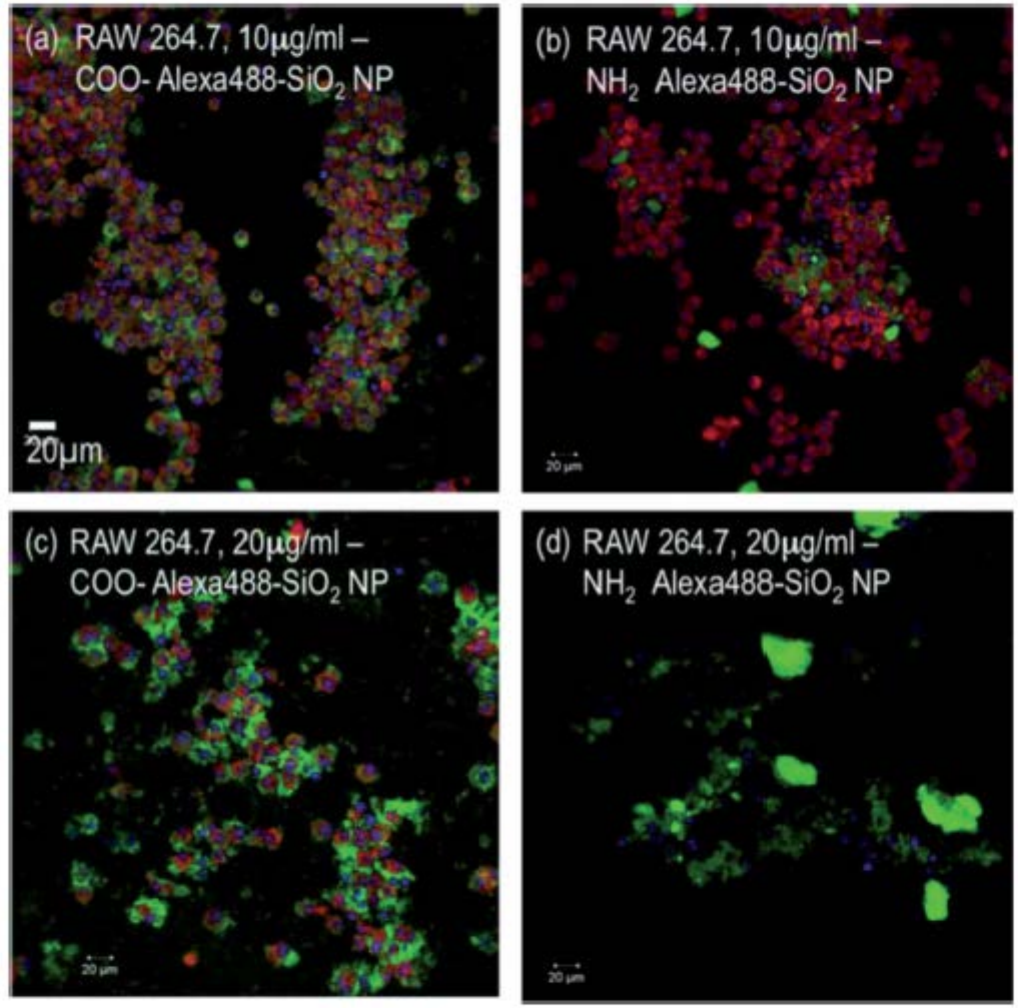


http://blogs.rsc.org/ce/2014/02/18/ph-controlled-formation-of-doped-yof-luminescent-particles/

IV. Fluorescent Silica Nanoparticles







10.1039/C3NR02639F

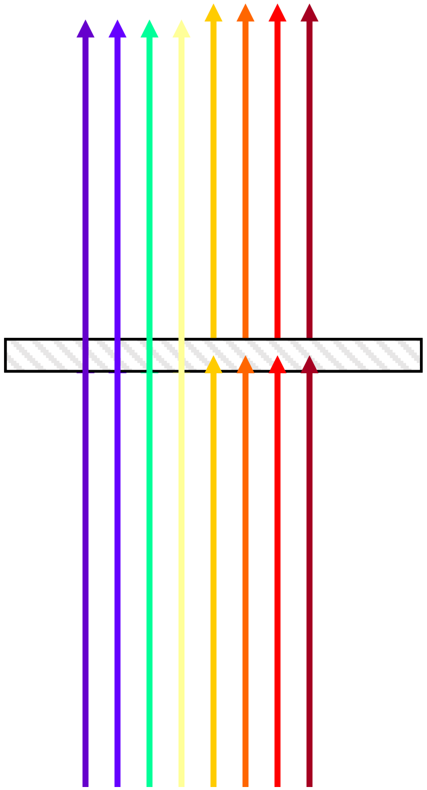
V. Detecting Fluorophores

- Dichroic and Filter System
- Use specialised filters to split colours to see specific fluorescent probes

V. Detecting Fluorophores

Long Pass Filter

- Typically permits transmission of all light above a set wavelength e.g. 500nm
- Used for single labelled samples and for maximum light gain
- Short Pass Filter

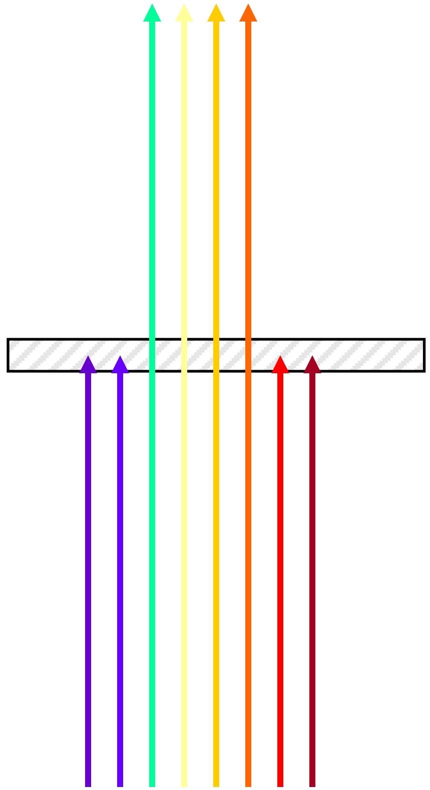


Short Pass
Long Pass

V. Detecting Fluorophores

Band Pass Filter

- Permits transmission of light between two defined wavelengths e.g. 530 $\pm$ 15nm
- Used for multiple labelled samples or to help reduce background fluorescence

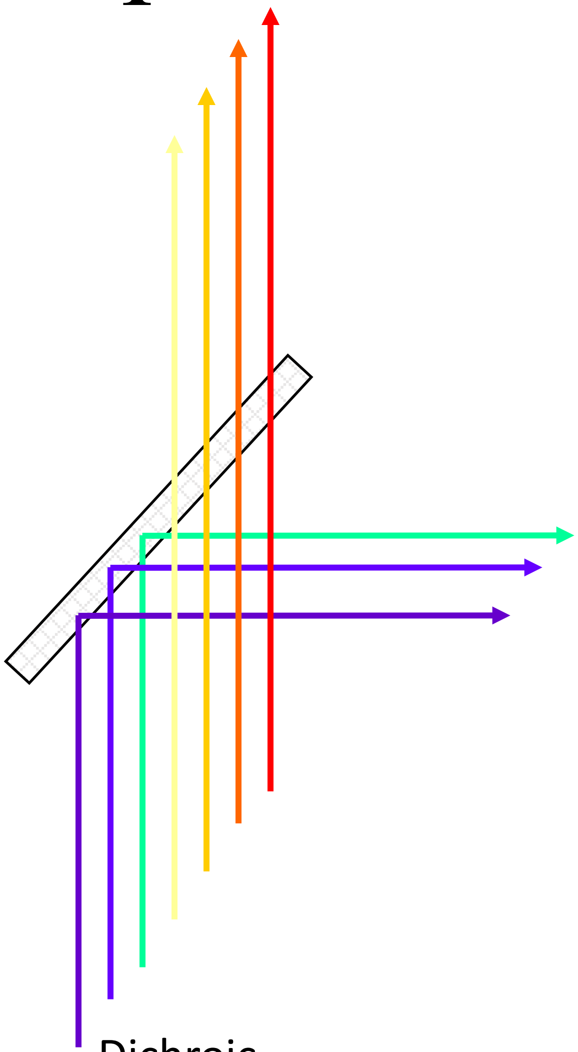


Band Pass

V. Detecting Fluorophores

Dichroic Filter

- Reflects light up to one wavelength and transmits light beyond specified wavelength or vice versa
- Used to excite sample with one wavelength, but also enables emission light to be directed to detector



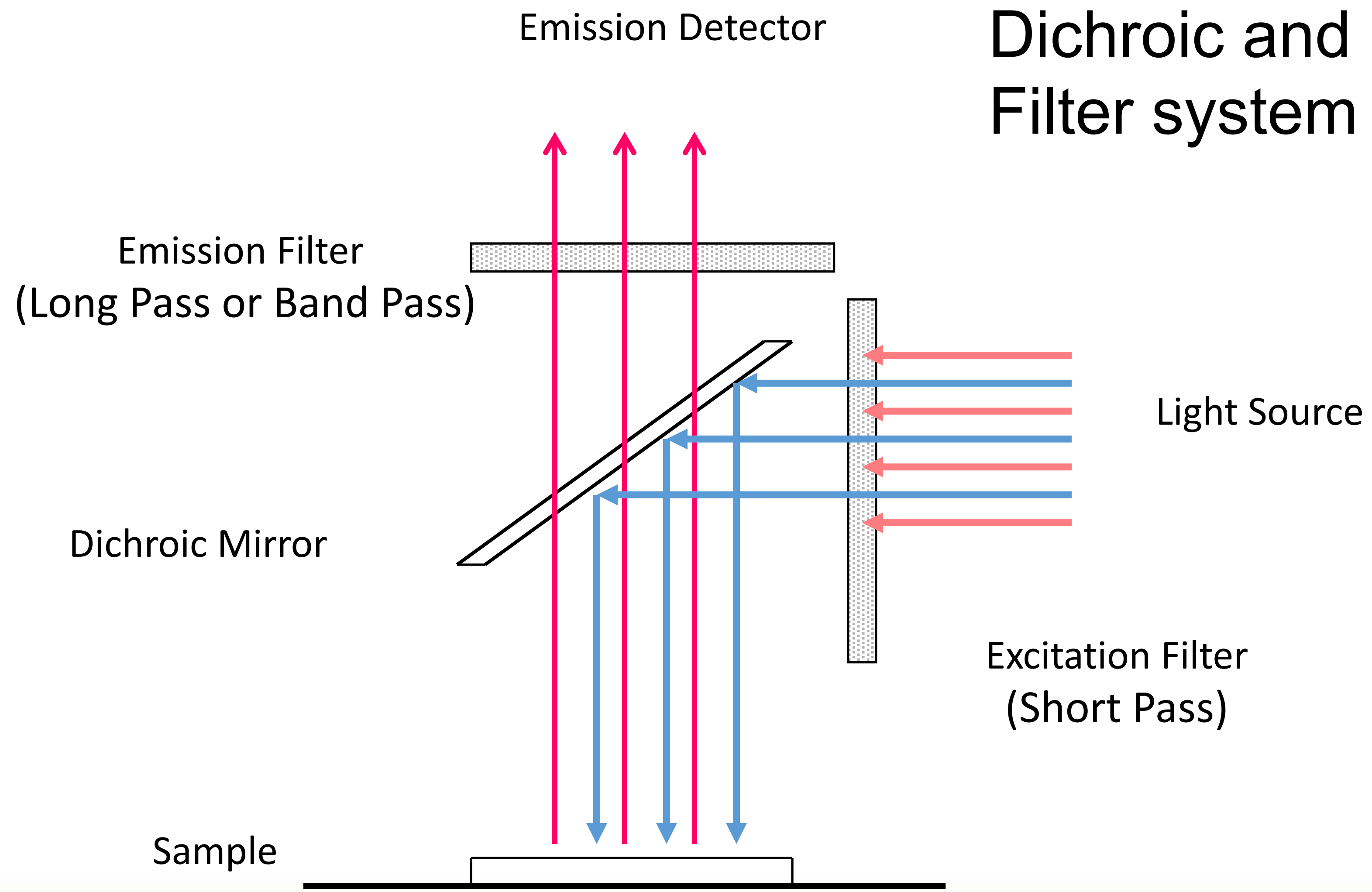
Dichroic

V. Detecting Fluorophores

No filter or dichroic is perfect!

Always use controls

V. Detecting Fluorophores



V. Detecting Fluorophores

Fluorescence Microscopy

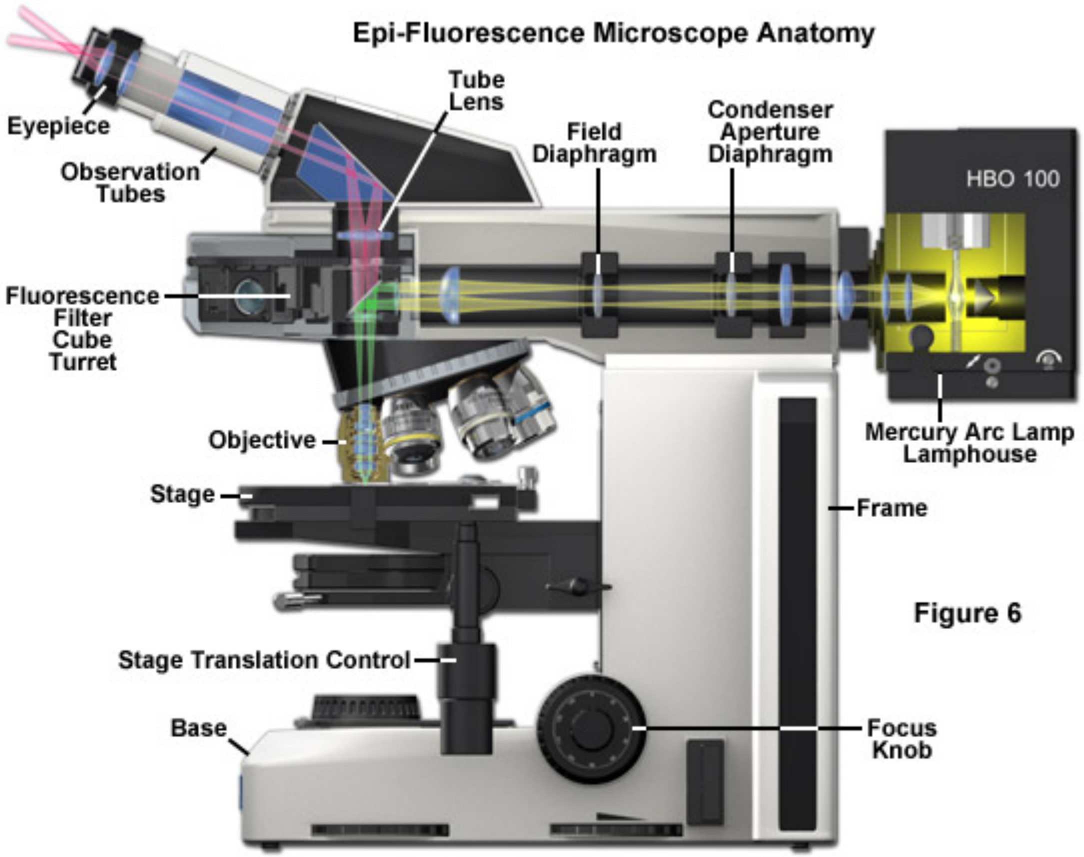


Figure 6

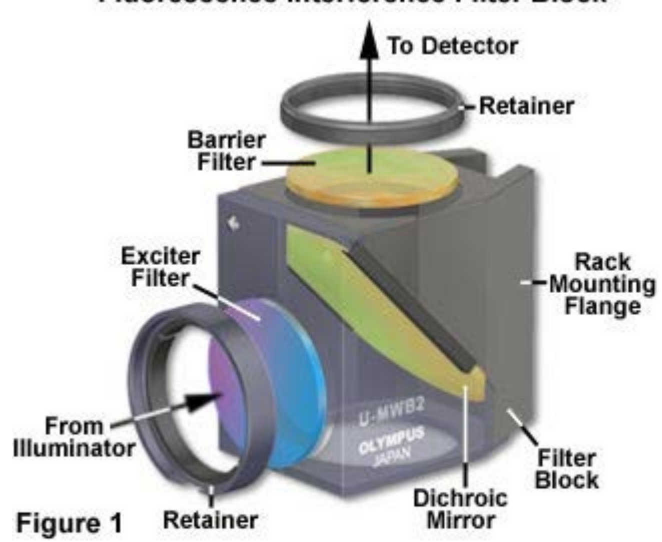
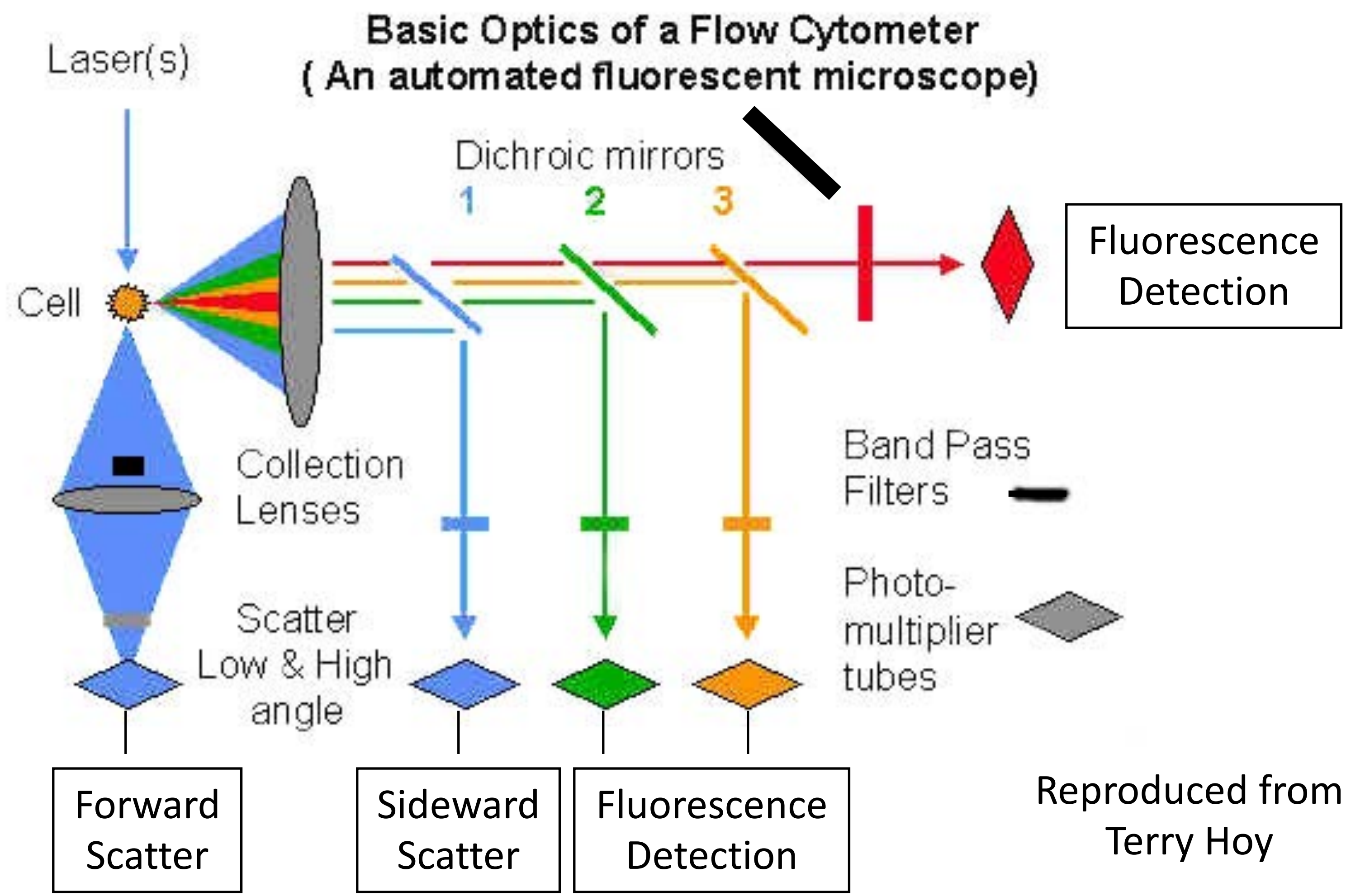


Figure 1

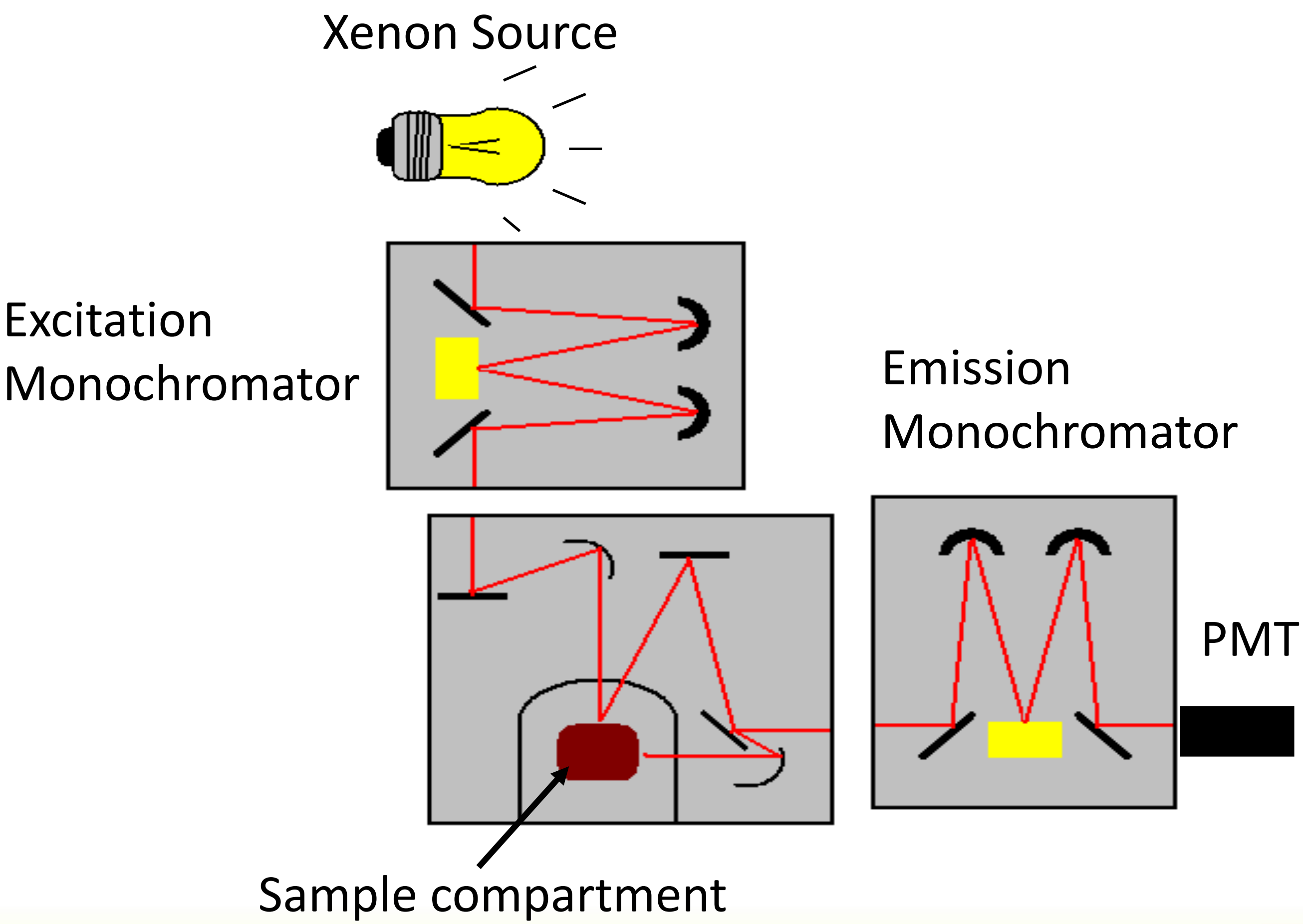
V. Detecting Fluorophores



Reproduced from Terry Hoy

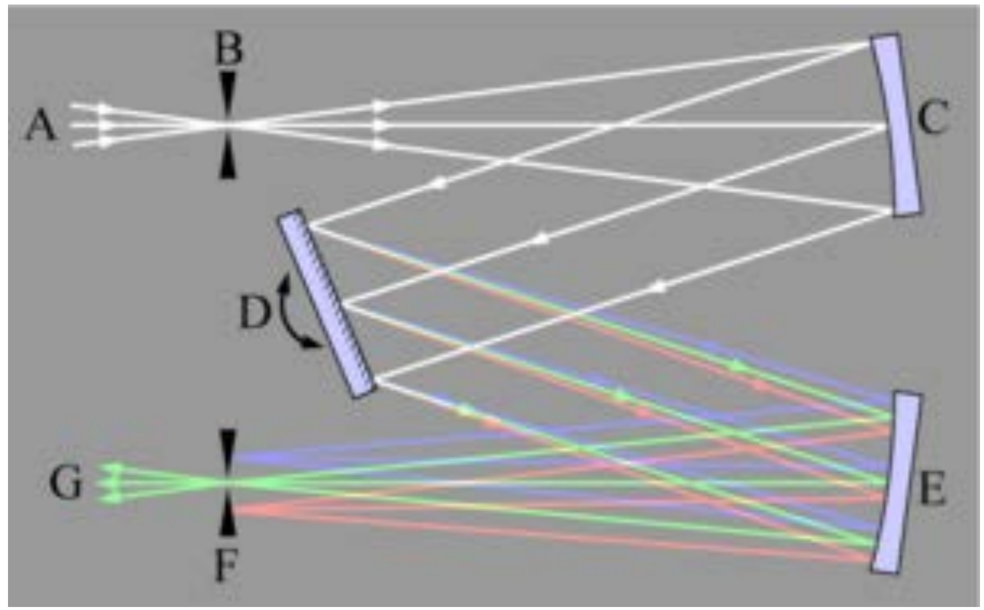
V. Detecting Fluorophores

Fluorescence Spectrophotometer

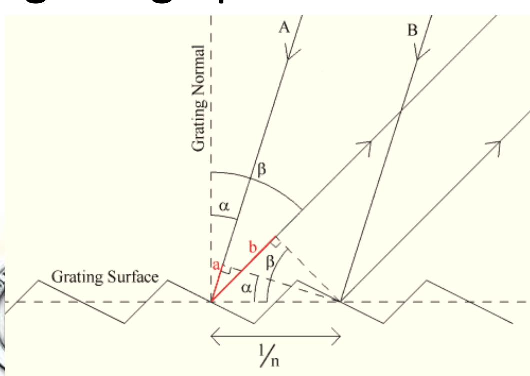


V. Detecting Fluorophores

Monochromator: only a small range of wavelengths are focused at the exit slit determined by angle of light incident on the diffraction grating



Principle of diffraction grating operation



V. Fluorescent Detectors

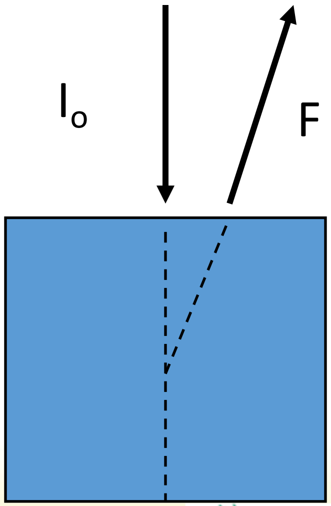
- Photomultiplier Tubes (PMT) and Photodiodes
  - PMTs are colour blind! They generate electron when photons are present, which in turn is converted into a digital signal. Therefore colours seen on the monitor is a pseudo colour.
- Other Fluorescence Detectors
  - Eyes
  - Photographic Film
  - Charge Couple Devices (CCD)
  - Photodiodes

VI. Fluorescence Measurements

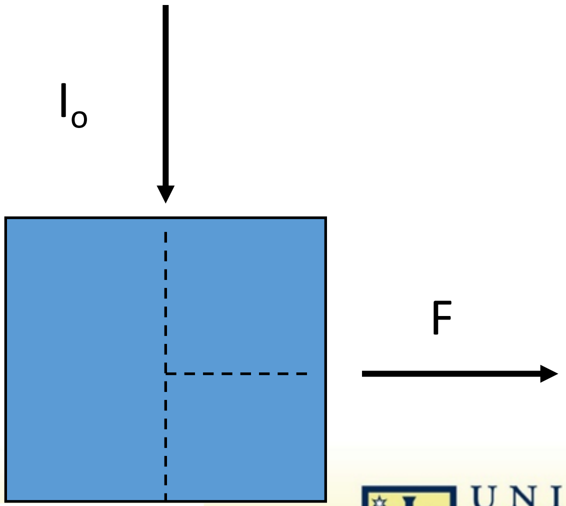
Collection geometry in sample compartment

- Front face – collection is at a 22 degree angle relative to the incident beam; appropriate for an optically absorbing / scattering sample; more stray light

- Right angle – collection is at a right angle to the incident light; appropriate for optically transparent sample; less stray light



Front Face



Right Angle

VI. Fluorescence Measurements

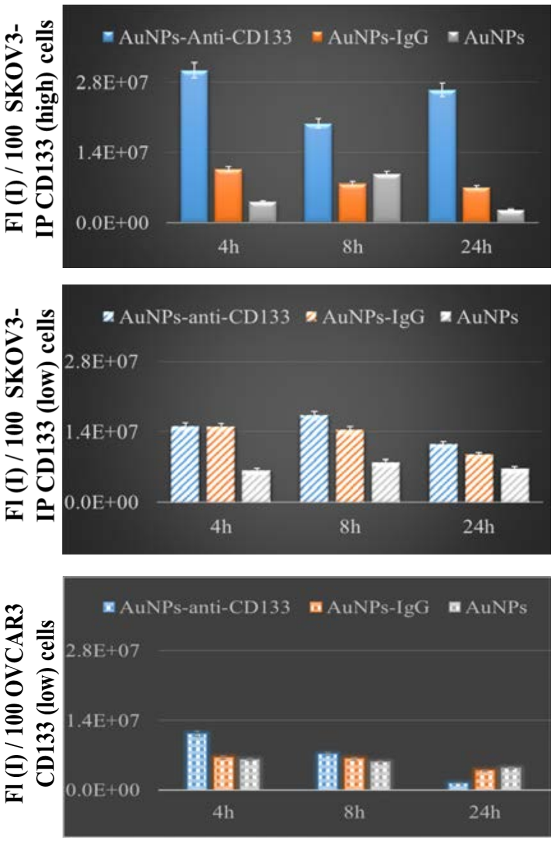
Blank scan

- Blank is identical to sample except it does not contain fluorophore
- Measuring the fluorescence of these samples allows the scattering (Rayleigh and Raman) to be assessed
- In addition, such samples can reveal the presence of fluorescence impurities, which can be subtracted

VII. Qualitative and Quantitative Assays Using Fluorophores

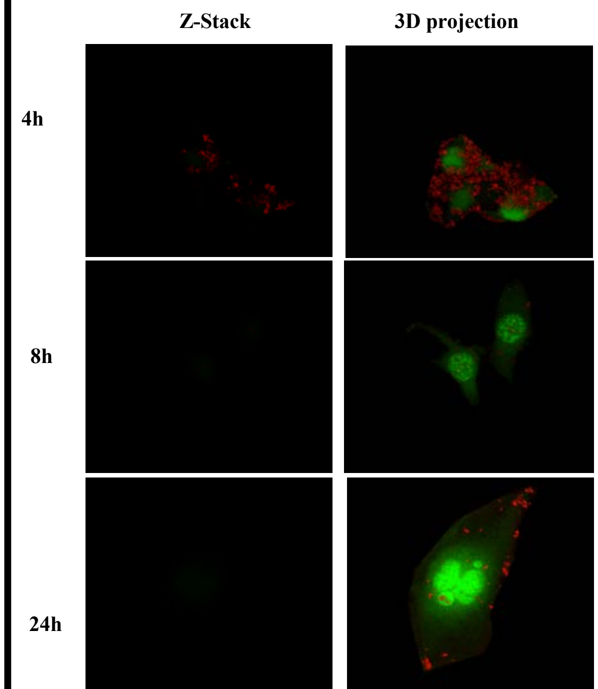
In Vitro Assay Probes for CD133+ Cells

A. Cell Specific Targeting



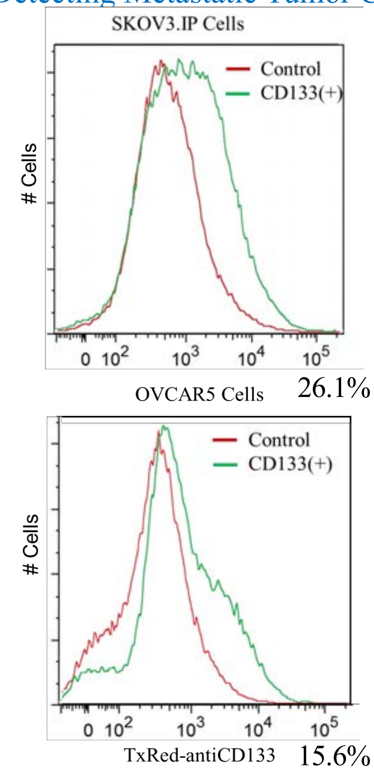
Nallathamby et al., in preparation

B. Trafficking in/on Cells



Nallathamby et al., in preparation

C. Detecting Metastatic Tumor Cell Subsets

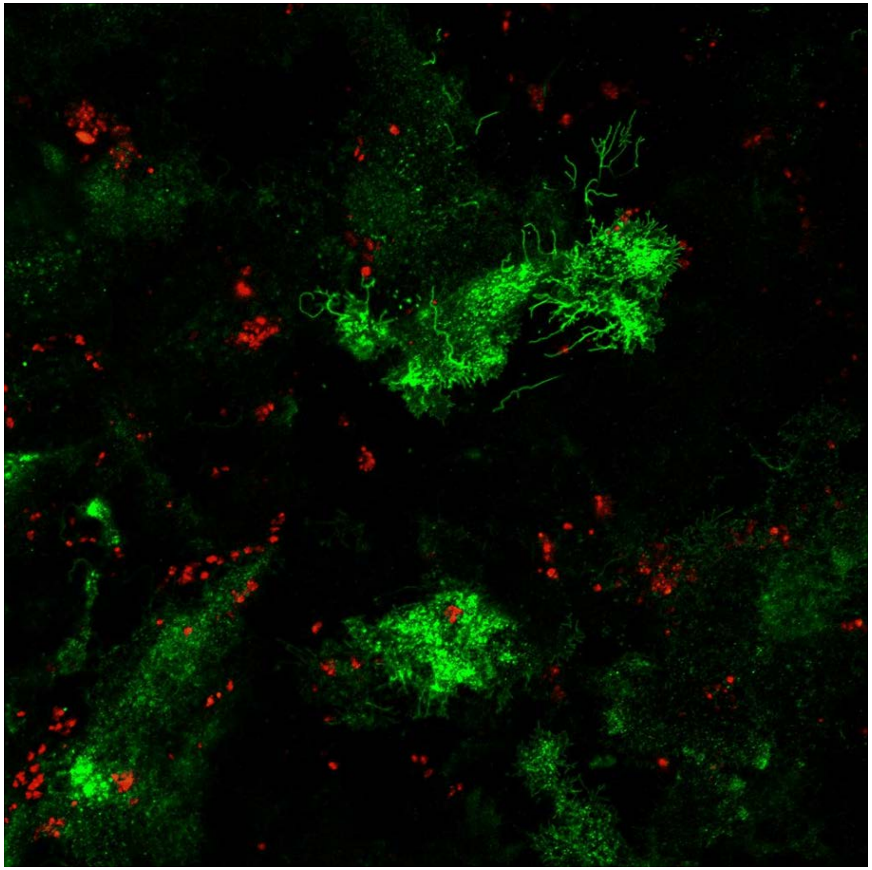


26.1% CD133 high cells

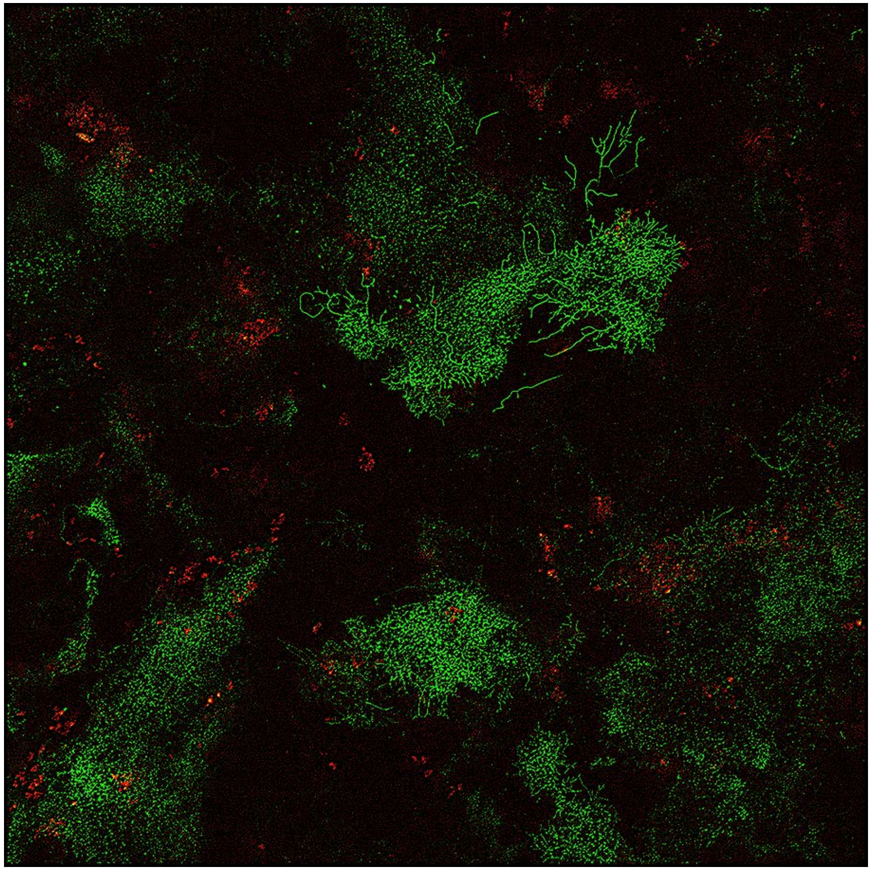
15.6% CD133 high cells

VII. Spatial Tracking of Molecules

RAW DATA



Super Resolution Analyzed Data



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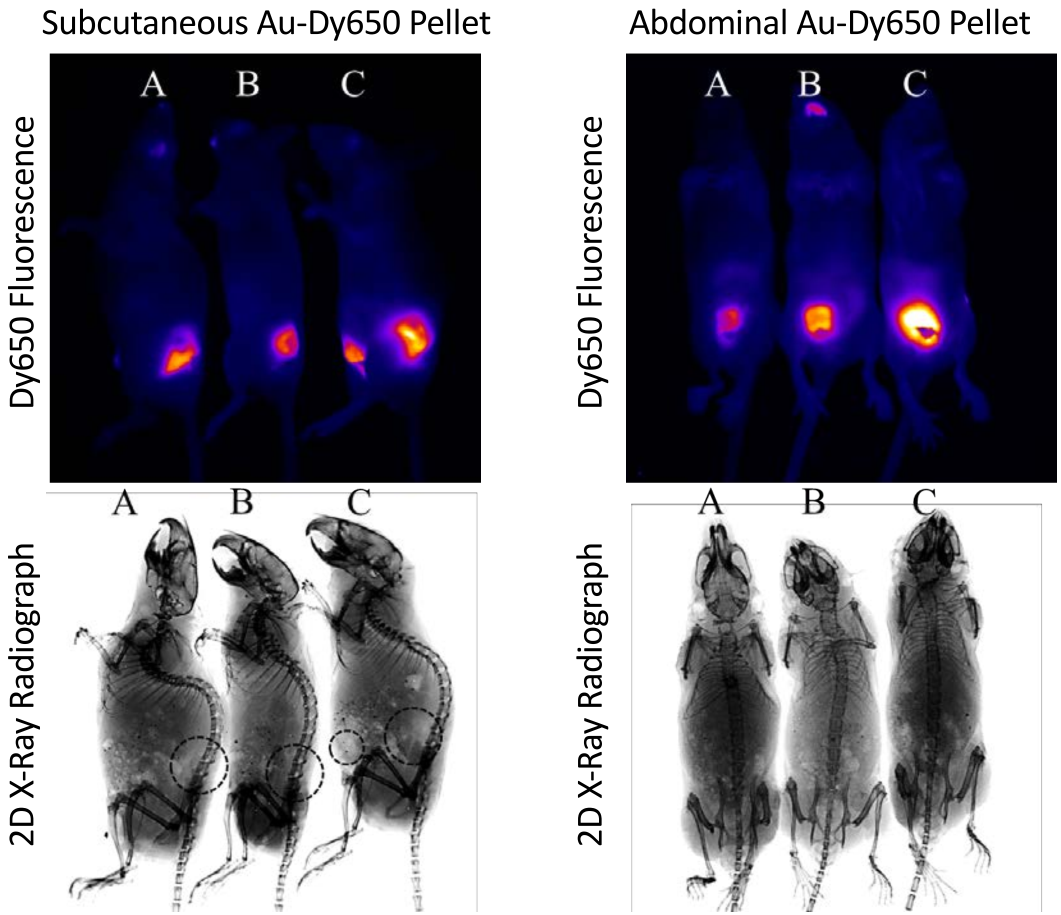
VII. Qualitative and Quantitative Assays Using Fluorophores

Mouse Tumor Phantoms to Demonstrate Fluorescence / X-ray Modality of AuDy650 Nanoprobes



A = 10 mM AuDy650 Pellet

B = 15 mM AuDy650 Pellet

C = 30 mM AuDy650 Pellet