Fluorescence Probes and Labels for Biomedical Applications

Ewald Terpetschnig

Fluorescence Based Biological Assay Formats

Fluorescence Intensity Assays

Fluorescence Polarization Assays

Fluorescence Energy Transfer Assays

Fluorescence Lifetime Assays

Luminescent, Time-Resolved Assays

Fluorescence Based Biological Assay Formats

Fluorescence Intensity Assays

- *Most Frequently Used Read-Out Parameter*
- *Prone to Interferences*
- *Not Suitable for Homogeneous Assay*

Fluorescence Based Biological Assay Formats

Fluorescence Polarization Assays

Widely Used for HTS and Clinical Assays

Self-Referenced Method

Only One Label for Assay Required

Fluorescence Based Biological Assay Formats

Fluorescence Energy Transfer Assays

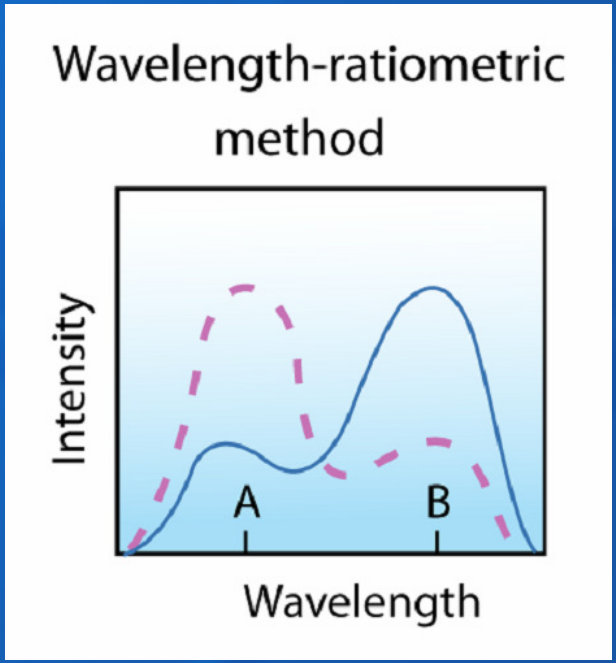
Two Labels for Assay Required

READ OUT:

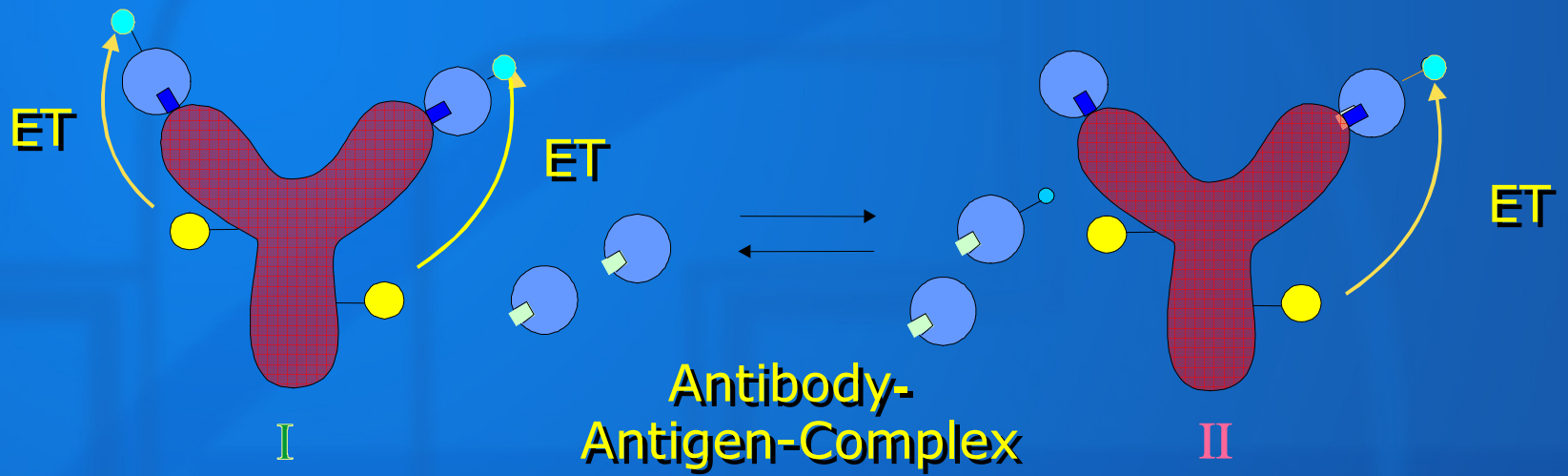
Intensity (dark acceptor)

Intensity-Ratiometric (2 fluorophores)

Lifetime (dark acceptor)



Competitive Energy Transfer Immunoassay



donor-labelled antibody

antigen

acceptor-labelled antigen

- Fluorescent Donor
- Non-fluorescent Acceptor $\tau\,(D_{bound}) < \tau\,(D_{free})$
- No Need to Separate D and A Signals
- Lifetime independent of Volume, Color- Quenching, etc

Fluorescence Based Biological Assay Formats

Fluorescence Liftetime Assays

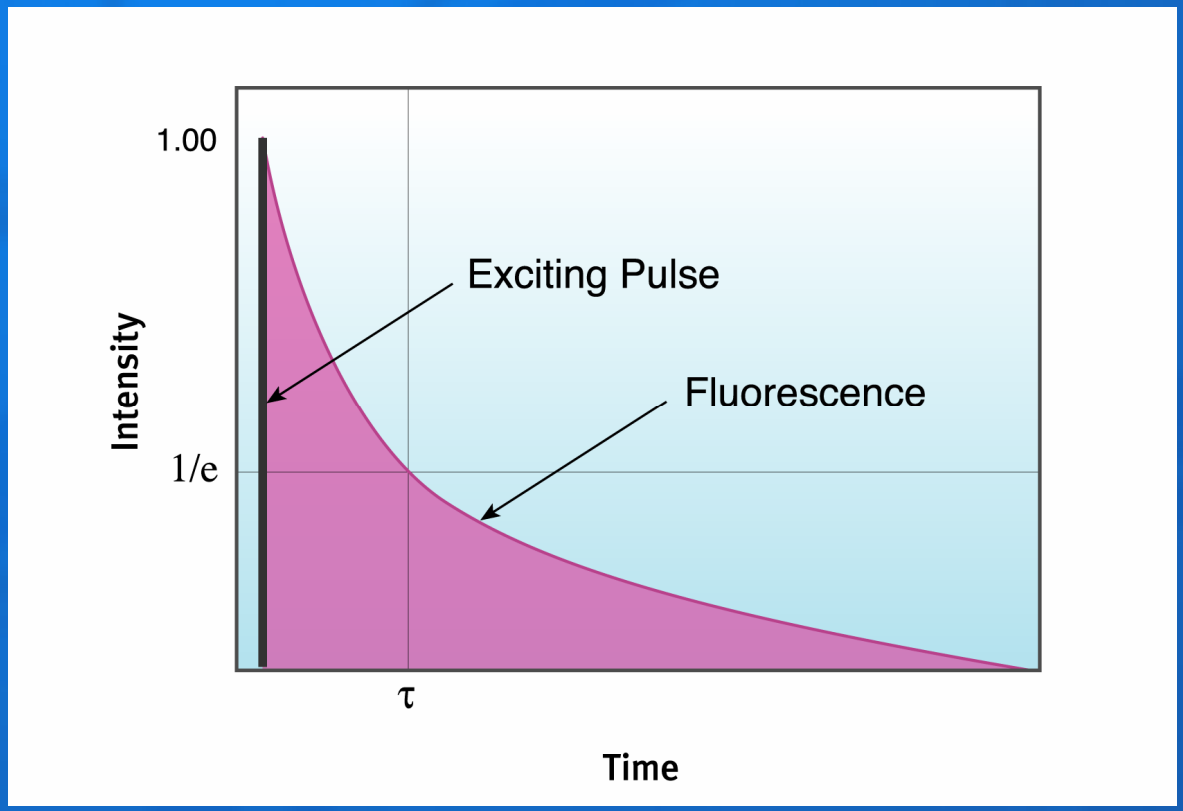
- *Independent of Fluorophore Concentration*
- *1- or 2-Label Assays*

2 Methods:

- *Phase-Modulation Method*
- *Pulse-Method*

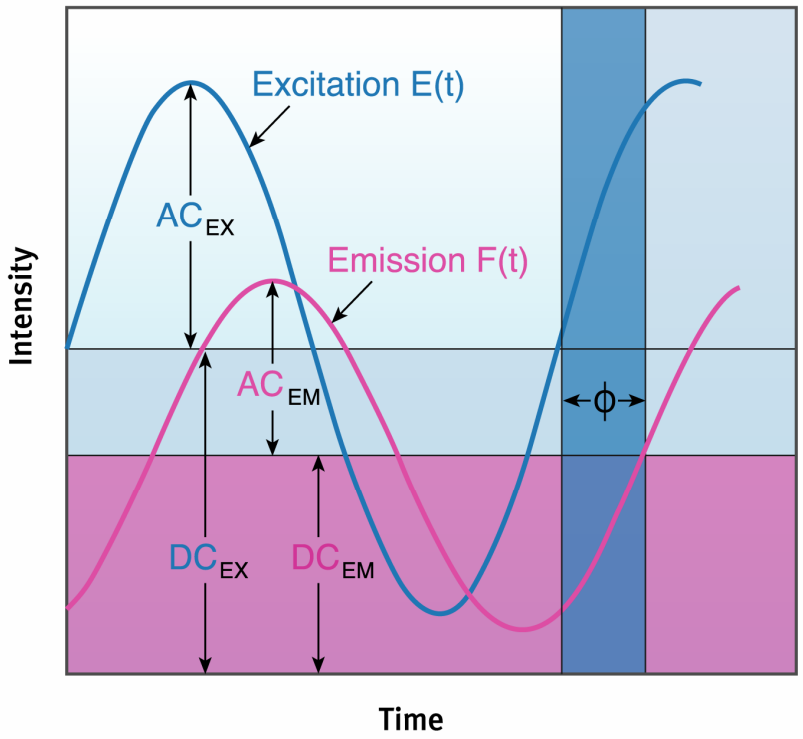
Two Ways to Measure Lifetime:

Time-Domain



$$I_t = \alpha\, e^{-t/\tau}$$

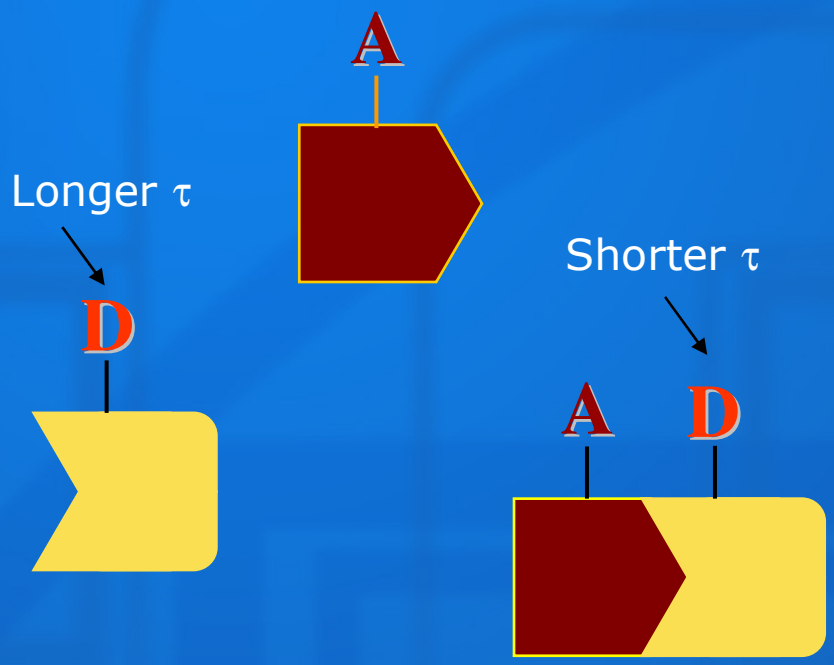
Frequency Domain



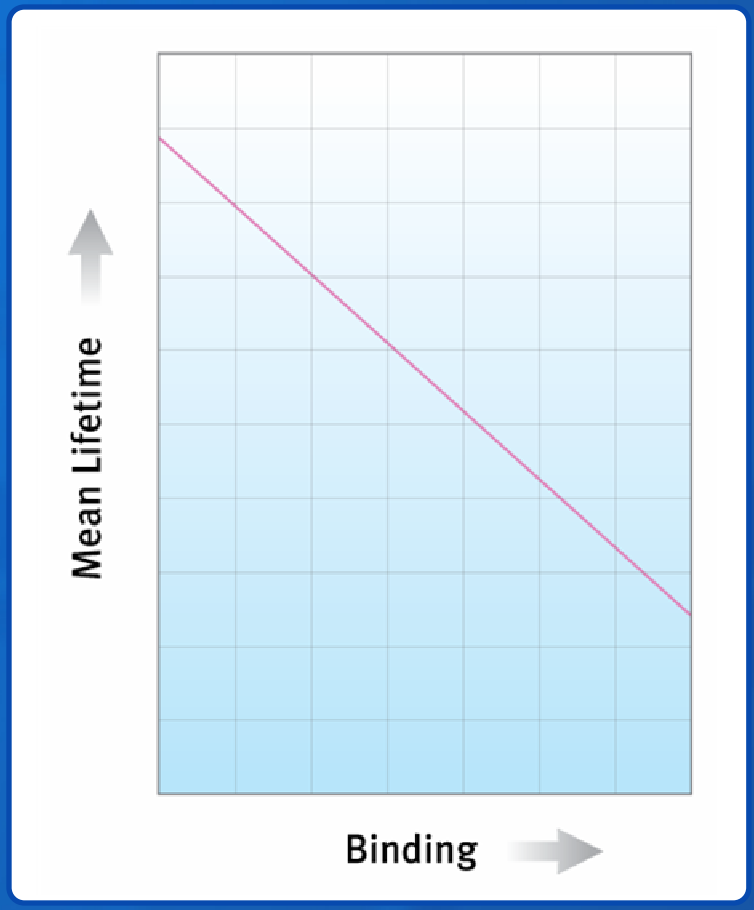
- Phase Shift $\tan \phi = \omega\tau$
- Demodulation $M = \frac{(AC/DC)_{EM}}{(AC/DC)_{EX}}$

$$M = \frac{1}{\sqrt{1+(\omega\tau)^2}}$$

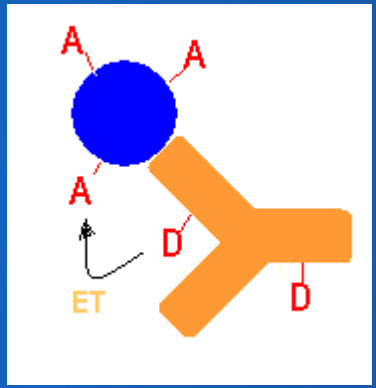
Assays Can Be Designed with Lifetime Readout



In FRET the Acceptor Shortens the Donor's Lifetime



Lifetime-Detected Energy Transfer (Binding Assay)



- Fluorescent Donor
- Nonfluorescent Acceptor A

  $\tau\ (D_{bound}) < \tau\ (D_{free})$
- No need to separate D and A signals
- Lifetime independent of volume, color- quenching, etc.

Classification:

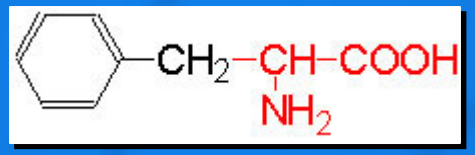
- Intrinsic Fluorophores
- Extrinsic Fluorophores

Intrinsic Fluorophores

Naturally Occurring Fluorophores

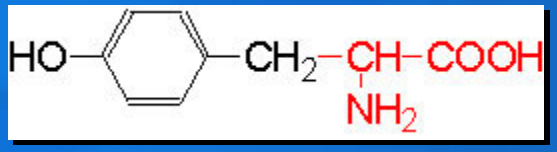
Proteins: Naturally Occurring Fluorophores

Aromatic amino acids



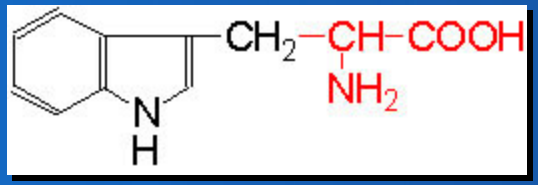
Phenylalanine (Phe – F)

Ex/Em 260 nm/282 nm



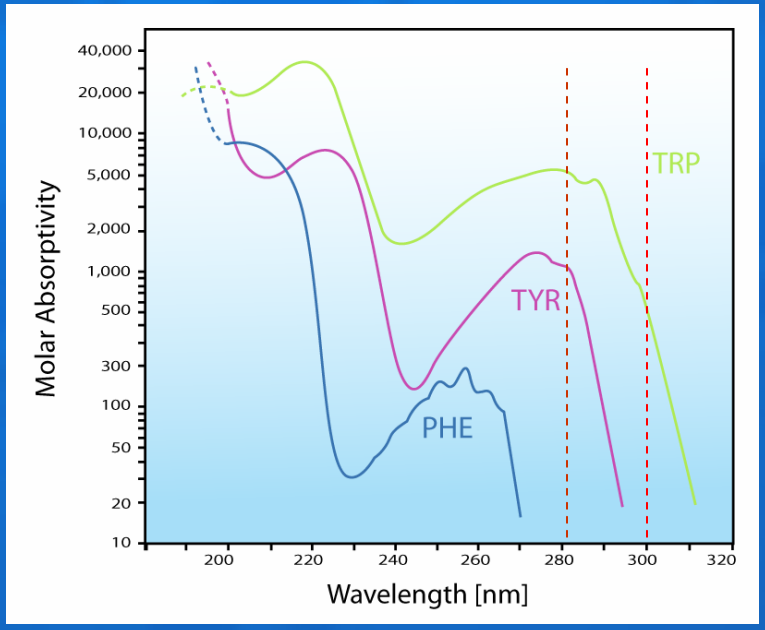
Tyrosine (Tyr – Y)

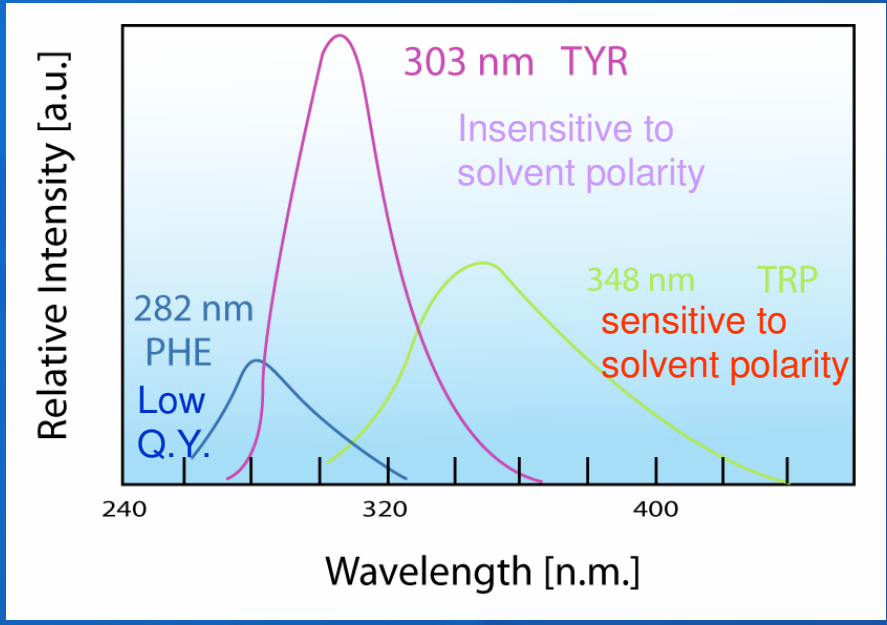
Ex/Em 280 nm/303 nm



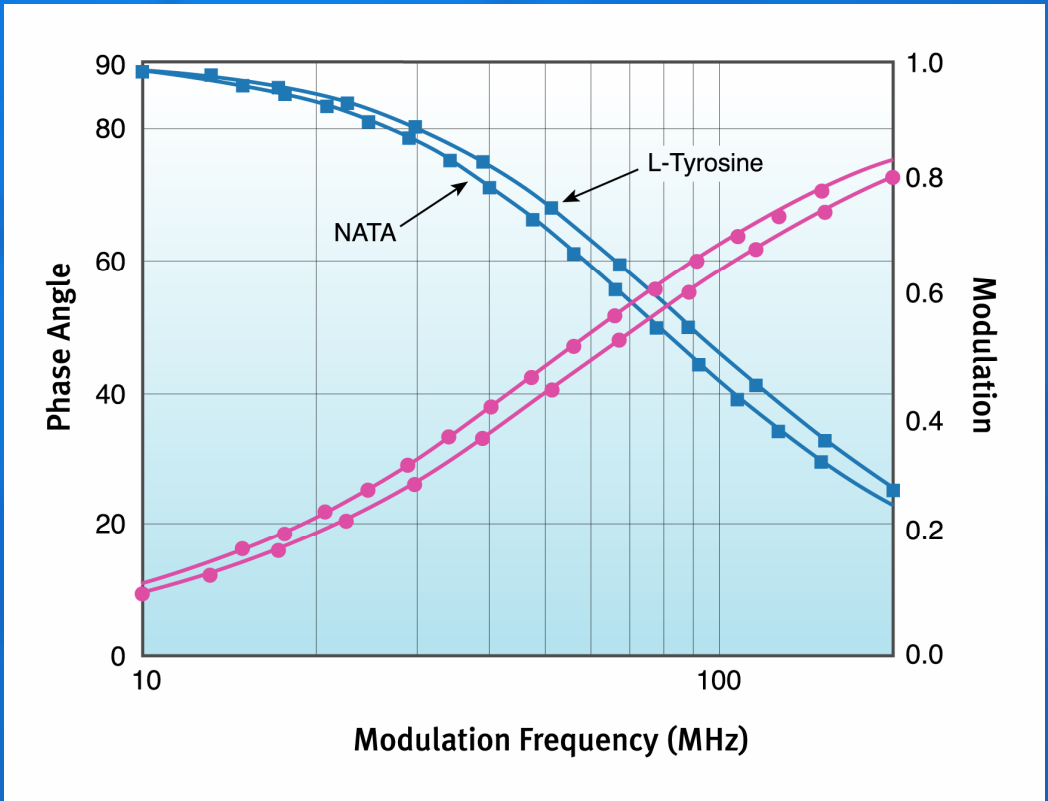
Tryptophan (Trp-W)

Ex/Em 280, 295nm/ 305-350 nm





Fluorescence Lifetimes of Protein-Related Fluorophores



Tyrosine:

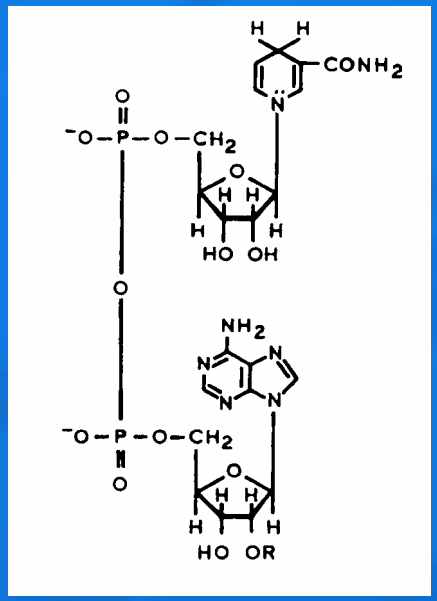
- λ_{Ex}: 280-nm LED
  Em: 320-nm LP
- τ = 3.2 ns (Water)

NATA:

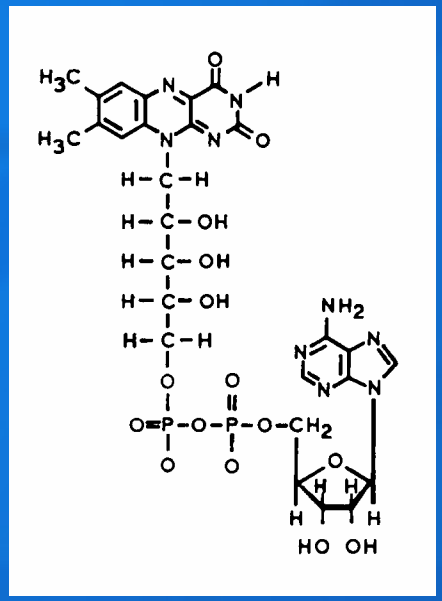
- λ_{Ex}: 300-nm LED
  Em: 320-nm LP
- τ = 3.09 ns (Water)

Naturally Occurring Fluorophores

Enzyme Cofactors



NADH
(Oxido-reductases)
Ex/Em 340/460 nm



FAD
(Metabolic enzymes)
Ex/Em 450nm/540 nm

Porphyrins

Ex/Em 550 nm/620 nm

Fe^{2+} (Heme)
myoglobin,
hemoglobin
cytochromes b and c,
cytochrome P450 and
cytochrome oxidase

Mg^{2+} chlorophylls

Extrinsic Fluorophores

Synthetic dyes or modified biochemicals that are added to a specimen to produce fluorescence with specific spectral properties.

Fluorescent Probes:

- Non covalent interaction

A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific analyte.

Fluorescent Labels:

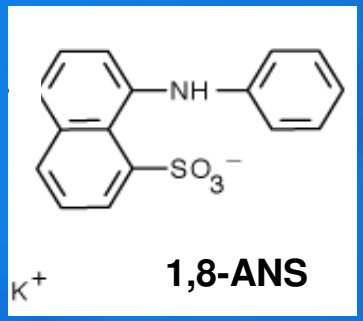
- Covalent interaction

Classes of Probes, Dyes and Labels:

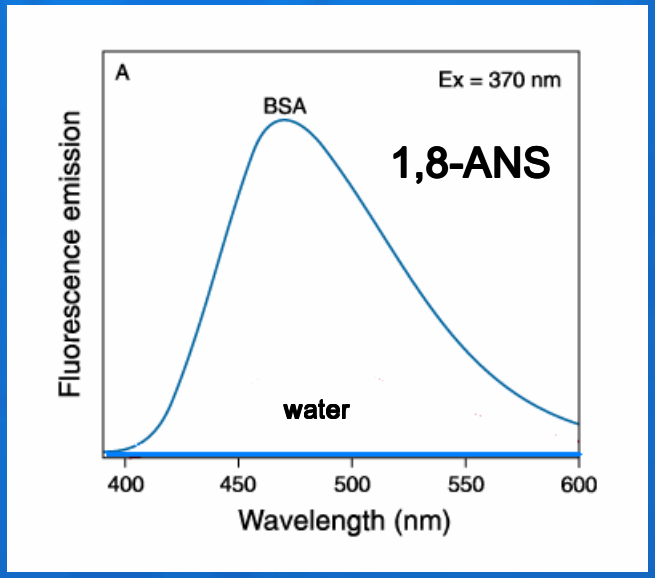
- Organic Dyes
- Metal-Ligand Complexes
- Quantum Dots and Nanoparticles
- Fluorescent Beads or Polymers
- Fluorescent Proteins

Fluorescent Probes

Non-covalent



1,8-ANS

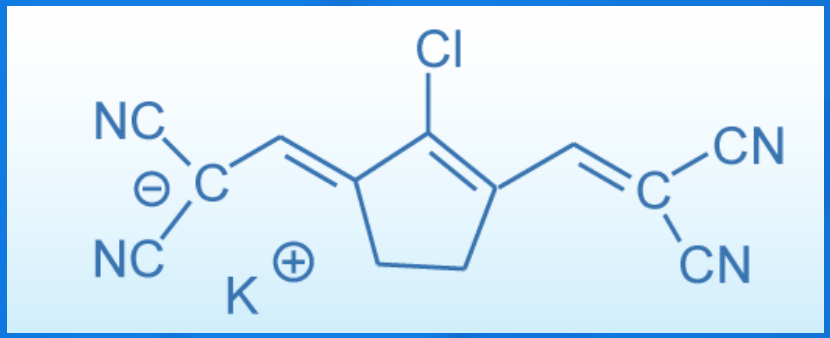


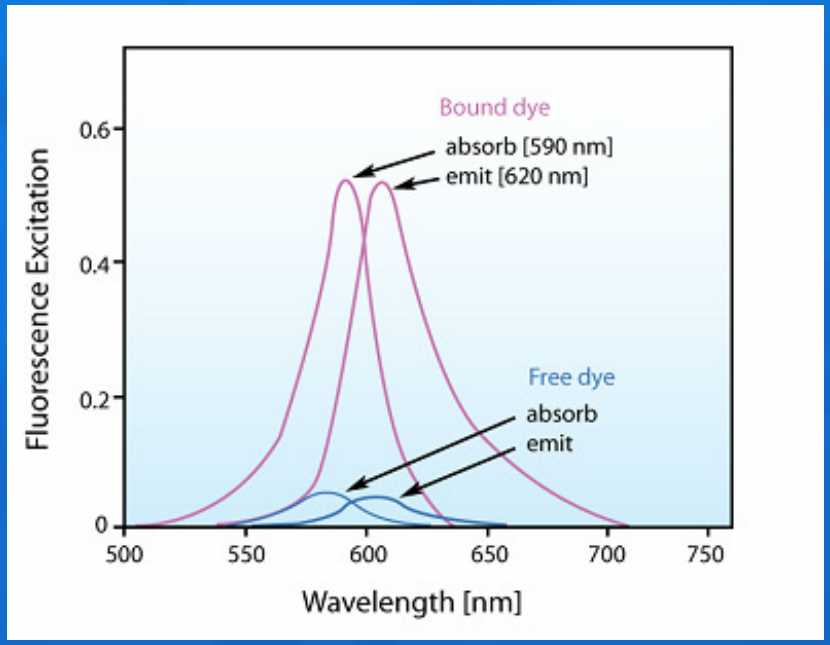
Developed by G. Weber in 1950's

Barely fluorescent water - fluorescence is strongly enhanced in hydrophobic environments

Valuable probes for studying membranes and proteins: QY ~ 0.25 (membranes) or ~0.7 (proteins)

Fluorescent Probes





Albumin Blue

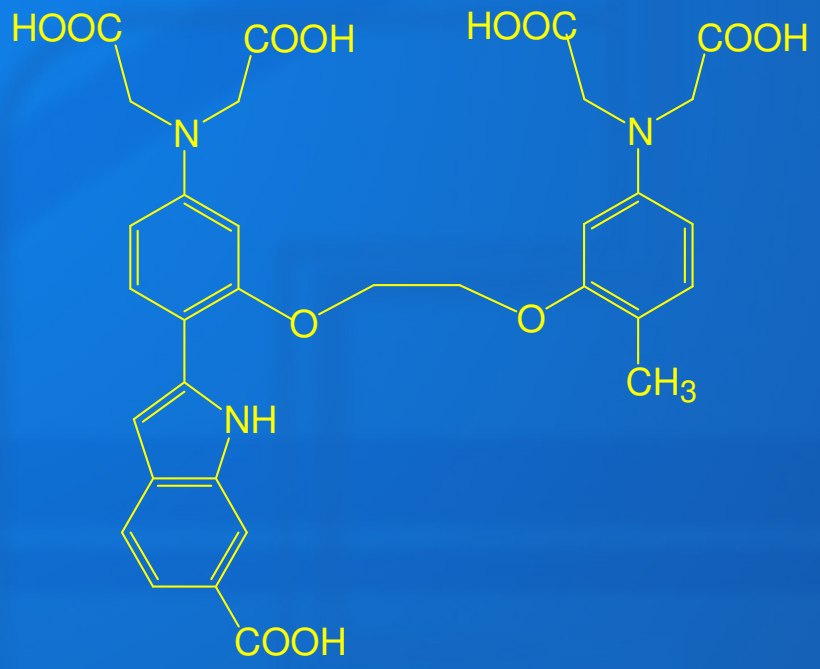
Measurement of albumin levels in biological samples including serum and urine.

Developed by Kessler & Wolfbeis

Specific- quantitative determination of Albumin in presence of other proteins

Albumin-specific also at concentrations below 100mg.L^{-1} - Microalbuminuria

Fluorescent Ion-Probes



Fluorescent Ion-Probes

Fluorescence probes have been developed for a wide range of ions:

Cations:

H^+, Ca^{2+}, Li^+, Na^+, K^+, Mg^{2+}, Zn^{2+}, Pb^{2+} *and others*

Anions:

Cl^-, PO_4^{2-}, Citrate, ATP, *and others*

How to choose the correct fluorescent probe

Dissociation Constant (Kd)

- Must be compatible with the concentration (pH) range of interest.
- Calibration: Kd of probe is dependent on pH, temperature, viscosity, ionic strength etc…….

Measurement Mode

Qualitative or quantitative measurements.

- Ion-probes showing spectral shifts - radiometric measurements
- Light source available

Indicator Form

Influences cell loading and distribution of the probe.

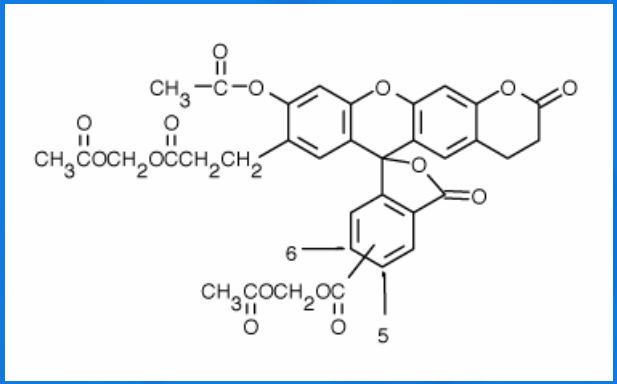
- Salts and dextran-conjugates - microinjection, electroporation
- AM-esters - passively loaded and cleaved by intracellular esterases

pH-Probes

Probe	pH Range	Measurement Mode
SNARF indicators	6.0-8.0	Em. ratio 580/640 nm
HPTS (pyranine)	7.0-8.0	Exc. ratio 450/405 nm
BCECF	6.5-7.5	Exc. ratio 490/440 nm
Fluoresceins and Carboxyfluoresceins	6.0-7.2	Exc. ratio 490/450 nm
Oregon Green dyes	4.2-5.7	Exc. ratio 510/450 nm
LysoSensor Yellow/Blue DND-160	3.5-6.0	Em. ratio 450/510 nm

Molecular Probes' pH indicator families, in order of decreasing pK_a

BCECF

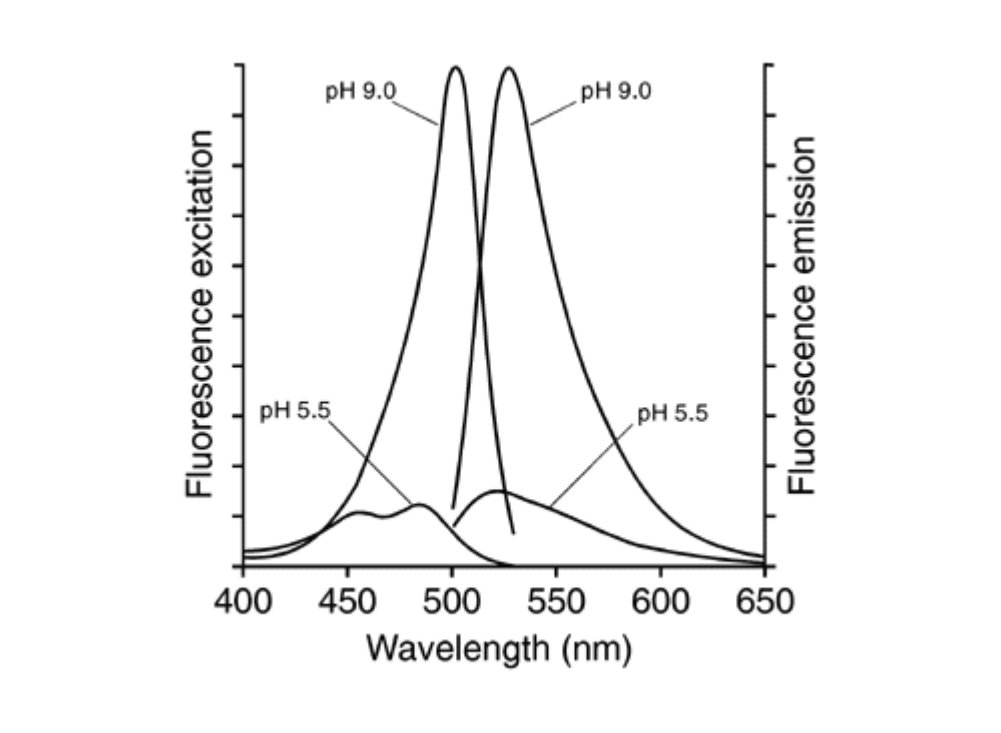


R. Tsien 1982

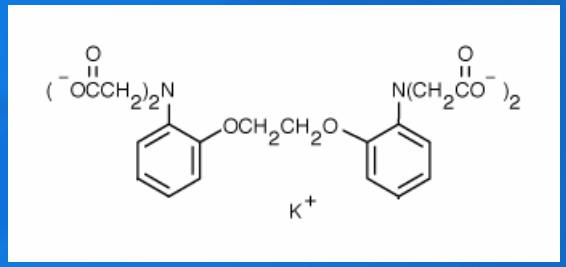
Most widely used fluorescent indicator for intercellular pH

Membrane-permeant AM: pKa ~ 6.98 is ideal for intracellular pH measurements

Excitation-ratiometric probe with Ip at 439 nm, which is used a the reference point



Calcium-Probes



BAPTA

Chelator with high selectivity for Ca^{2+} in presence of excess [mM] Mg^{2+}

K_d for Ca^{2+}

No Mg^{2+}: 160 nM
1 mM Mg^{2+}: 700 nM

Calcium-Probes

UV

FURA (Fura-2, Fura-4F, Fura-5F, Fura-6F, Fura-FF)
INDO (Indo-1, Indo 5F)

Ratiometric

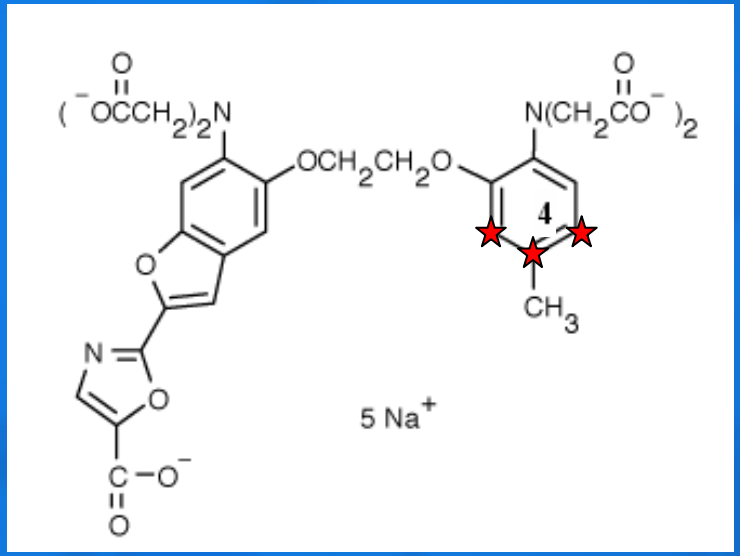
VISIBLE

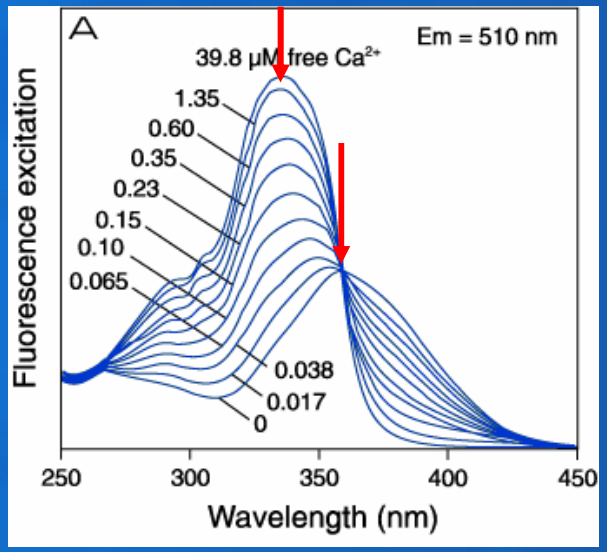
FLUO (Fluo-3, Fluo-4, Fluo5F, Fluo-5N, Fluo-4N)
RHOD (Rhod-2, Rhod-FF, Rhod-5N)
Calcium Green, Calcium Orange, Calcium Crimson
Oregon Green 488-BAPTA

Non-Ratiometric

FURA-2

Excitation-Ratiometric



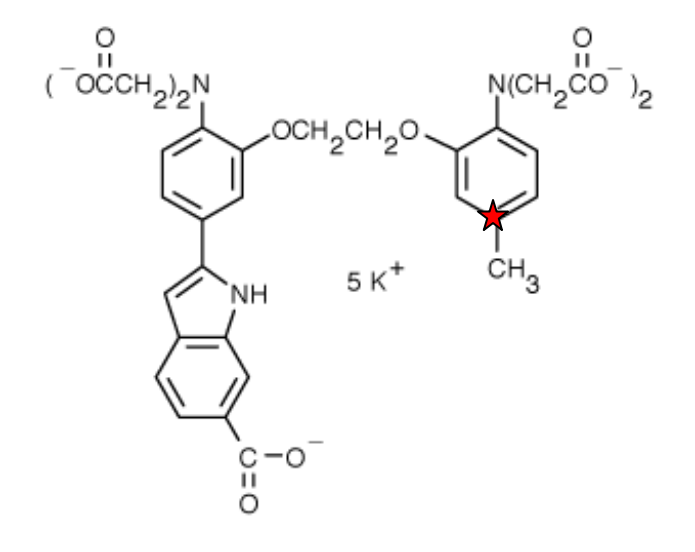


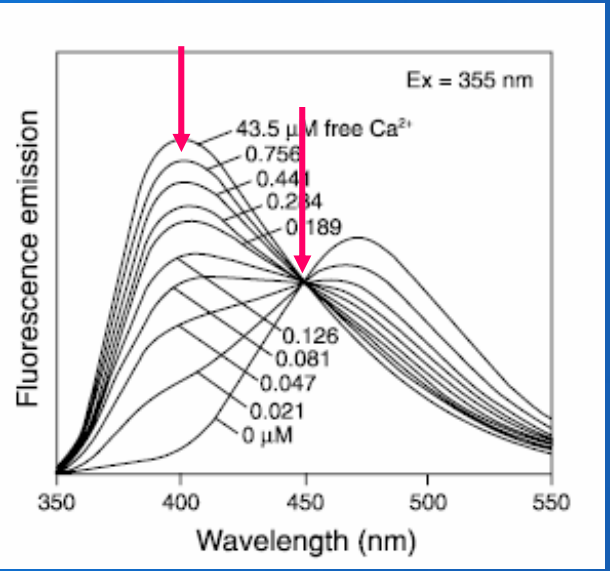
Indicator	$K_d(Ca^{2+})$
Fura-2	0.14 μM
Fura-5F	0.40 μM
Fura-4F	0.77 μM
Fura-6F	5.30 μM
Fura-FF (5,6)	35 μM

- Most used in conventional microscopic imaging
- Good excitation shift with Ca^{2+}
- Ratioed between 340 and 350 and 380 to 385 nm
- Fura-4F, Fura-5F, Fura-6F and Fura-FF provide increased sensitivity to intracellular Ca^{2+} concentration in the 0.5–35 μM range

Indo-1

Emission-Ratiometric



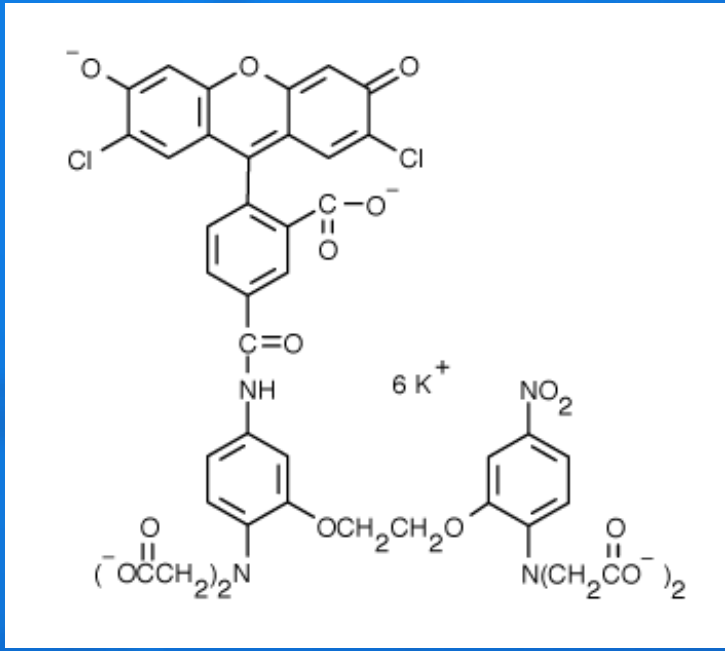


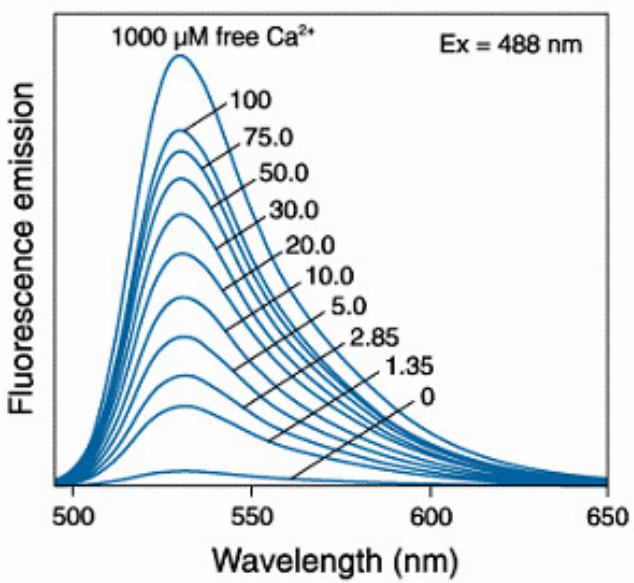
Indicator	$K_d(Ca^{2+})$ (µM)
indo-1	0.23
indo-5F	0.47

- Most used in laser flow cytometry
- Ratioed between 450 and 405 nm
- Photobleaches faster than Fura-2
- Excitation with UV laser or Ti-Sapphire at 350 nm

Calcium Green-5N

Non-Ratiometric

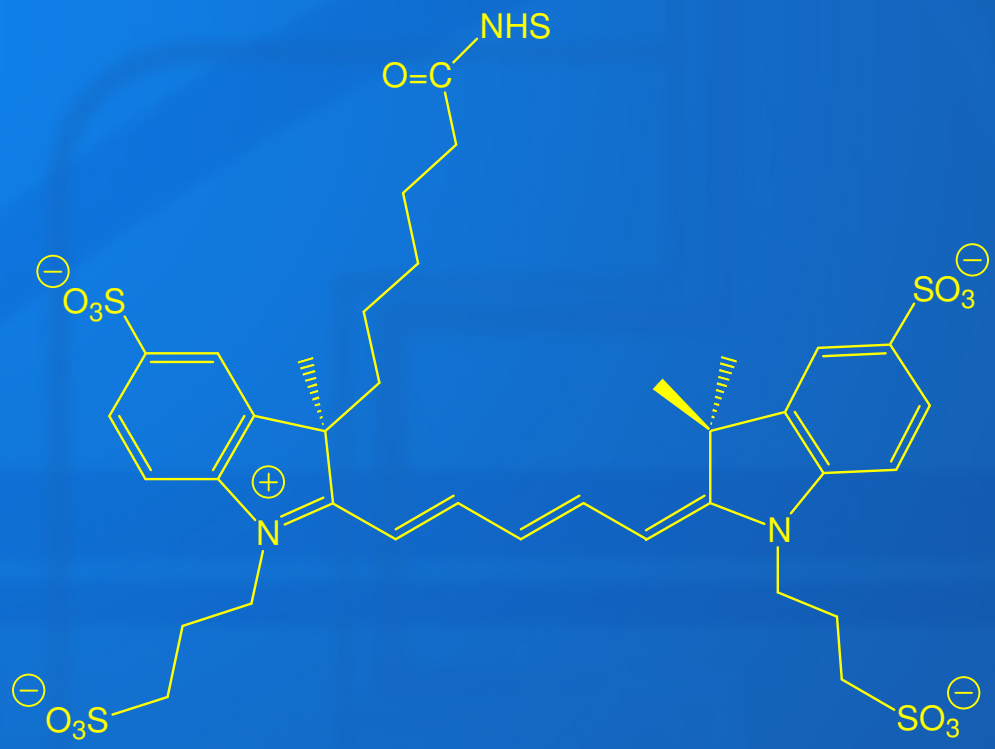




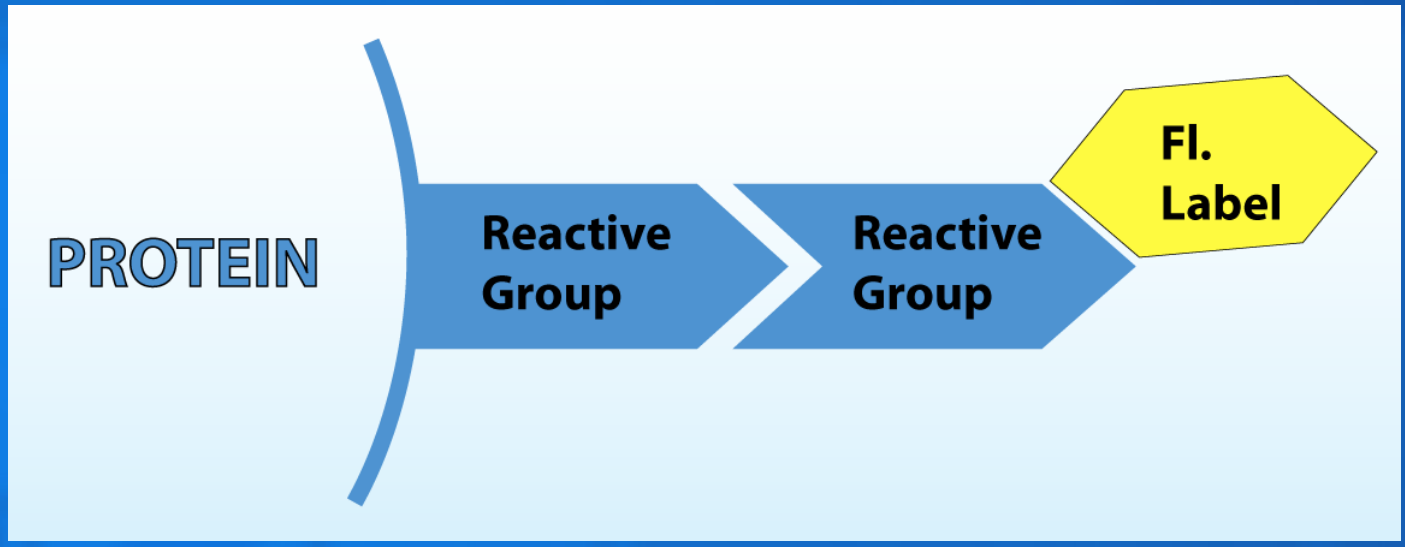
Indicator	$K_d(Ca^{2+})$
Calcium Green-5N	14 uM

- Low affinity Ca-probe
- Low fluorescence in absence of Ca^{2+}
- Tracking rapid Ca^{2+}-release kinetics

Fluorescent Labels



Selecting the Label



Reactive groups on proteins

- NH_2 Lysine N-terminus
- SH Cysteine

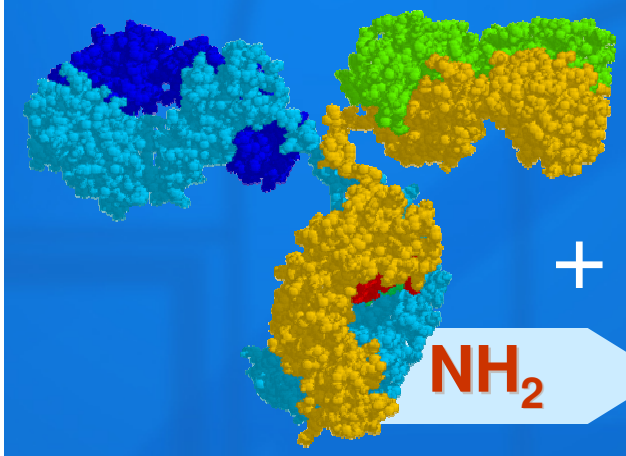
Depends on the reactive group on the protein

- Light source
- Spectral properties
- Autofluorescence
- Photostability

Labeling should not alter the biological activity of biomolecules

Protein Labeling

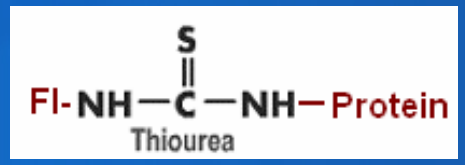
Amino-Modification:



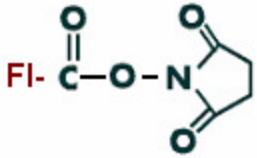
Protein-NH_2

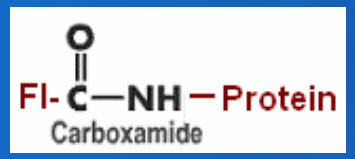
Lysine
N-terminus

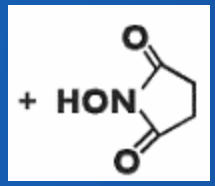
isothiocyanate:
Fl-N=C=S



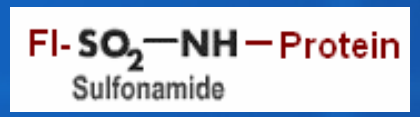
succinimidyl ester:





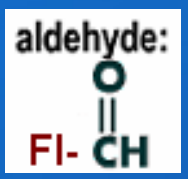


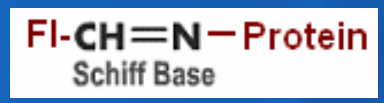
sulfonyl chloride:
Fl-SO_2Cl



+ HCl

aldehyde:

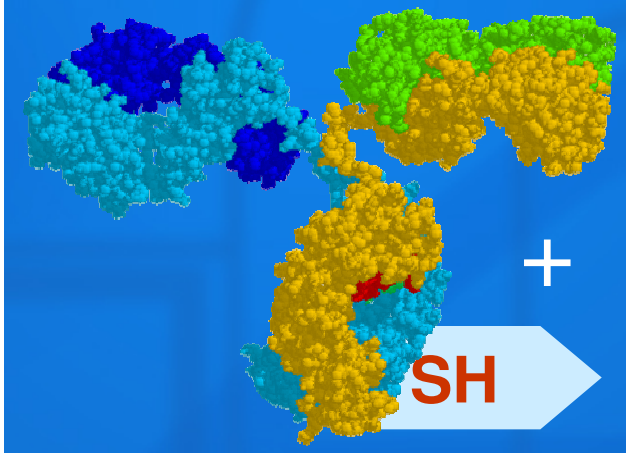




reduction

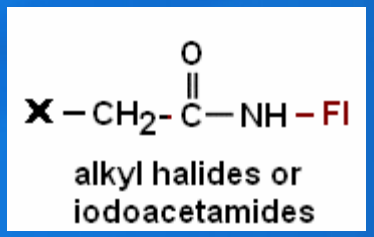
Protein Labeling

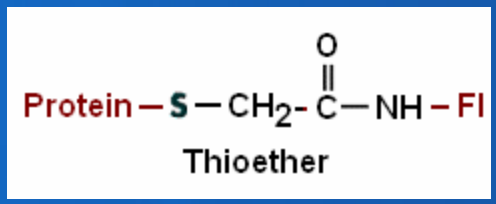
Thiol-Modification:

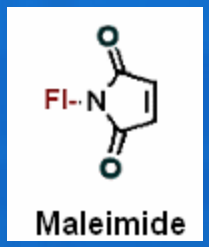


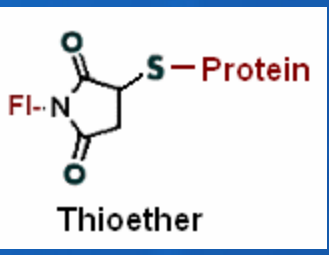
Protein-SH

Cysteine

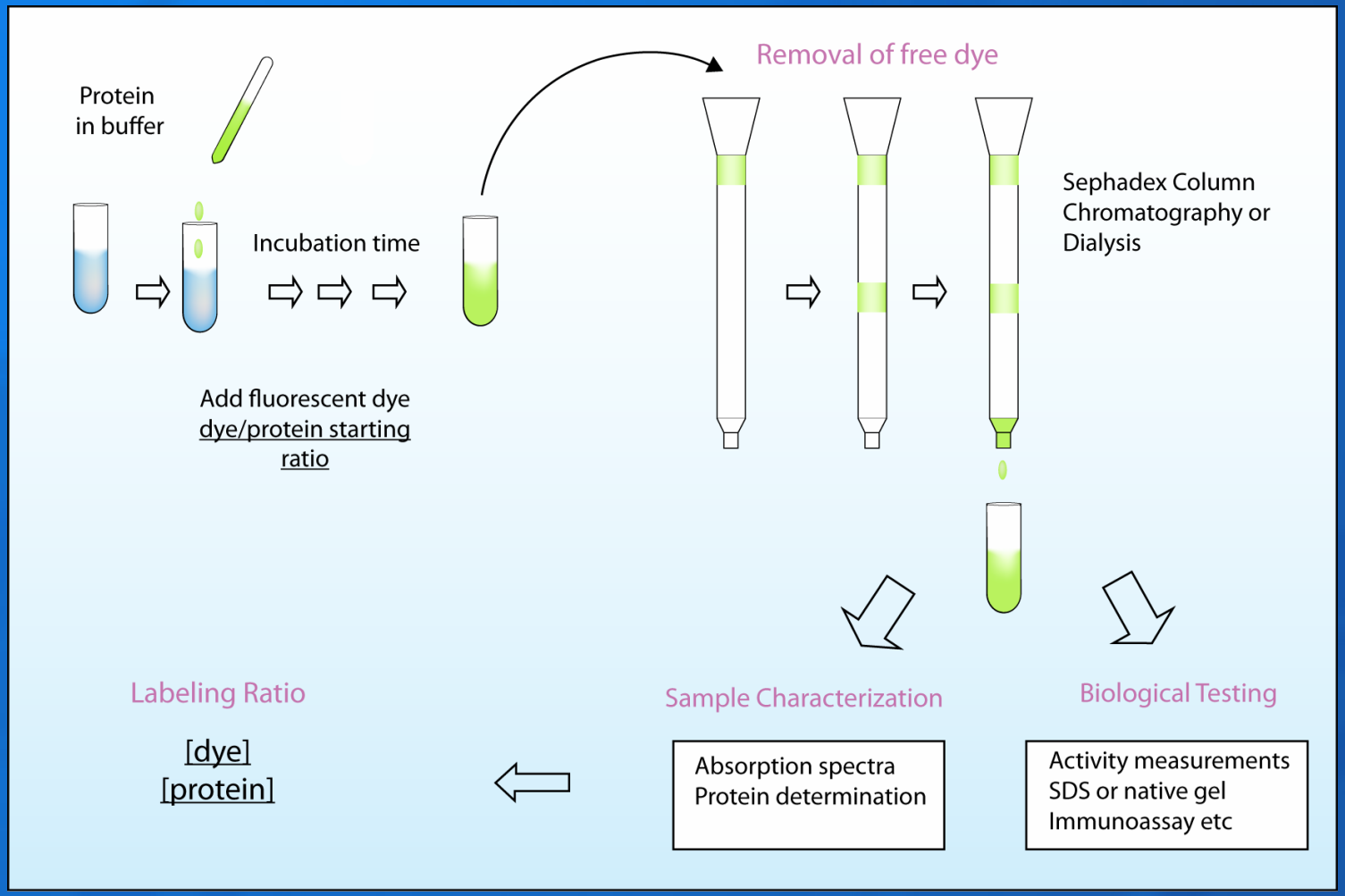




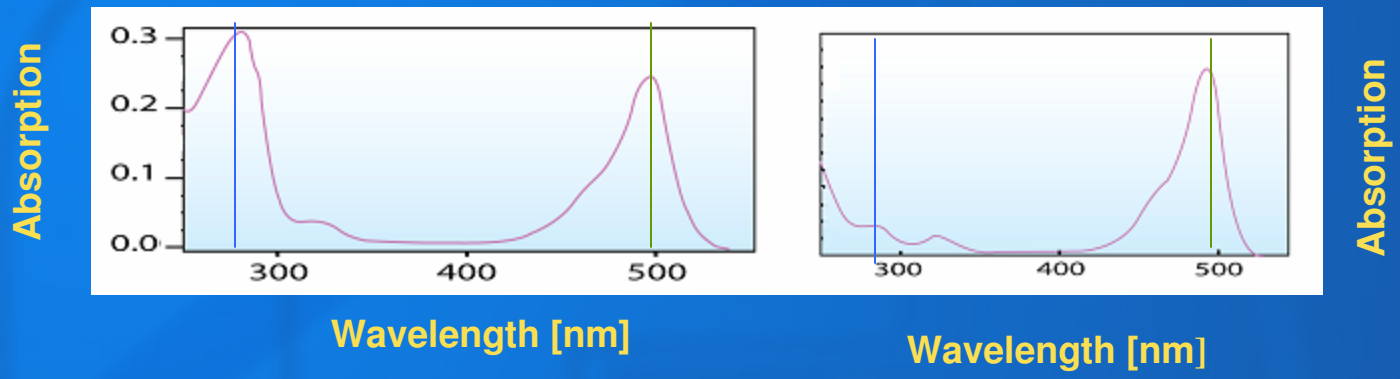




Labeling Procedure



Determination of Dye-to-Protein Ratios



Dye-Protein

Dye

$$D/P = A_{conj(\lambda max)} \cdot \varepsilon_{Prot} / (A_{conj(280)} - x \cdot A_{conj(\lambda max)}) \cdot \varepsilon_{dye}$$

$$x = A_{dye(280)} / A_{dye(\lambda max)}$$

Protein:

Photometric measurement

Bradford, Lowry assay

Organic Dye Classes

Xanthenes

Fluoresceins - pH-sensitive, not photostable

Rhodamines - tendency to aggregate in aqueous solution

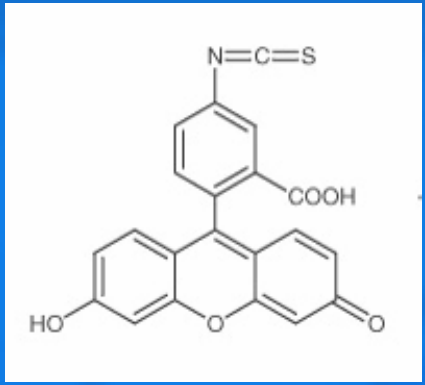
Coumarins - low extinction coefficients, phototoxic

BOPIDYs - non-polar and relatively insoluble in water

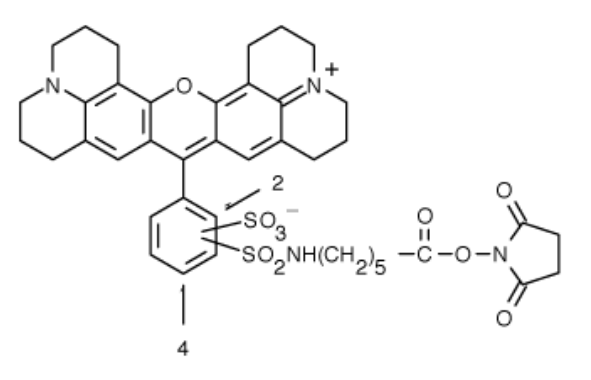
Phthalocyanines - photostable but difficult to synthesize and purify

Cyanines - high extinction coefficients and reasonable quantum yields

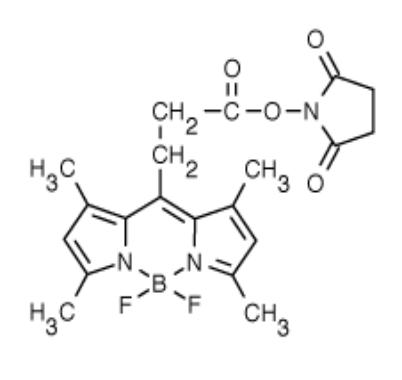
Examples of Extrinsic Labels



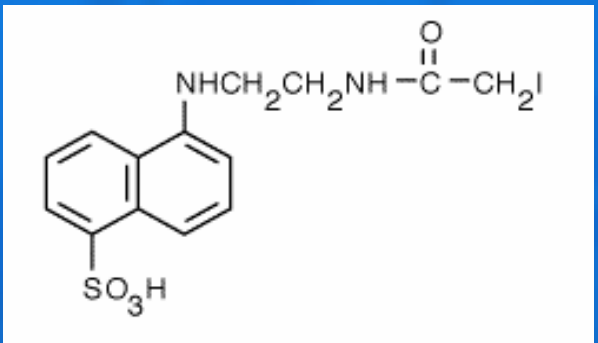
FITC
(488/512), τ ≈ 4.0



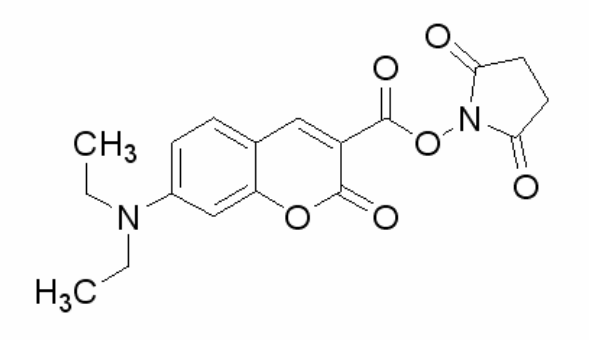
Texas Red-NHS
(595-615), τ ≈ 3.5 ns



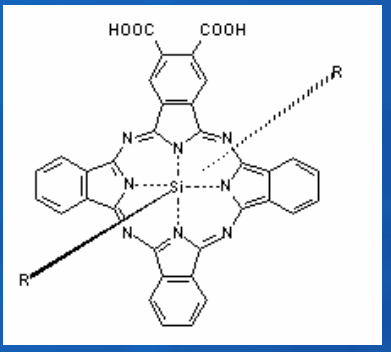
BODIPY
(493/503), τ = 6 ns



IAEDANS
(360/480), $\tau \approx$ 15 ns

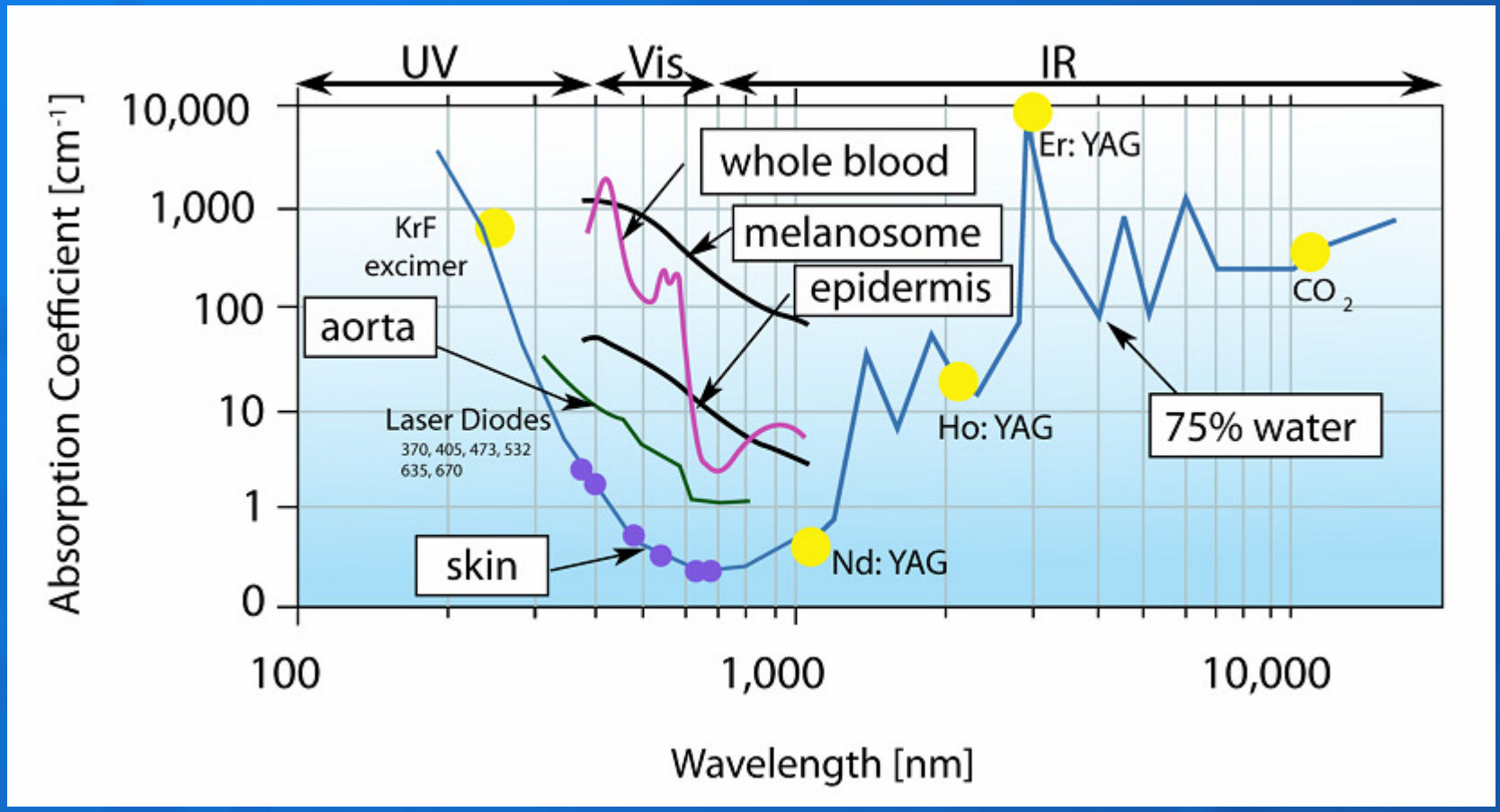


Coumarin-3-carboxylic acid
-NHS
(445/482), $\tau \approx$ 2 -3 ns



LaJolla Blue
(680/700)

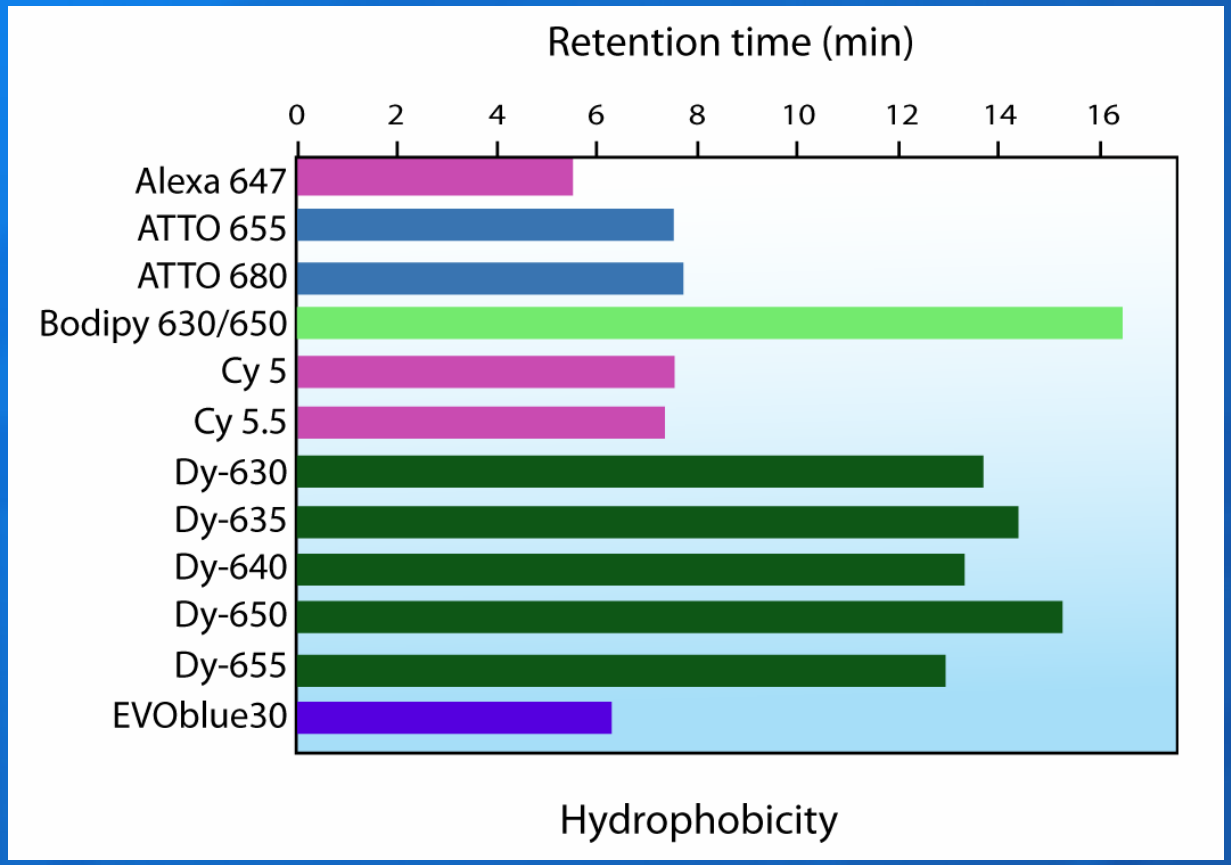
Absorption of Biological Material



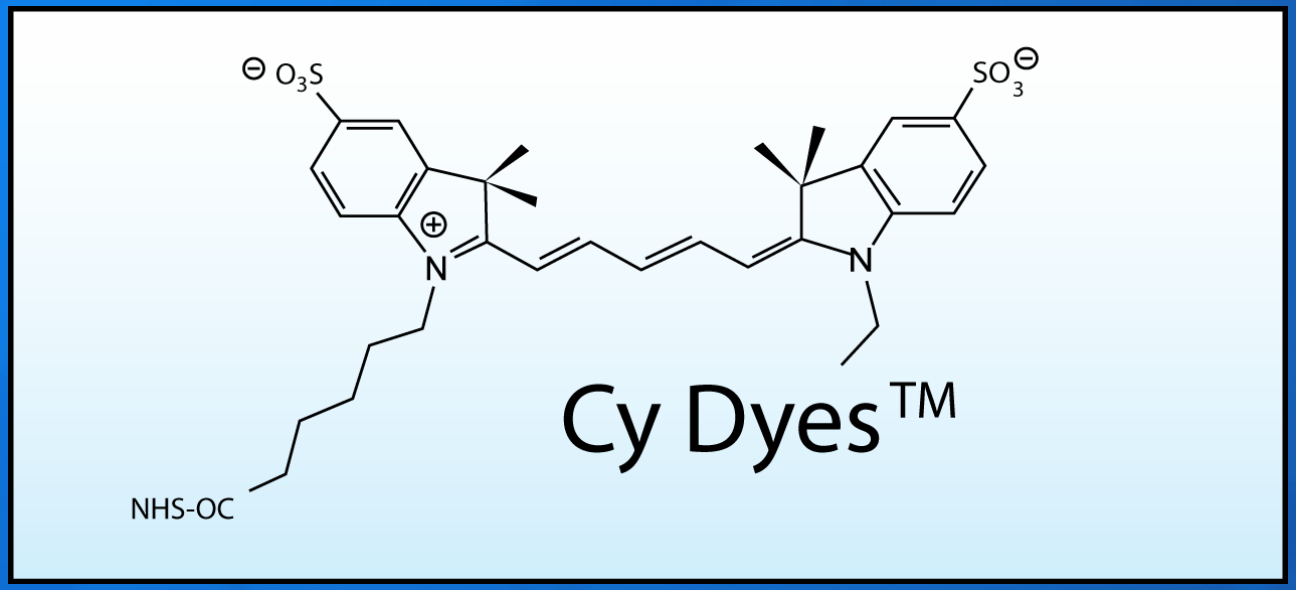
Spectral Properties and Quantum Yields of Representative Long-Wavelength Absorbing and Emitting Dyes

Dye	λ_{max} (abs) [nm]	λ_{max} (em) [nm]	ε ($M^{-1}cm^{-1}$)	Lifetime τ [ns]	Q.Y. (H_2O)
Cy5	649	670	250,000	1.0	0.3
Dy650	649	670	120,000	0.64	0.05
Alexa 647	647	666	265,000	1.0	0.33
DyLight 649	646	674	250,000	1.0	0.33
HiLyte 647	649	674	250,000	1.0	0.28
BODIPY	629	646	97,000	3.9	0.7
Atto	661	678	100,000	1.8	0.3

HPLC-Retention Times of Red Fluorescent Dyes

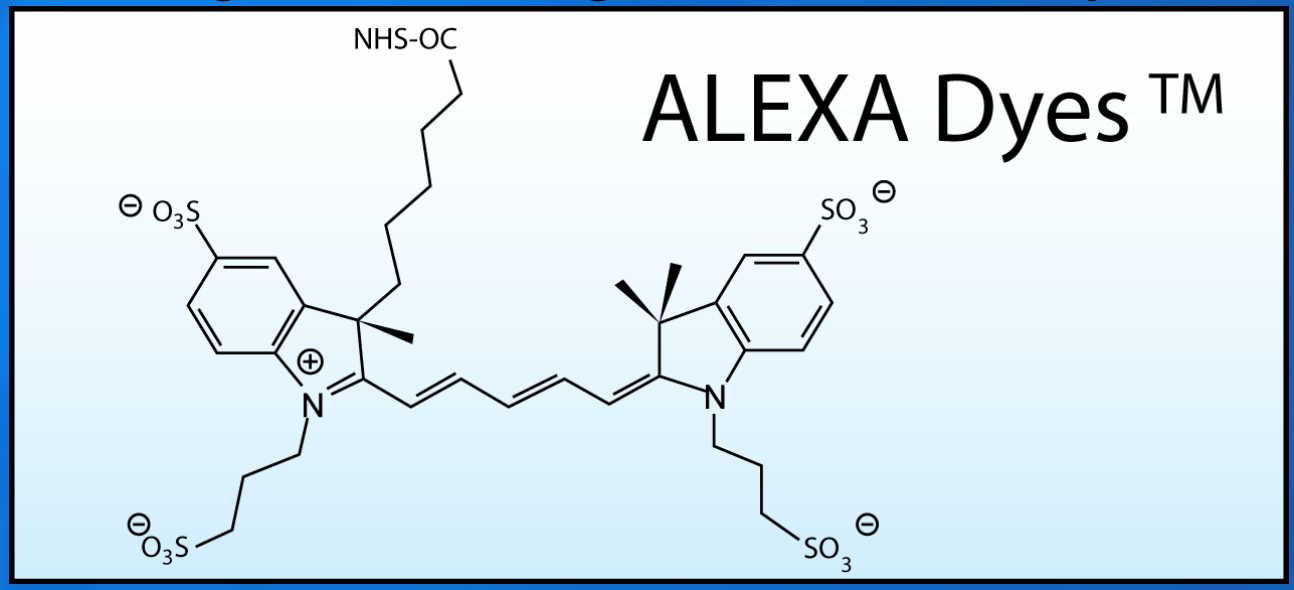


Structure and Spectral Properties of Long-Wavelength Cy Dyes™



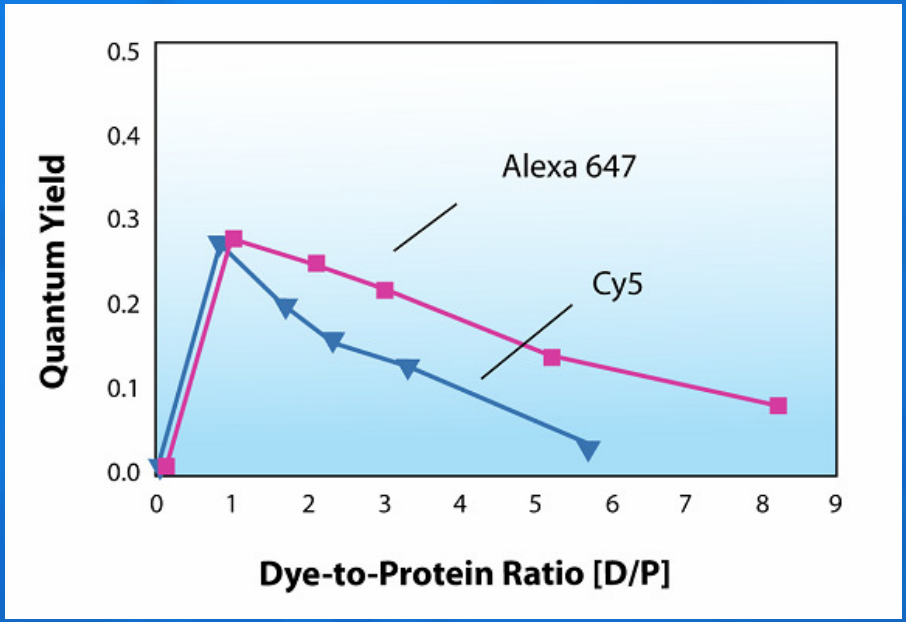
Dye-Conjugate	λ_{max} (abs) [nm]	λ_{max} (em) [nm]	ε ($M^{-1}cm^{-1}$)	Q.Y.
Cy5	649	670	250,000	0.2-0.28
Cy5.5	675	694	250,000	0.28
Cy7	743	767	250,000	0.28

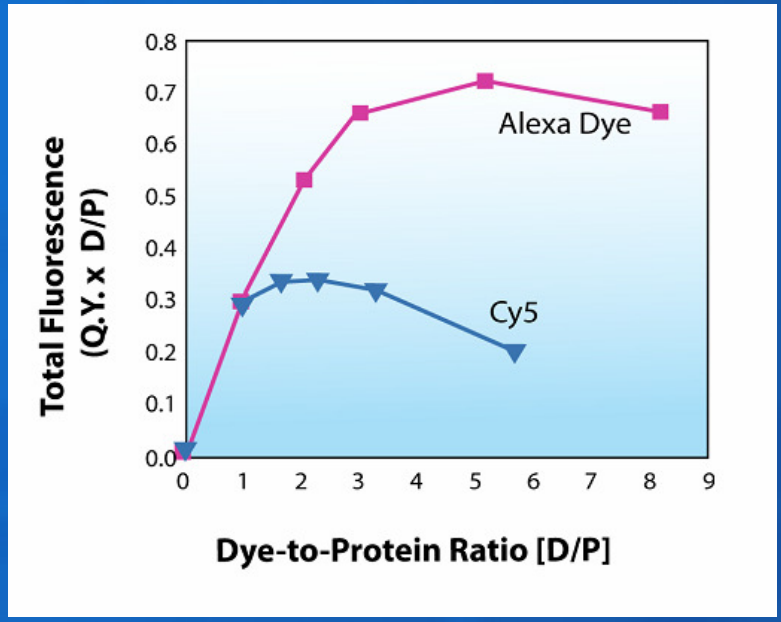
Structure and Spectral Properties of Long-Wavelength ALEXA Dyes™



Dye-Conjugate	λ_{max} (abs) [nm]	λ_{max} (em) [nm]	ε ($M^{-1}cm^{-1}$)	Q.Y.
AF647	650	668	250,000	0.33
AF660	668	668	132,000	0.37
AF750	749	782	240,000	0.12

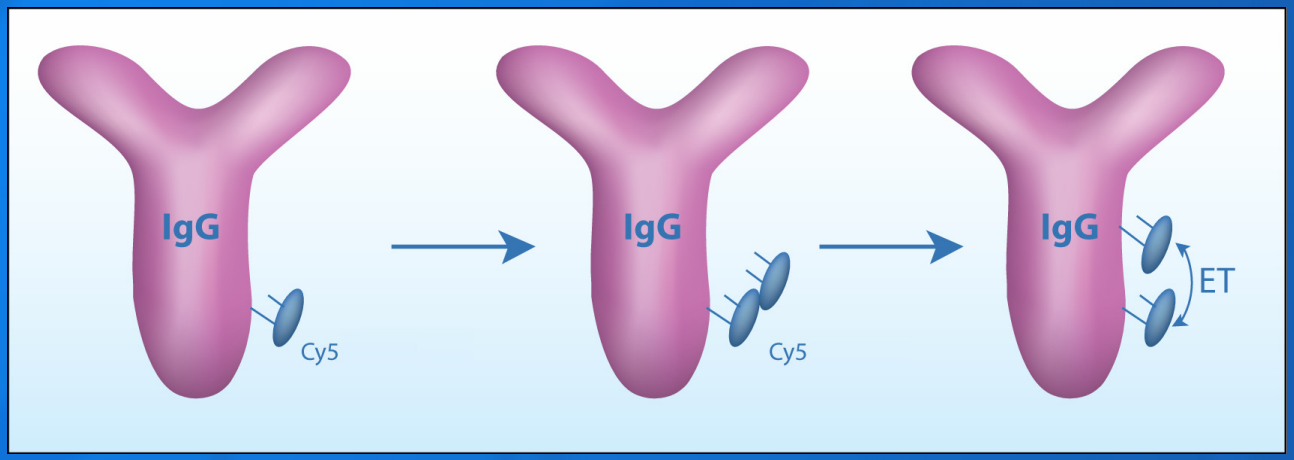
Quantum Yields and Total Fluorescence of IgG-Conjugates of Cy5 (▼), and Alexa-647(■) for Various D/P Ratios





Cy5 has anomalous tendency to aggregate

Cy5 - Anomalous Tendency to Aggregate

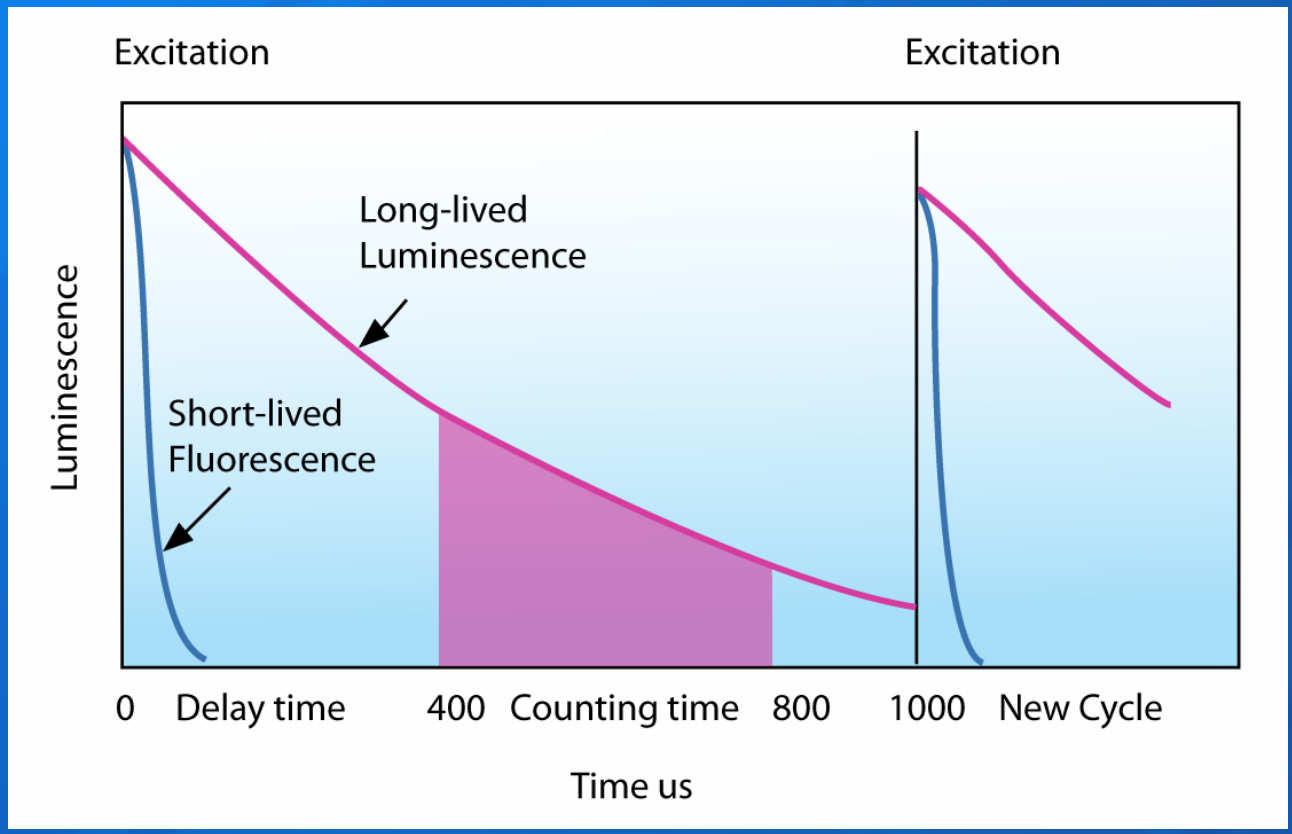


1st Cy5 molecule associates with the surface of a protein and reacts

2nd molecule interacts with labeled Cy5 and then reacts with next closest amino-group

Gruber et al., *Bioconjugate Chem.* **2000,** *11,* 696–704

Time-Resolved Luminescence Measurement

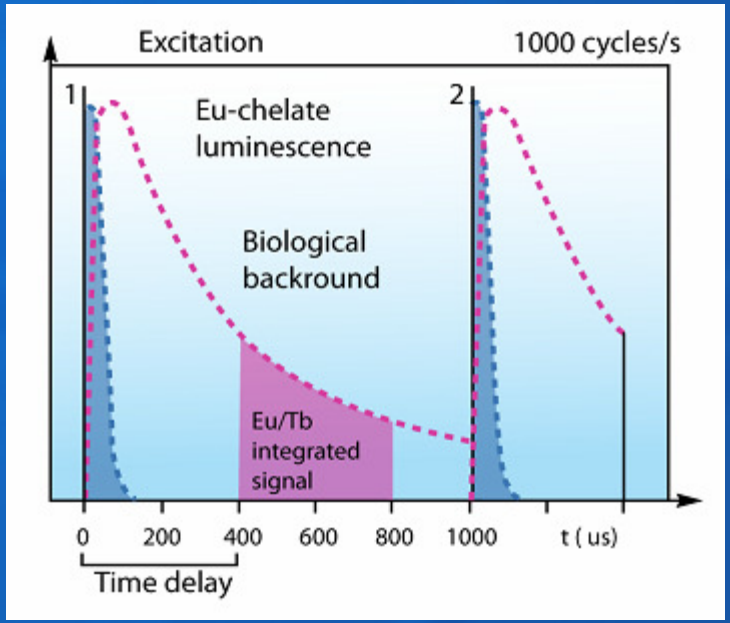


Luminescent Lanthanides (Eu^{3+}, Tb^{3+})

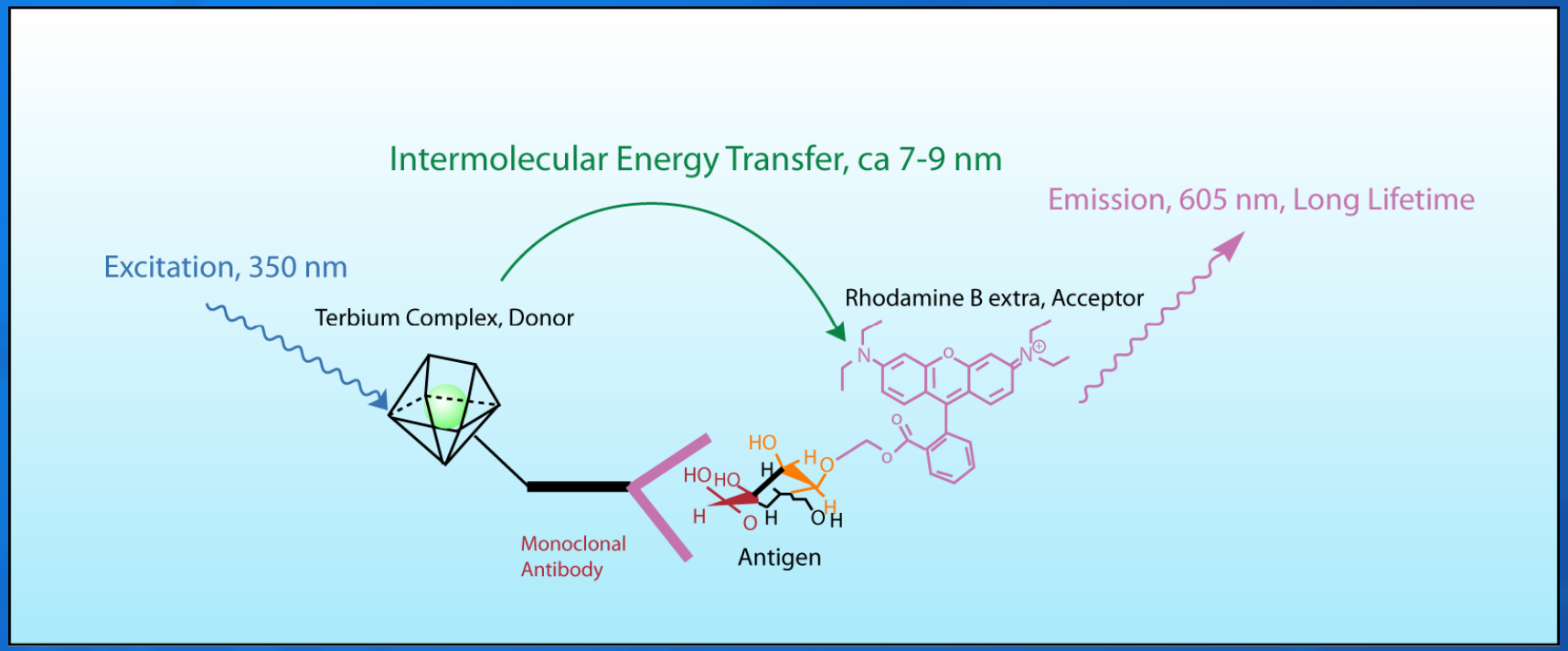
Advantages vs. Organic Fluorophores:

Enable Discrimination of Short-Lived Autofluorescence by "Gating" - More Sensitive and Reliable Measurements

- Narrow Emission Bands
- Long Luminescent Lifetimes
- Large Stokes' Shifts



Homogeneous Time-Resolved Fluorescence Immunoassays (HTRF)



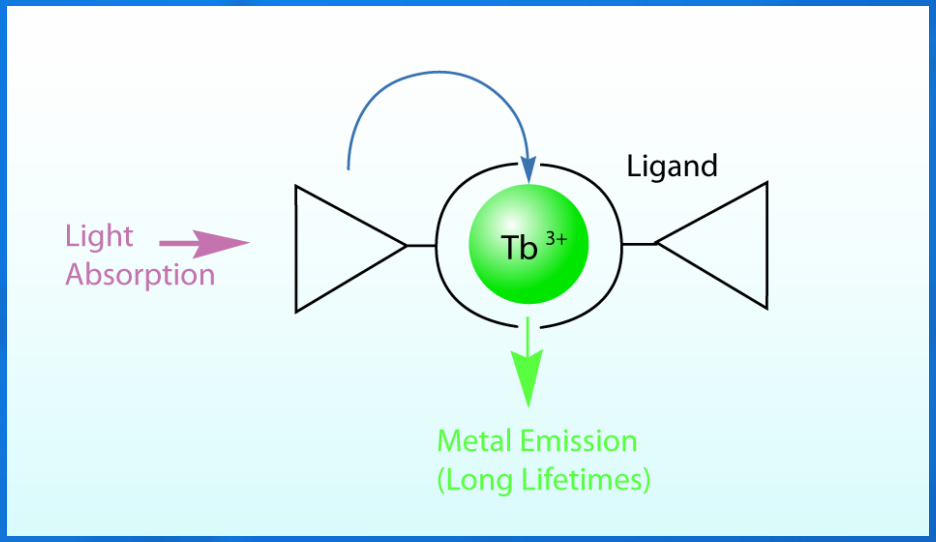
Minimal Sample Preparation - No Wash Steps

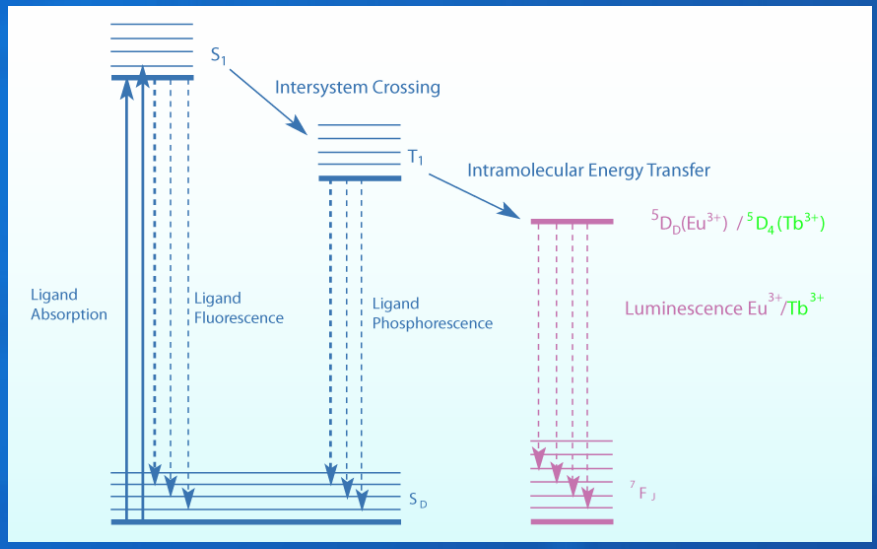
Applications: Drug Discovery

Clinical Diagnostics

What Is The Mechanism?

Antenna Effect



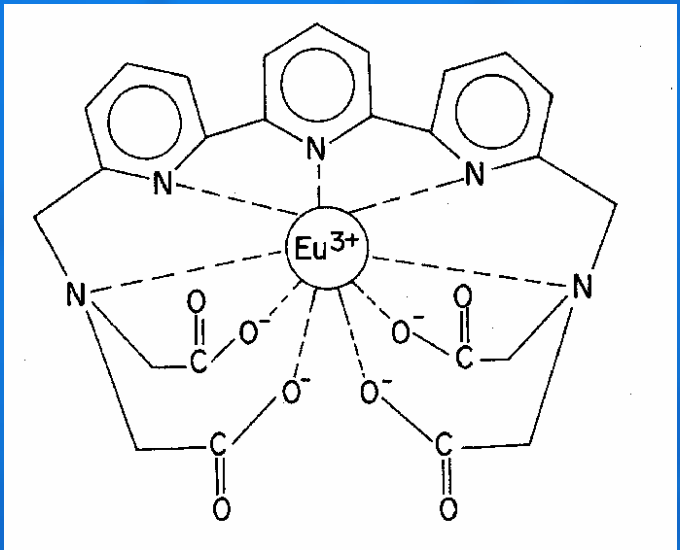


"Antenna Effect": Strong Absorption and Good Energy Transfer

Ln(III) Protected from Quenching

Thermodynamically Stable and Kinetically Inert Complexes

Lanthanide Complexes for Homogeneous Time-Resolved Immunoassays



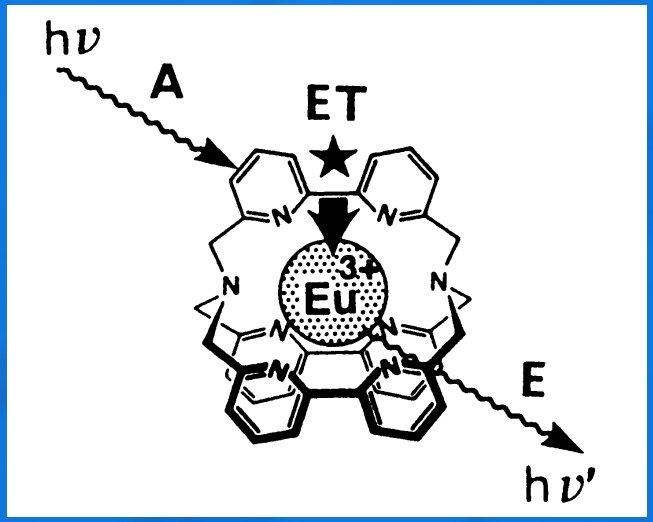
LANCE Ultra™

PerkinElmer
precisely.

- Eu^{3+} - Luminescence
- ϕ = 11 - 15 %, $\lambda_{excitation}$ ~ 320 nm

- CH- and CH_2 -Group are Replaced by CD and CD_2
- Acceptors : Allophycocyanine (APC) or cyanine dyes

Lanthanide Complexes for Homogeneous Time-Resolved Immunoassays



Eu-Cryptate™

Luminescent Only in Presence of F^-

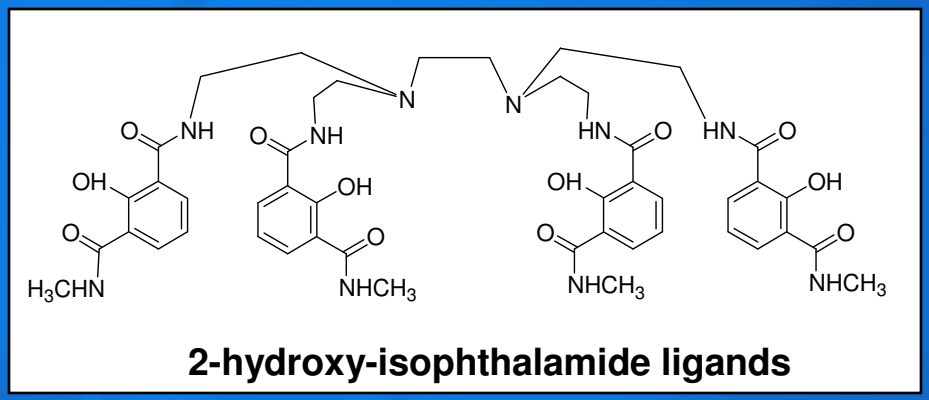
ϕ = 2 %

Not Stable in Water but "Kinetically Inert"

1-3 Water Molecules are Coordinated to Ln(III) (Limited Protection)

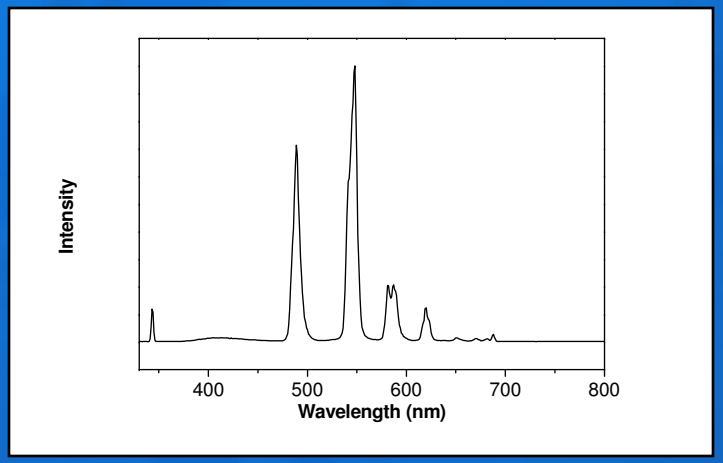
Acceptor : XL665 or C2 a low-MW acceptor

Highly Luminescent Lanthanide Complexes



2-hydroxy-isophthalamide ligands

Ligand **Tb^{3+}** **Eu^{3+}**

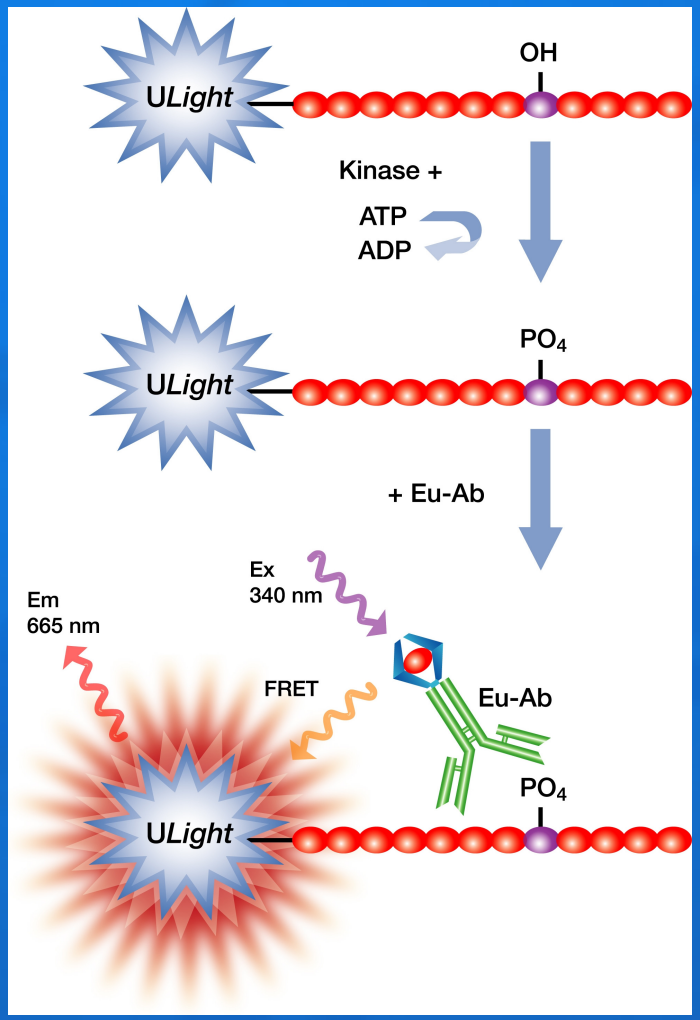


Lumiphore, Inc.

Quantum Yield: 60 % !!!

Lifetime τ = 2.56 ms

TR-FRET HTS Assay



5 µl enzyme

384-well Plate

+ 5 µl U*Light*-peptide
(+ ATP)

Incubation at RT for 1h

+ 10 µl Eu-anti-phosphosubstrate
antibody (+ EDTA)

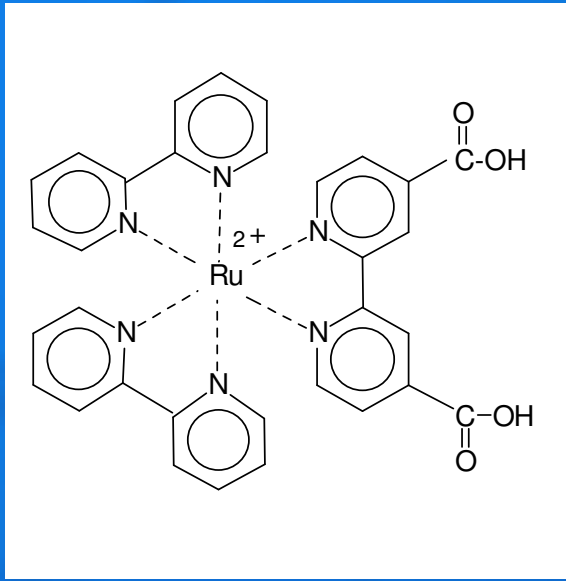
Incubation at RT for 1h

Read

(Excitation at 320 nm, Emission at 665 nm)

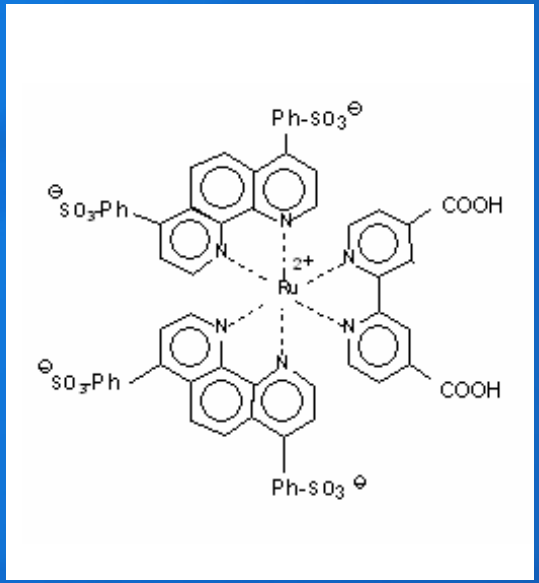
Courtesy of PerkinElmer

Spectral Data and Lifetimes for a Representative Metal-Ligand Complexes (MLCs)



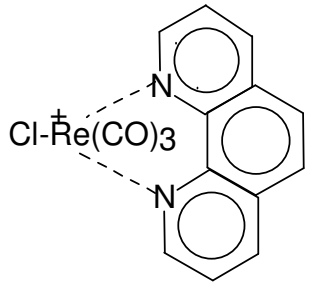
$Ru(bpy)_2(dcbpy)$

λ_{max}(abs) (water) = 467 nm
λ_{max}(em) = 655 nm
Q.Y. = 0.05
r_0 = 0.23
τ = 366 ns



$Ru(SO_3dphphen)_2(dcbpy)$

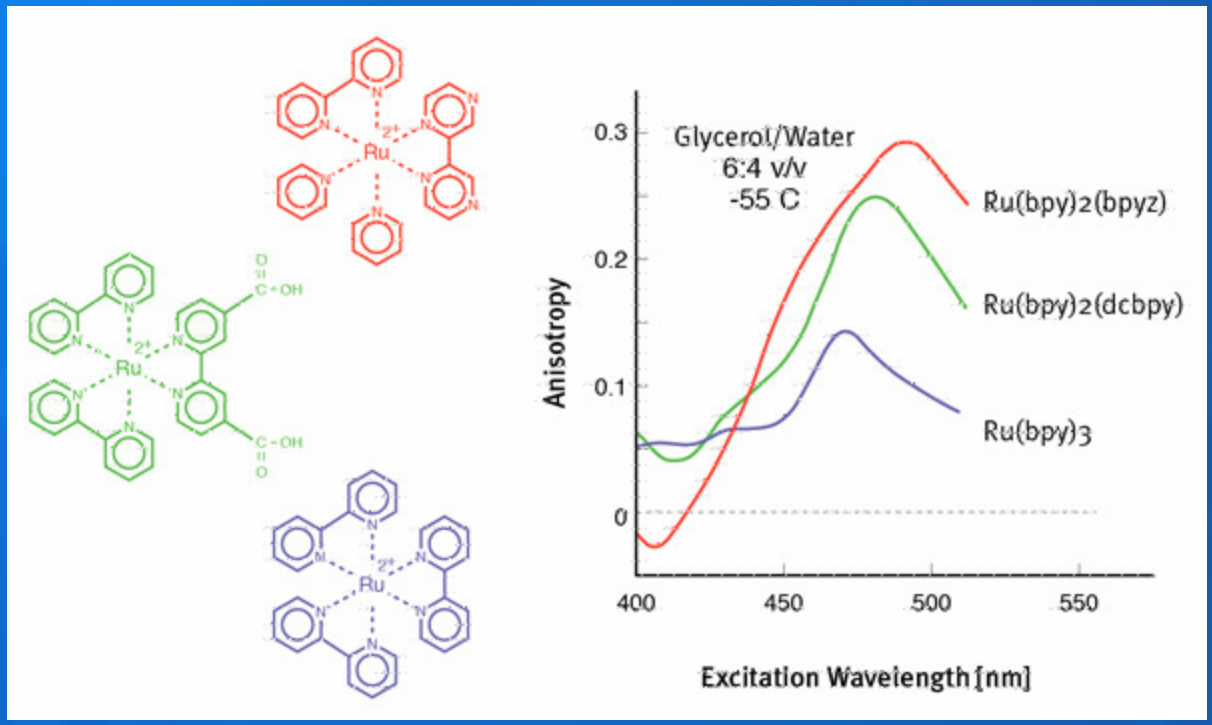
λ_{max}(abs) (water) = 4640 nm
λ_{max}(em) = 643 nm
Q.Y. = 0.06
τ = 0.8 µs
τ = 2 µs (HSA)



$Re(CO)_3Cl(phen)$

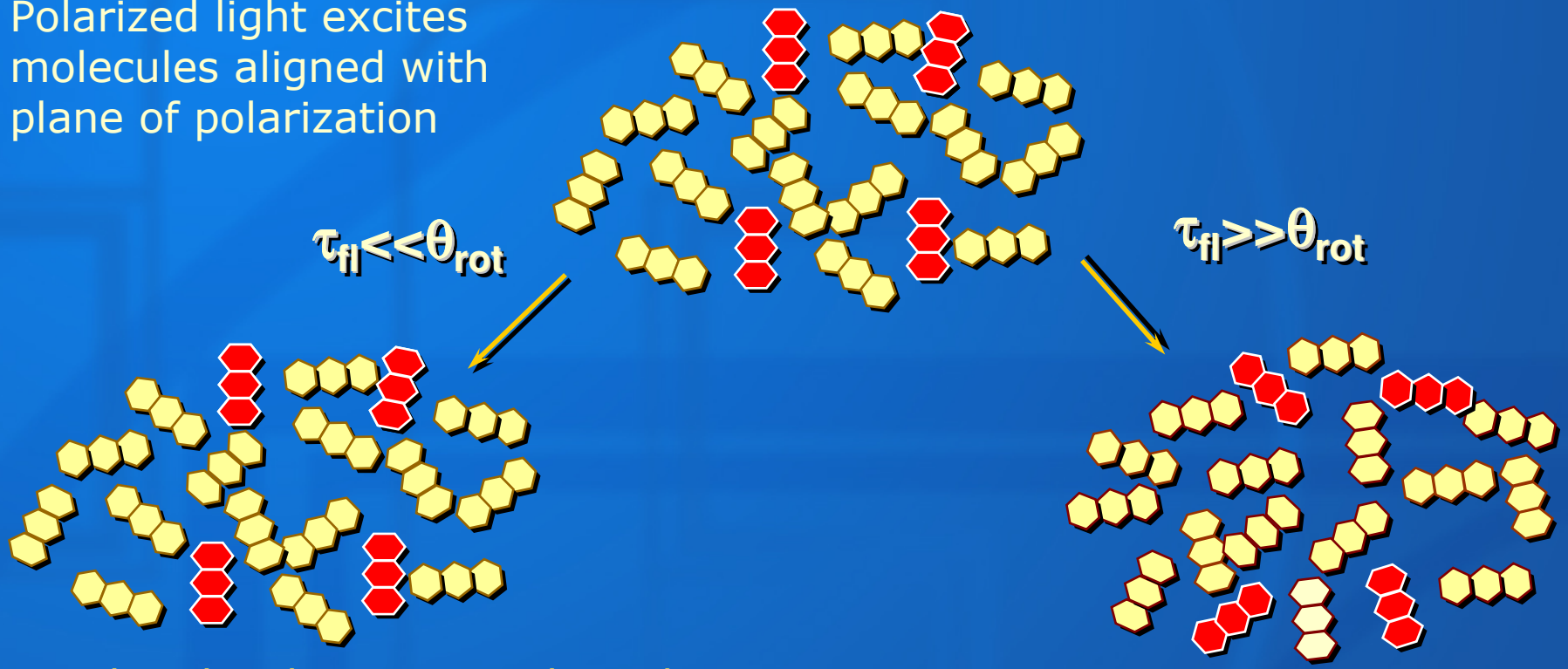
λ_{max}(abs) (water) = 275 nm
λ_{max}(em) = 589 nm
Q.Y. = 0.2
r_0 = 0.3
τ = 110 ns

Excitation Polarization Spectra of Representative MLCs



Fluorescence Polarization:
A Race between Emission and Molecular Motion

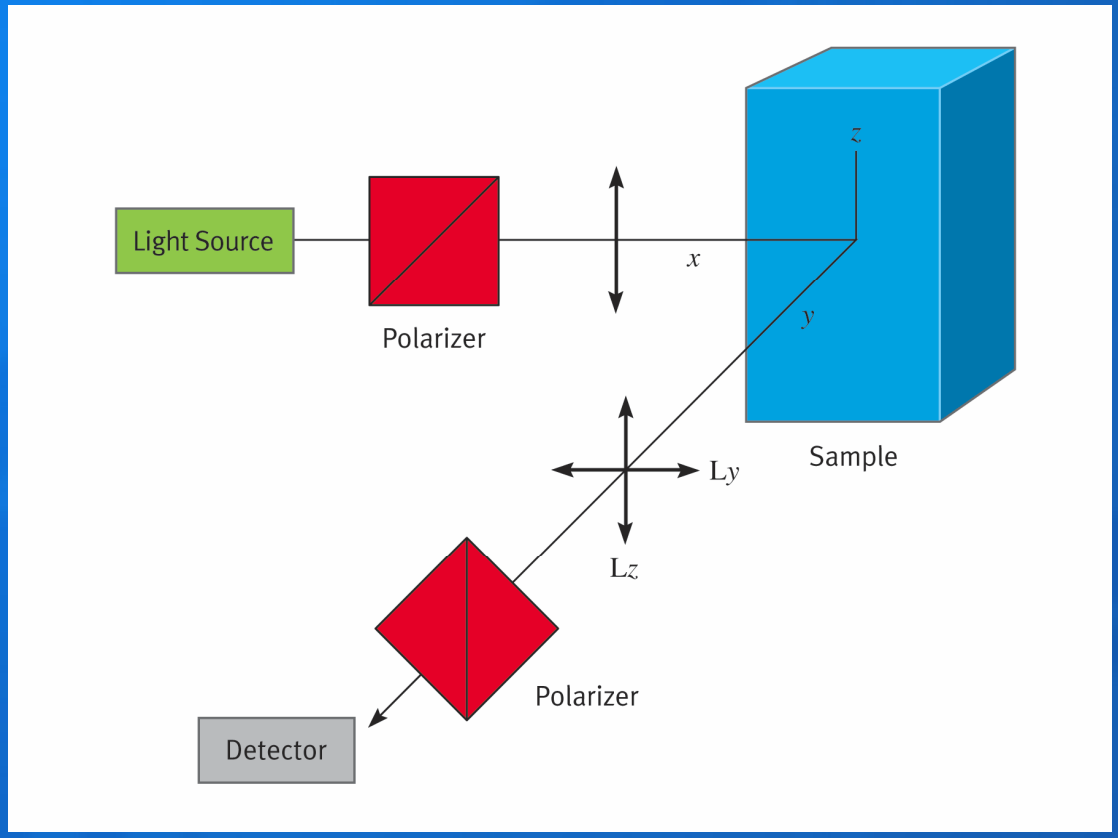
Polarized light excites molecules aligned with plane of polarization



Excited molecules remain aligned. Fluorescence is polarized.

Orientation of excited molecules randomizes. Fluorescence is depolarized.

Fluorescence Polarization Measurement



Fluorescence Polarization

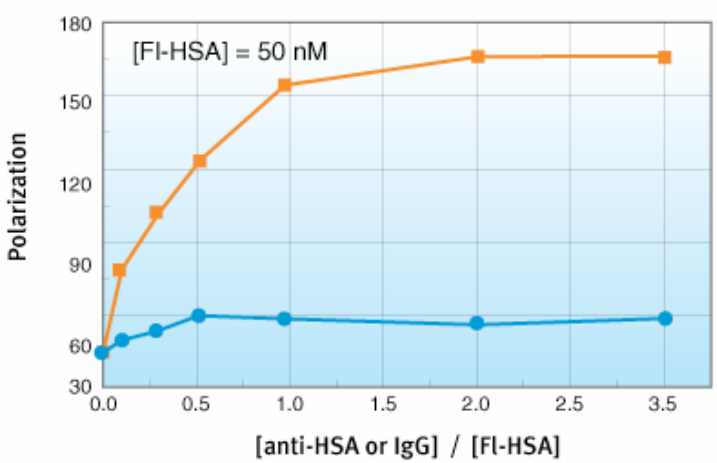
$$\text{Polarization } (P) = \frac{I_v - I_h}{I_v + I_h}$$

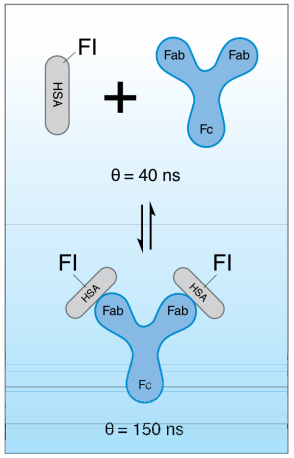
$$\text{Anisotropy } (r) = \frac{I_v - I_h}{I_v + 2 I_h}$$

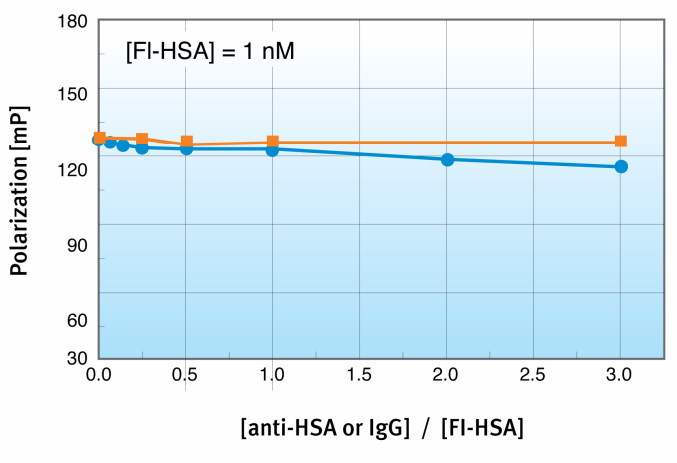
$$P = \frac{3 r}{2 + r}$$

$$r = \frac{2 P}{3 - P}$$

Role of Lifetime in FP







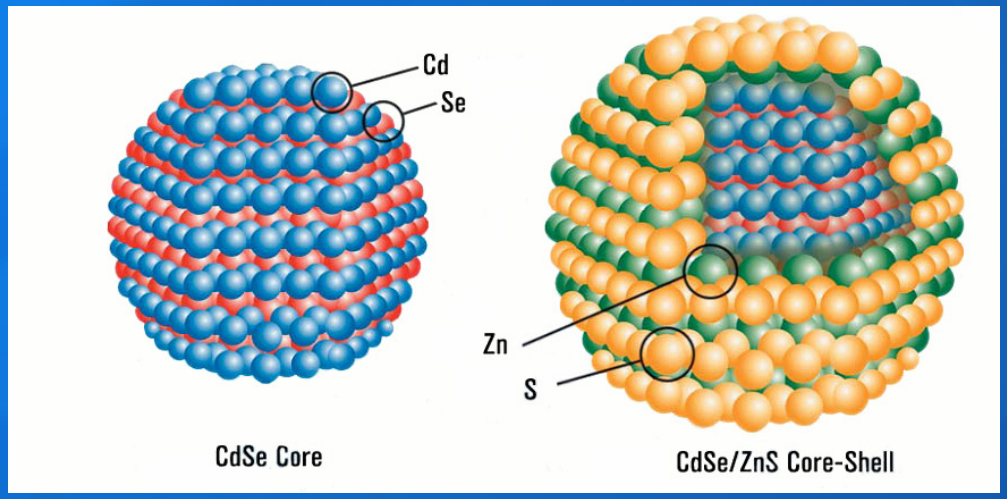
$$r = \frac{r_0}{(1 + \tau/\theta)}$$

τ_{fl} ~ 300 ns

τ_{fl} = 4ns

θ = 150 ns

Quantum Dots



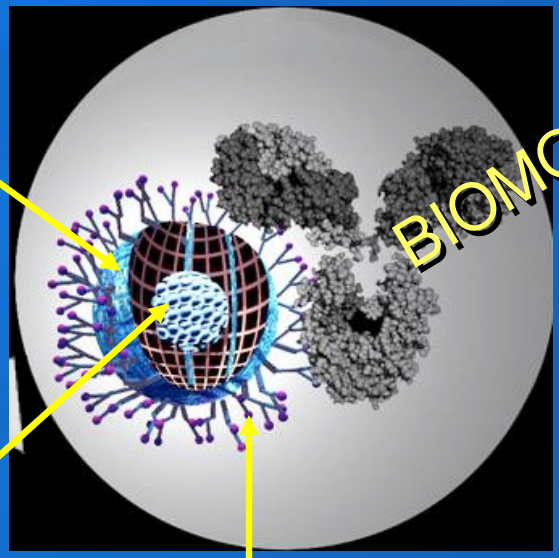
Quantum Dots

Nanometer-Scale Atom Clusters

CORE

Cadmium selenide (**CdSe**), or Cadmium telluride (**CdTe**)
few hundred – few thousand atoms

The semiconductor material is chosen based upon the emission wavelength, however it is the **size** of the particles that **tunes the emission wavelength**.



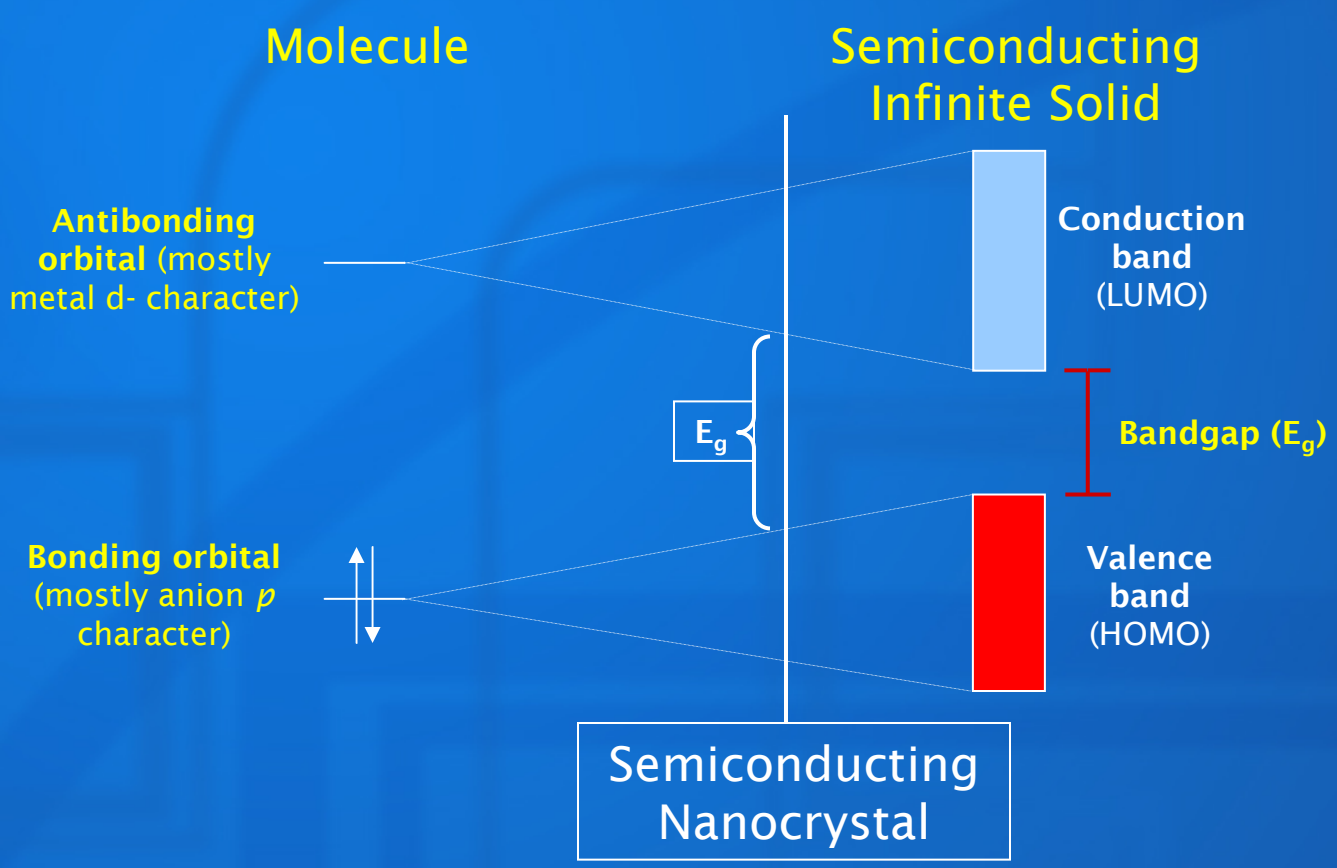
SHELL

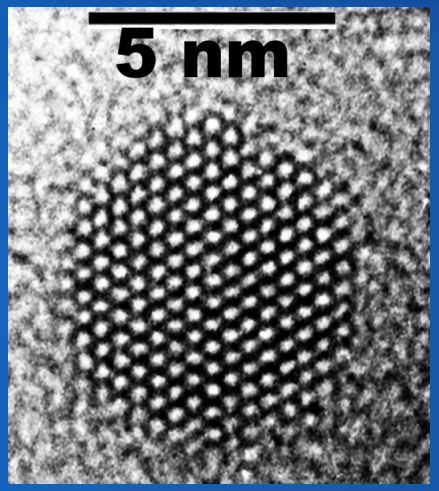
In the core emission is typically weak and always unstable.
The shell material (**ZnS**) has been selected to be almost entirely unreactive and completely insulating for the core.

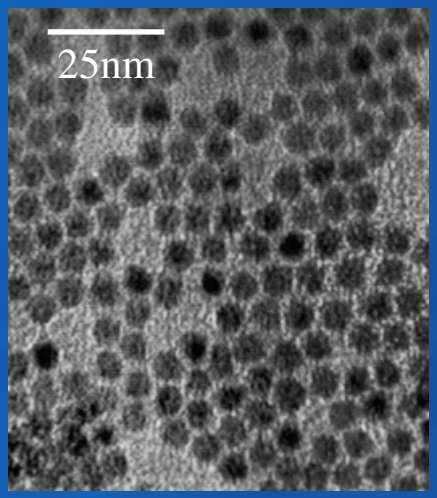
COATING

A layer of organic ligands covalently attached to the surface of the shell. This coating provides a **surface for conjugation** to biological (antibodies, streptavidin, lectins, nucleic acids) and nonbiological species and makes them "water-soluble"
.

Semiconductor Nanocrystals

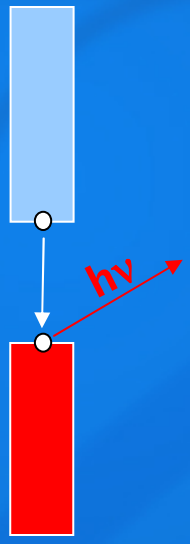


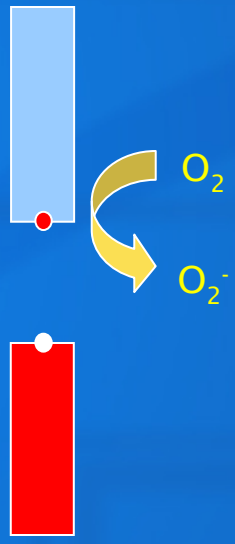


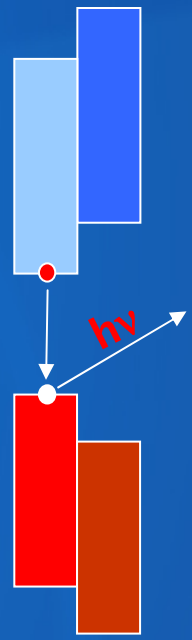


Bandgap of nanocrystal is size-dependent, larger than for bulk material

Preventing Photobleaching In Quantum Dots







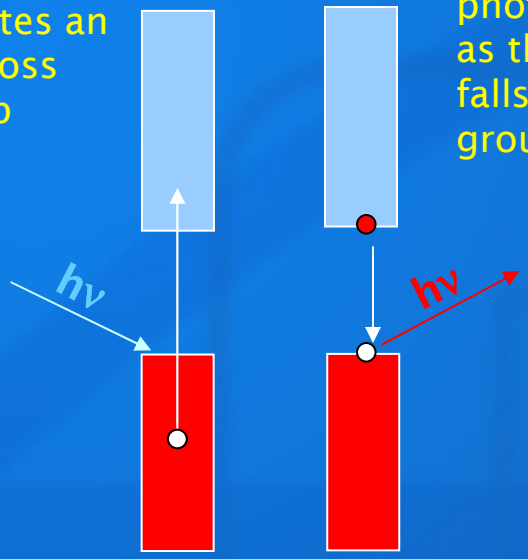
Plain core QDots show emission, but oxidation results in permanent loss of emission, so the population would gradually bleach away.

In Core/shell QDots the electron remains in the lower-energy core orbitals, and never reaches particle surface to react

The shell keeps the high-energy excited electron away from oxygen

Semiconductor Nanocrystal Fluorescence

A high-energy photon excites an electron across the bandgap



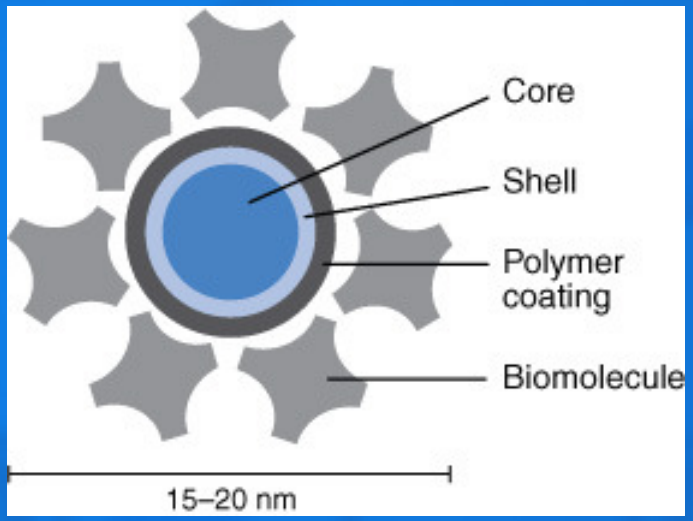
A bandgap-energy photon is emitted as the electron falls back to the ground state

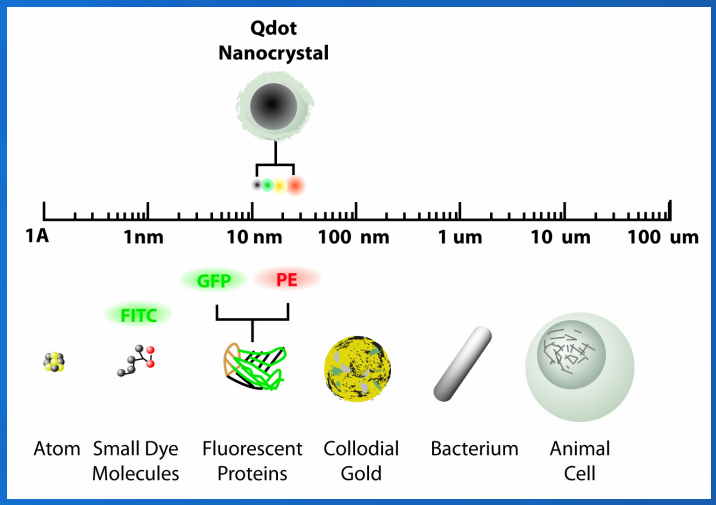
Size-dependent bandgap means size-dependent color

Courtesy of Invitrogen

Quantum Dots

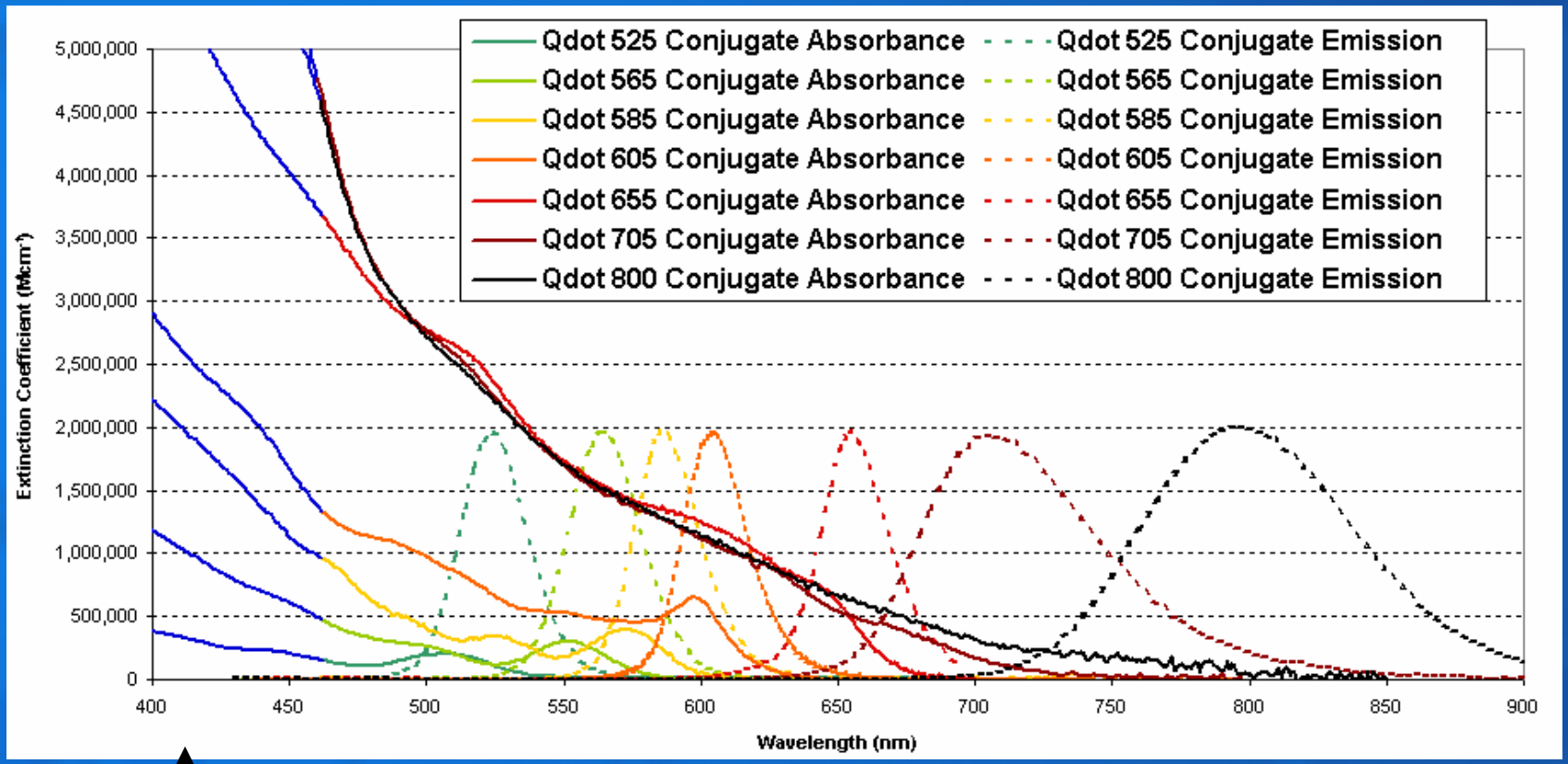
Nanometer-Scale Atom Clusters





Quantum Dot Material System	Emission Range	Quantum Dot Diameter Range	Quantum Dot Type	Standard Solvents	Example Applications
CdSe	465nm - 640nm	1.9nm - 6.7nm	Core	Toluene	Research, Solar Cells, LEDs
CdSe/ZnS	490nm - 620nm	2.9nm - 6.1nm	Core-Shell	Toluene	VisibleFluorescence Applications, Electroluminescence, LEDs
CdTe/CdS	620nm - 680nm	3.7nm - 4.8nm	Core-Shell	Toluene	Deep Red Fluorescence Apps.

Qdot Optical Spectra



Violet excitation

Absorbance × Quantum Yield = Brightness
photons in **fraction converted** **photons out**

Broad range of emissions

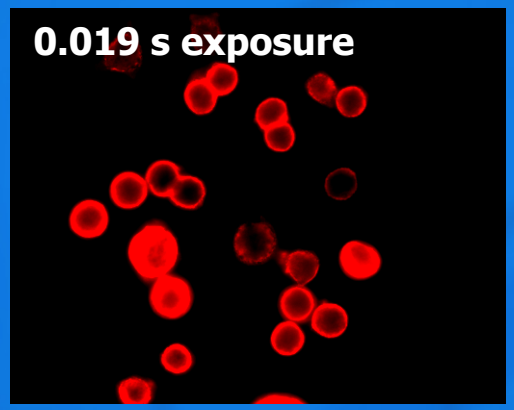
High absorbance means increased brightness

Single-color excitation, multicolor emission for easy multiplexing

Courtesy of Invitrogen

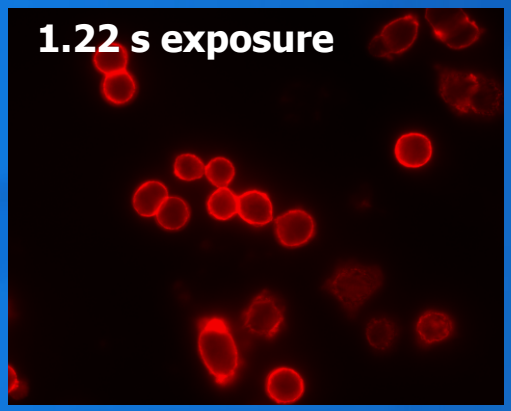
Brightness Means Sensitivity

Qdot® nanocrystals



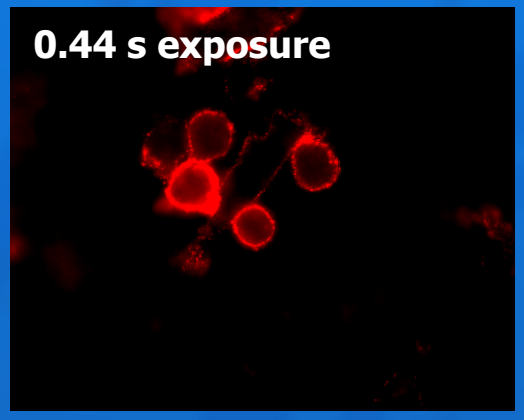
60X

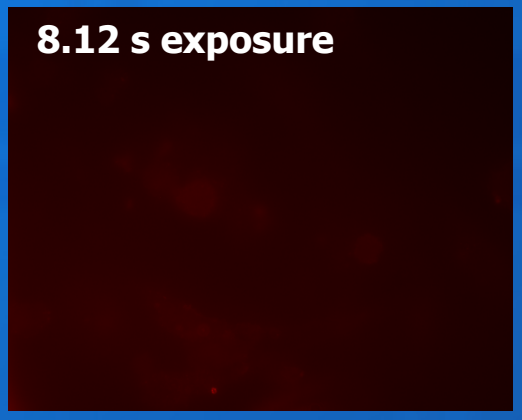
Cy5 organic dye



Anti-Her2/neu + anti-mouse Ig conjugates

- SK-BR-3 Cells: High Her2/neu expression
- Nanocrystals up to 50x brighter





- MDA-MB-231 cells: Low Her2/neu expression
- Nanocrystals easy to detect but dye undetectable

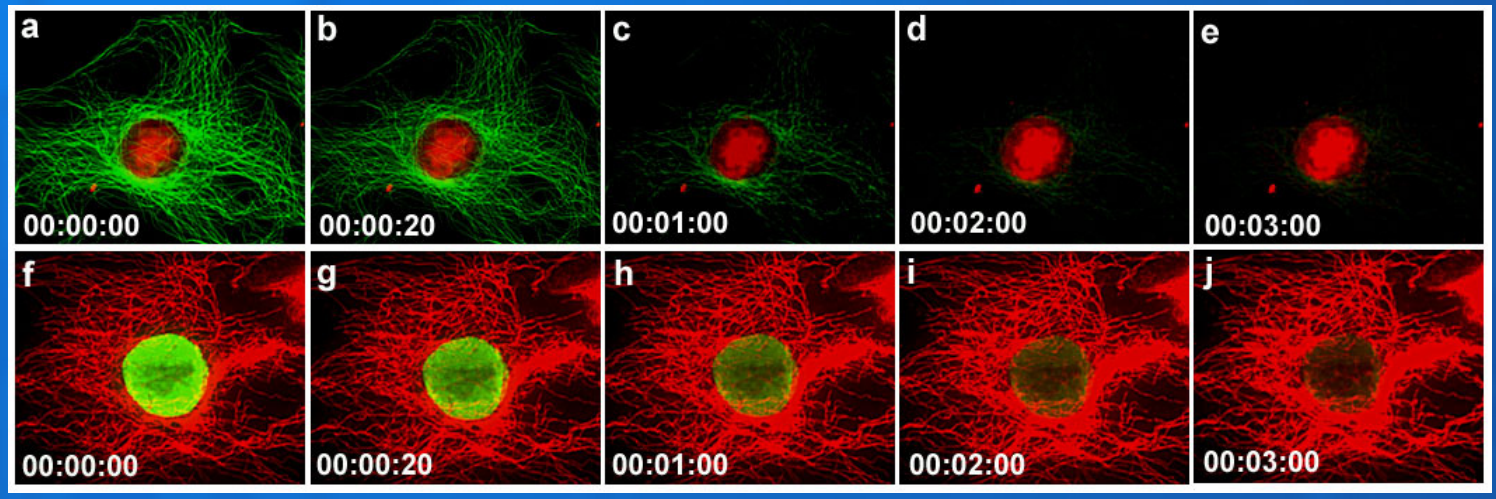
Courtesy of Invitrogen

Photostability in Microscopy

3T3 Cells

Top panel (a-e)
Nucleus: Qdot® 605 conjugate
Microtubules: Alexa Fluor® 488 conjugate

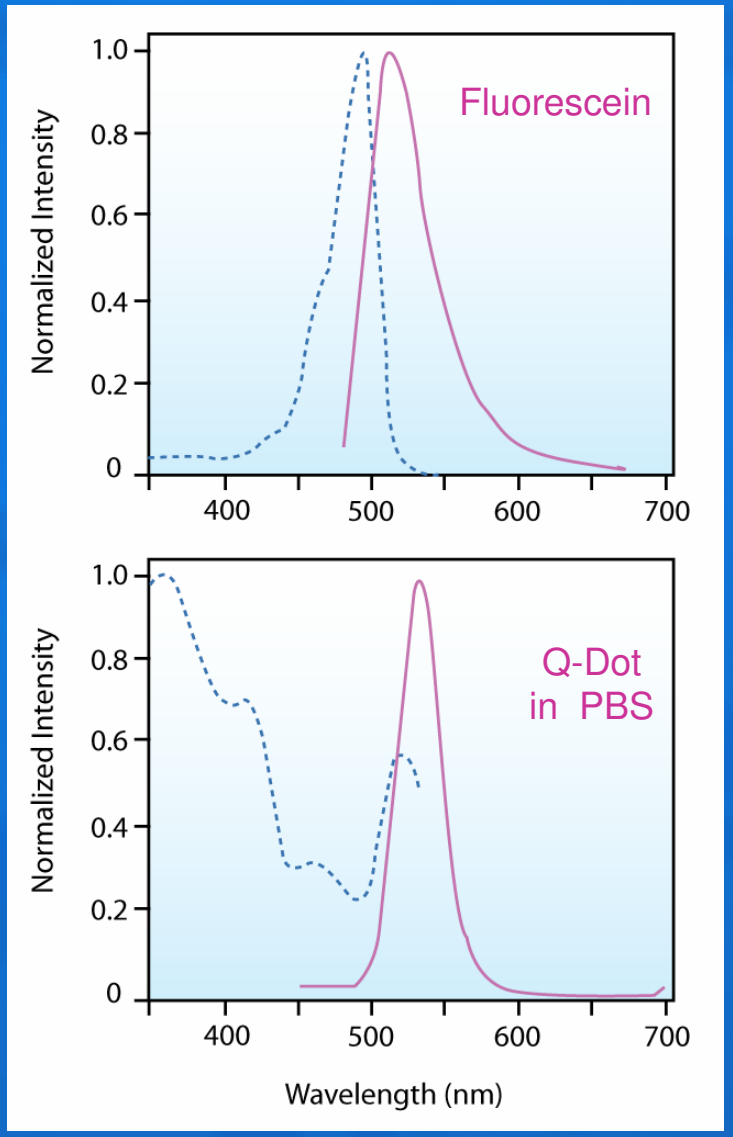
Bottom panel (f-j)
Nucleus: Alexa Fluor® 488 conjugate
Microtubules: Qdot® 605 conjugate



Photostability results in sensitivity, ease of use, and sample permanence

Courtesy of Invitrogen

Qdot Summary



Advantages:

Broad absorption spectra, making it possible to excite all colors of QDs simultaneously with a single light source - **Multiplexing**

Narrow and symmetrical emission spectra

Emission tunable with size and material composition

Exhibit excellent **photo-stability**

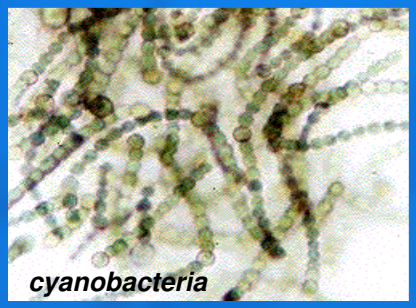
Disadvantages:

Large size and **high mass** limit their use in applications requiring high diffusional mobility

QDot	$\lambda_{max\ (abs)}$ [nm]	$\lambda_{max\ (em)}$ [nm]	ε ($M^{-1}cm^{-1}$)	Q.Y.
655	350	655	9,000,000	~0.5
705	350	705	13,000,000	~0.5
800	350	800	13,000,000	~0.5

Fluorescent Proteins

Phycobiliproteins



From red algae and cyanobacteria (blue-green algae).

Absorb strongly between 470 and 650 nm.

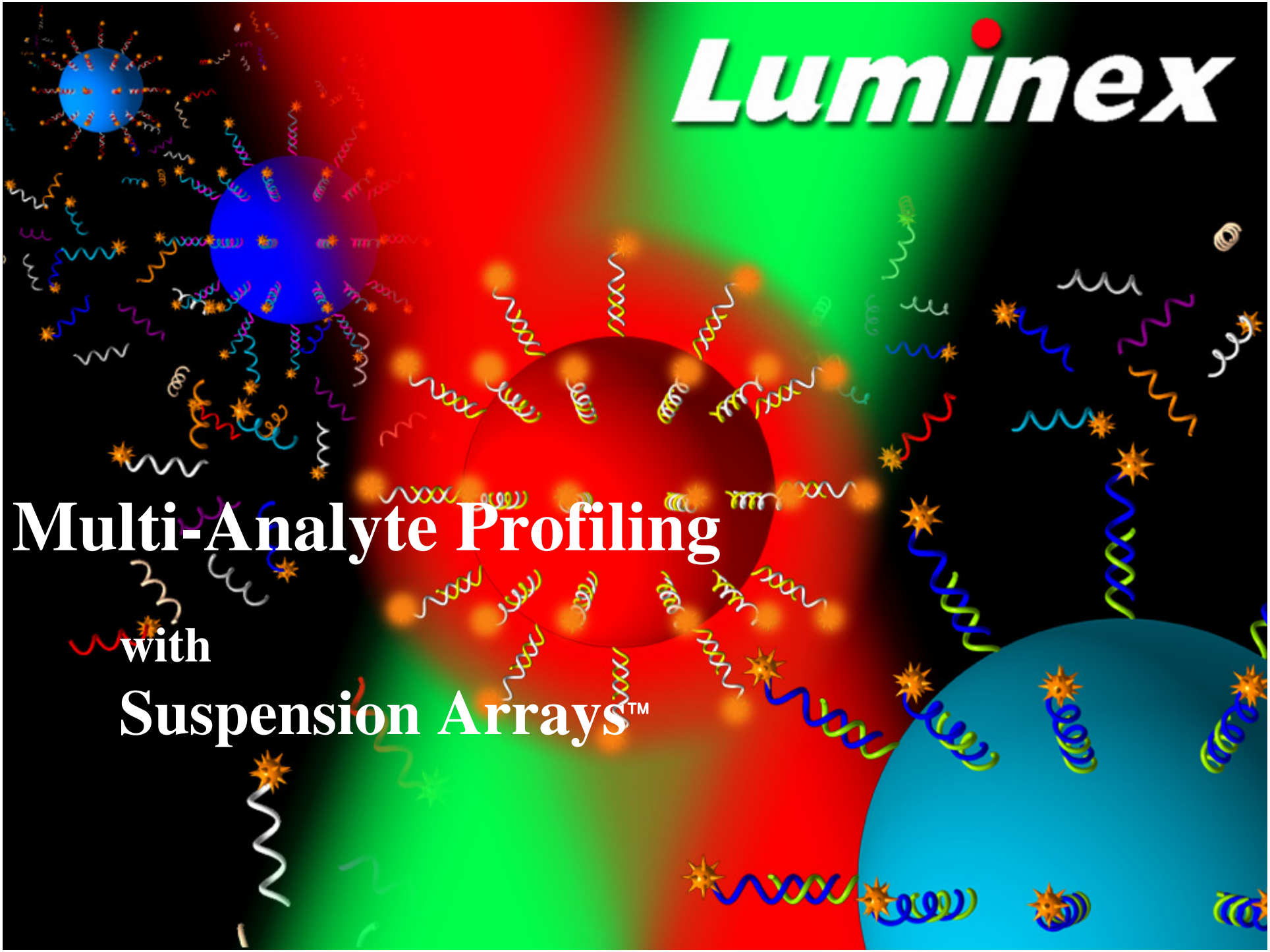
Highly fluorescent *in vitro*

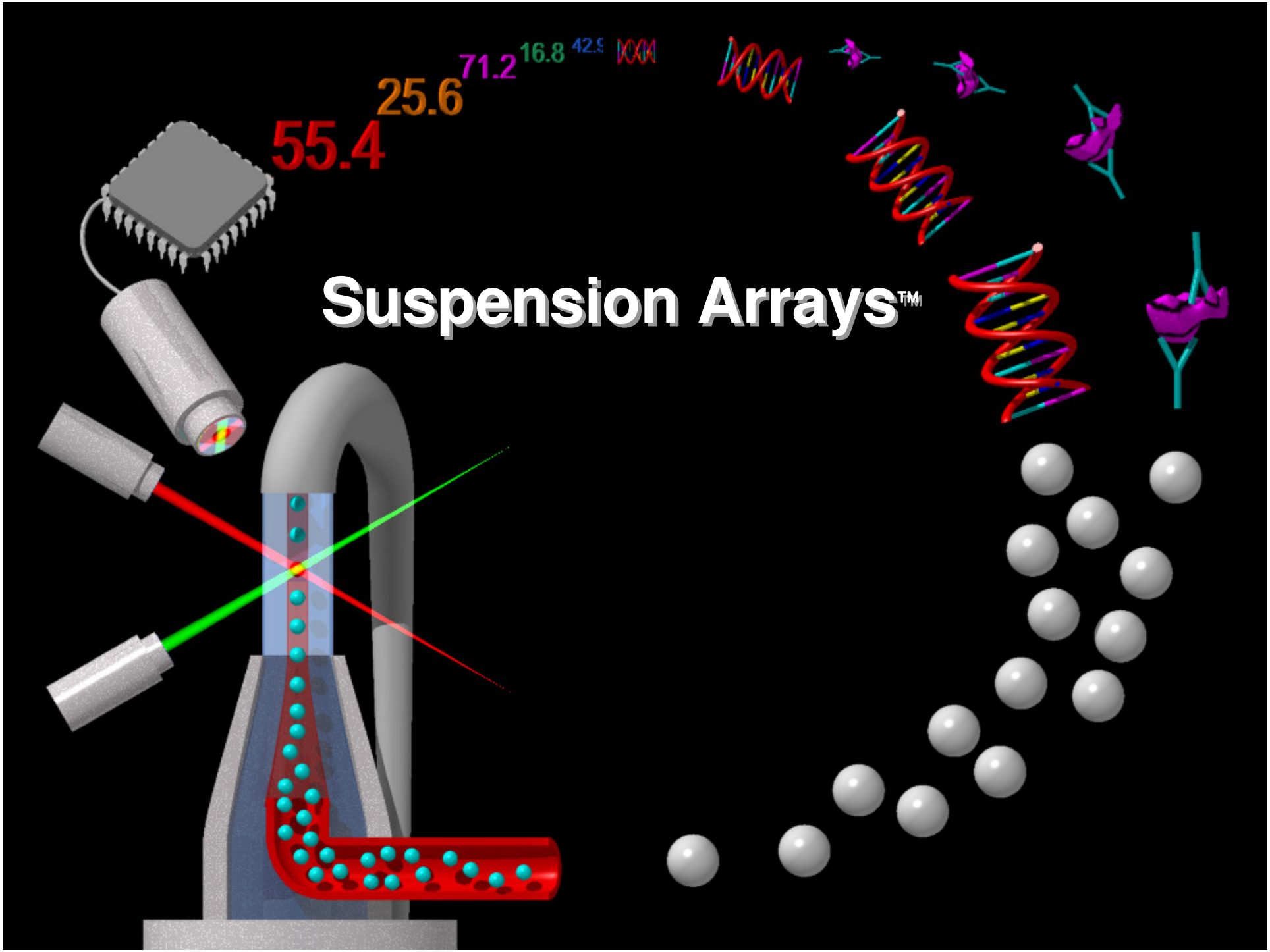
Hold the record for the highest extinction coefficients and largest number of photons emitted before bleaching

Used as ET acceptors and in tandem-constructs for multi-color analysis (flow cytometry)

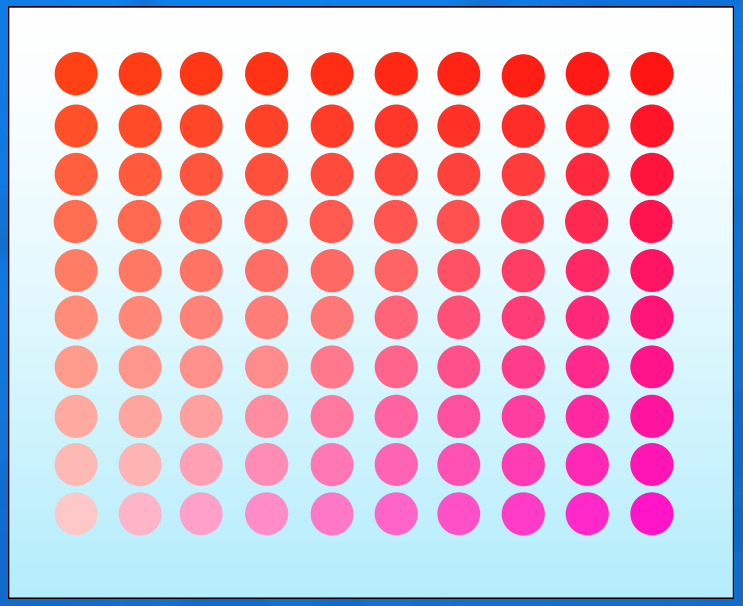
Four main classes of phycobiliproteins

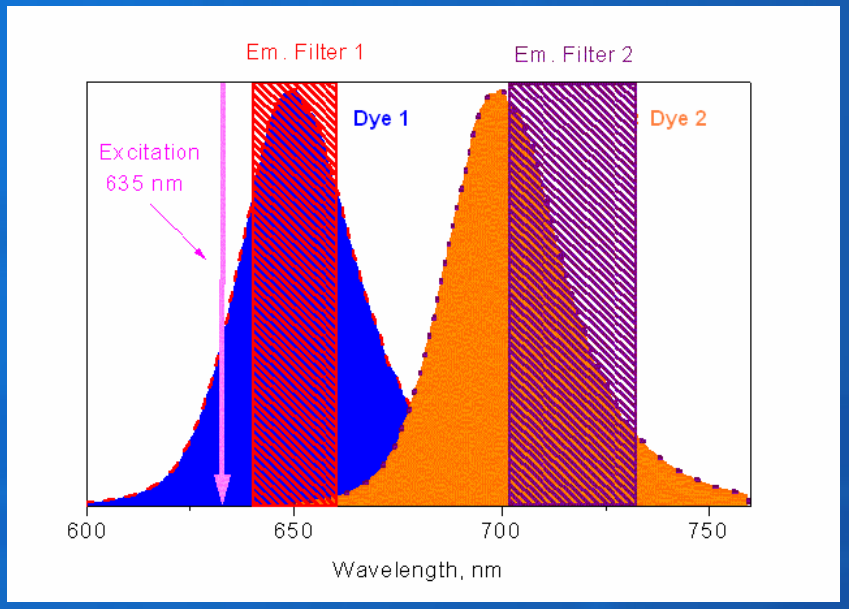
Protein	Subunit Composition	Approx mol. wt.	ε ($M^{-1}cm^{-1}$)	Total bilins per protein	λ_{max} (abs) [nm]	λ_{max} (em) [nm]
Allophycocyanin	$(\alpha\beta)_3$	100,000	700,000	6	650	660
R-Phycocyanin	$(\alpha\beta)_3$	110,000	1,000,000	9	555,618	642
B-Phycoerythin	$(\alpha\beta)_{6Y}$	240,000	2,400,000	34	543,562	576
R-Phycoerythrin	$(\alpha\beta)_{6Y}$	240,000	2,200,000	34	495,536,565	576

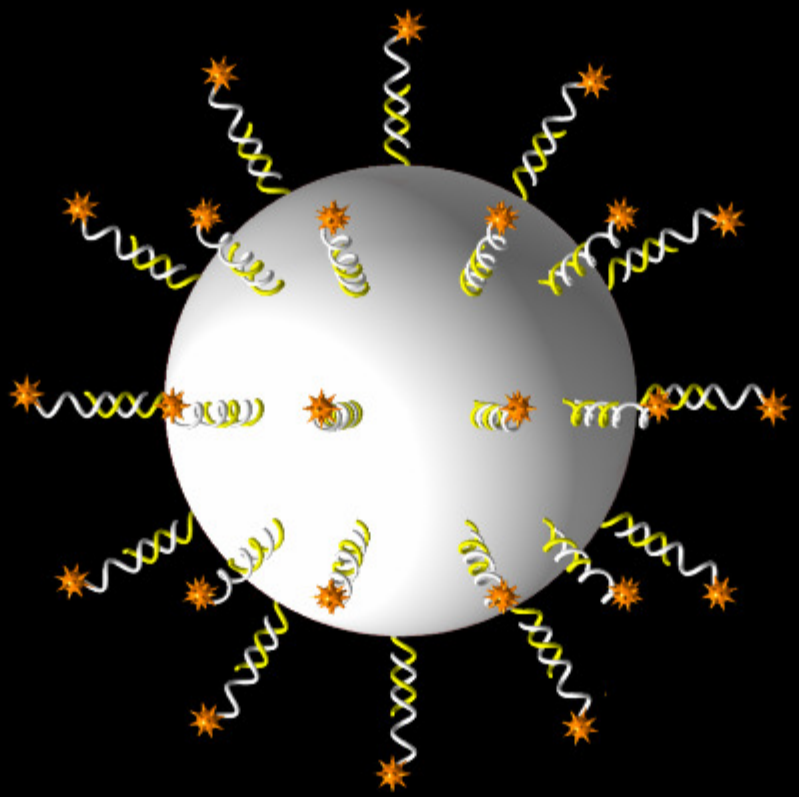

Luminex
Multi-Analyte Profiling
with
Suspension Arrays™

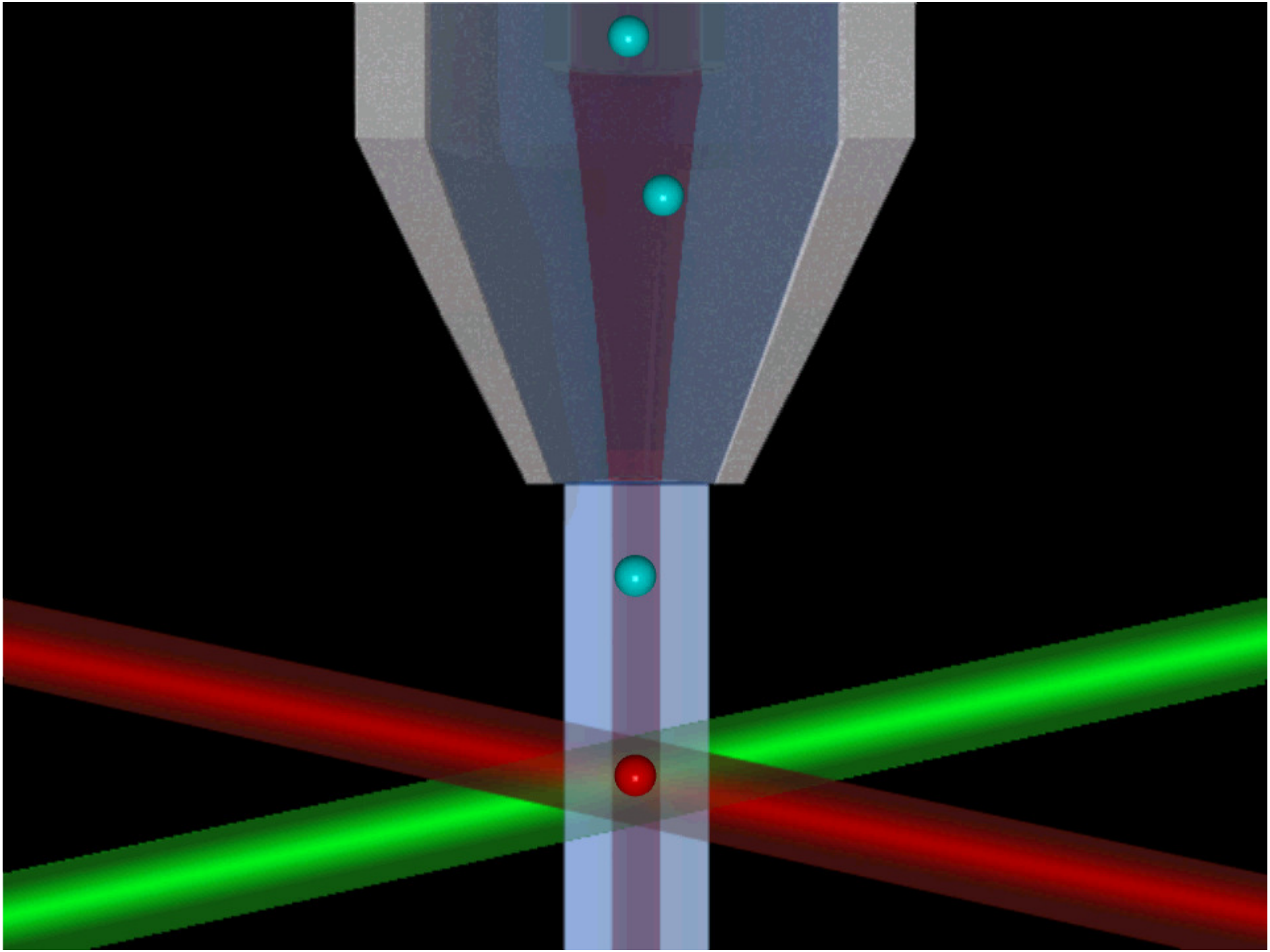

55.4
25.6
71.2
16.8
Suspension Arrays™

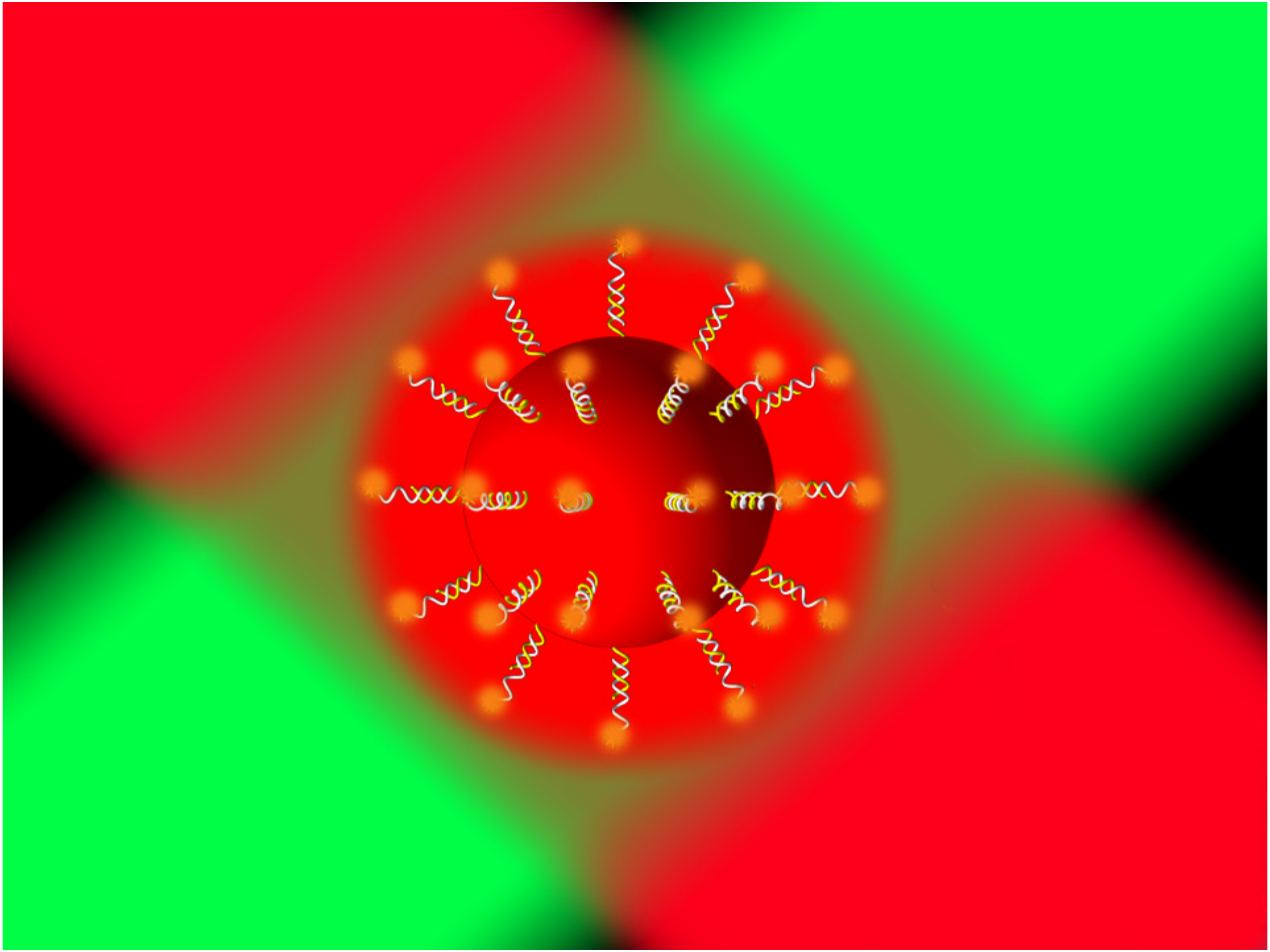
100-Plex Bead System



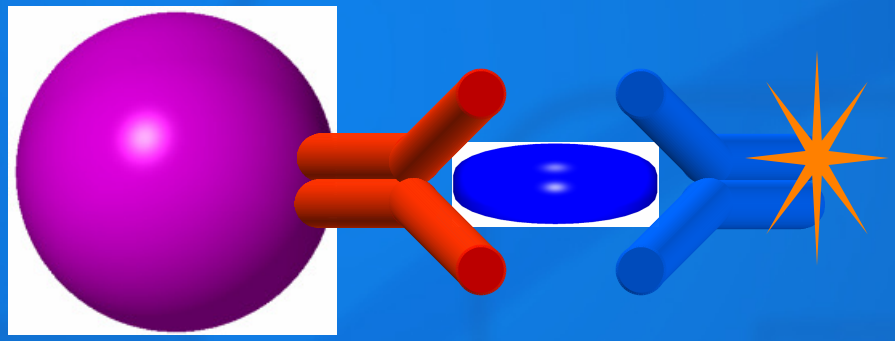


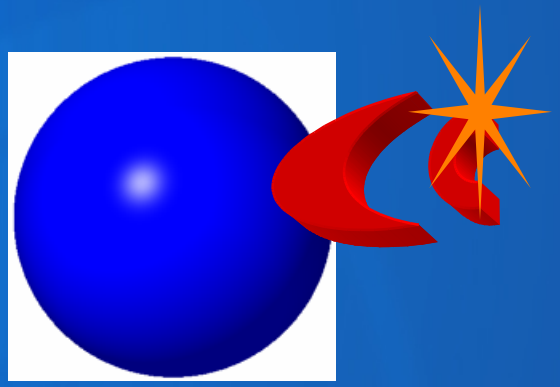


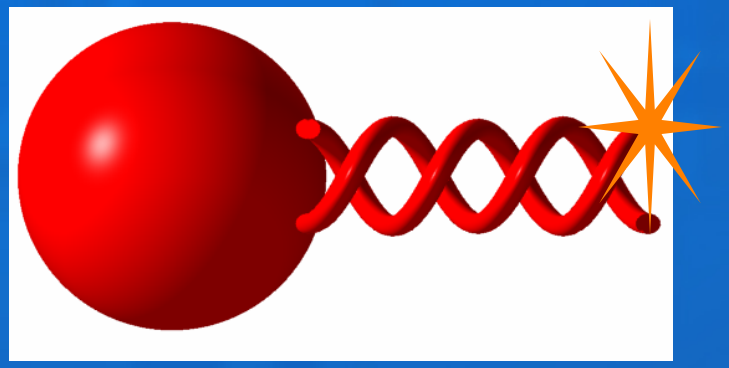


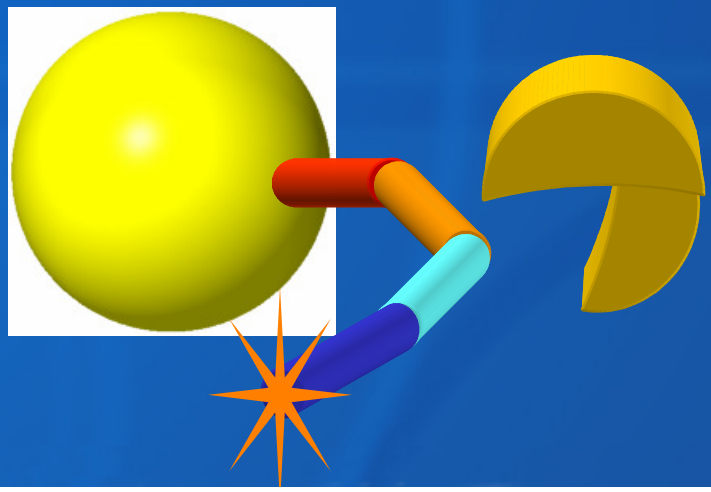


Applicable Formats

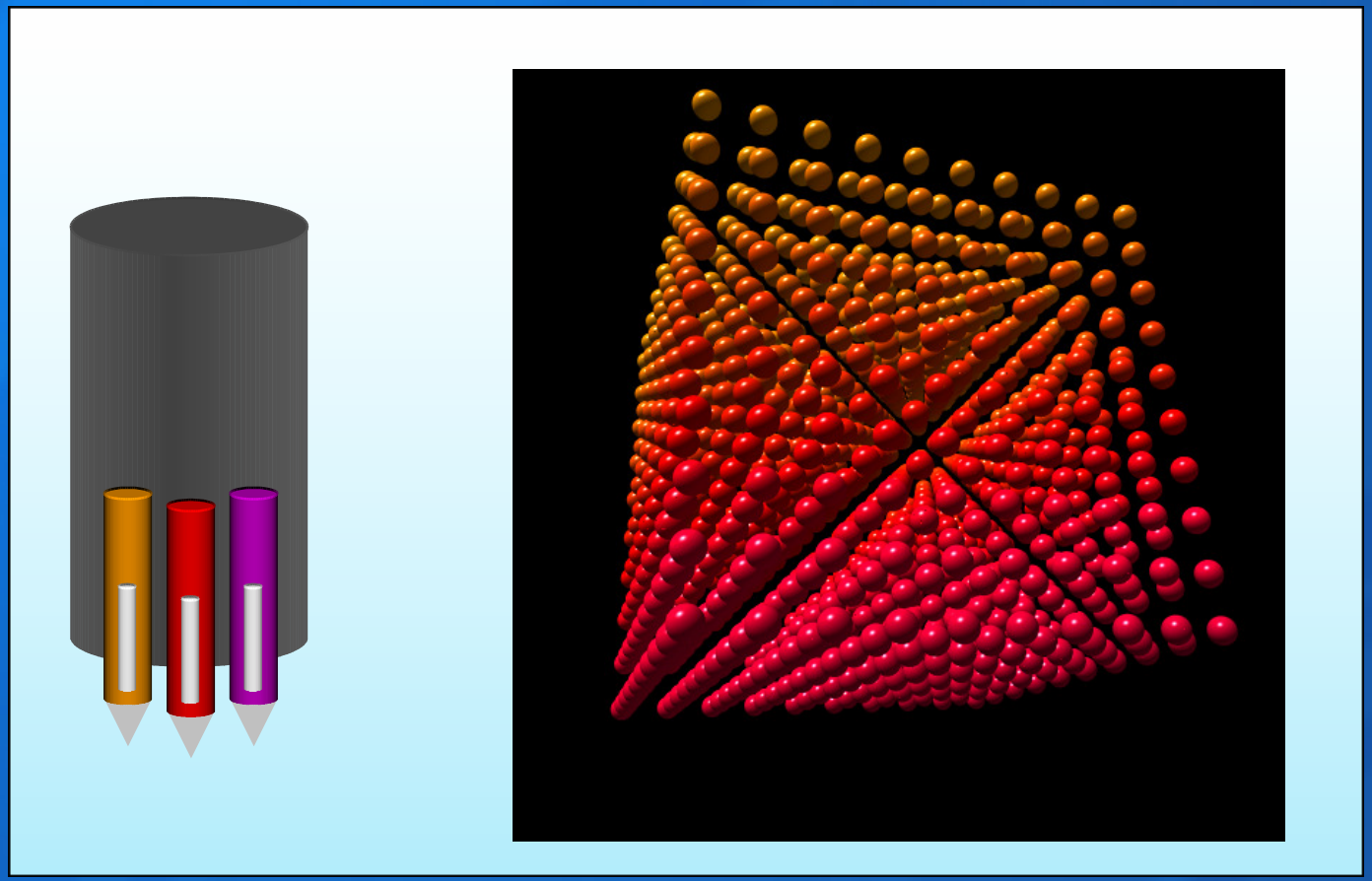








1000 Bead Set System



Thank You