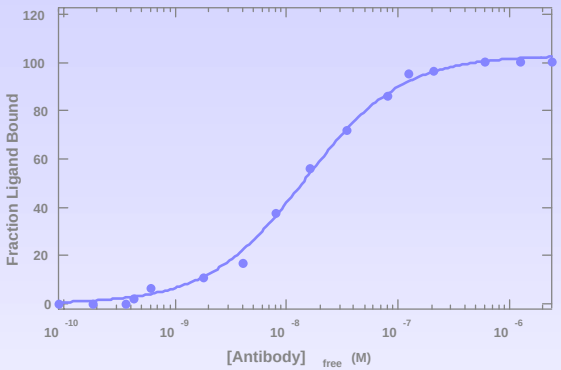
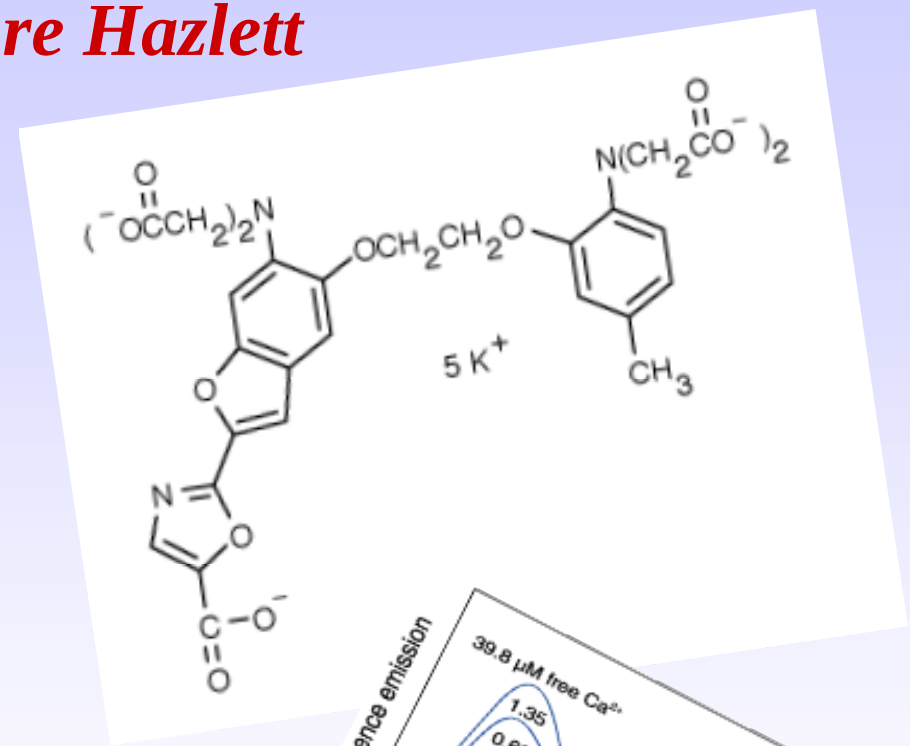
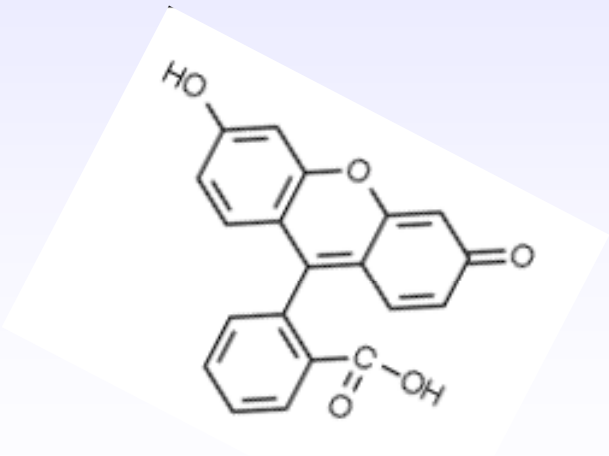
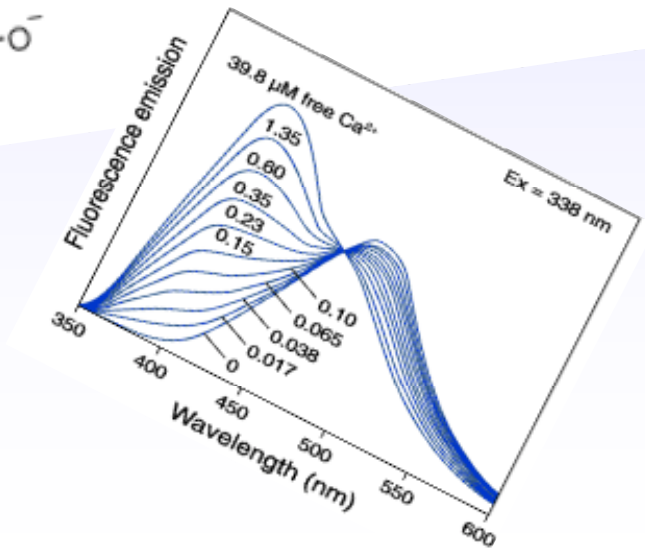
Applications of Fluorescence Methodologies in the Biochemical and Chemical Sciences

Theodore Hazlett









Principles of Fluorescence Techniques
Genova, Italy
June 14-16, 2004

Where is Fluorescence Used?

Microscopy

Drug Discovery

Chemical Analysis

Enzymatic Assays

Contamination Detection

Environmental Monitoring

Molecular Organization

Why is Fluorescence Used?
Advantages of Fluorescence Based Analysis Systems

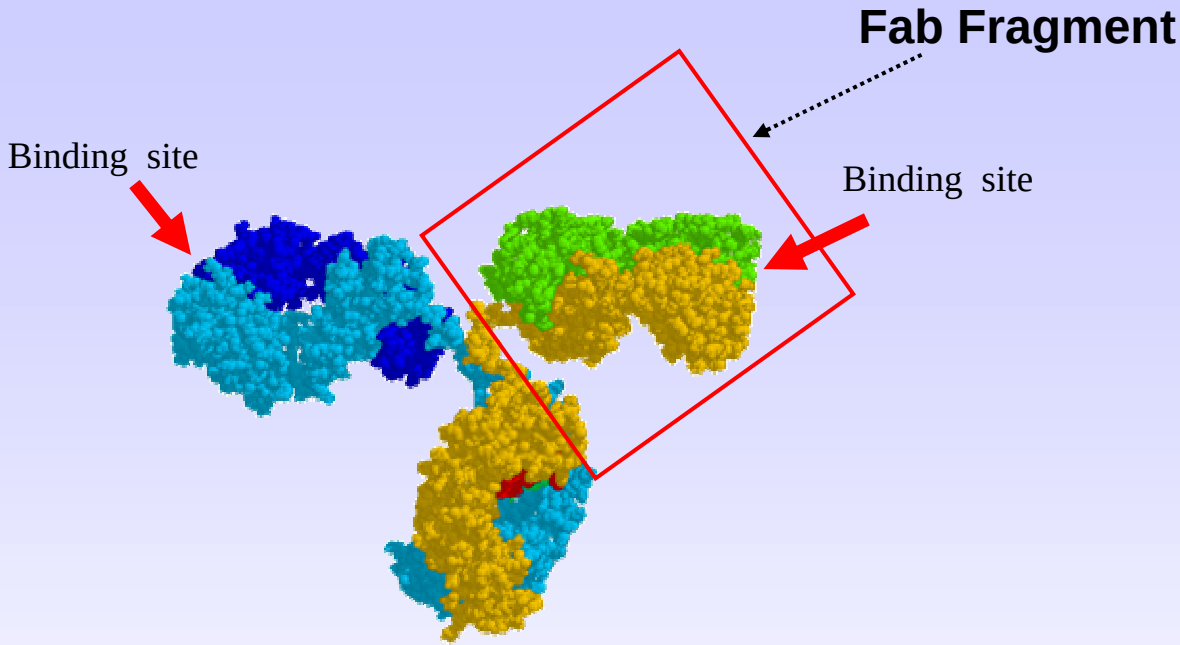
1. **Sensitive**
   - **High signal to noise ratio**
   - **Single molecule sensitivity**
2. **Flexible**
   - **Broad array of possible parameters to measure (Intensity, Spectra Shifts, Anisotropy, Decay parameters, FRET, …)**
3. **Information can be multidimensional**
   - **Use of multiple probes or probes that report a variety of environmental parameters.**
4. **Real-time data acquisition**
   - **System dynamics**
   - **Kinetic events**
5. **Monitoring can be distant**
   - **Chambers for harsh environmental conditions**

Biochemical & Chemical Assays for Detection

The Ideal Assay

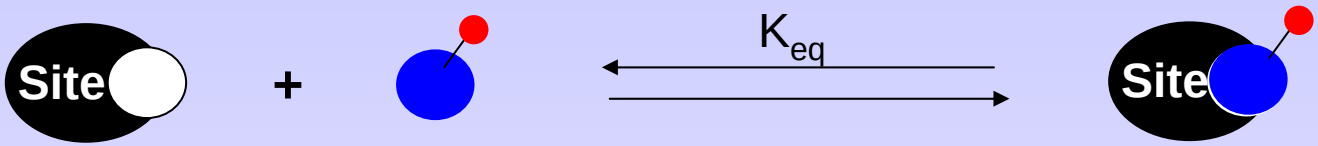
1. Appropriate sensitivity for real-world samples
2. Recognition only to the target molecule
3. High signal/noise ratio
4. Low volume requirements
5. Fast reaction completion
6. Fast sampling

A Robust General System: Immunoassays



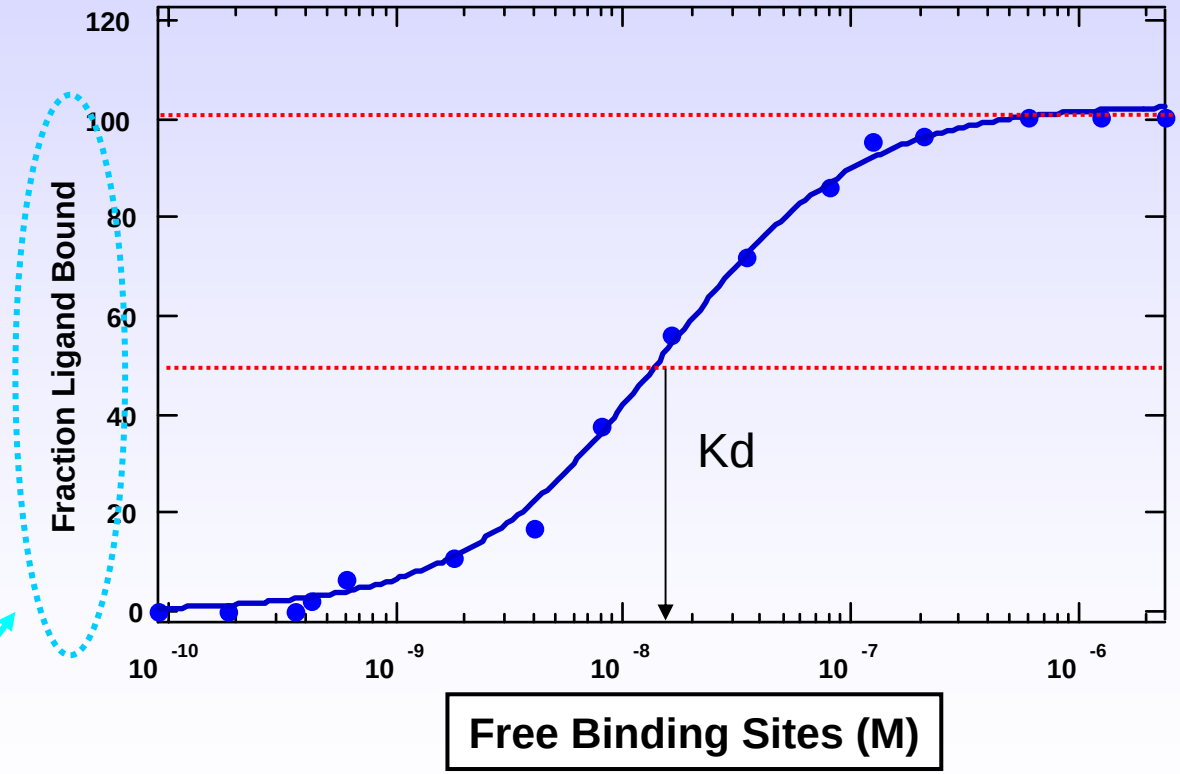
Mouse IgG: The two heavy chains are shown in yellow and light blue. The two light chains are shown in green and dark blue..*J.Harris, S.B.Larson, K.W.Hasel, A.McPherson, "Refined structure of an intact IgG2a monoclonal antibody", Biochemistry 36: 1581, (1997).*

Many Recognition Assays Rely on an Equilibrium Condition



$$K_d = \frac{[Sites_{free}][Ligand_{free}]}{[Sites \bullet Ligand]}$$

$$F_b = \frac{m \cdot S_{free}}{K_d + S_{free}} + c$$



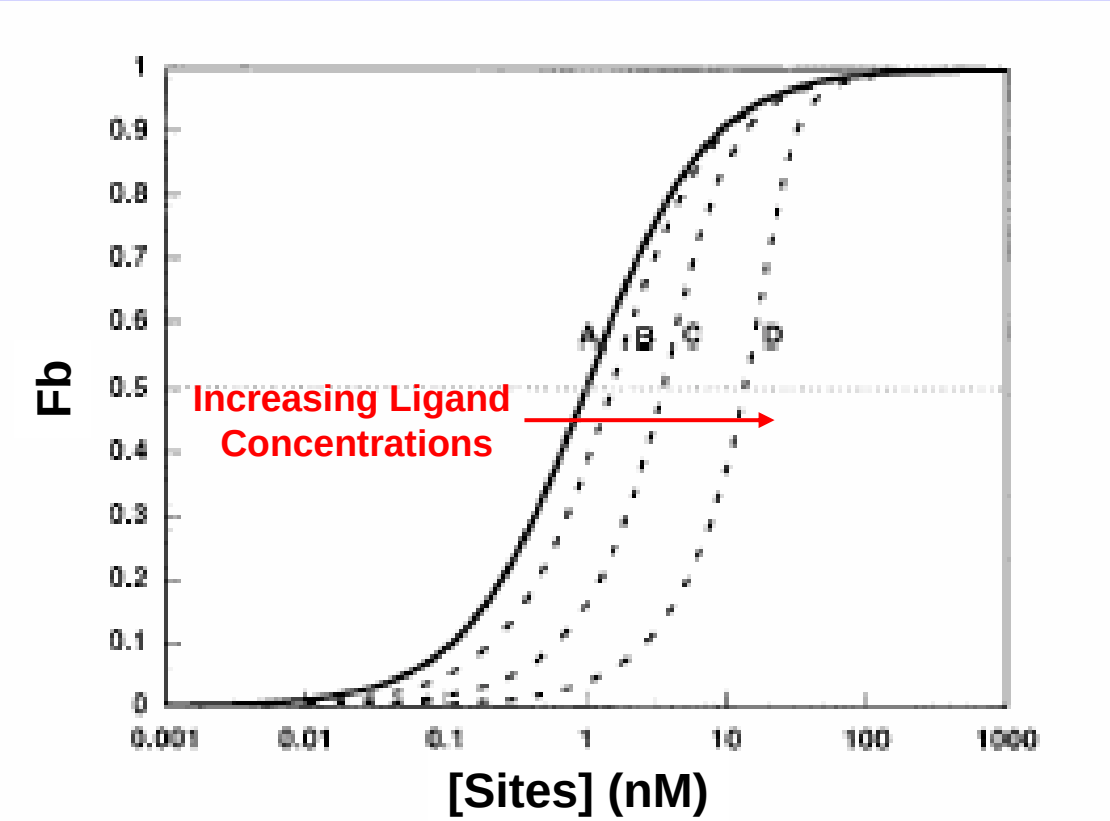
Critical Point

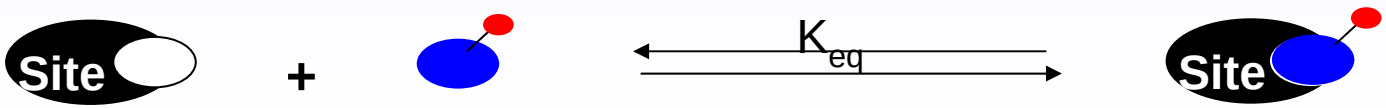
What should you titrate?

Adapted from S. Tetin, K. Swift, & , E, Matayoshi

Common Errors in Binding Studies

Concentrations Too High !

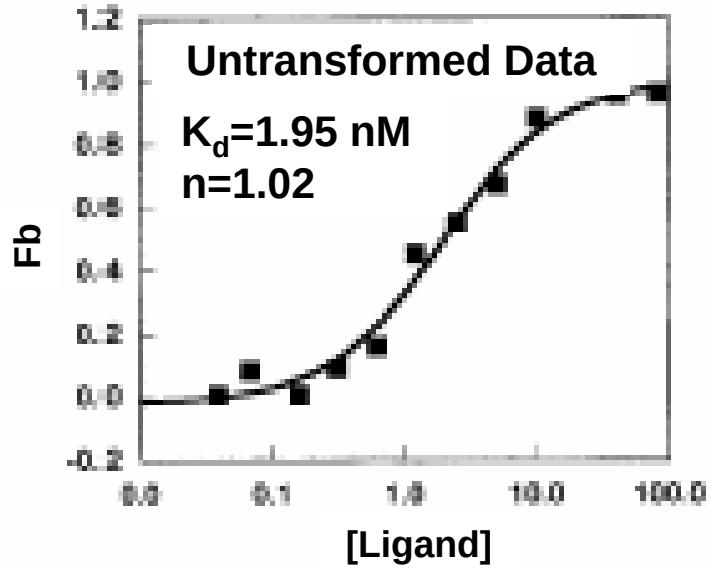


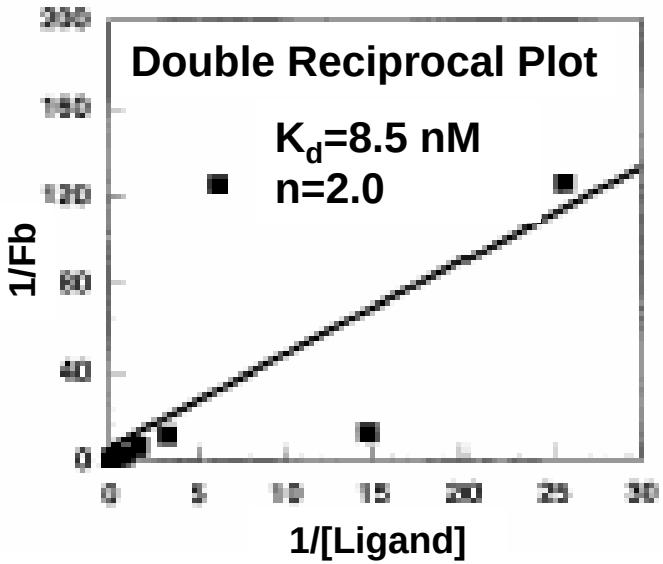


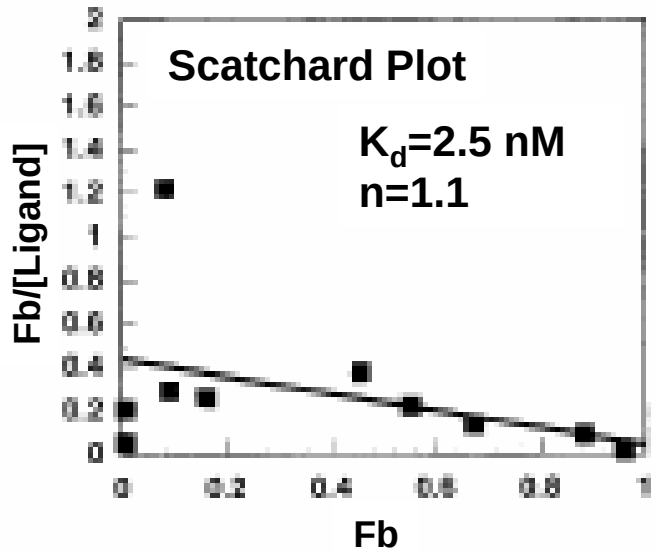
Tetin & Hazlett (2000) Methods 20, 341-361

Errors in analysis: data transformation

Theophylline & anti-Theophylline IgG. K_d = 2 nM & n=1



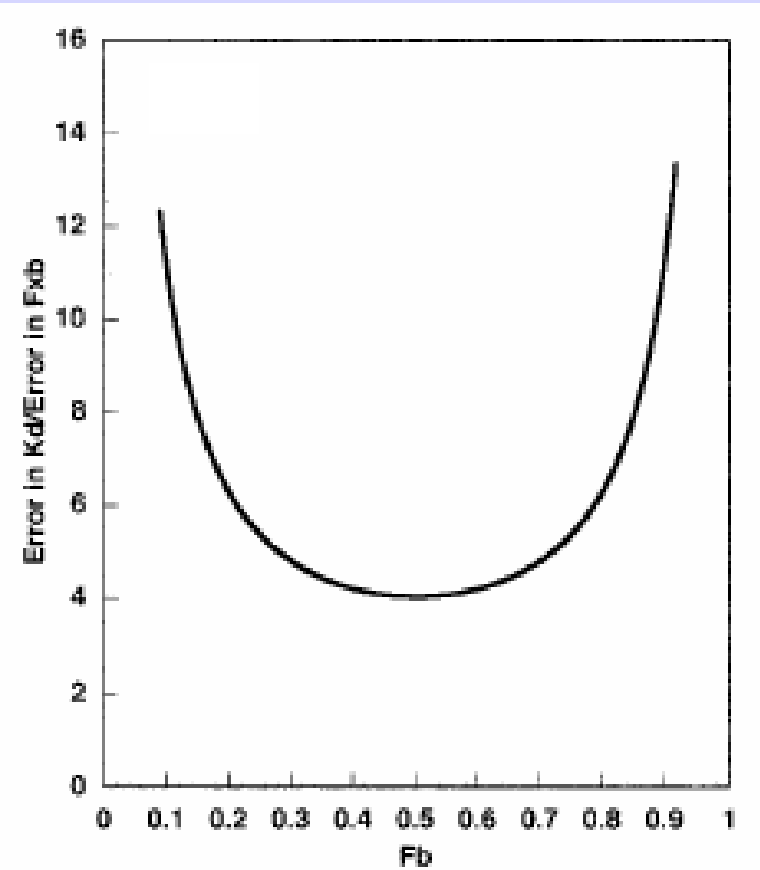




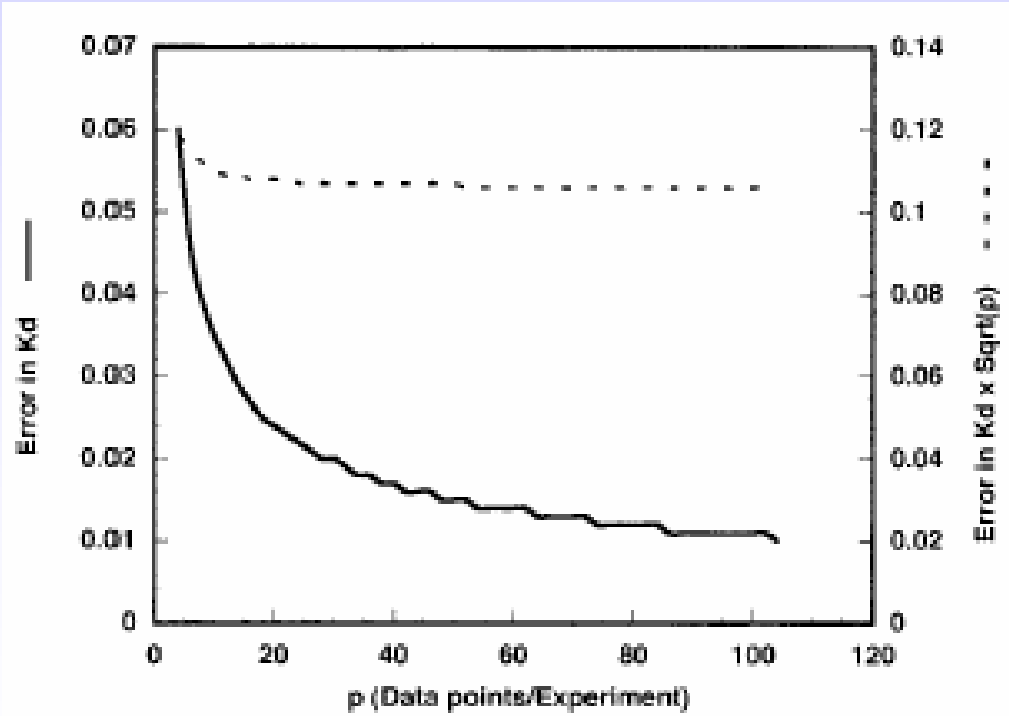
Tetin & Hazlett (2000) Methods 20, 341-361

Error in Estimates of K_d

K_d error as a function of the fraction bound



Error in K_d as a function the number of data points

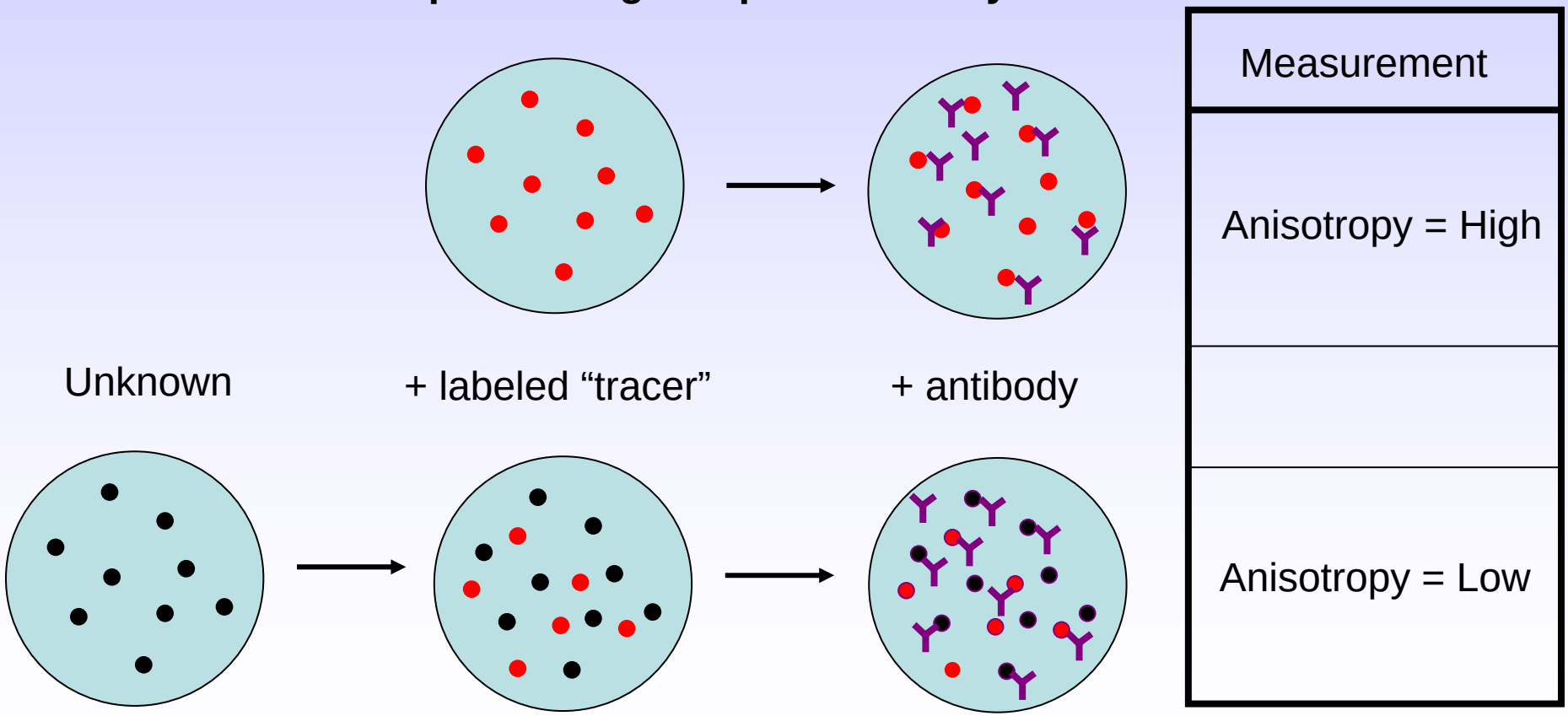


Tetin & Hazlett (2000) Methods 20, 341-361

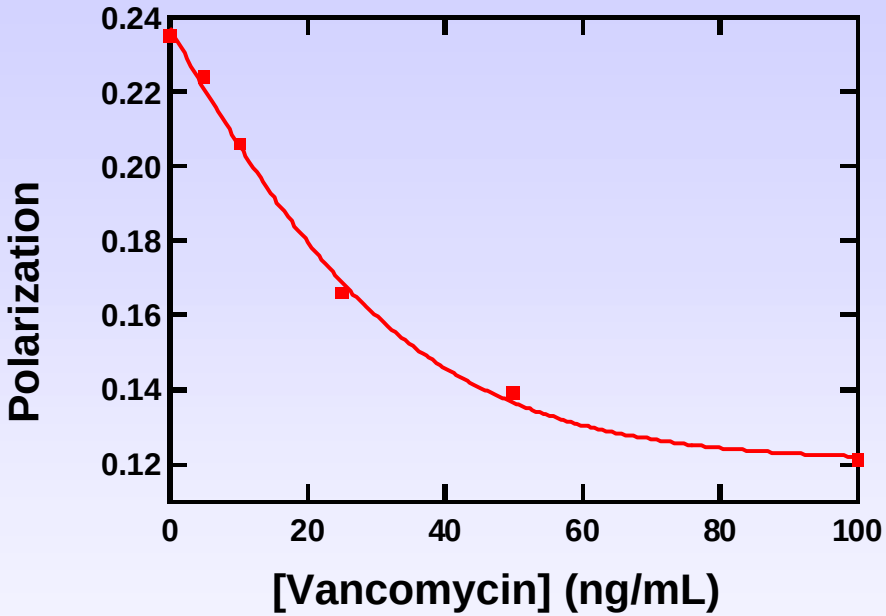
How do we create an assay?

The test samples do not have labeled material, do they?

1. The simple binding competition assay:



Abbott Laboratory Polarization Assay



Vancomycin (ng/mL)	Polarization	[Tracer] bound (%)
0	0.234	100
5	0.223	91
10	0.207	76
25	0.167	41
50	0.139	16
100	0.119	0

Calibration curve for a vancomycin immunoassay. Vancomycin tracer and varying concentrations unlabeled vancomycin were added to sera and polarization values were collected using the TDx assay instrumentation. Curve was fit to a 4-parameter logistic function (empirical fit).

Considerations

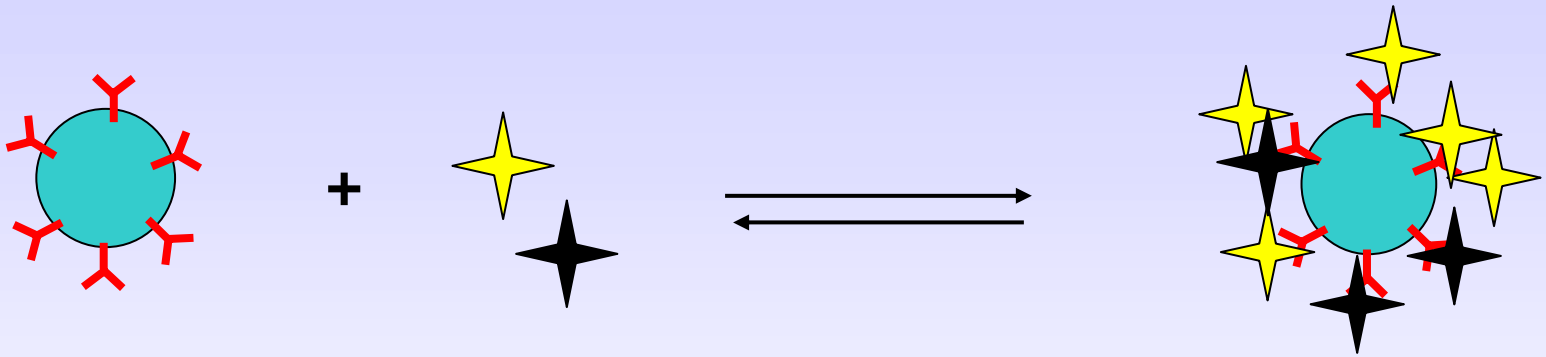
Speed
Accuracy
Background contributions
Measuring errors

S.Y. Tetin et. al. Analytical Biochemistry 307 (2002) 84–91

Solid Support Immunoassays

Nanoparticle Immunoassays employing fluorescence correlation spectroscopy

Competitive Immunoassay



Particle with IgG attached

Fluorescent Antigen & Antigen

Complex Slower Diffusion & Bright

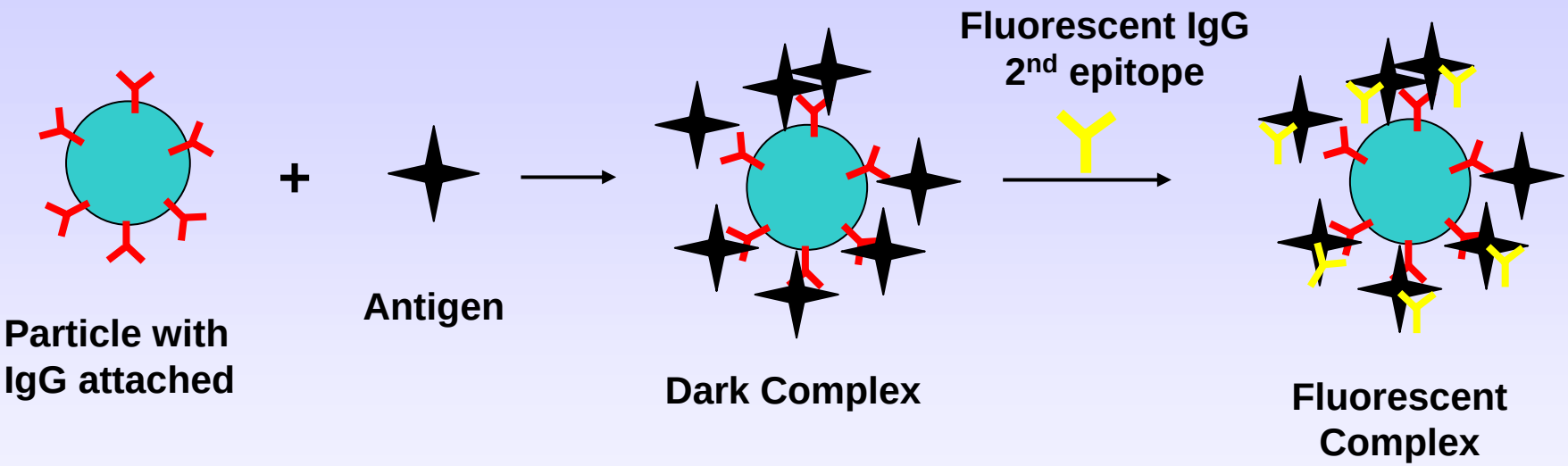
Potential Advantages

Fast separation of soluble, unattached fluorescent antigen

Large, dilute volumes can be assayed (depends on K_d)

Adapted from Meyers-Almes (2001), in Fluorescence Correlation Spectroscopy, Rigler & Elson, Eds.

Sandwich Immunoassay

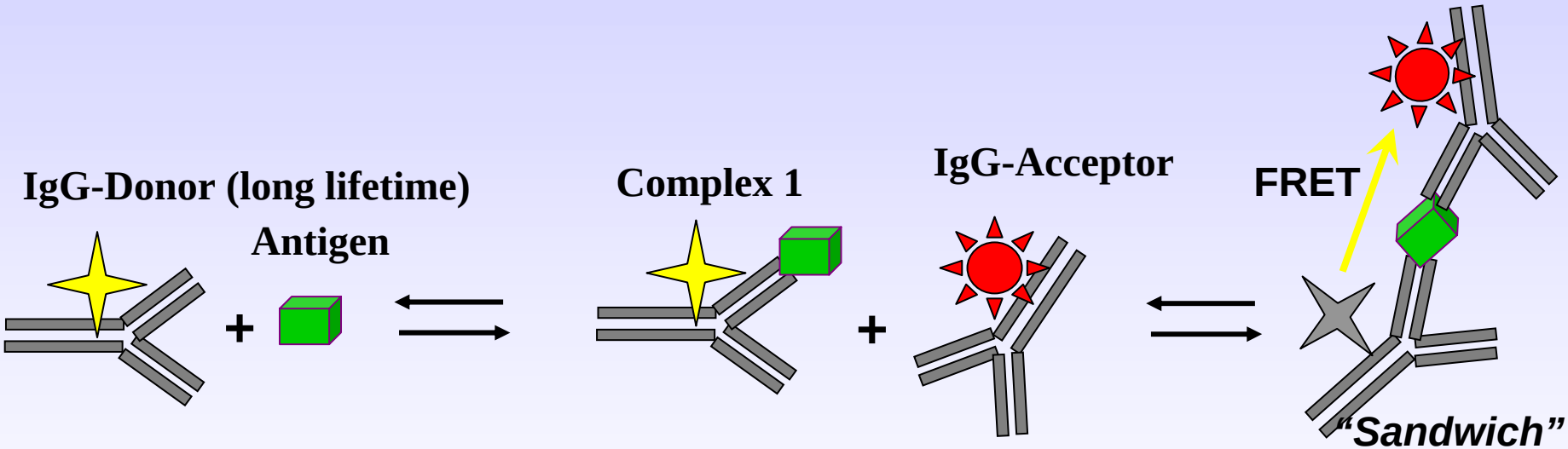


Multiple epitopes can be targeted with other IgG to enhance fluorescence contribution of one antigen.

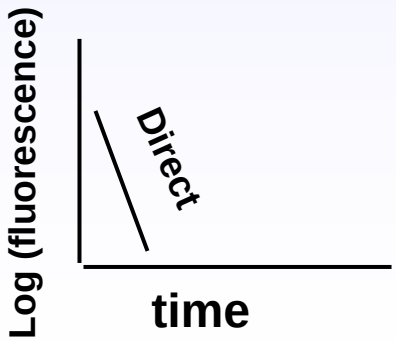
Homogeneous Lifetime Assay

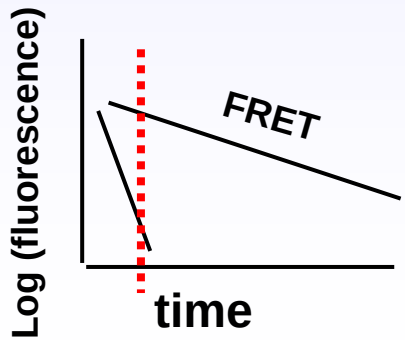
Homogeneous – No need for separation, free and bound materials are together in solution during the assay

Lifetime – Gated detection that eliminates "short" lifetime components

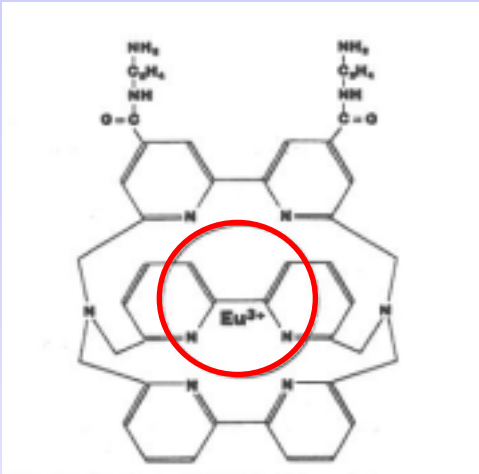


The emission is collected at wavelengths of the acceptor.



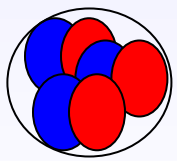


Donor

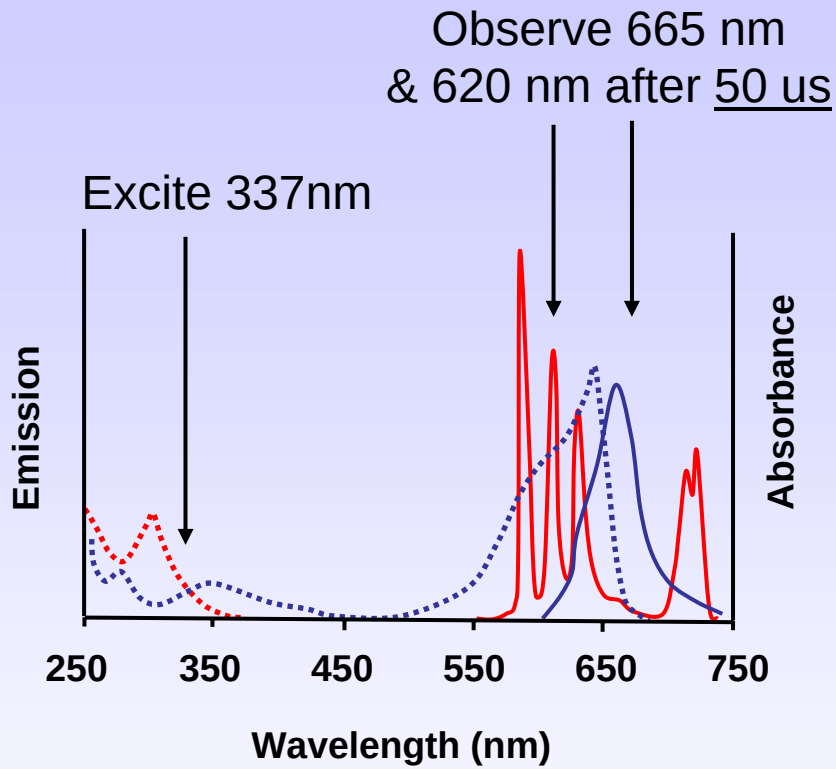


Eu^{3+} trisbipyridinediamine
TBP(Eu^{3+}) cryptate
Long Lifetime (ms) & stable

Acceptor



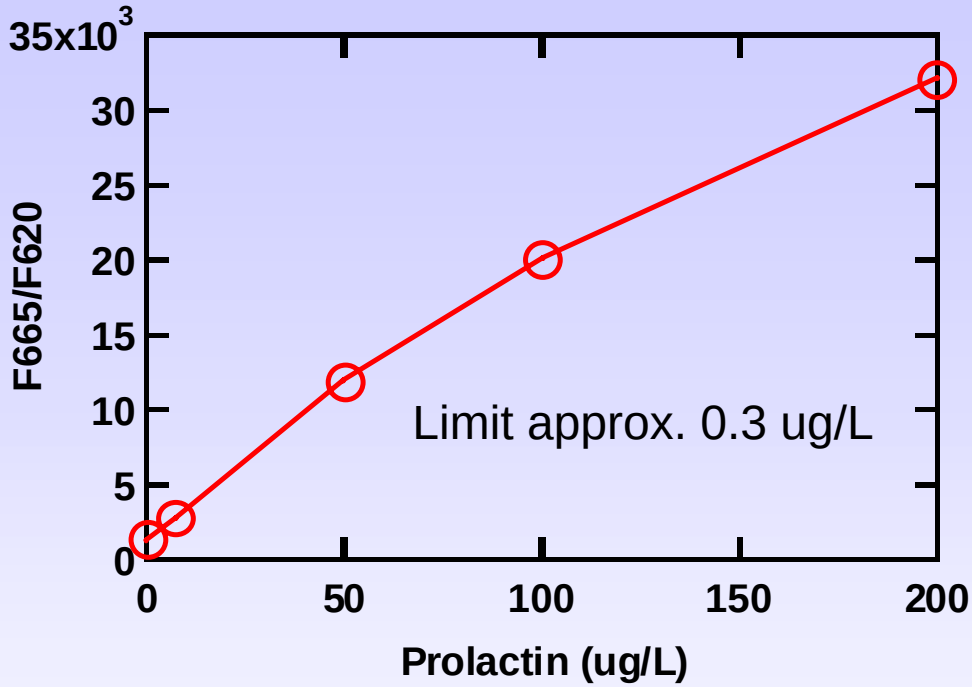
Allophycocyanin (APC)
Phycobiliprotein from thylakoids of red algae, α–β trimer.



TBP(Eu^{3+}) and APC are exciting at 337 nm
TBP(Eu^{3+}) is monitored at 620 nm
FRET excited APC is monitored at 665 nm

G. Mathis (1993) Clin. Chem. 39, 1953

$$Ratio = \frac{F_{665}}{F_{620}} \approx \frac{\varepsilon^{665}_{APC*} \cdot [APC*]}{\varepsilon^{620}_{TBP(Eu)} \cdot [TBP(Eu)]}$$



F_{620} will essentially be constant in an assay and will serve to correct for sample interference with the emission or excitation (inner filter effects).

- Prolactin Immunoassay*
- Thyroid-stimulating hormone immunoassay

also

- Reverse transcriptase activity assay
- DNase activity assay

adapted from G. Mathis (1993) Clin. Chem. 39, 1953

Fluorescent Sensors for Ions and Molecules

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

Anions:

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

Cations:

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

and Neutral Molecules:

Saccharides, others?

Sensors can respond through Collisional Quenching

Measurement: Steady-State Intensity and Lifetime Quenching

Example: MEQ (or the cell permeant derivative DiH-MEQ: 6-methoxy-*N*-ethyl-1,2-dihydroquinoline).

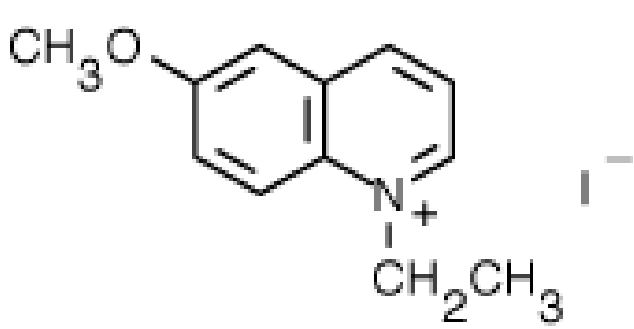
Cl^- detection

Calculations:

Stern-Volmer Quenching

$$X_0/X_i = 1 + [Cl^-]_i K_{SV}$$

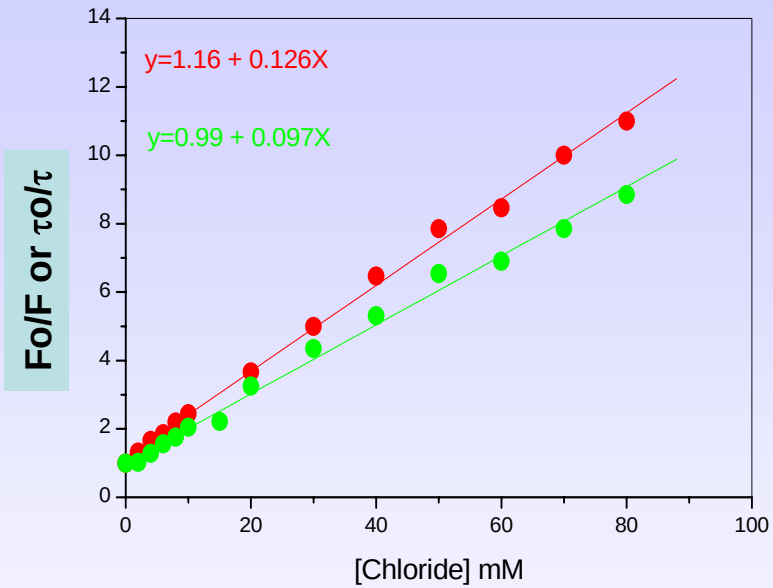
$$K_{sv} = 100\ M^{-1}$$



MEQ

Calibration for DiH-MEQ Quenching

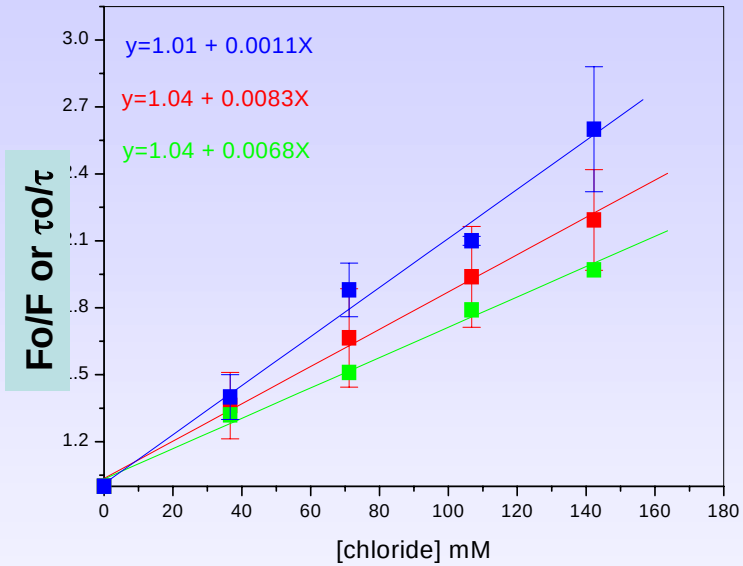
In Solution



Stern-Volmer plots for chloride quenching of MEQ in solution (Fluorescence intensity data (●) and fluorescence lifetime data (●)).

Forward angle light scattering (FALS) was used as a measure of the cellular swelling (increase in FALS -> Increase in swelling).

In "Porous" CHO Cells



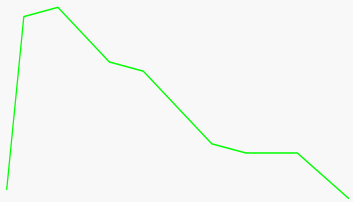
The Stern-Volmer plots for quenching of MEQ in CHO cells using the Flow Cytometer. The CHO cells were loaded with 50 mM DiH-MEQ and resuspended in buffers containing 7 mM nigercin and 10 mM tributyltin (stops active transport) and an appropriate concentration of chloride (Fluorescence intensity data (■), Intensity/FALS (■) and fluorescence lifetime data (■)).

Pilas, Sanchez, Hazlett and Durack, Biophys. J. 76, A449

CHO cells resting concentrations of Cl^- ions

Data	$[Cl^-]_i$ mM
Fluorescence Intensity	53.5 +/- 7.2
Fluorescence Intensity / FALS	78.0 +/- 5.4
Fluorescence Lifetime	91.0 +/- 5.5

CHO Cells Response to Hypotonic Shock



Typical changes in MEQ/FALS fluorescence (red), FALS (blue) and fluorescence lifetime (green) for CHO cells responding to hypotonic shock. CHO cells were loaded with 50 μM DiH-MEQ and resuspended in PBS. At 5 minutes H_2O (1:1 proportion) was added to the cells to induce hypotonic shock.

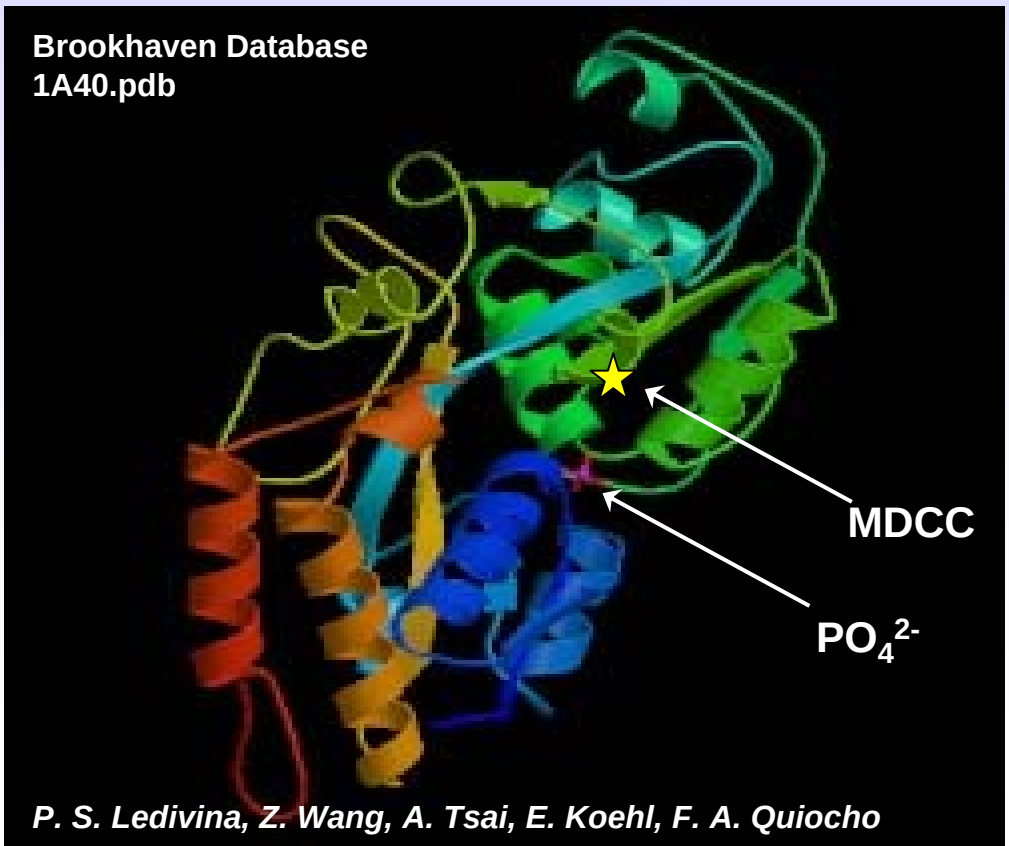
Pilas, Sanchez, Hazlett and Durack, Biophys. J. 76, A449

Sensors can be Based on Biomolecules

Measurement: <u>Steady-state intensity</u>, possibly <u>Lifetime,</u> possibly <u>Spectral changes</u>

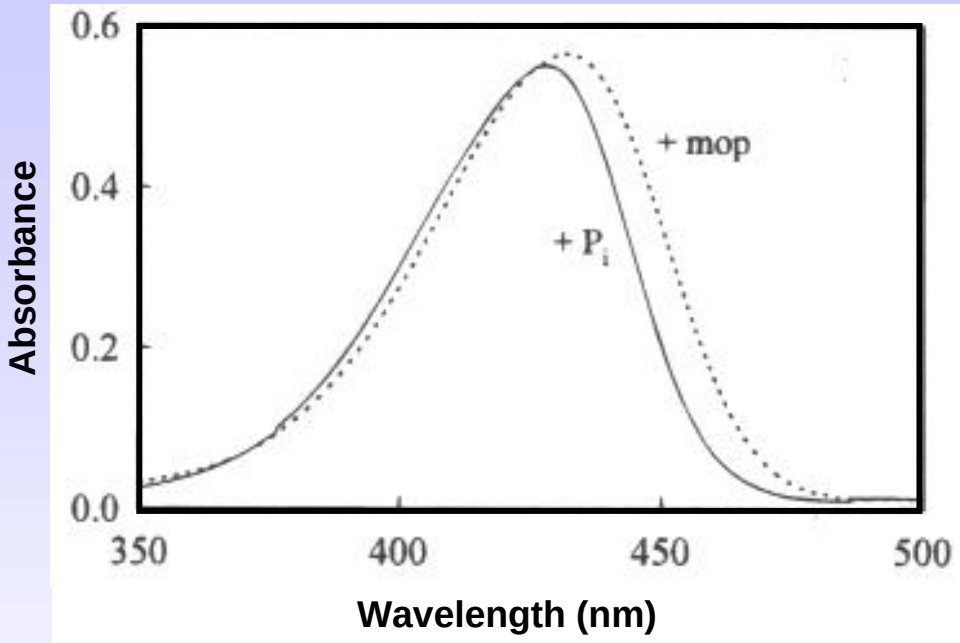
Example: A protein biosensor for PO_4^{2-}

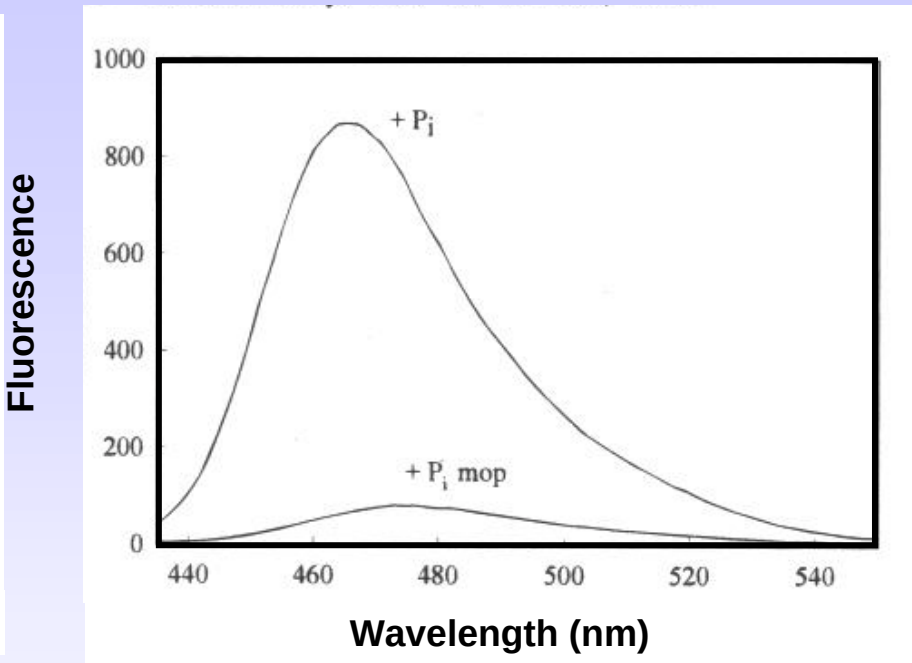
MDCC-PBP is a fluorescently tagged (MDCC is a coumarin maleimide derivative and reactive toward thiols) fluorophore phosphate binding protein (PBP) from E.coli. Its biological function is too scavenge the periplasm for inorganic phosphate under low phosphate (starvation) conditions.



Phosphate-Binding Protein with MDCC labeled to cysteine residue at position 197 (A197C mutation).

Phosphate Binding Protein-MDCC





8-fold increase in Intensity
8-fold increase in lifetime

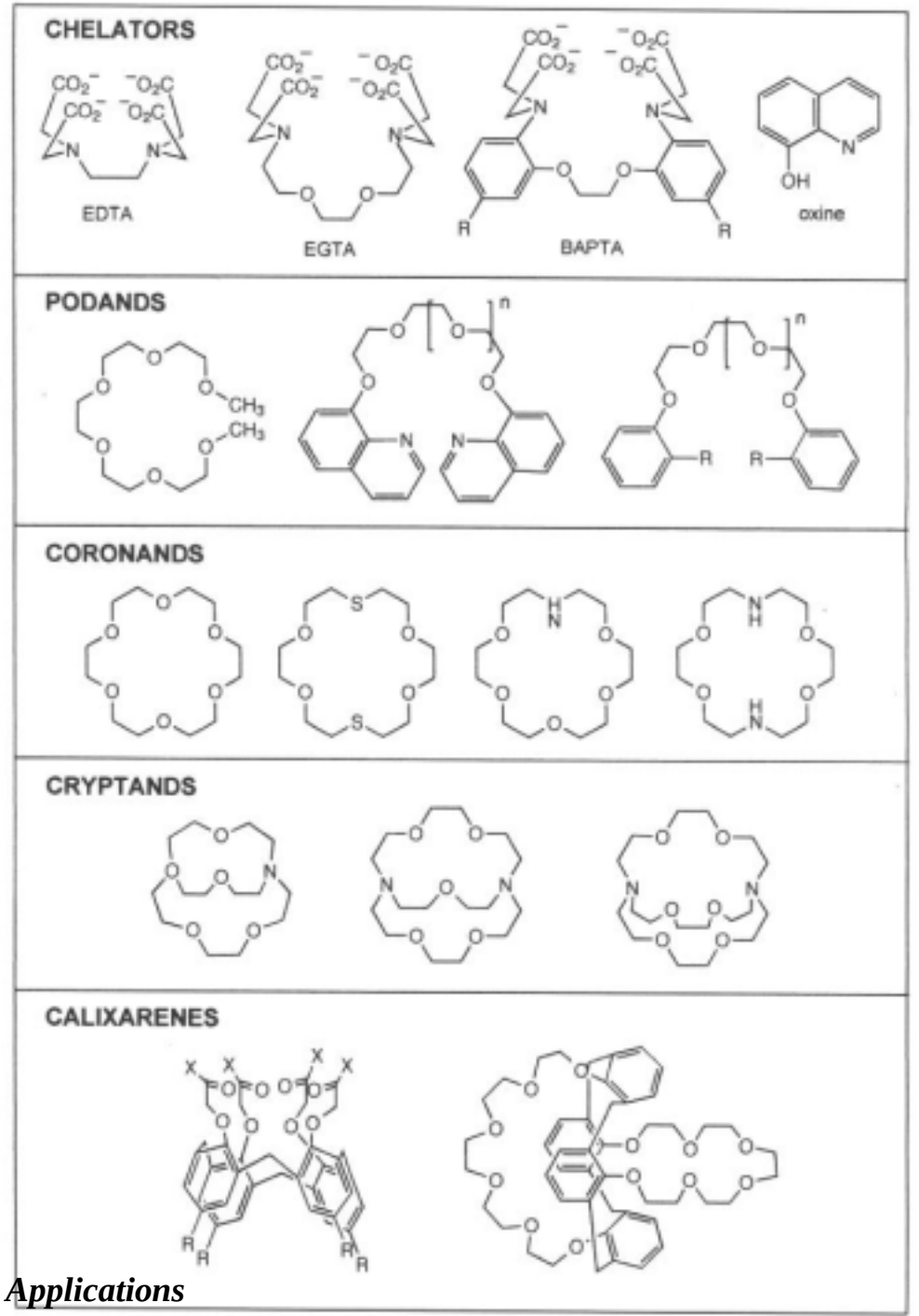
K_d = 0.1 uM

Brunie, Hunter, Howell, Martin, Hazlett, Corrie, Webb (1998) Biochemistry, 37:10370

Cation-Binding Molecules

A large variety of compounds have been synthesized which bind cations based on the ion's size, charge and coordination chemistry.

Cation	Diameter (A)	Coord. No
Mg^{2+}	1.32	4 or 5
Cu^{2+}	1.44	4 or 6
Zn^{2+}	1.48	4 or 6
Na^{+}	1.94	6
Cd^{2+}	1.94	4 or 6
Ca^{2+}	1.98	7 or 9
Pb^{2+}	2.40	4 or 6
K^{+}	2.66	6

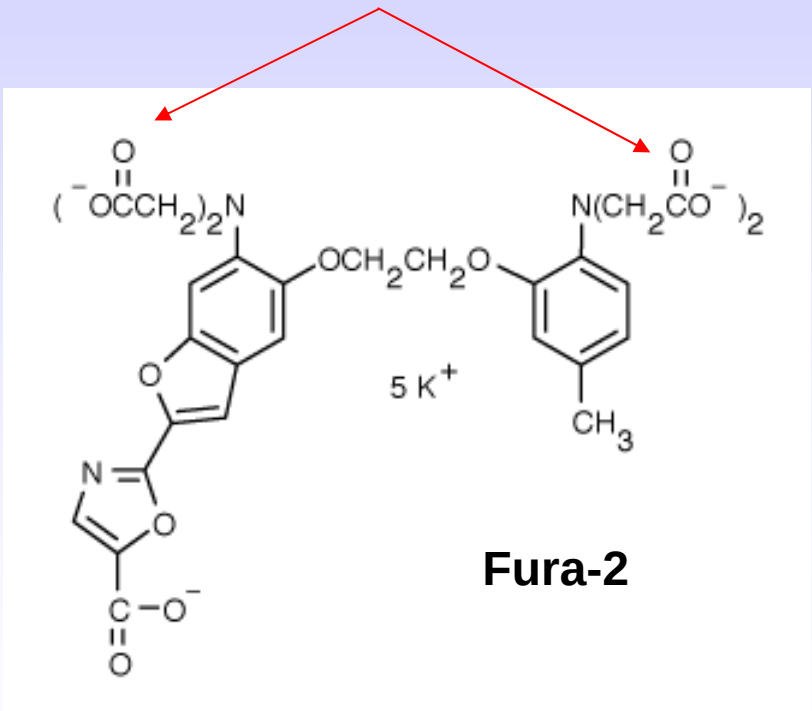


B. Valeur (2002), Molecular Fluorescence, Principles and Applications

Sensors Based on Chelating

Calcium probes that shift in their excitation energy on binding Ca^{2+}

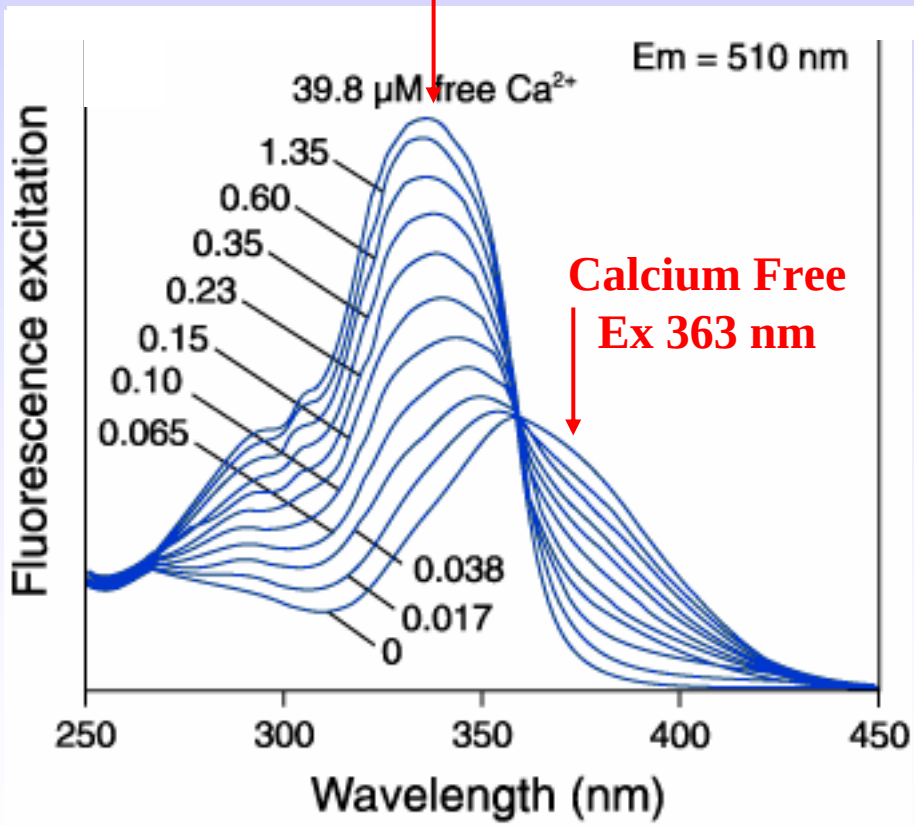
Calcium ion chelating groups



Fura-2

Oxazole based fluorophore

Calcium Bound
Ex 335 nm



Calcium Free
Ex 363 nm

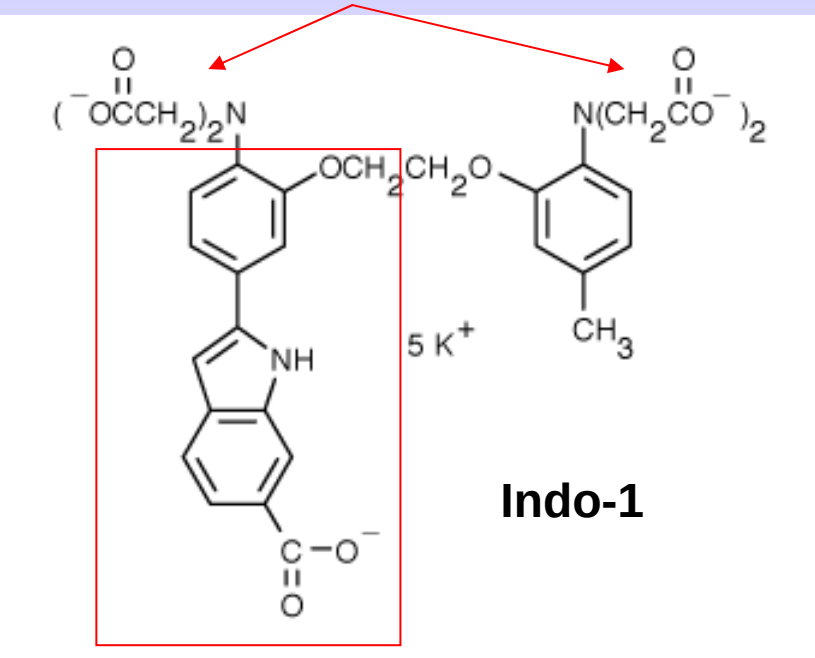
Abs max = 335/363 nm, ε = 33,000 $M^{-1}cm^{-1}$

K_d = 145 nM

Molecular probes, www.probes.com

Calcium probes that shift in their emission on binding Ca^{2+}

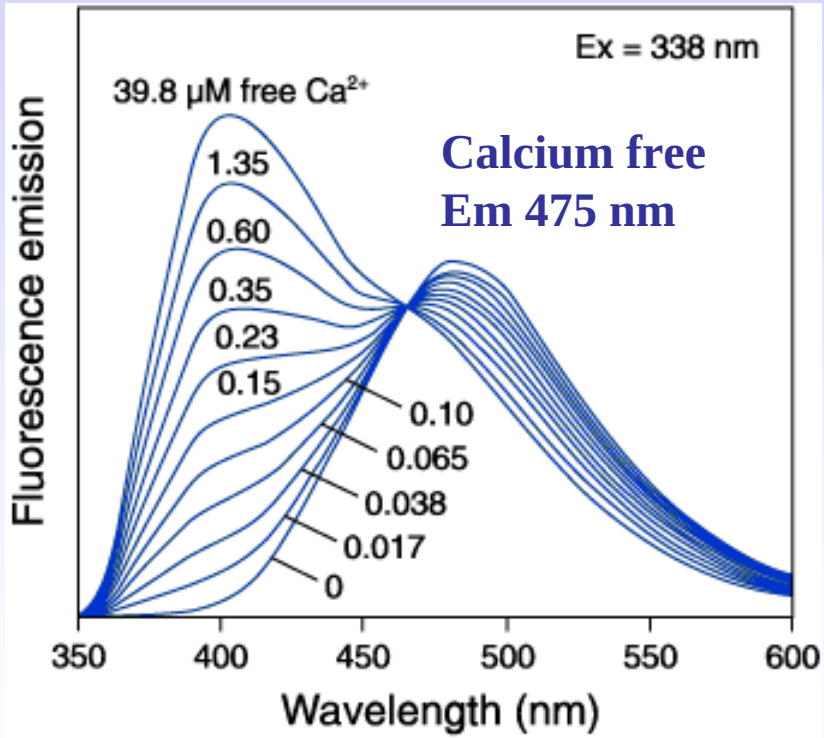
Calcium ion chelating groups



Indole based fluorophore

Abs max = 338 nm, ε = 33,000 $M^{-1}cm^{-1}$

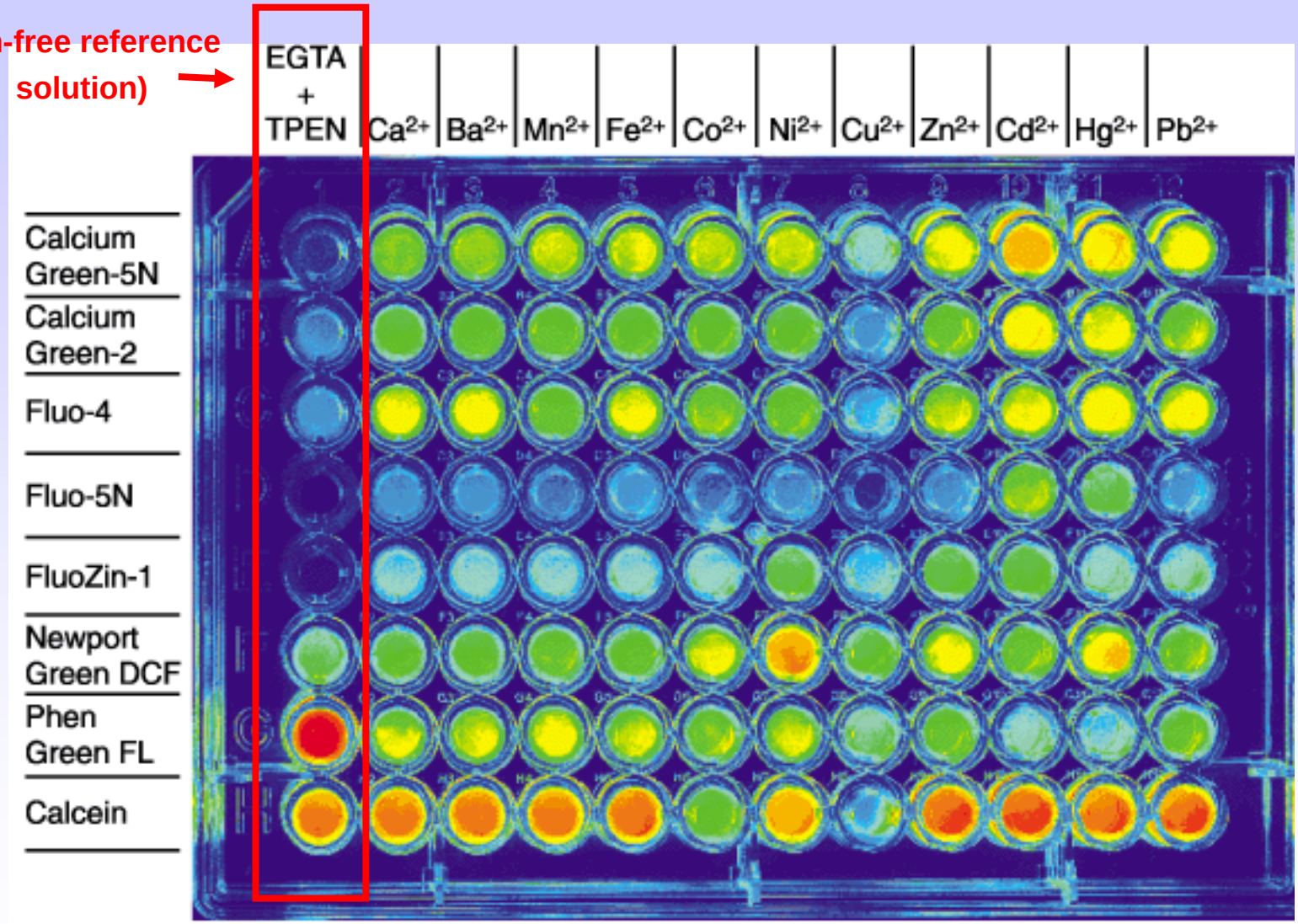
Calcium bound
Em 405 nm



Kd = 230 nM

Molecular probes, www.probes.com

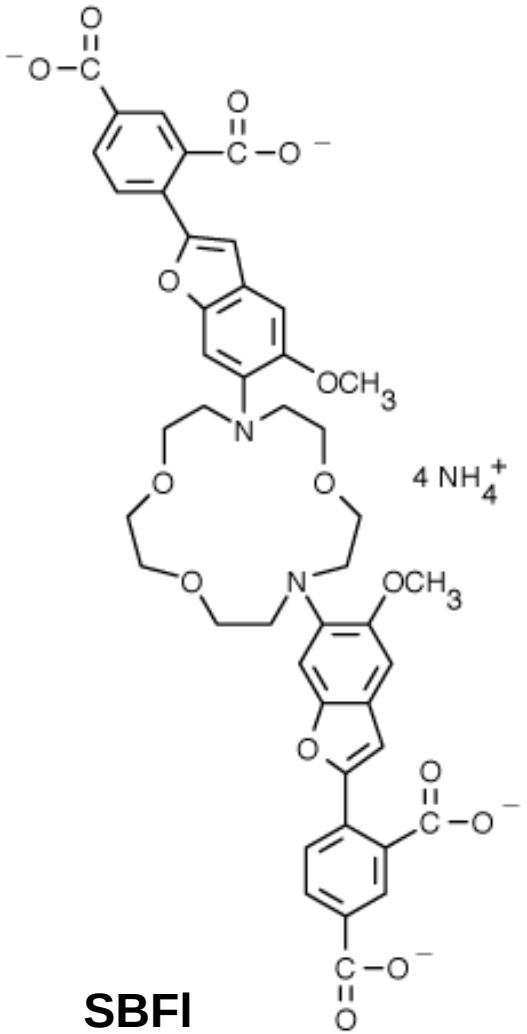
Ion Probes Specificity



(Fluorescence: high = red > orange > yellow > green > blue = low).

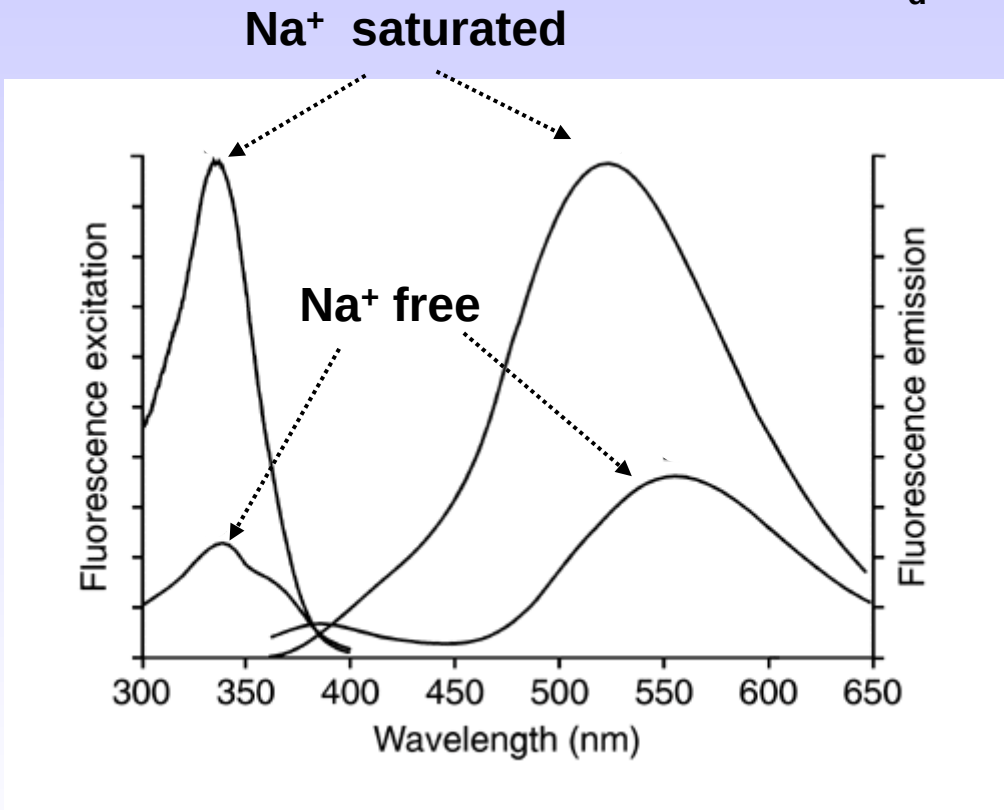
Molecular probes, www.probes.com

Capturing Na^+ Ions with a Crown Ether



SBFI

Na^+ K_d = 3.8 mM
K^+ K_d = 68 mM



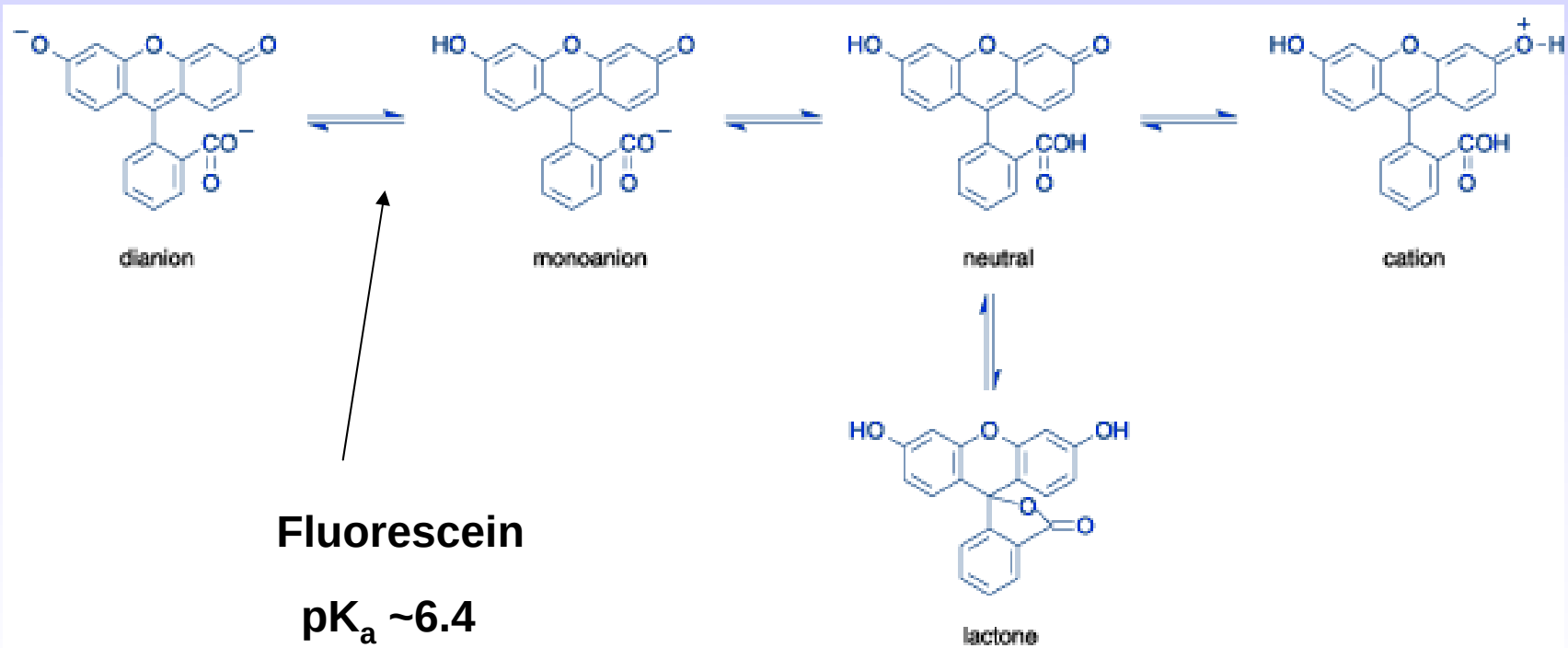
Enhancement with Na^+, with a small emission shift

1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10-trioxa-7,13- diazacyclopentadecane-7,13-diylbis(5- methoxy-6,2-benzofurandiyl)]bis-, tetraammonium salt

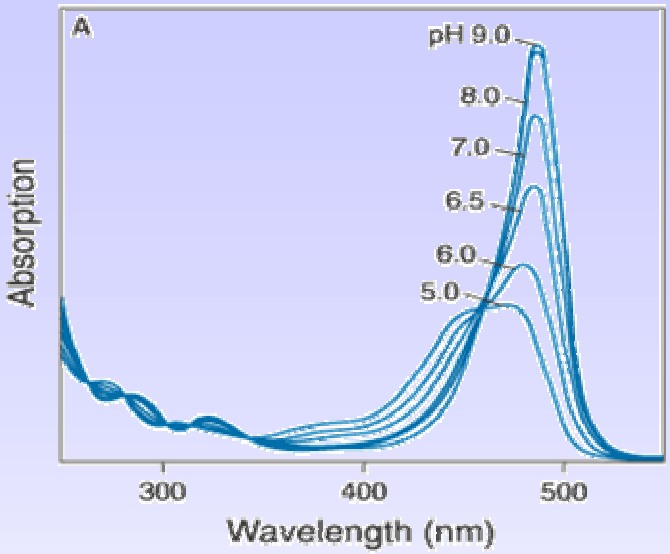
Molecular probes, www.probes.com

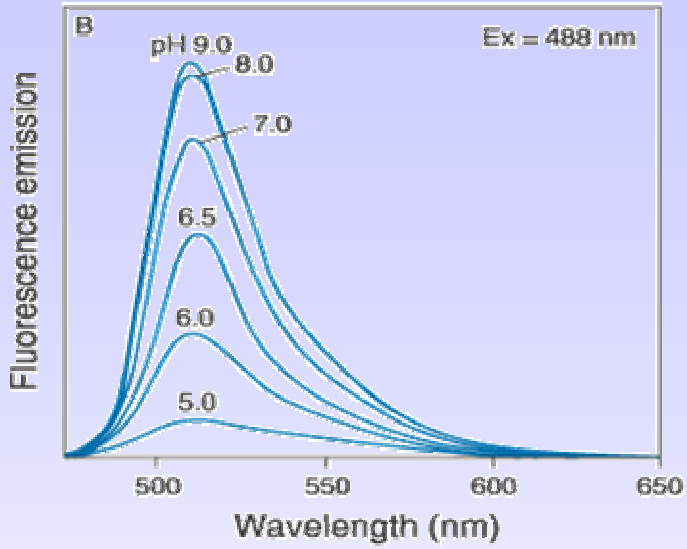
Measuring H^+

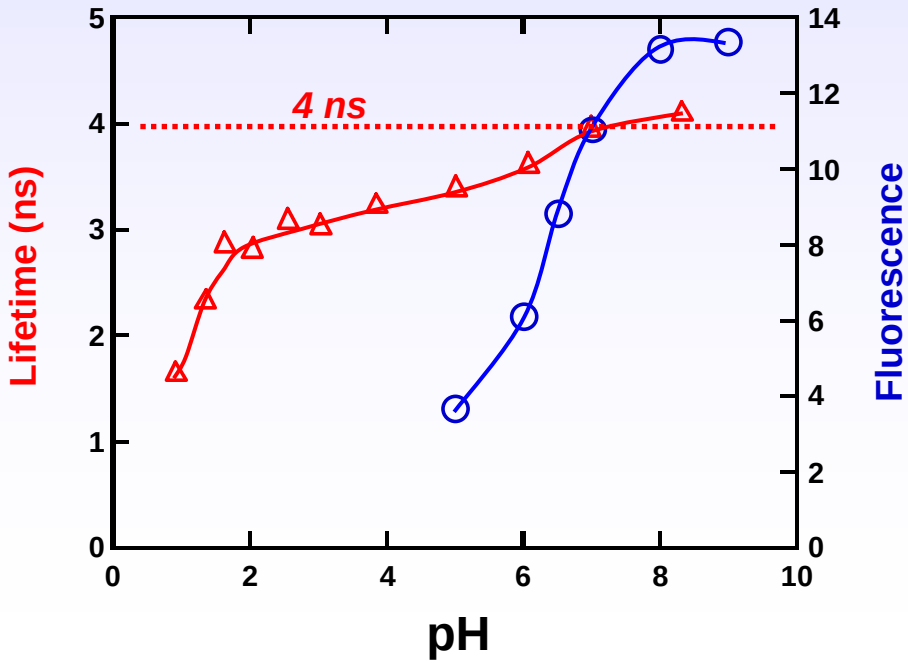
Probes with titratable groups can have pH dependent spectra



Fluorescein Data

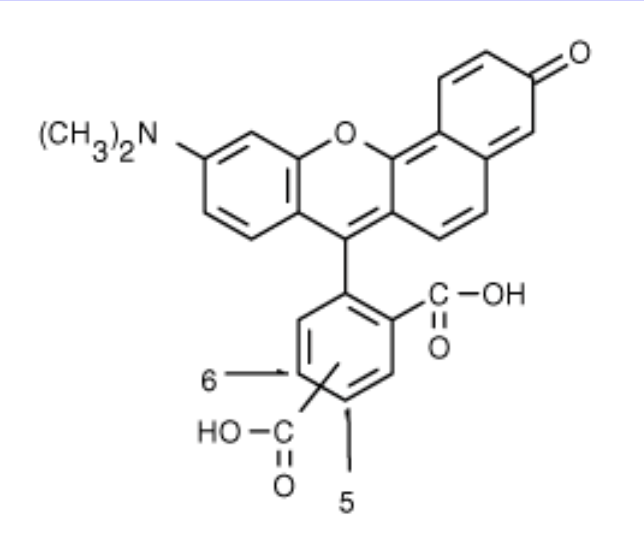




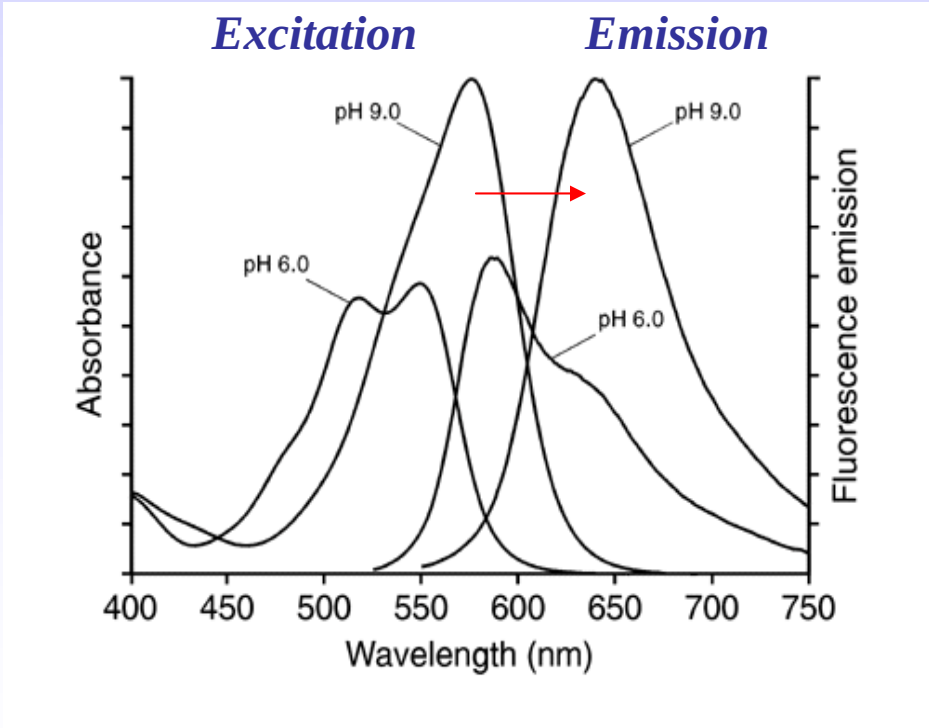


Ratiometric pH Sensors

Titratable hydroxy groups in SNARF-1 can be used a pH sensors



$pK_a = 7.5$



Properties of carboxy-SNARF-1

- ✓**Abs** 548 nm (ε = 27,000 $cm^{-1}M^{-1}$),
- ✓**Em** 587 nm (pH 6.0)
- ✓**Abs** 576 nm (ε = 48,000 $cm^{-1}M^{-1}$),
- ✓**Em** 635 nm (pH 10.0)

- ✓Φ = 0.1-0.3 in membrane
- ✓**Energy**: Generally insensitive
- ✓**Decay**: 1-2 ns
- ✓**Polarization**: High 0.49

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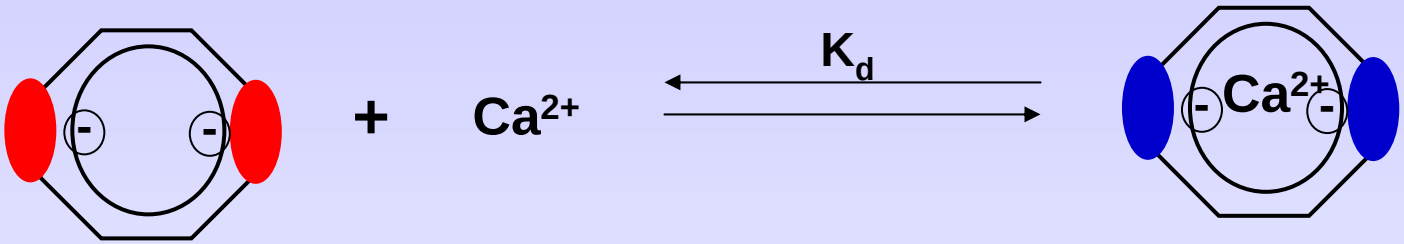
Analyzing Ratiometric Data for a 1 to 1 Stoichiometry



Fluor Ion Fluor*Ion

$$K_d = \frac{[Ion][Fluor]}{[Fluor \bullet Ion]}$$

$$[Fluor]_{total} = [Fluor] + [Fluor \bullet Ion]$$

$$[Ion]_{total} = [Ion] + [Fluor \bullet Ion]$$

The contributions of each state (Ion bound and free Fluor) can be written:

$$\lambda 1 = \varepsilon_{Fluor\_\lambda 1} \cdot [Fluor] + \varepsilon_{Fluor \cdot Ion\_\lambda 1} \cdot [Fluor \bullet Ion]$$

$$\lambda 2 = \varepsilon_{Fluor\_\lambda 2} \cdot [Fluor] + \varepsilon_{Fluor \cdot Ion\_\lambda 2} \cdot [Fluor \bullet Ion]$$

$$Ratio = \frac{\lambda 1}{\lambda 2} = \frac{\varepsilon_{Fluor\_\lambda 1} \cdot [Fluor] + \varepsilon_{Fluor \cdot Ion\_\lambda 1} \cdot [Fluor \bullet Ion]}{\varepsilon_{Fluor\_\lambda 2} \cdot [Fluor] + \varepsilon_{Fluor \cdot Ion\_\lambda 2} \cdot [Fluor \bullet Ion]}$$

Also, experimentally we can obtain the limits of our system.

(1) Free Fluor

$$R_0 = \frac{\lambda 1}{\lambda 2} = \frac{\varepsilon_{Fluor\_\lambda 1}}{\varepsilon_{Fluor\_\lambda 2}}$$

(2) Saturation (all Fluor is bound with Ion)

$$R_\infty = \frac{\lambda 1}{\lambda 2} = \frac{\varepsilon_{Fluor-Ion\_\lambda 1}}{\varepsilon_{Fluor-Ion\_\lambda 2}}$$

$$Ratio = \frac{\lambda 1}{\lambda 2} = \frac{\varepsilon_{Fluor\_\lambda 1} \cdot [Fluor] + \varepsilon_{Fluor-Ion\_\lambda 1} \cdot [Fluor \bullet Ion]}{\varepsilon_{Fluor\_\lambda 2} \cdot [Fluor] + \varepsilon_{Fluor-Ion\_\lambda 2} \cdot [Fluor \bullet Ion]}$$

[1]

$$K_d = \frac{[Ion][Fluor]}{[Fluor \bullet Ion]}$$

$$Ratio = \frac{\varepsilon_{Fluor\_\lambda 1} \cdot Kd + \varepsilon_{Fluor-Ion\_\lambda 1} \cdot [Ion]}{\varepsilon_{Fluor\_\lambda 2} \cdot Kd + \varepsilon_{Fluor-Ion\_\lambda 2} \cdot [Ion]}$$

[2]

Divide through by $\varepsilon_{Fluor\_\lambda 2}$

$$Ratio = \frac{R_0 \cdot Kd + \frac{\varepsilon_{Fluor-Ion\_\lambda 1}}{\varepsilon_{Fluor\_\lambda 2}} \cdot [Ion]}{Kd + \frac{\varepsilon_{Fluor-Ion\_\lambda 2}}{\varepsilon_{Fluor\_\lambda 2}} \cdot [Ion]}$$

[3]

Collect K_d on one side and [Ions] on the other

$$Kd\,(Ratio - R_0) = [Ion] \cdot \left(\frac{\varepsilon_{Fluor-Ion\_\lambda 1}}{\varepsilon_{Fluor\_\lambda 2}} - Ratio \cdot \frac{\varepsilon_{Fluor-Ion\_\lambda 2}}{\varepsilon_{Fluor\_\lambda 2}}\right)$$

[4]

Multiply both sides by $\frac{\varepsilon_{Fluor\_\lambda 2}}{\varepsilon_{Fluor-Ion\_\lambda 2}}$

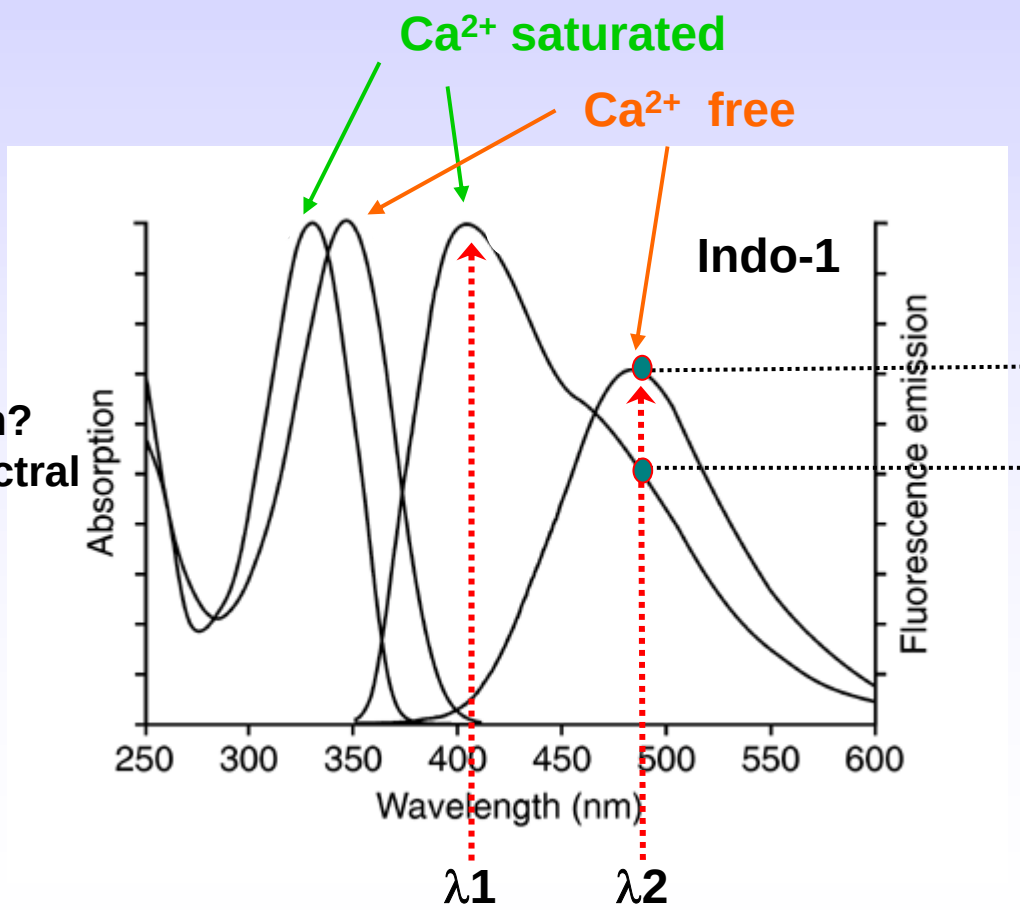
$$Kd\,(Ratio - R_0) \cdot \frac{\varepsilon_{Fluor\_\lambda 2}}{\varepsilon_{Fluor-Ion\_\lambda 2}} = [Ion] \cdot (R_\infty - Ratio)$$

And finally….

$$[Ion] = \frac{Kd \cdot \varepsilon_{Fluor\_\lambda 2} \cdot (R - R_0)}{\varepsilon_{Fluor-Ion\_\lambda 2} \cdot (R_\infty - R)}$$

Assuming [Ion] approximates [Ion]$_{total}$

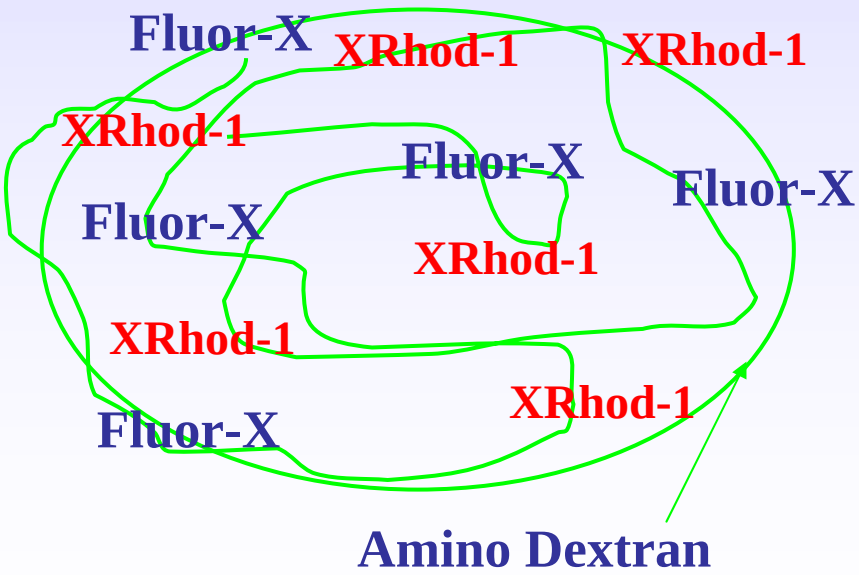
What excitation wavelength?
Why not use the entire spectral shape?
Why not use just λ1?

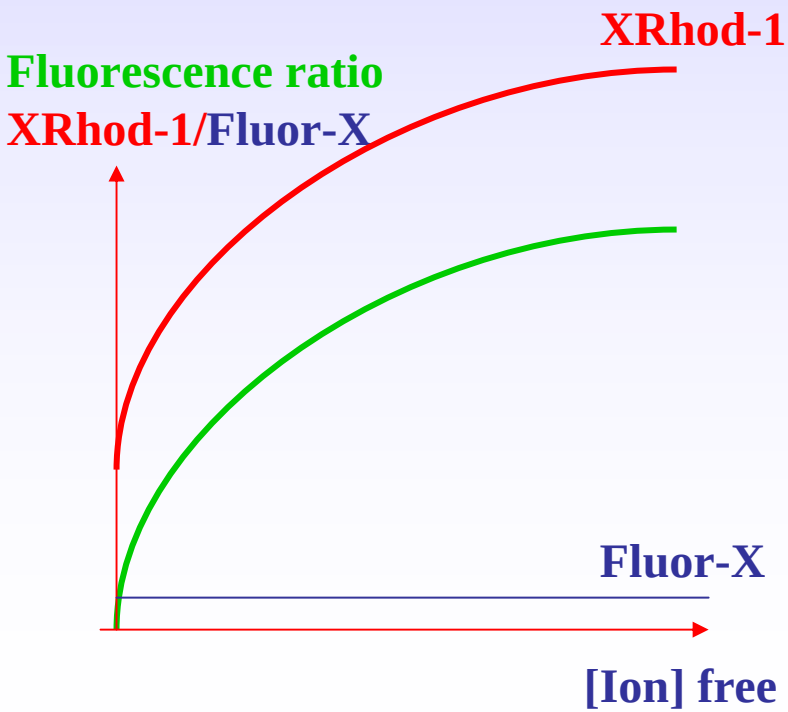


Creating a Ratiometric probe by linking indicator dyes to dextran:

✓**Dextran reduces protein binding artifacts**
Isolate probe from intracellular proteins (ion affinity modified by binding)

✓**Ratiometric indicator**
Co-label dextran with a probe (Fluor-X) that is insensitive to calcium ions





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Another Analysis: Generalized Polarization (GP)

We assume a 2 -state system : bound to unbound

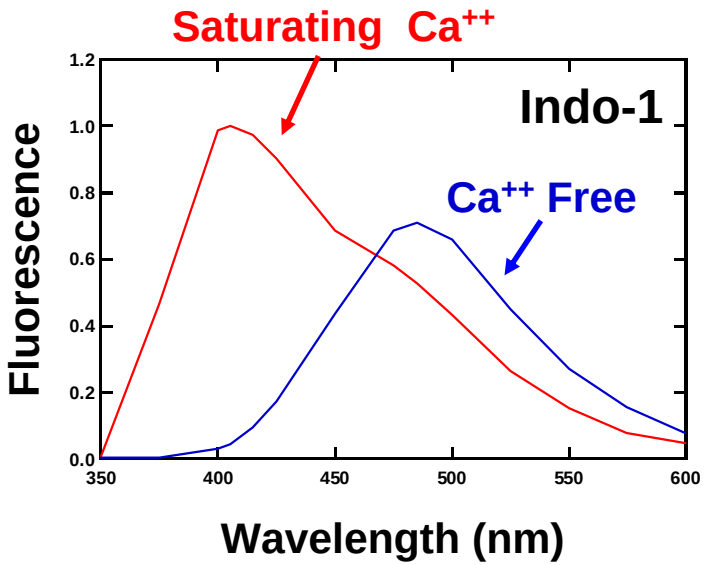
The GP Function:

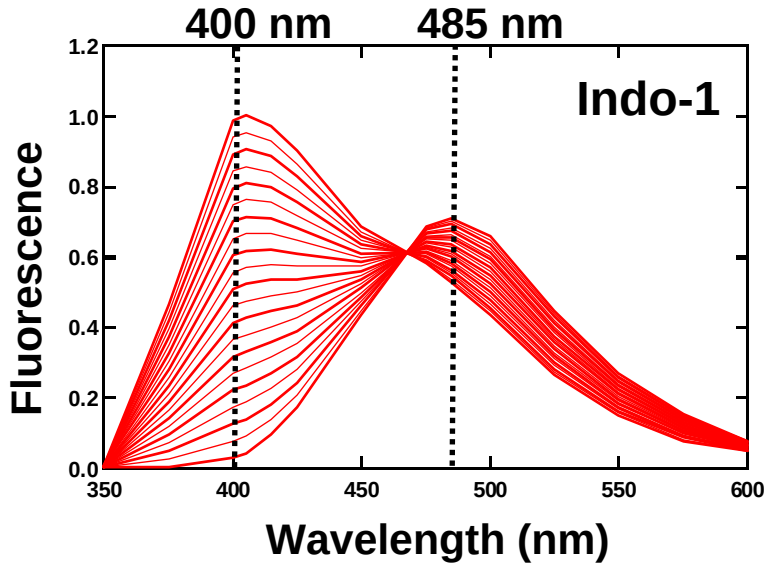
$$GP = (I_{"Blue"} - I_{"Red"})/(I_{"Blue"} + I_{"Red"})$$

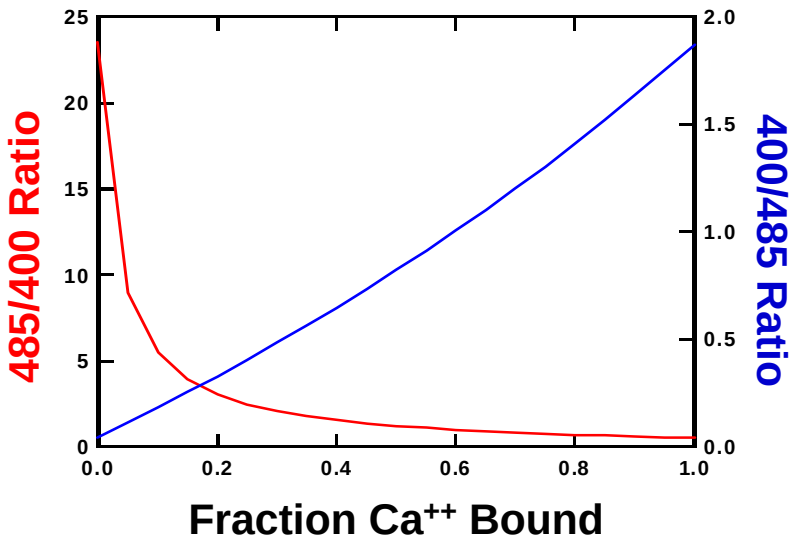
The GP function has several advantages over strict ratio but the information content is the same:

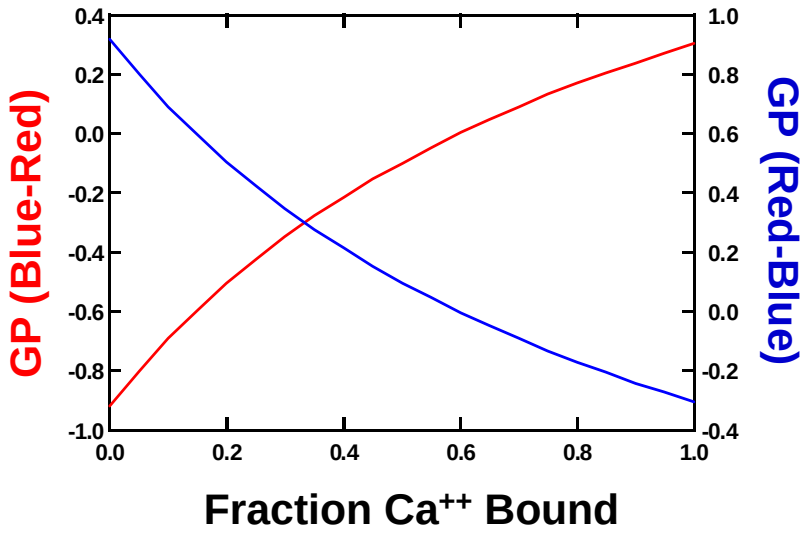
Range -1.0 to 1.0 (more intuitive than ratio)

$$GP_{sample} = \sum_{i=1}^{n} f_i \cdot GP_i$$ (mathematically simple)





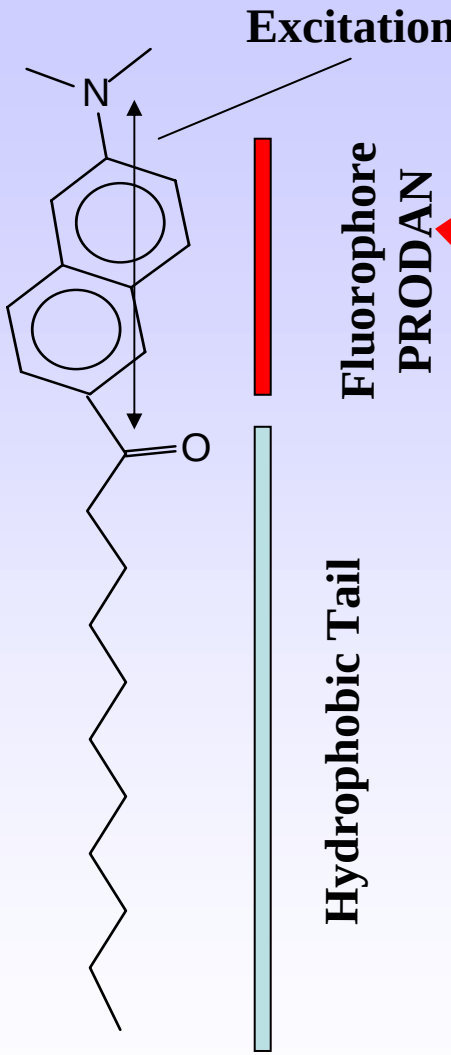


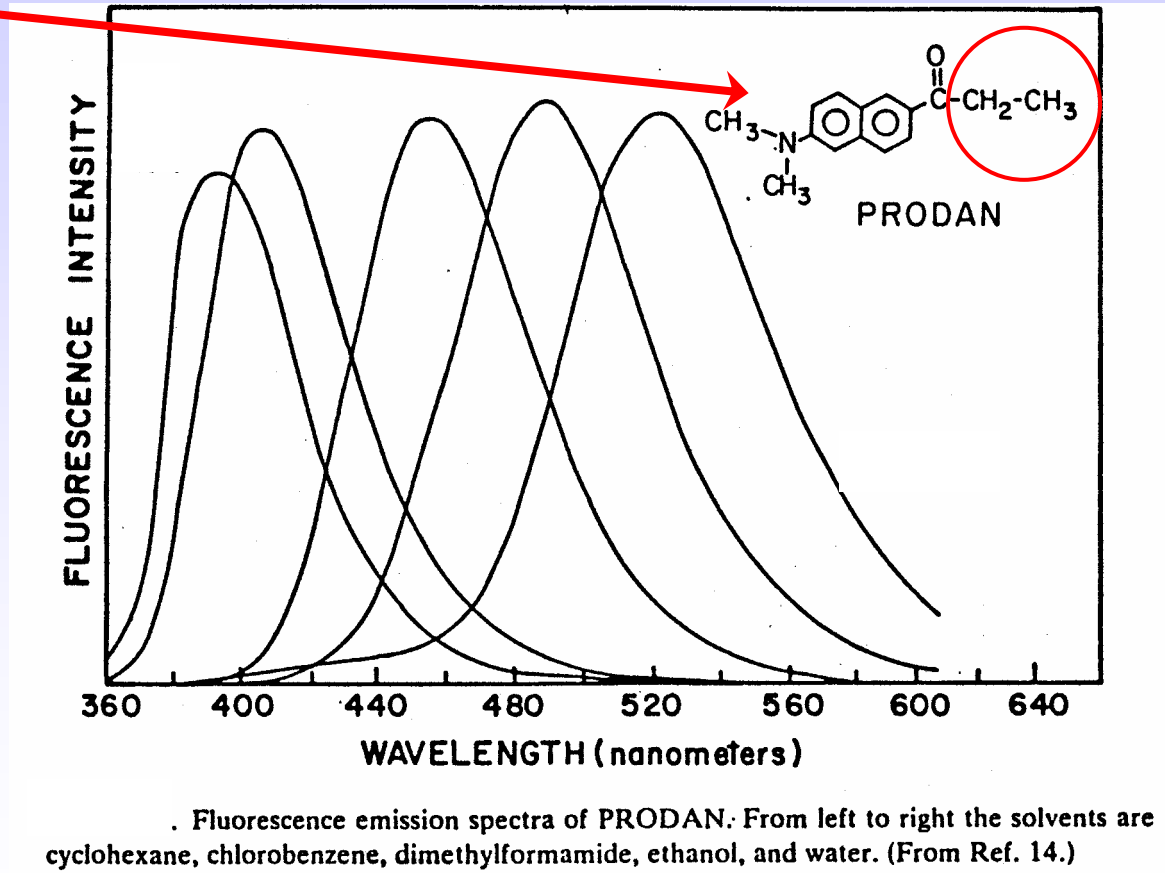


$$[Ion] = \frac{Kd \cdot \varepsilon_{Fluor\_\lambda 2} \cdot (R - R_0)}{\varepsilon_{Fluor-Ion\_\lambda 2} \cdot (R_\infty - R)}$$

$$[Ion] = \frac{Kd \cdot (GP_{sample} - GP_F)}{Q_{Ca2+\bullet F/F} \cdot (GP_{Ca2+\bullet F} - GP_{sample})}$$

A Polarity Sensing Probe





. Fluorescence emission spectra of PRODAN. From left to right the solvents are cyclohexane, chlorobenzene, dimethylformamide, ethanol, and water. (From Ref. 14.)

Laurdan

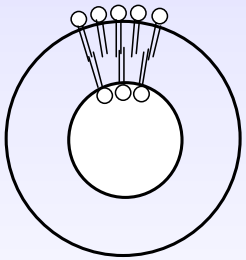
Weber and Farris (1979) Biochemistry 18, 3075

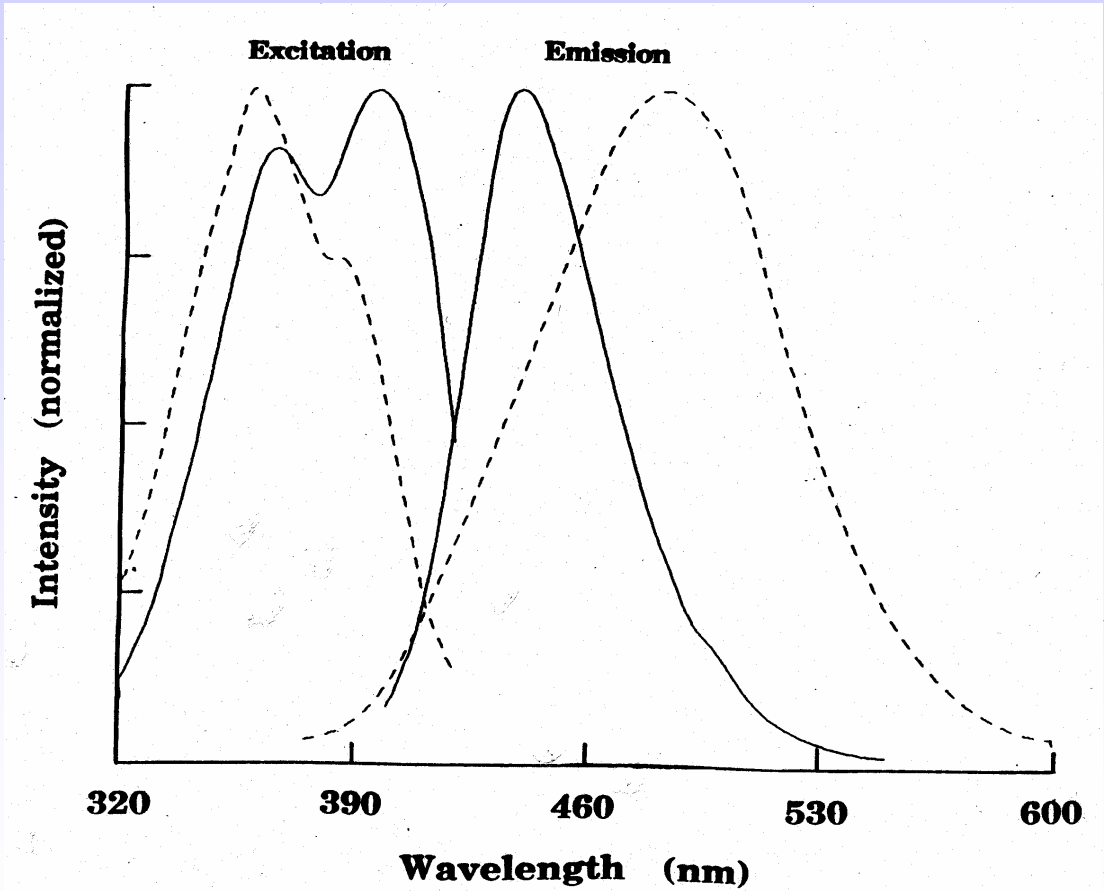
Laurdan Emission Properties

DPPC vesicles at 20°C ——

DPPC vesicles at 60°C - - - -

Vesicles
(SUVs, LUVs
& GUVs)





Laurdan in DPPC Vesicles

Parassassi, Stasio, d'Ubaldo, & Gratton (1990) Biophys. J. 57, 1179

Processing Laurdan Spectral Shifts

Membrane studies using this probe have been generally carried out using GP analysis.

Our2 -state system consists of an unrelaxed and relaxed state.

Our GP function is:

$$GP = (I_{435} - I_{490})/(I_{435} + I_{490})$$

Which will sample the blue (435 nm) and red (490 nm) shifted parts of the spectra.

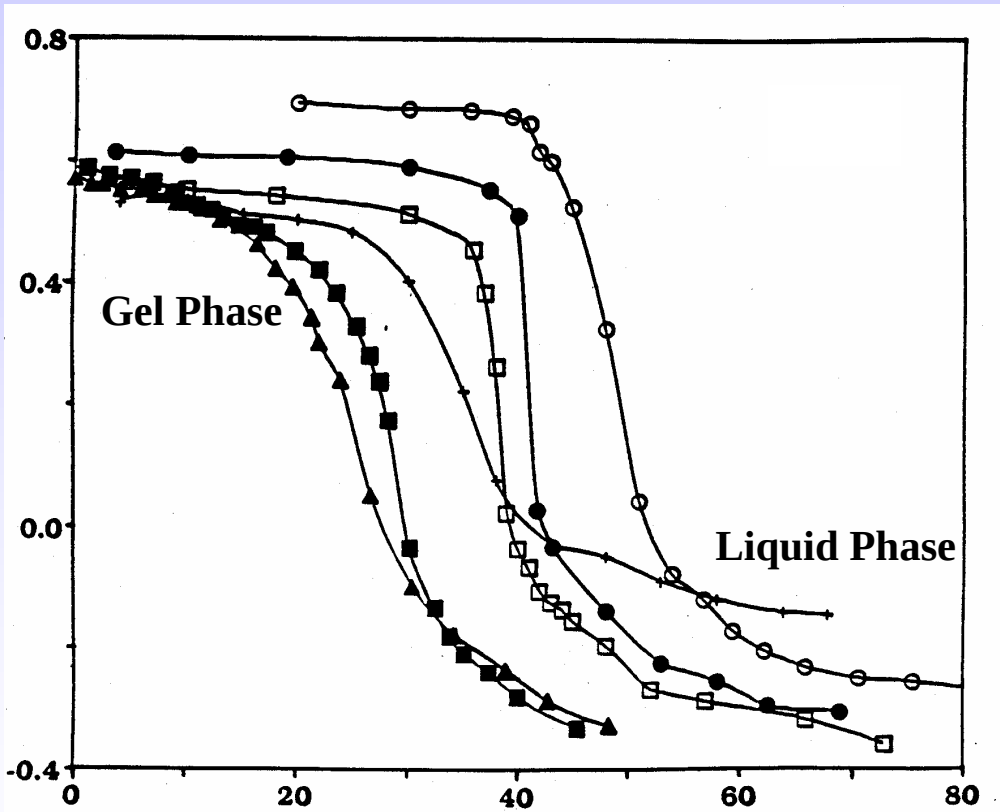
Parassassi, Stasio, d'Ubaldo, & Gratton (1990) Biophys. J. 57, 1179
Parassassi, Stasio, Ravaganan, Rusch, & Gratton (1991) Biophys. J. 60, 179

Lipid Phase Transition
Multilamellar Vesicles (MLVs)

blue-shifted

GP

red-shifted

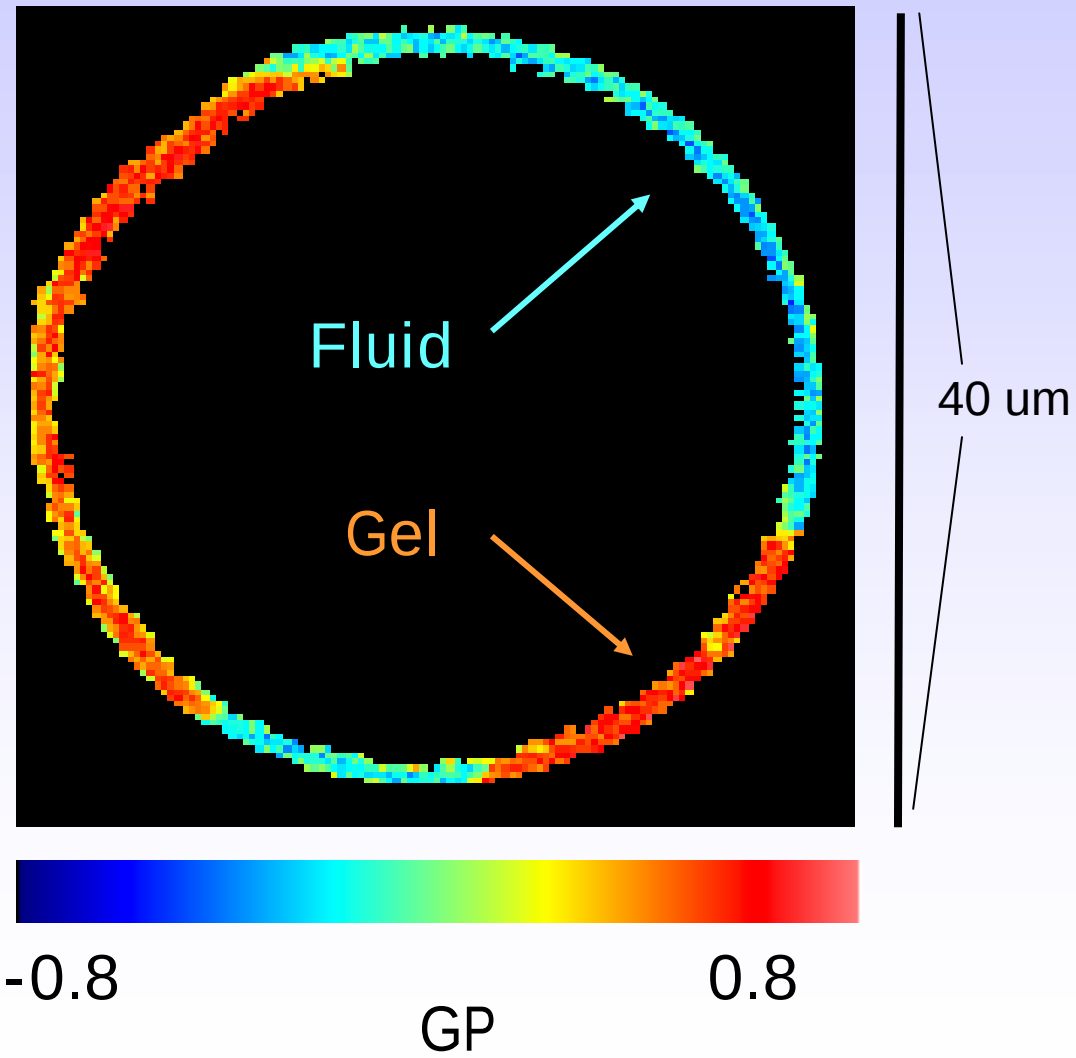


DPPC
DPPS:DPPC (2:1)
DPPG:DPPC (2:1)
DMPA:DMPC (2:1)
DPPG:DLPC (1:1)
DPPC:DLPC (1:1)

Temperature (°C)

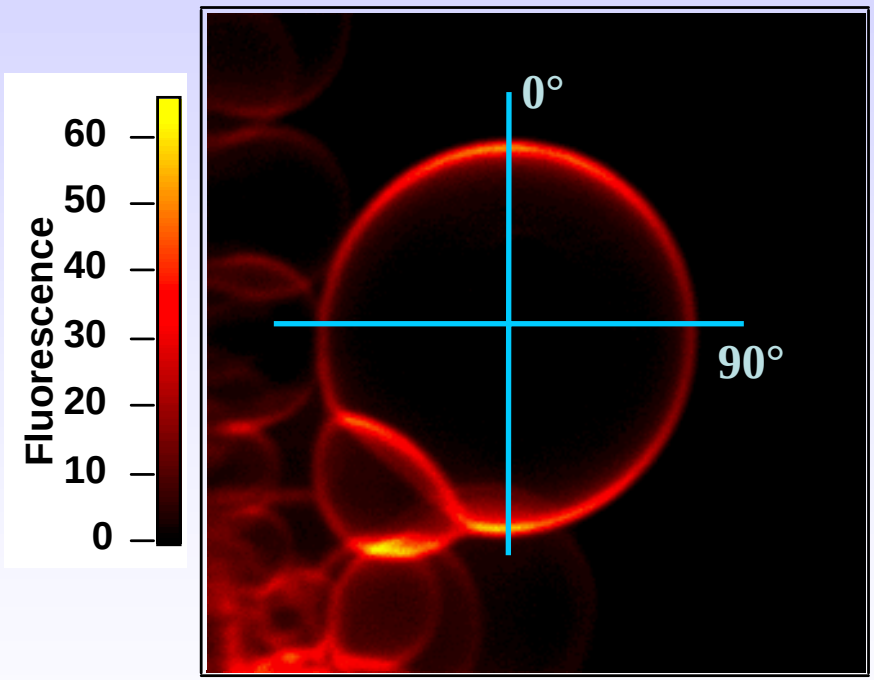
Parassassi, Stasio, Ravaganan, Rusch, & Gratton (1991) Biophys. J. 60, 179

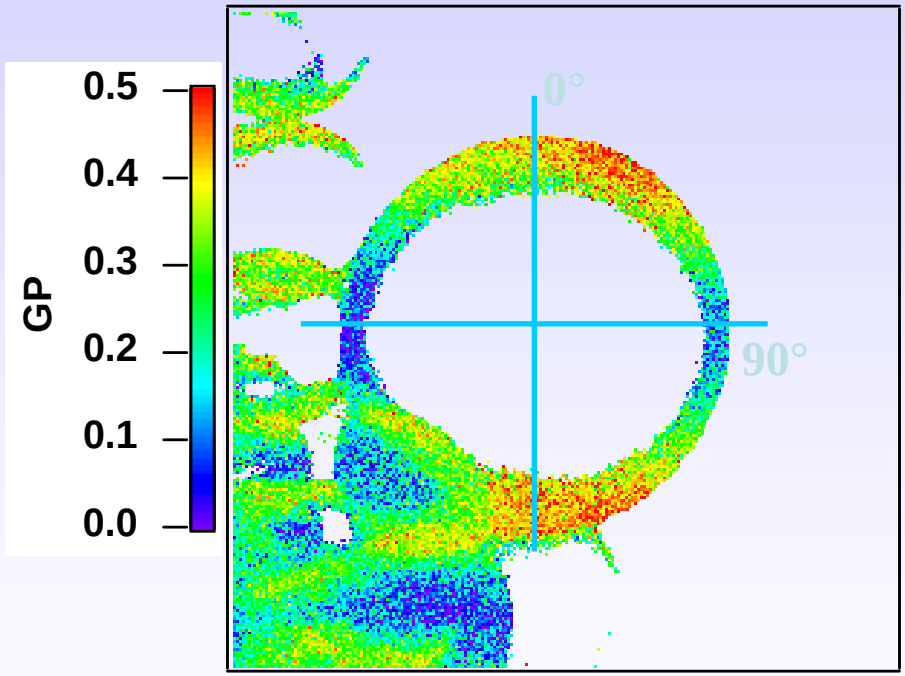
Domain Coexistence on Giant Vesicles (Laurdan GP Image)



DMPC Giant Vesicles (+ Laurdan)

Excitation light polarized along the 0° axis

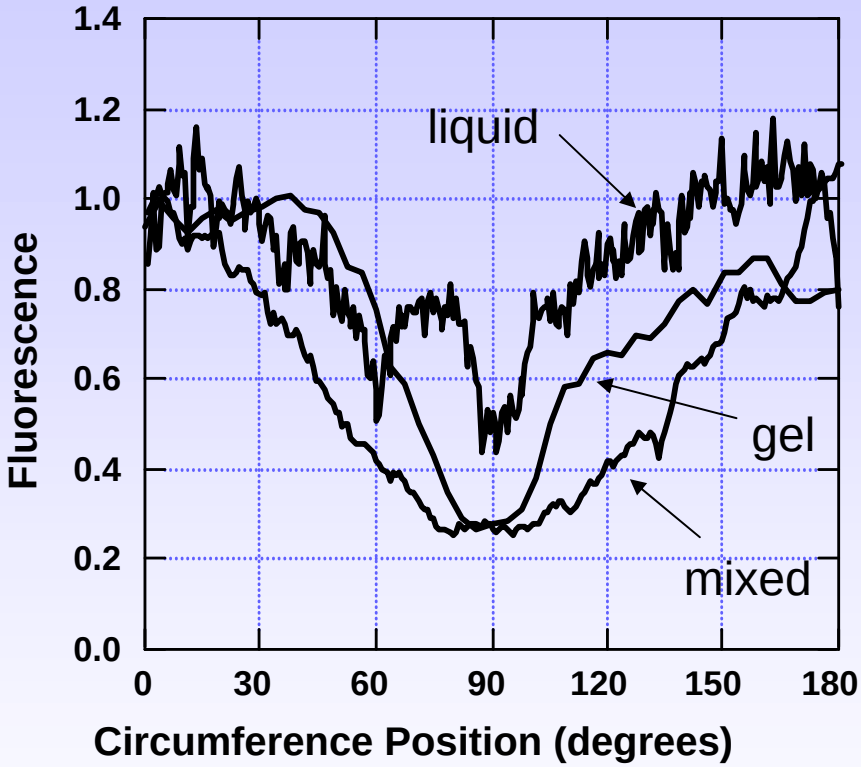


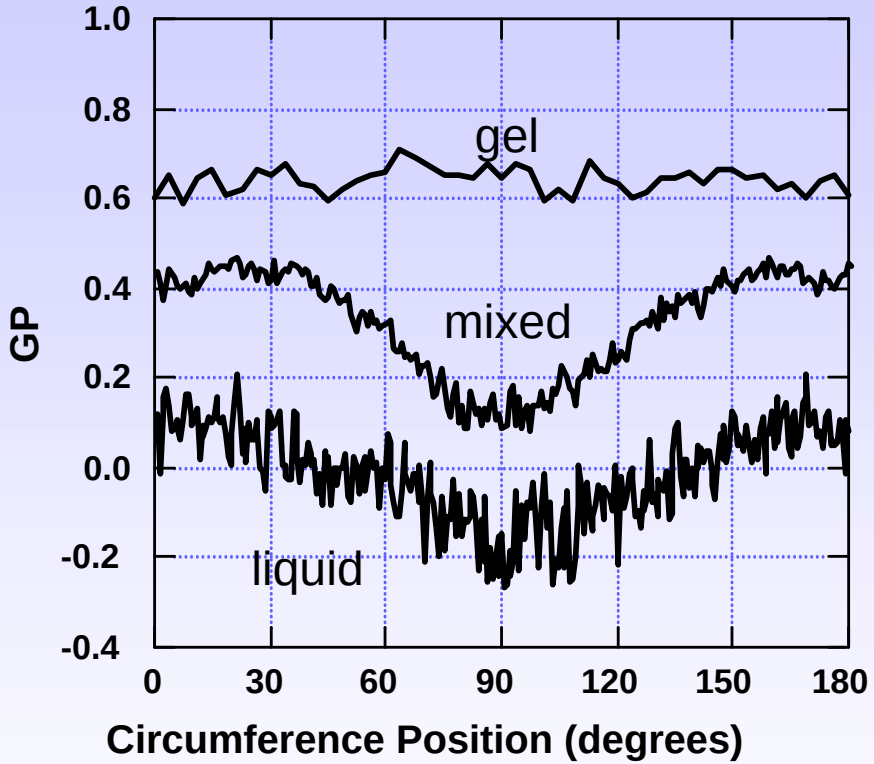


Bagatolli, Sanchez, Hazlett, E. Gratton (2002). Methods in Enzymology, 360,481.

DMPC Giant Vesicles (+ Laurdan)

Excitation light polarized along the 0° axis





Gel Phase ——

Gel & Liquid Phases – – – –

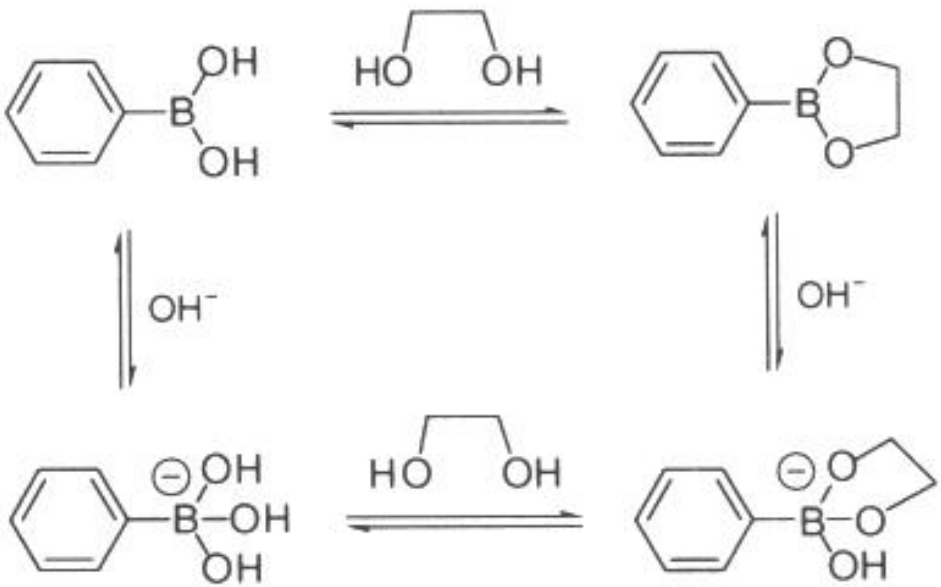
Liquid Phase .....

Bagatolli, Sanchez, Hazlett, E. Gratton (2002). Methods in Enzymology 360:481

Fluorescent Reagents for Saccharides

•Same considerations that we have for metal binding but more subtle steric alignments are needed.

•Boronic acids have been employed for detecting hydroxyl groups with specific relative orientations. Some selectivity for specific sugars and enantiomers.

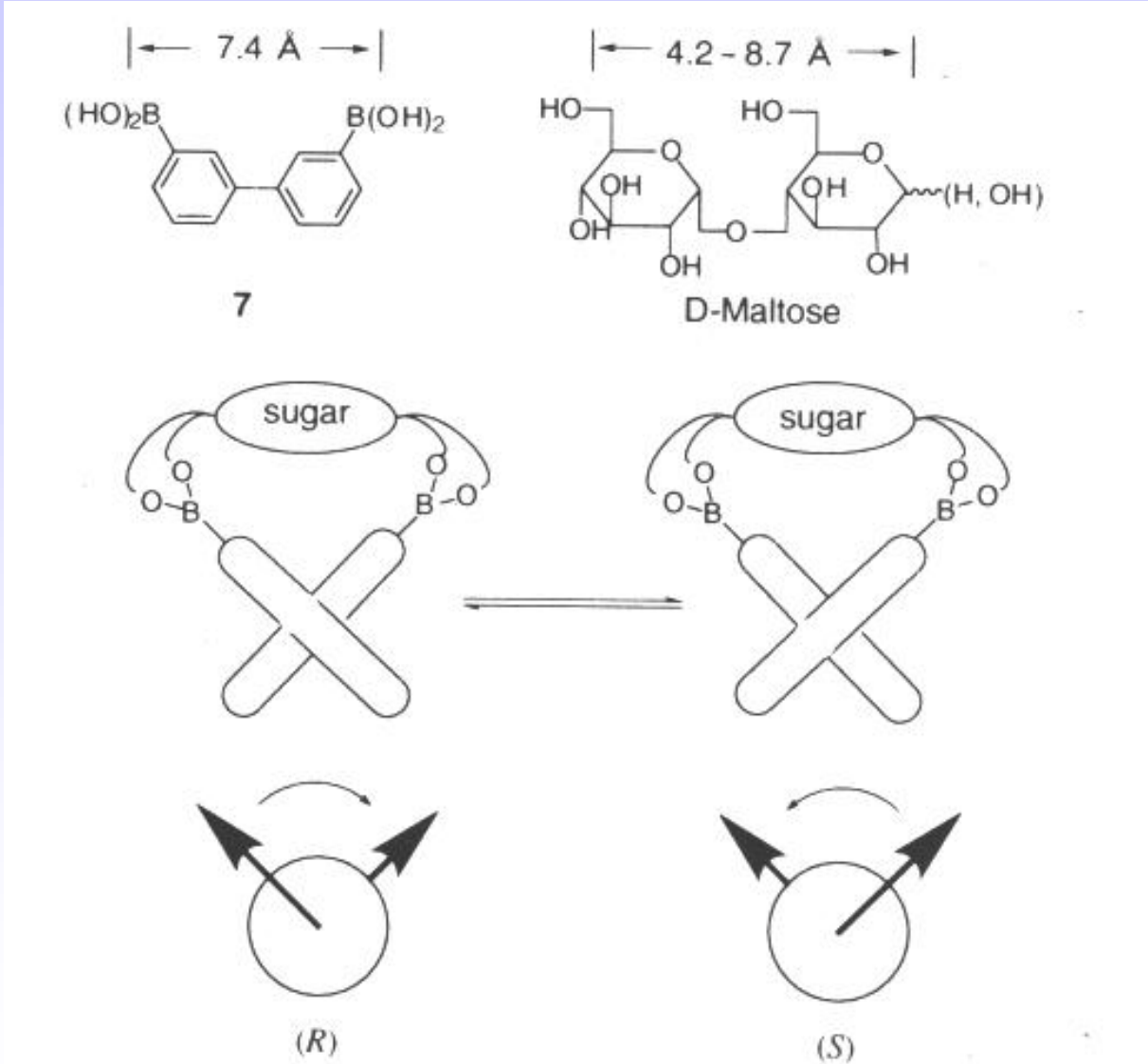


Boronate ester formation between a boronic acid and a diol in water

Selectivity of Boronic Acids

Distance & Steric Considerations

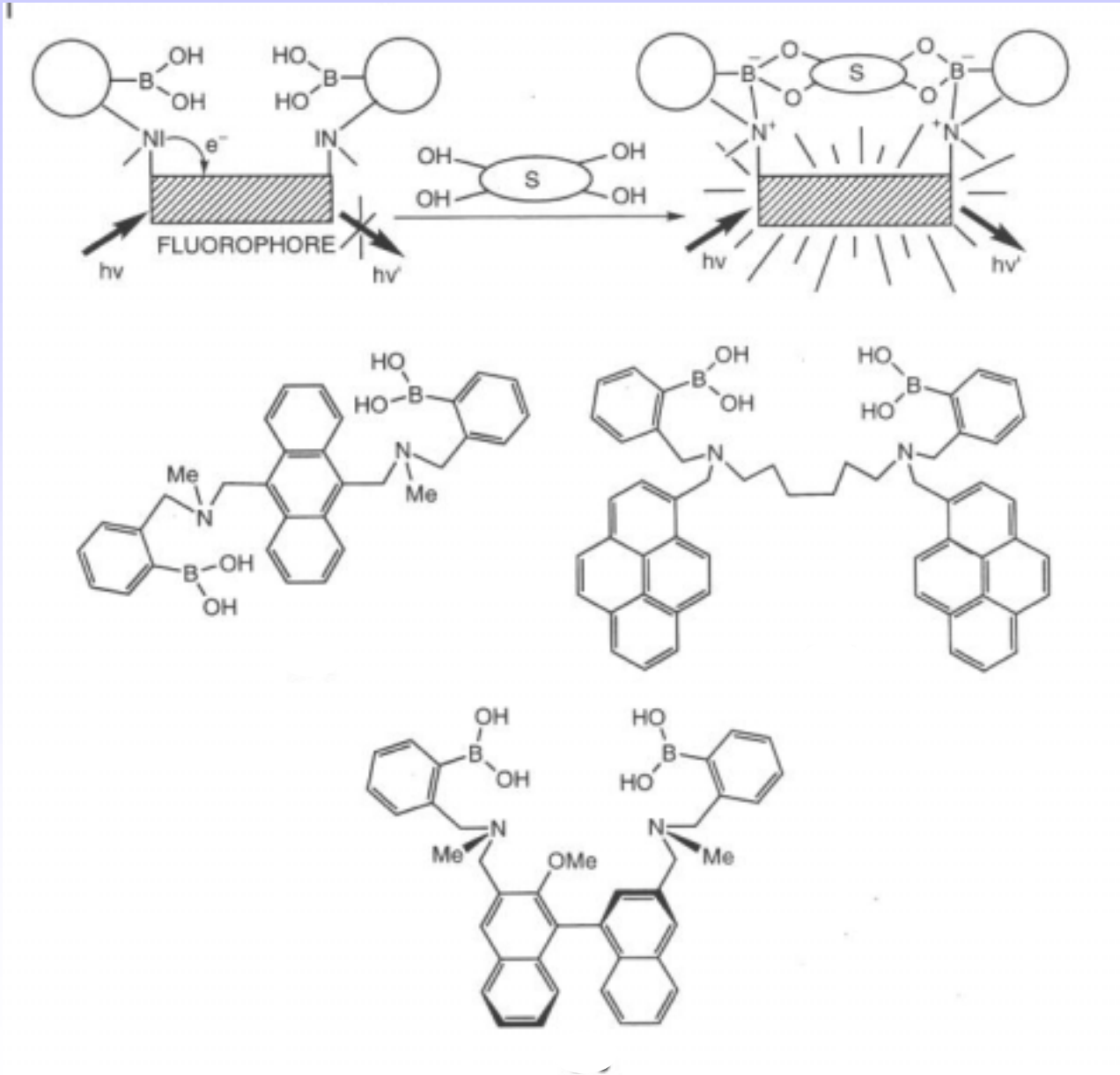
Relative Positions of the Diols (Chirality)



Shinkai & Robertson (2001) in New Trends in Fluorescence Spectroscopy , B. Valeur & J.-C. Brochon (Eds.)

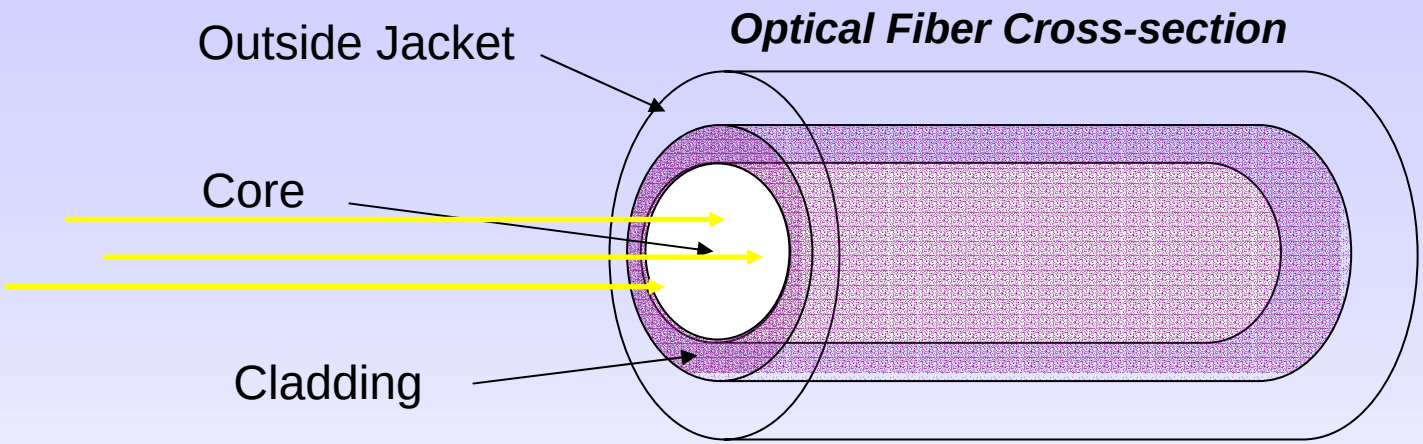
Fluorescent Derivatives of Boronic Acids

Photoinduced electron transfer mechanism



B. Valeur (2002) Molecular Fluorescence, Principles and Applications

Fiber-Optic Fibers in Fluorescence



Single Mode Fibers:

- low temporal dispersion
- insensitive to bending
- insensitive to temperature

$$V = \frac{2\pi}{\lambda} \cdot \rho \cdot \sqrt{\left(\eta_{core}^2 - \eta_{cladding}^2\right)}$$

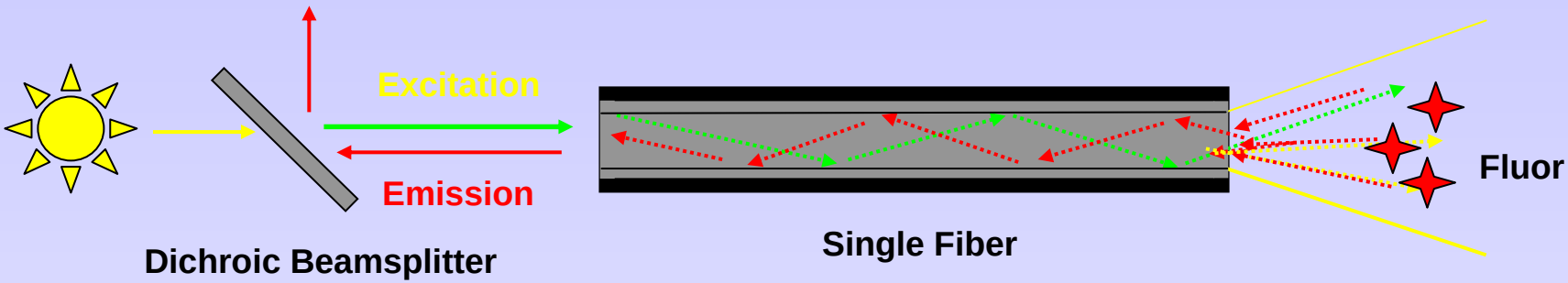
Single mode at V-Numbers <2.4

Multimode Fibers

- better for high intensities
- plastic claddings (easier access to core)

R.B.Thompson (1991) in Topics in Fluorescence Vol2

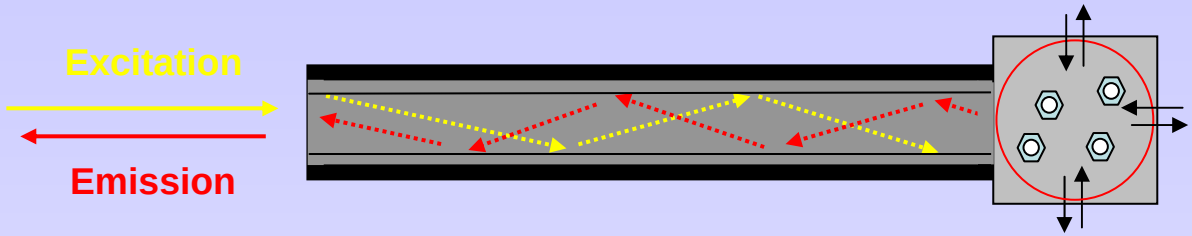
Passive Sensors



Passive sensors excite and collect from the solution or surface they are in contact with. Different geometries of the double fiber system can increase the collection efficiency .

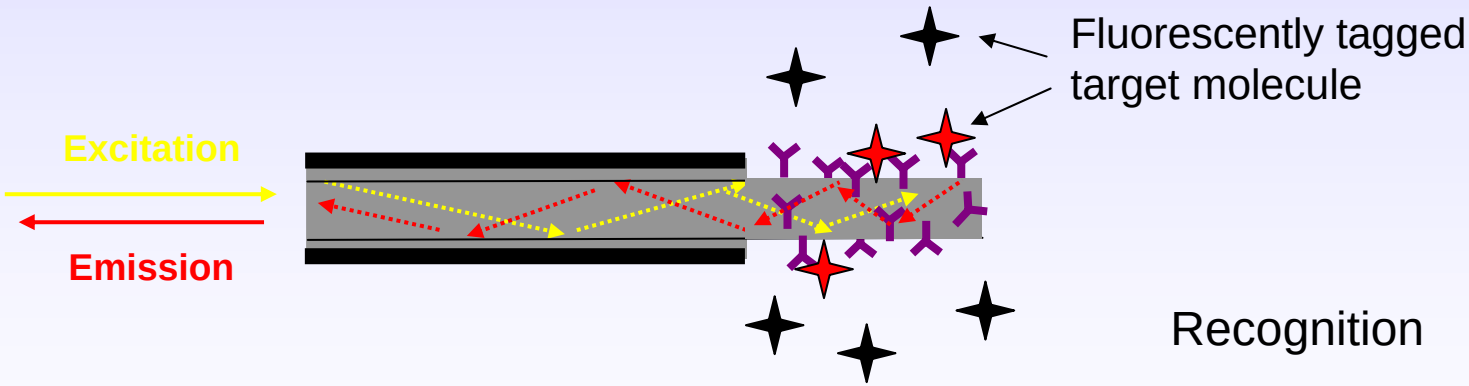
Used for Ion sensing, pH sensing, Immunoassays…

Active Optical Sensors



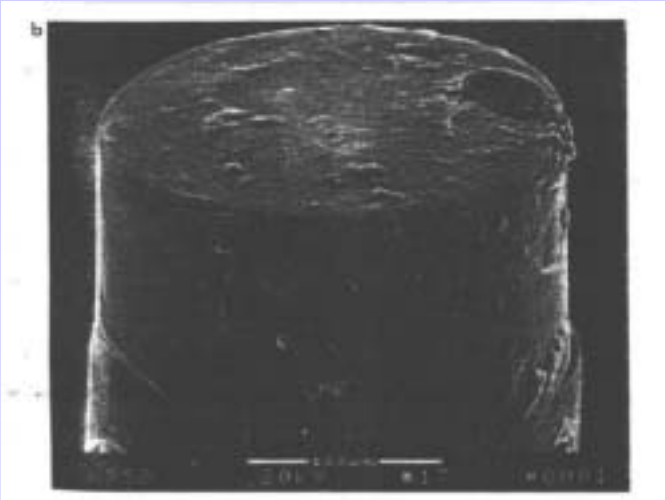
Semipermeable Membrane or Permeable Matrix e.g. polymers (polystyrene, polyacylamide) or glasses (SiO_2, TiO_2).

Fluorophore sensors are then bound or restricted to the tip region where they can interact with the solution.

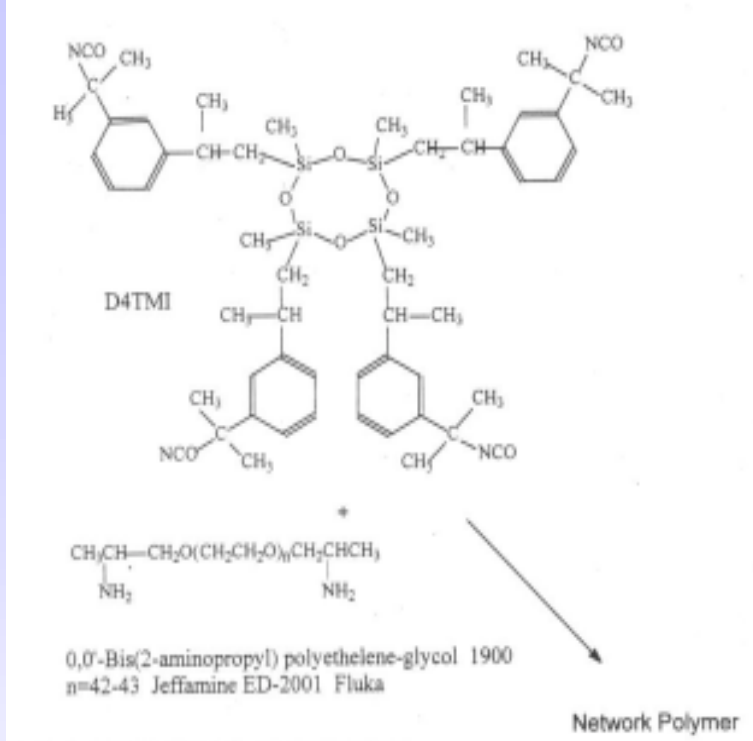


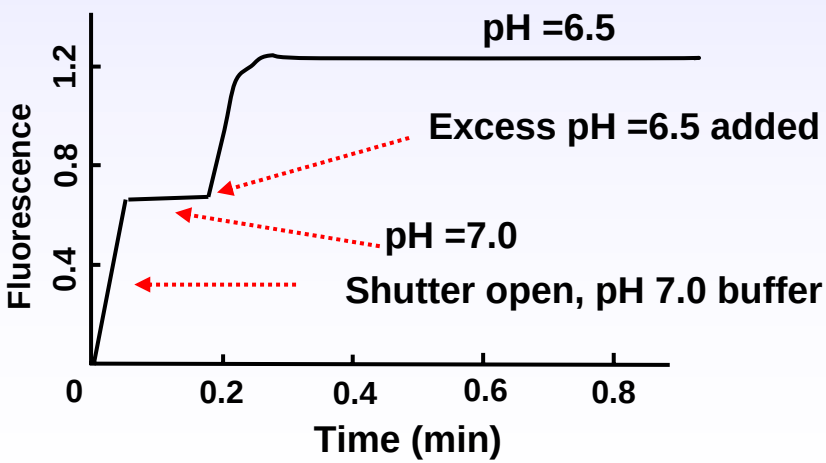
Target molecule or recognition molecule is attached to the core where the <u>evanescent wave</u> will excite only molecules near the surface will be excited.

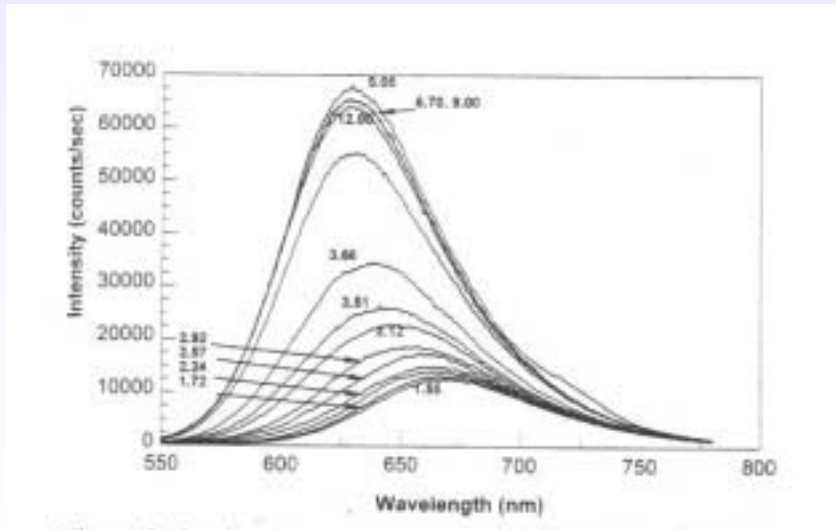
B. Valeur (2002), Molecular Fluorescence, Principles and Applications



Fiber coated with
Eosin-Phenol Red Polymer (10um layer)







Jordan, Walt,& Milanovich (1987) Anal. Chem.59, 437

Clarke, Xu, Demas,& DeGraff (2000) Anal. Chem. 69, 863

Tagging Fluorophores to Reagents and Biomolecules

Probes for Nucleic Acids

Hoechst 33342 (AT rich) (uv)	346	460
DAPI (uv)	359	461
POPO-1	434	456
YOYO-1	491	509
Acridine Orange (RNA)	460	650
Acridine Orange (DNA)	502	536
Thiazole Orange (vis)	509	525
TOTO-1	514	533
Ethidium Bromide	526	604
PI (uv/vis)	536	620
7-Aminoactinomycin D (7AAD)	555	655

Probes for Proteins

Probe	*Excitation*	*Emission*
FITC	488	525
PE	488	575
APC	630	650
PerCP	488	680
Cascade Blue	360	450
Coumerin-phalloidin	350	450
Texas Red	610	630
Tetramethylrhodamine-amines	550	575
CY3 (indotrimethinecyanines)	540	575
CY5 (indopentamethinecyanines)	640	670

DNA Probes

- AO
  - Metachromatic dye
    - concentration dependent emission
    - double stranded NA - Green
    - single stranded NA - Red
- AT/GC binding dyes
  - AT rich: DAPI, Hoechst, quinacrine
  - GC rich: antibiotics bleomycin, chromamycin A_3, mithramycin, olivomycin, rhodamine 800

Probes for Oxidation States

Probe	*Oxidant*	*Excitation*	*Emission*
DCFH-DA	(H_2O_2)	488	525
HE	(O_2^-)	488	590
DHR 123	(H_2O_2)	488	525

DCFH-DA - *dichlorofluorescin diacetate*
HE - *hydroethidine*
DHR-123 - *dihydrorhodamine 123*

Molecular probes, www.probes.com

Naturally Occurring Fluorophores in Proteins

Amino acids

Tyrosine (ex/em 280 nm/305 nm)
Tryptophan (ex/em 280, 295nm/ 305-350 nm
Phenylalanine Ex/Em 260 nm/282 nm

Unnatural Amino acids

7-azatryptophan (ex/em 320nm/403nm)
5-hydroxy tryptophan (ex/em 310nm/339 nm)

***Cofactors*: NADH (oxido-reductases) Ex/Em 340/460 nm, Porphyrins (ex/em 550 nm/620 nm), FAD+ (metabolic enzymes (ex/em 450 nm/540 nm)**

Fluorescent proteins:

CFP, GFP, YFP, RFP

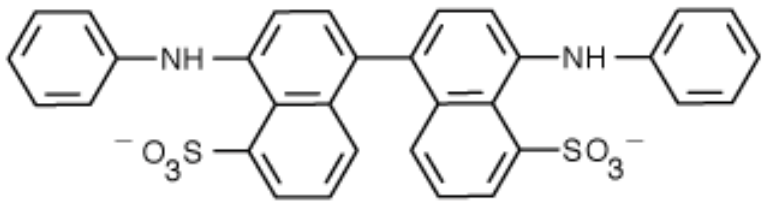
G. Marriott

Types of fluorescent probes:

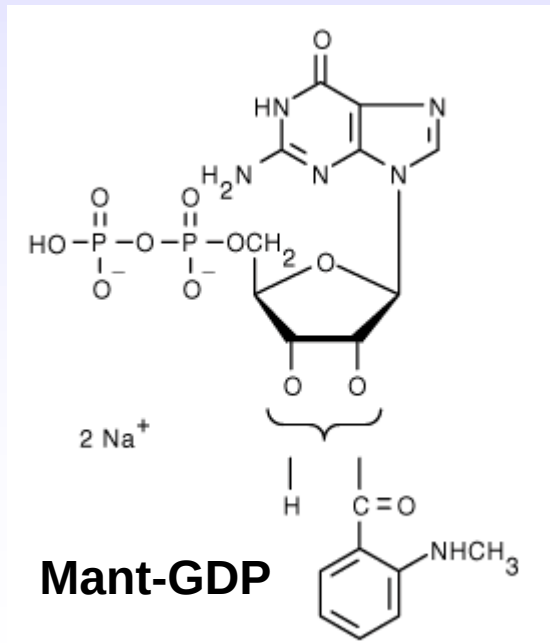
Extrinsic probes : (not present in the natural molecule/macromolecule)

1. Non-covalent Attachments

Examples: bis-ANS binds to hydrophobic patches on proteins, Ethidium Bromide with dsDNA & IgG/Antigen interactions, Fluorescent derivatives of cofactors (mant-GDP).



bis-ANS



Mant-GDP

2. Covalent Attachments

This class of probe must have reactive groups capable of reacting with specific chemical groups.

The conjugate must be tested for its biological activity, spectroscopic properties and non-specific binding properties

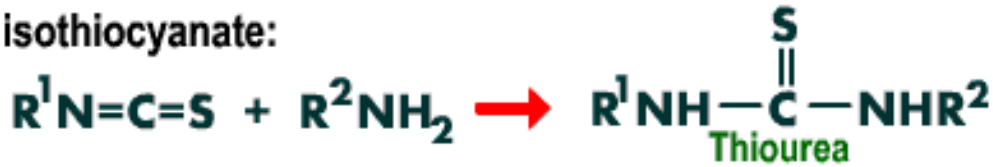
Reactive probes targeting amino groups:

Protein modifications

α-amino group (rare- only one!), pKa = 8.5 (50% protonation at pH 8.5)
ε-amino group (lots) pKa= 10.5 (50% protonation at 10.5, 1% at pH 8.5!)

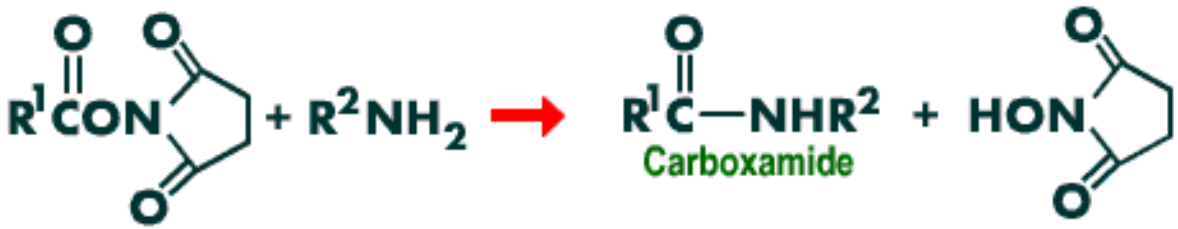
Reaction of Primary Amine with:

isothiocyanate:



Not very reactive
Label at pH>9.0

succinimidyl ester:



Very reactive
Label at pH 7.5

sulfonyl chloride:

Moderately reactive
Label at pH 8.5

G. Marriott

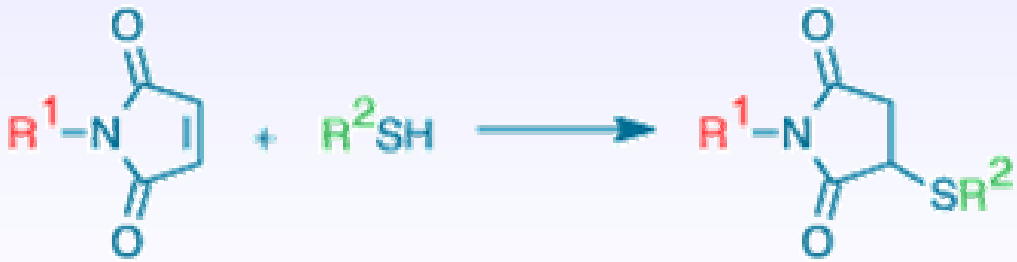
Reactive probes targeting thiol groups:

Protein modifications

Free thiol groups (less common than amines, often found as disulfides, commonly added through site-directed mutation of proteins, pKa = 5)

Alkyl halide or Haloacetamide (X = I, Br, Cl)

Thioether



Maleimide

Thioether

Reagents targeting Aldehyde and Ketones