Labeling and imaging living cells and tissues

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Workflow

1. Pick your cells/tissue/incubation conditions*
2. Pick your dye(s) (keep in mind excitation/emission channels available, multiplexing)
3. Pick loading method (single cell, multicellular, retention)
4. Load cells: how much dye, how long incubation, wash or not
5. Image

*dfilwt

Media

Common media such as DMEM use carbonic acid/bicarbonate pH buffering where gaseous CO_2 balances the H_2CO_3 / HCO_3^- content of the medium.

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

pH goes to 8 for bicarbonate buffered media in absence of CO_2. Neurons die within 5 minutes at pH 8.

Alternatives:

(1) HEPES: insensitive to CO_2 but photosensitizes H_2O_2 generation. Opti-MEM is HEPES buffered.

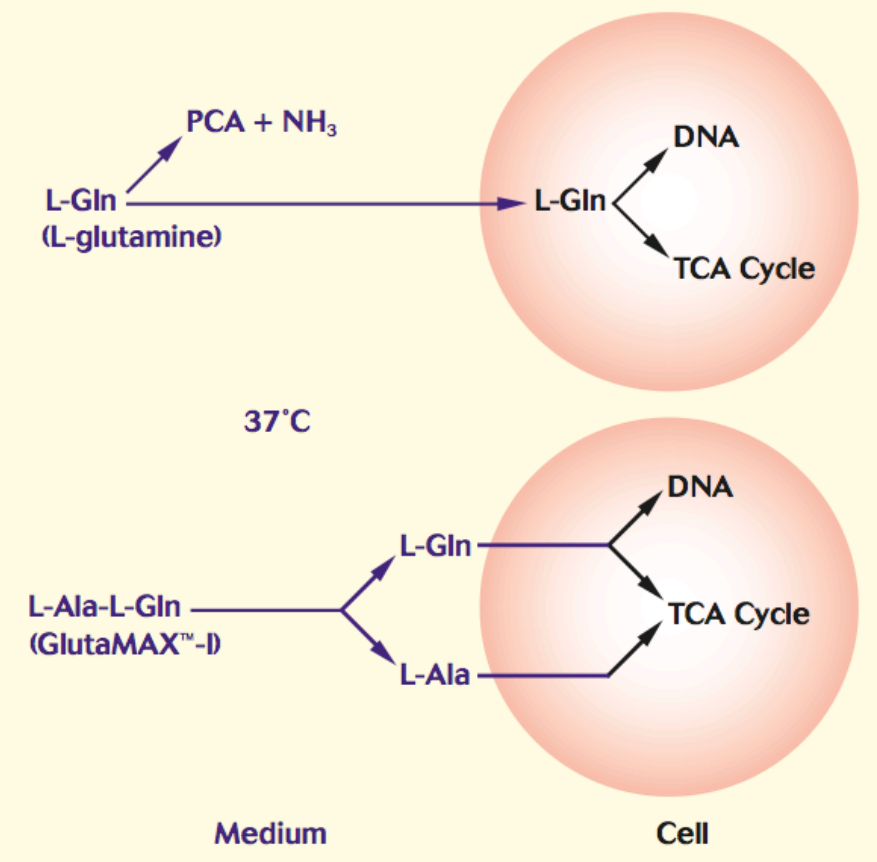
(2) Leibovitz L15. Uses high levels of the free base forms of arginine, cysteine and histidine to set the desired pH.

Serum:

Use serum-free media for dye loading. One of the stated reasons for inclusion of serum in culture media is “to bind and neutralize toxins” (such as dyes).

Ammonia

L-glutamine is an essential nutrient in cell cultures for energy production as well as protein and nucleic acid synthesis. However, L-glutamine in cell culture media spontaneously degrades, generating ammonia.



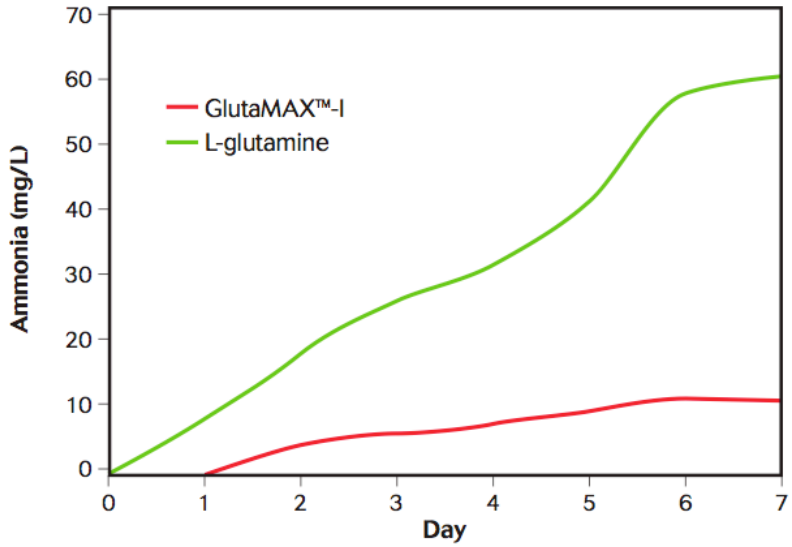
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 8, Issue of April 25, pp. 2669-2676, 1979
Printed in U.S.A.

Evidence That Glutamine, Not Sugar, Is the Major Energy Source for Cultured HeLa Cells*

(Received for publication, February 27, 1978, and in revised form, August 10, 1978)

Lawrence J. Reitzer, Burton M. Wice, and David Kennell

From the Department of Microbiology and Immunology, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110



D-MEM was supplemented with GlutaMAX™-I or L-glutamine, aliquoted into vials and stored at 37°C. Samples were taken daily and frozen at −20°C. Levels of ammonia were determined by HPLC.

Importance of culturing primary lymphocytes at physiological oxygen levels

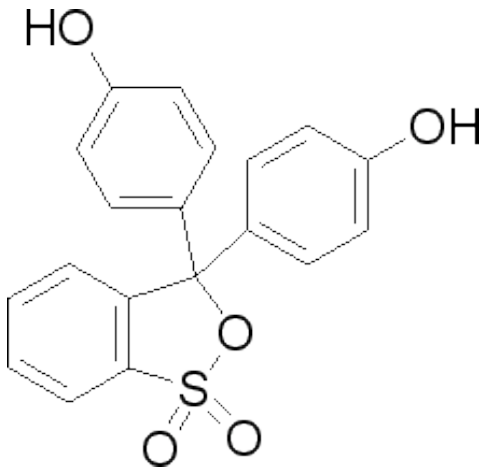
Kondala R. Atkuri*†, Leonard A. Herzenberg*‡, Anna-Kaisa Niemi§, Tina Cowan§, and Leonore A. Herzenberg*

Departments of *Genetics and §Pathology, Stanford University School of Medicine, Stanford, CA 94305

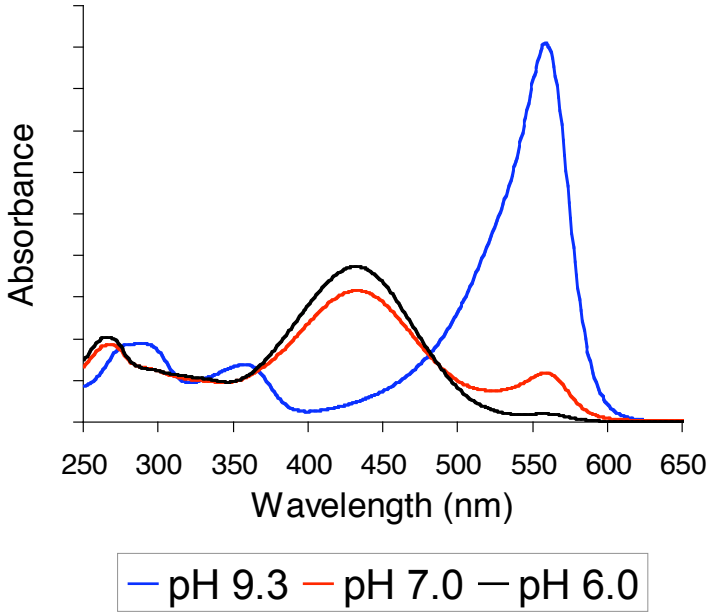
"Although studies with primary lymphocytes are almost always conducted in CO_2 incubators maintained at atmospheric oxygen levels (atmosO_2; 20%), the physiological oxygen levels (physO_2; 5%) that cells encounter *in vivo* are 2–4 times lower. We show here that culturing primary T cells at atmosO_2 significantly alters the intracellular redox state (decreases intracellular glutathione, increases oxidized intracellular glutathione), whereas culturing at physO_2 maintains the intracellular redox environment (intracellular glutathione/oxidized intracellular glutathione) close to its *in vivo* status".

Proc Natl Acad Sci USA (2007) **104:**4547–4552

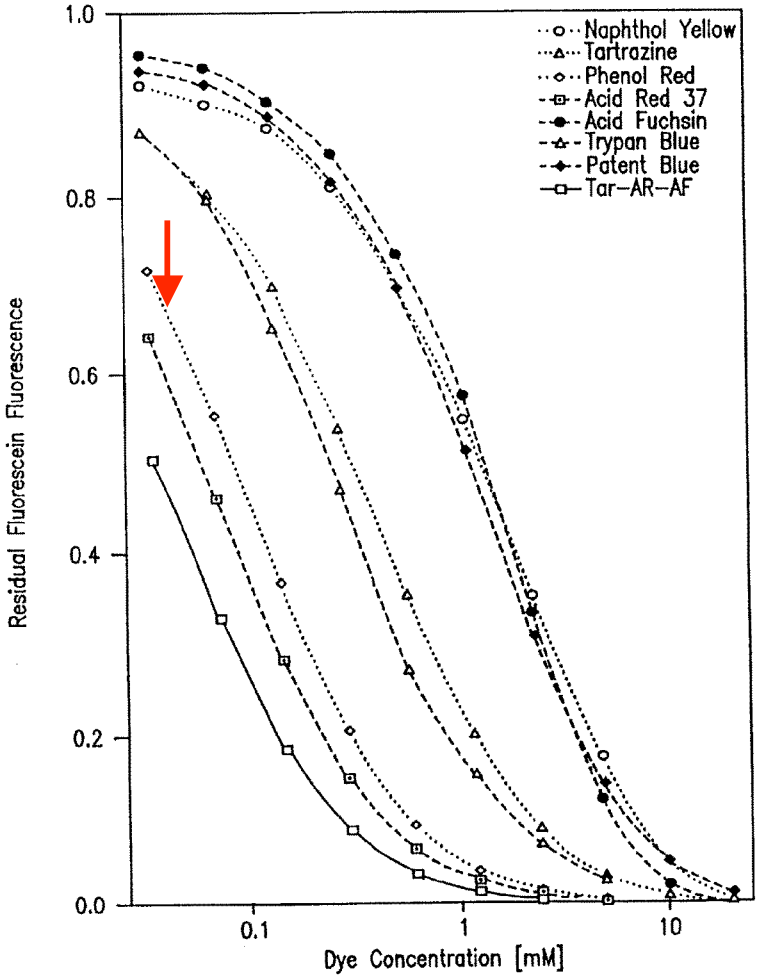
Phenol Red?



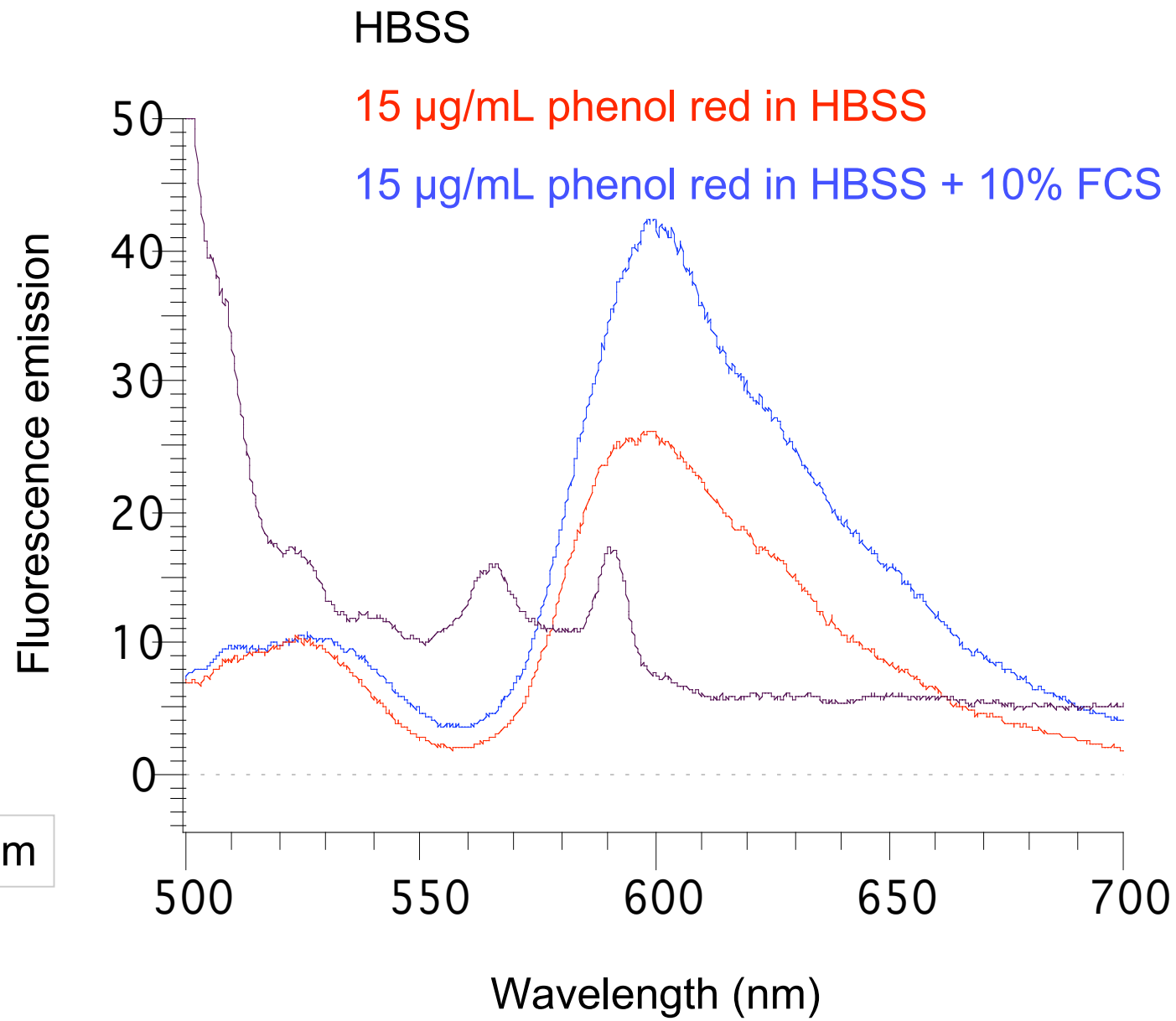
Phenol Red (phenolsulfonphthalein)
Typical concentration in media is 15 µg/mL (~40 µM)



10 µM fluorescein in 100 µL
CytoFluor microplate reader

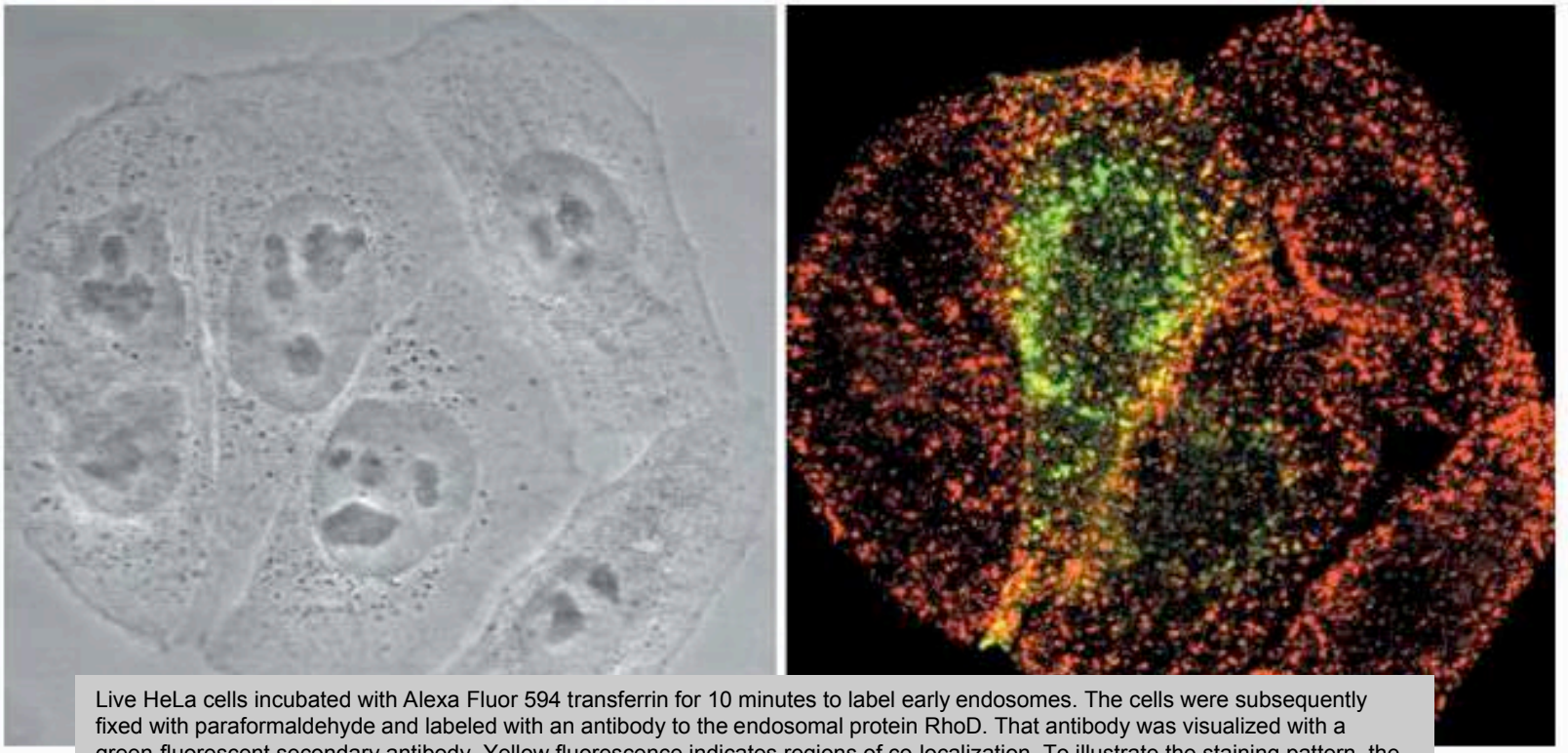


Fluorescence emission spectra @ working concentration



Ex = 488 nm

Stokes shift enables darkfield imaging



Live HeLa cells incubated with Alexa Fluor 594 transferrin for 10 minutes to label early endosomes. The cells were subsequently fixed with paraformaldehyde and labeled with an antibody to the endosomal protein RhoD. That antibody was visualized with a green-fluorescent secondary antibody. Yellow fluorescence indicates regions of co-localization. To illustrate the staining pattern, the cells were imaged by both fluorescence (upper panel) and differential interference contrast (DIC) microscopy (lower panel). The image was contributed by Harry Mellor, University of Bristol.

- You only see what you stain (or what is autofluorescent)
- Use DIC for cell morphology context, particularly on punctate targets like lysosomes, endosomes, phagocytosis
- Fluorescent counterstaining for context; Hoechst 33342 (nucleus), calcein AM (cytoplasm), CellMask Orange (plasma membrane)

Background Signals

Instrument
Stray light, detector noise

Reagent
Unbound or nonspecifically bound probes

Sample
Solute and solvent autofluorescence, scattered excitation light (particle size and wavelength dependent)

- 1/0 is better S/N than 1000/1

Sources of Autofluorescence

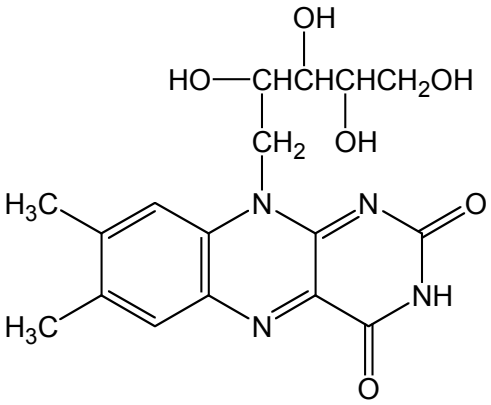
Source	Organism/Tissue	Ex (nm)	Em (nm)
Flavins	CHO cells	380, 460	520
	Rat hepatocytes	468	525
	Neural cells (rat, bovine)	488	540-560
	Goldfish inner ear	450	540
	Periplaneta americana	<350	530
NAD(P)H	Rat cardiomyocytes	395	509
	S. cerevisiae	366	440-470
	CHO cells	360	440-450
Lipofuscins	Medulla (rat, human, rhesus monkey)	460-490	520
	Rat heart	450-490	550
	Muscle, myocardium, hepatocytes	360	540-560
	Human brain	435	481-673
	Rat liver	345	430
	Rat retina	390-490	>510
Collagen and elastin	Aorta, coronary artery (human)	476	>515
	Skin (human)	442	470-520

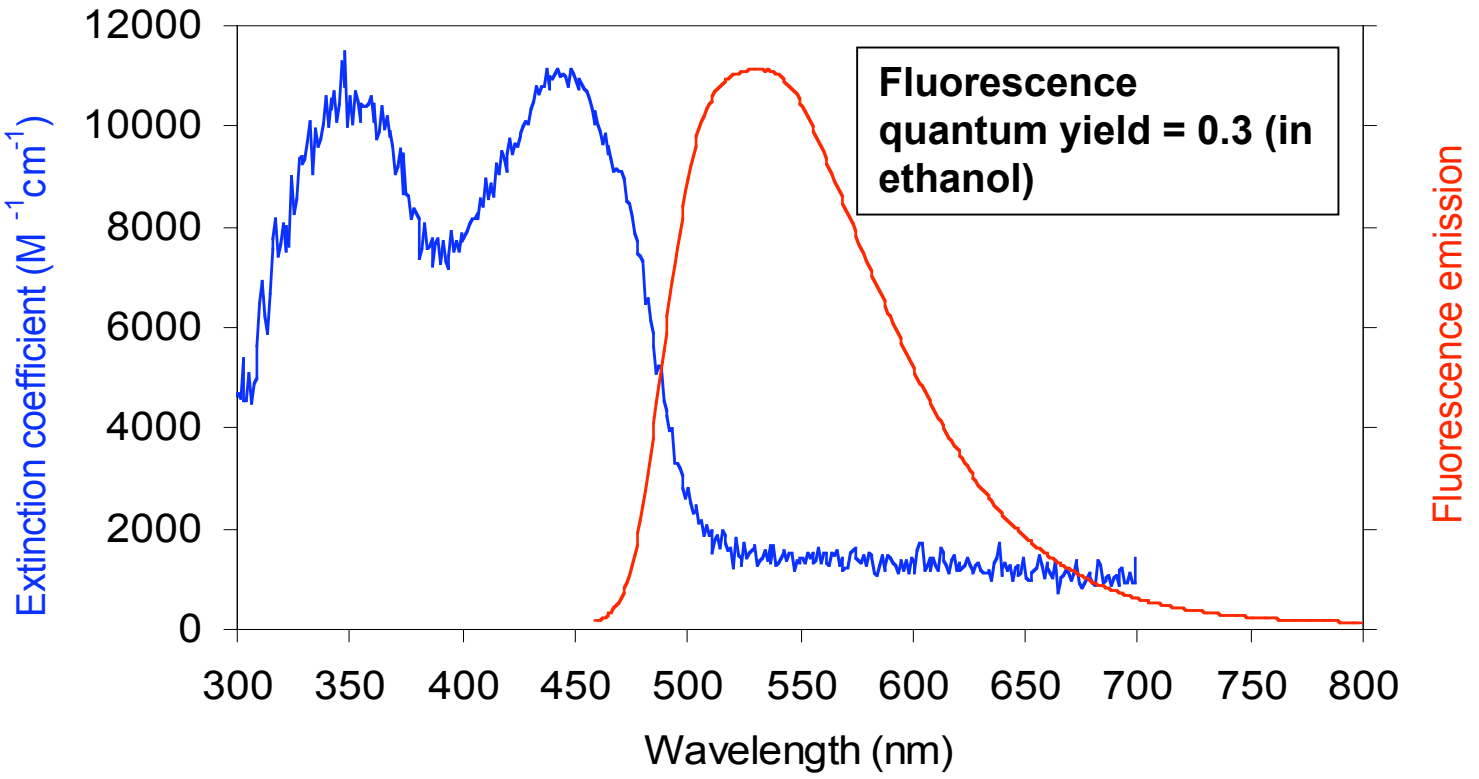
No Ex > 500 nm
No Em >600 nm

*Table from Analytical Biochemistry, **291:**175 (2001)*

▶ Autofluorescence (ex/em= 488/530 nm) of a typical 3T3 cell is equivalent to about 34,000 fluorescein molecules

Autofluorescence: Riboflavin





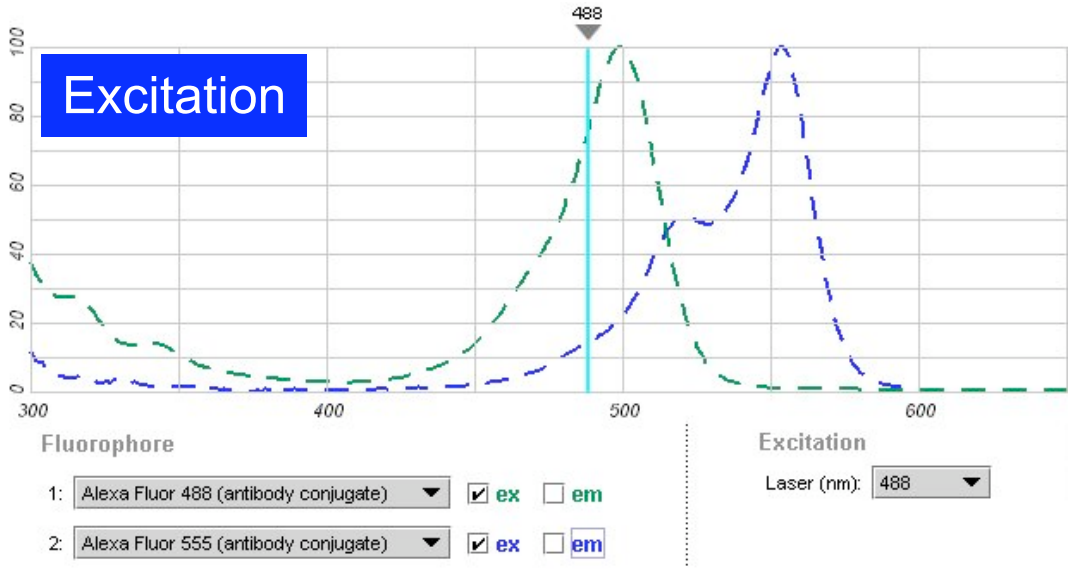
Medium	MEM	DMEM	RPMI 1640	Medium 199	Ham's F12
Riboflavin content (mg/L)	0.1	0.4	0.2	0.01	0.04

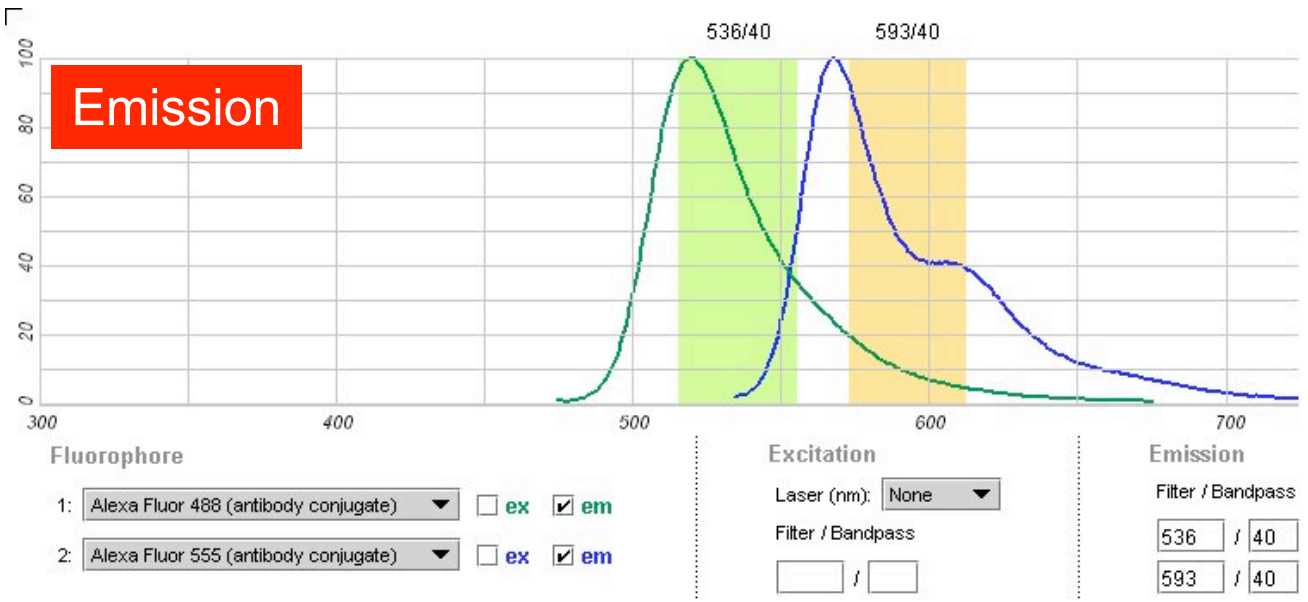
Cell culture medium affects GFP photostability: a solution
Nature Methods (2009) **6:**859–860

Matching dye spectra to excitation sources and filters

Online spectra viewer utility

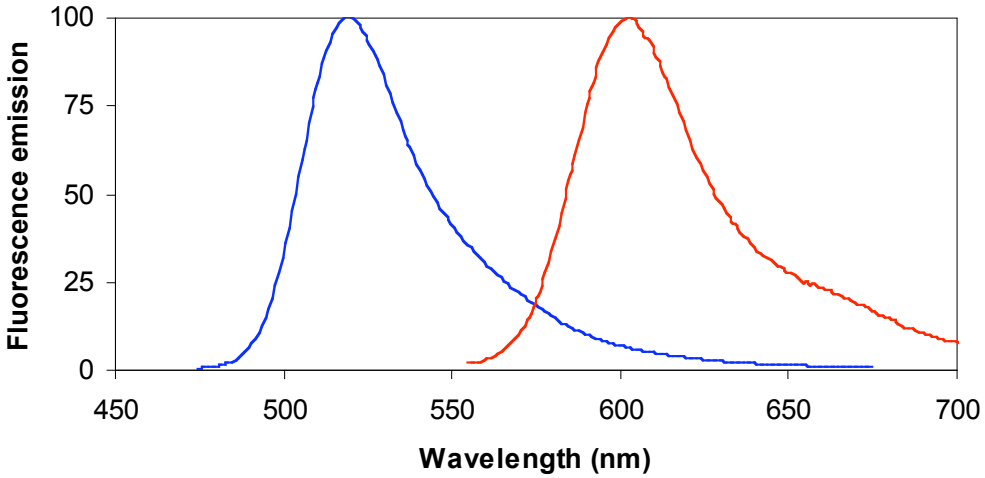
http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html



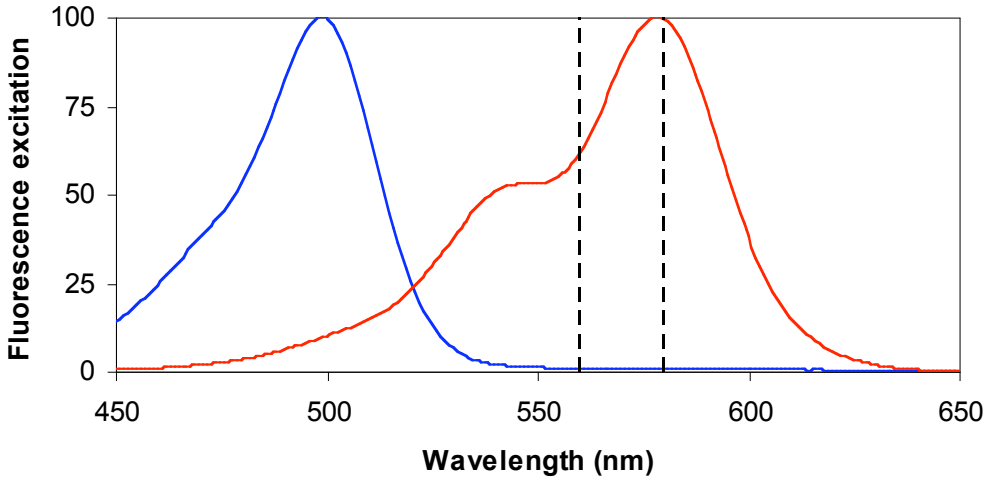


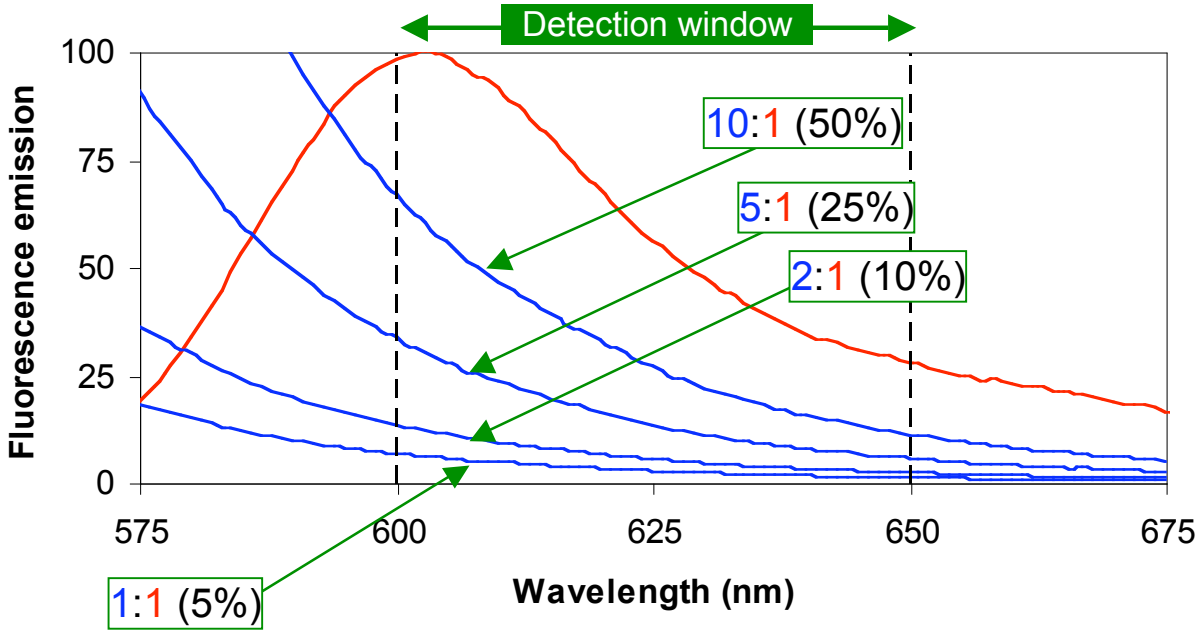
Fluorescence Spectra: Crosstalk

Emission spectra 1:1



Excitation spectra 1:1





❖ AF488 (blue) signal expressed as % AF568 (red) within detection window (50% means 1 AF488 photon per 2 AF568; 5% = 1 AF488 per 20 AF568

❖ Dynamic range of protein expression: 1 –10^8 copies per cell
❖ Brightest dyes on lowest abundance targets

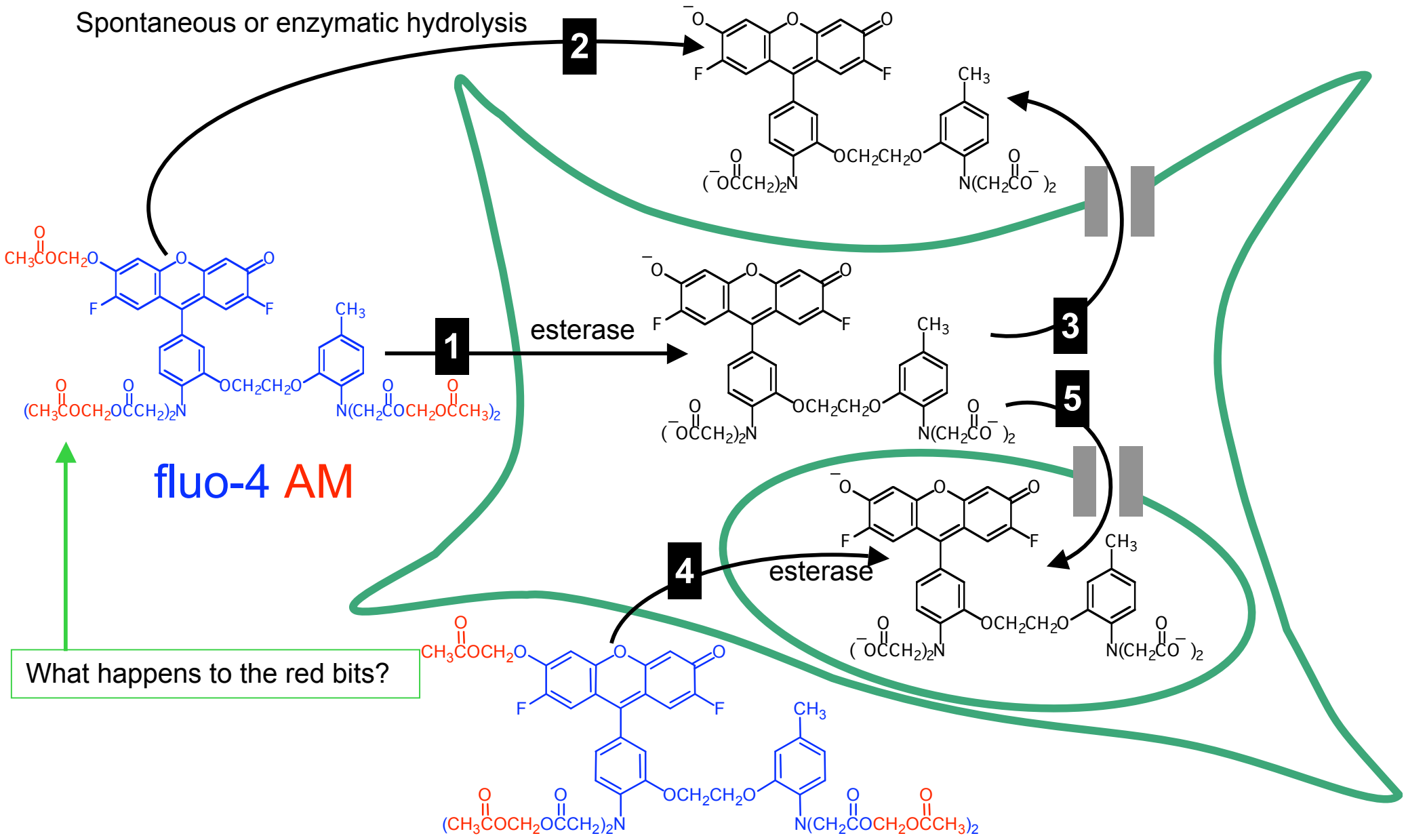
Dye Loading Issues

- Is the experimental specimen a pure or mixed cell culture or a tissue?.
- If the specimen is multicellular, is disseminated (all cells) or selective (one cell or a subgroup of cells) loading desired?
- What is the size of the cells to be labeled?
- What is the size of the probe?
- Impact of loading on cell viability and function.
- Precision of amount delivered and location of delivery (e.g. focal application of neuronal tracers)

Loading Methods Survey

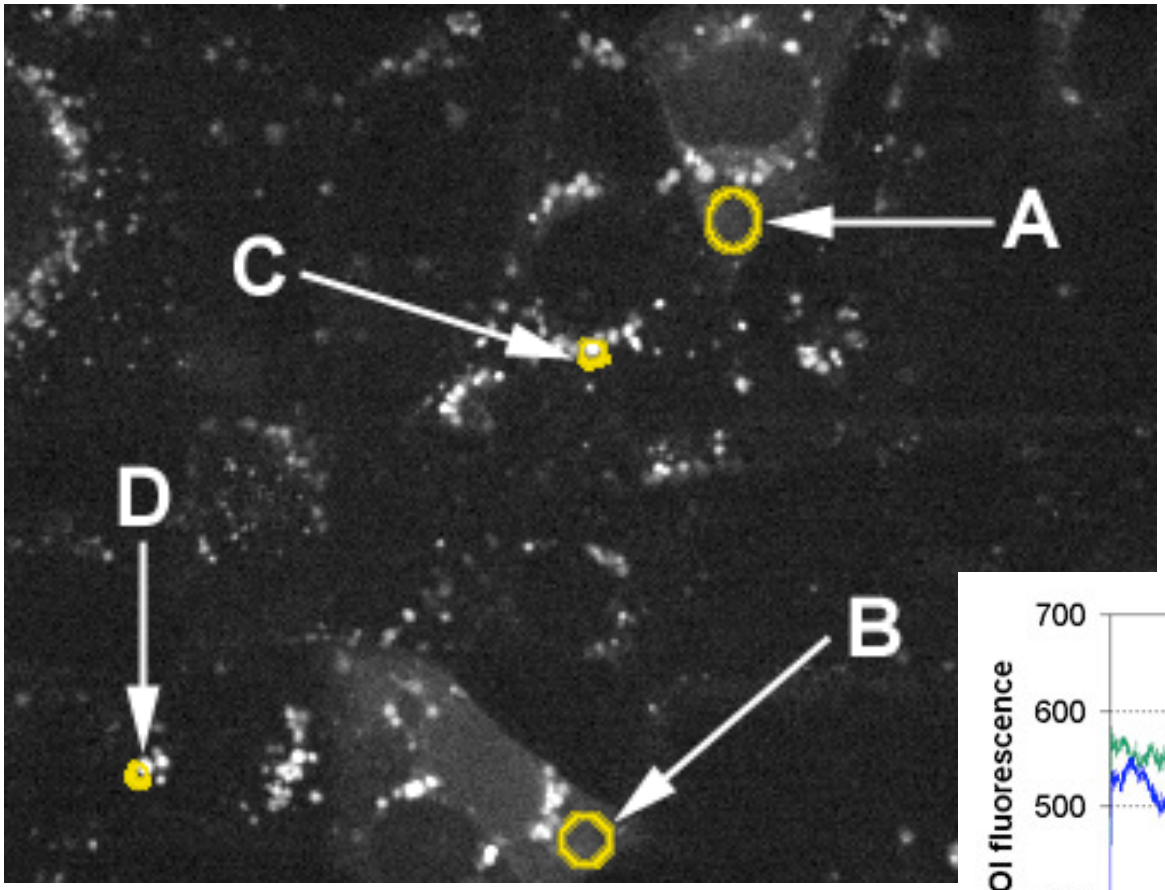
Method	Single or Multicellular	Size	Features
Direct permeability	Multicellular	Low MW (<1 kDa)	Restricted to relatively nonpolar molecules
ATP-gated cation channels	Multicellular	Low MW (<1 kDa)	Restricted to specific cell types (neurons, dendritic cells, macrophages)
Endocytic uptake	Multicellular	High MW (>10 kDa)	Usually receptor-mediated, e.g. transferrin, EGF, LDL
Membrane permeant esters (AM esters)	Multicellular	Low MW (<1 kDa)	Difficult to precisely control destination
Peptide-mediated uptake (poly Arg, TAT).	Multicellular	High MW (>10 kDa)	Intracellular concentrations generally low (10 –100 nM)
Osmotic permeabilization	Multicellular	High MW (>10 kDa)	Cell viability compromised
Transient permeabilization	Multicellular	High MW (>10 kDa)	Agents include streptolysin O digitonin, staphylococcal α-toxin
Electroporation	Multicellular or Single cells	High MW (>10 kDa)	Most versatile (single cells to tissues)
Ballistic microprojectile ("gene gun") delivery	Multicellular	High MW (>10 kDa)	Excellent tissue penetration
Microinjection	Single cells	<10 kDa	Pneumatic or electrophoretic ("iontophoresis")
Whole-cell patch pipet delivery	Single cells	<10 kDa	Precise control of intracellular solute concentrations

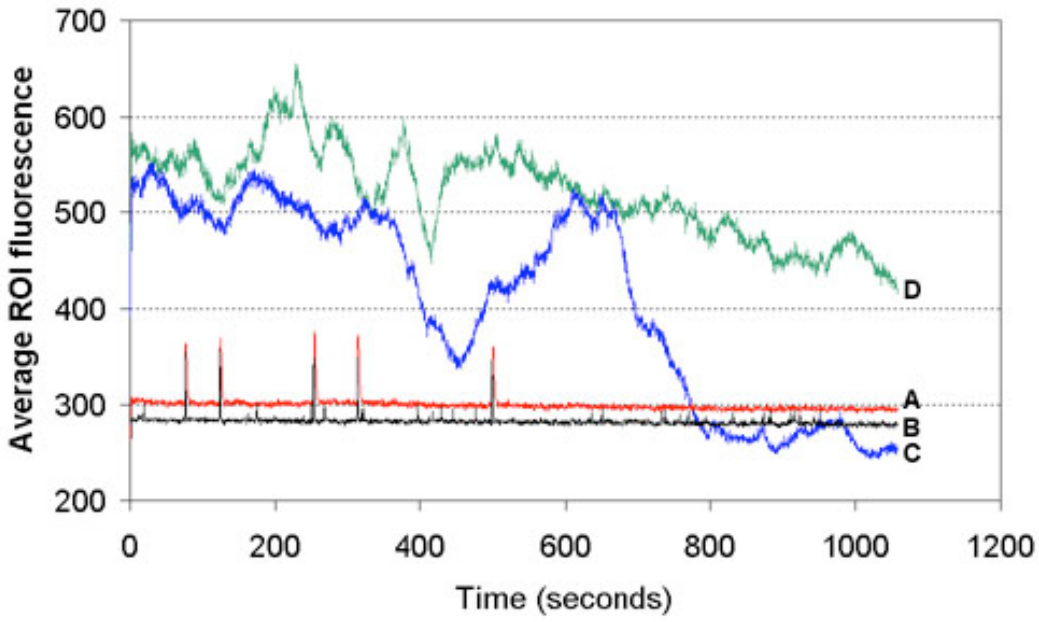
AM Ester Loading



When you add dyes to living cells, all hell can (and does) break loose……

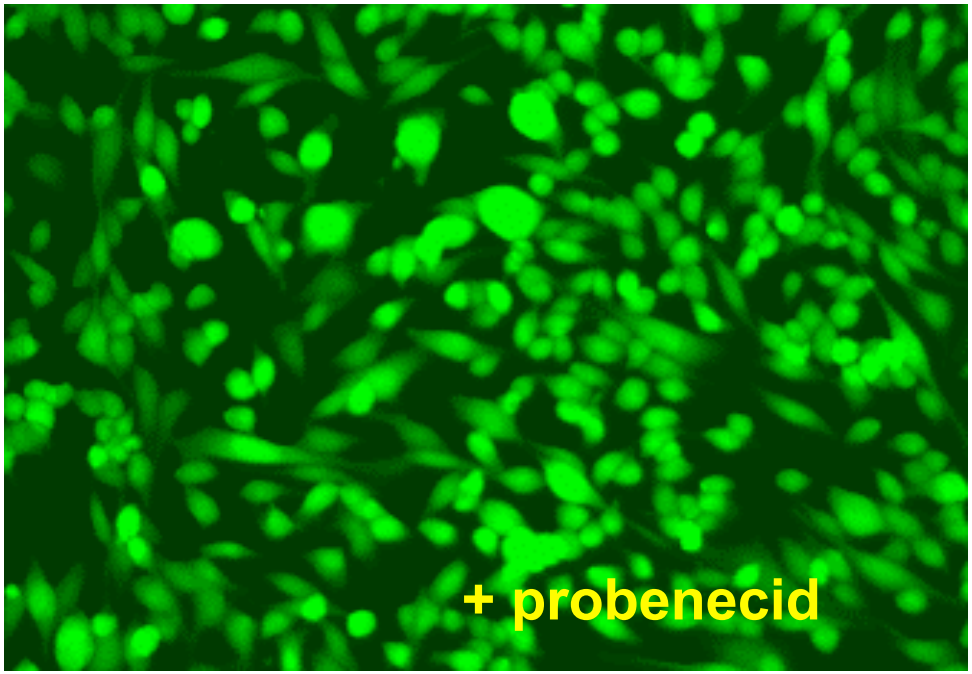
Compartmentalization: fluo-4 in HL-1 cardiomyocytes

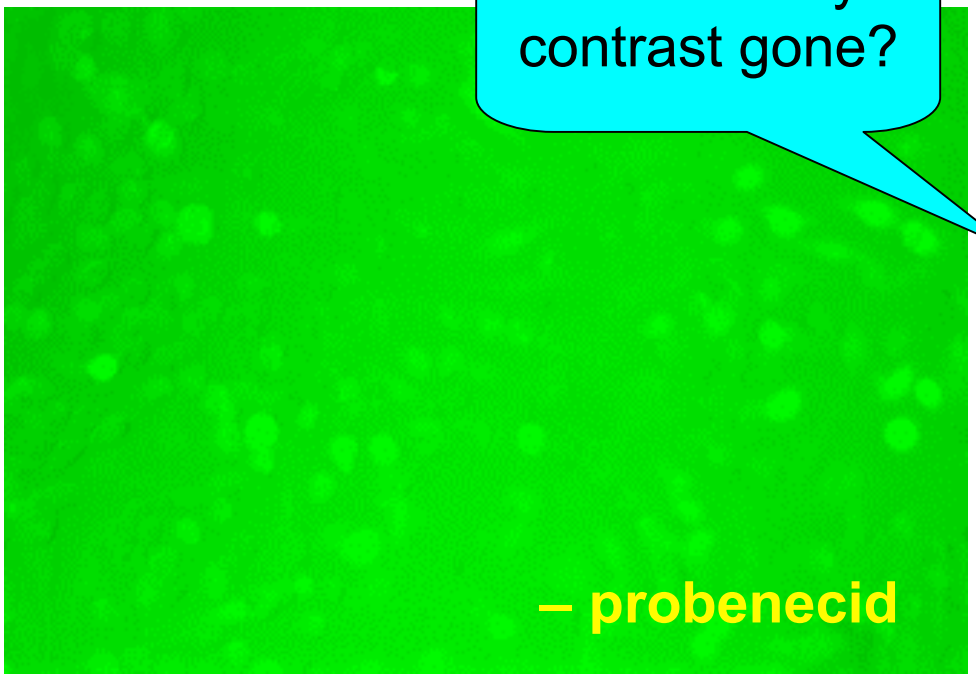




Data from 2004 3D course
PerkinElmer UltraView
LSCM, 60X, 1.2 NA, water
immersion objective

CHO M1 cells, 4 μM Fluo-4 AM, 60 minutes @37C





- probenecid blocks extrusion of intracelluar fluo-4 via organic anion transporter multidrug resistance transporter assays

How much label? Target abundance and localization

✓ Nuclear DNA: 7 pg (~ 6 × 10^9 base pairs per cell). Dye intercalation capacity = 1.2 × 10^9 (1 dye: 5 base pairs).

? EGF receptors: 10,000 copies per cell

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 31, Issue of August 3, pp. 29361–29367, 2001

Printed in U.S.A.

Individual Rotavirus-like Particles Containing 120 Molecules of Fluorescent Protein Are Visible in Living Cells*[S]

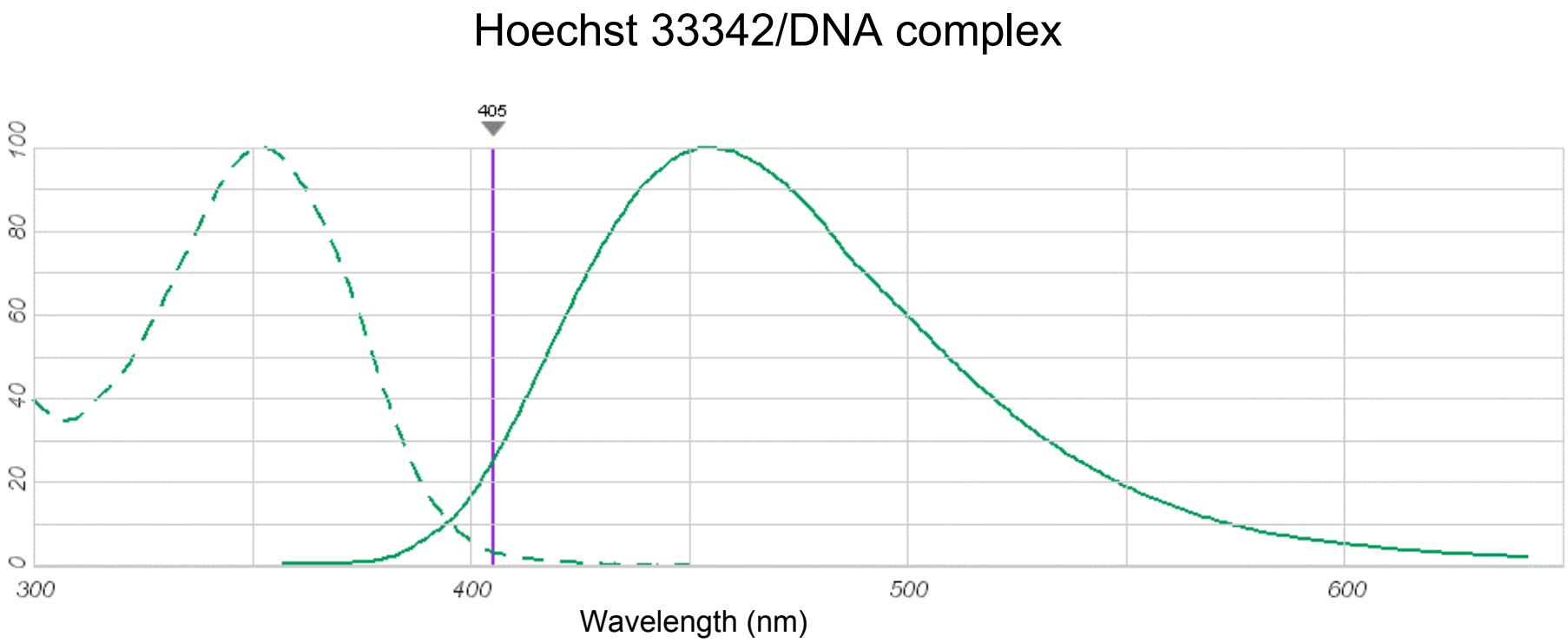
75 nm diameter

Received for publication, March 2, 2001, and in revised form, May 11, 2001
Published, JBC Papers in Press, May 16, 2001, DOI 10.1074/jbc.M101935200

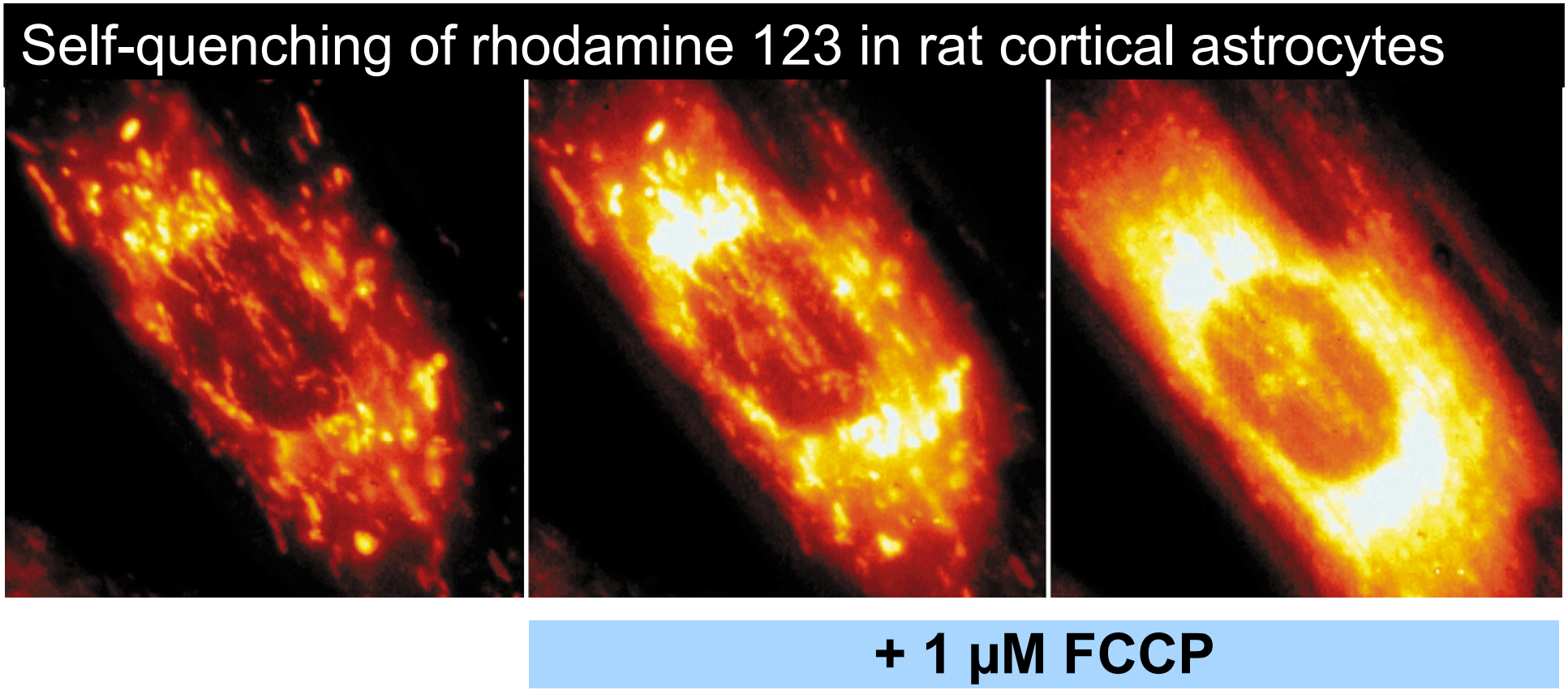
Annie Charpilienne‡, Mohamed Nejmeddine‡§, Mabel Berois‡, Nathalie Parez¶, Emmanuelle Neumann‖, Elizabeth Hewat‖, Germain Trugnan§, and Jean Cohen‡ **

- Compartmentalization increases concentration: 1 million molecules in a HeLa cell (volume = 1.2 pL or 1200 μm^3) is about 1.4 μM (1.4×10^{-6} M). 1 million molecules in 0.5mL is 3.3×10^{-15} M.
- Intracellular concentrations of dye can easily exceed 100 μM even when extracellular incubation concentration < 0.1 μM
- Even more extreme effects for membrane-bound organelles (N_{mito}/N_{cyto} >1000)

Because nuclear DNA is so abundant, this works……

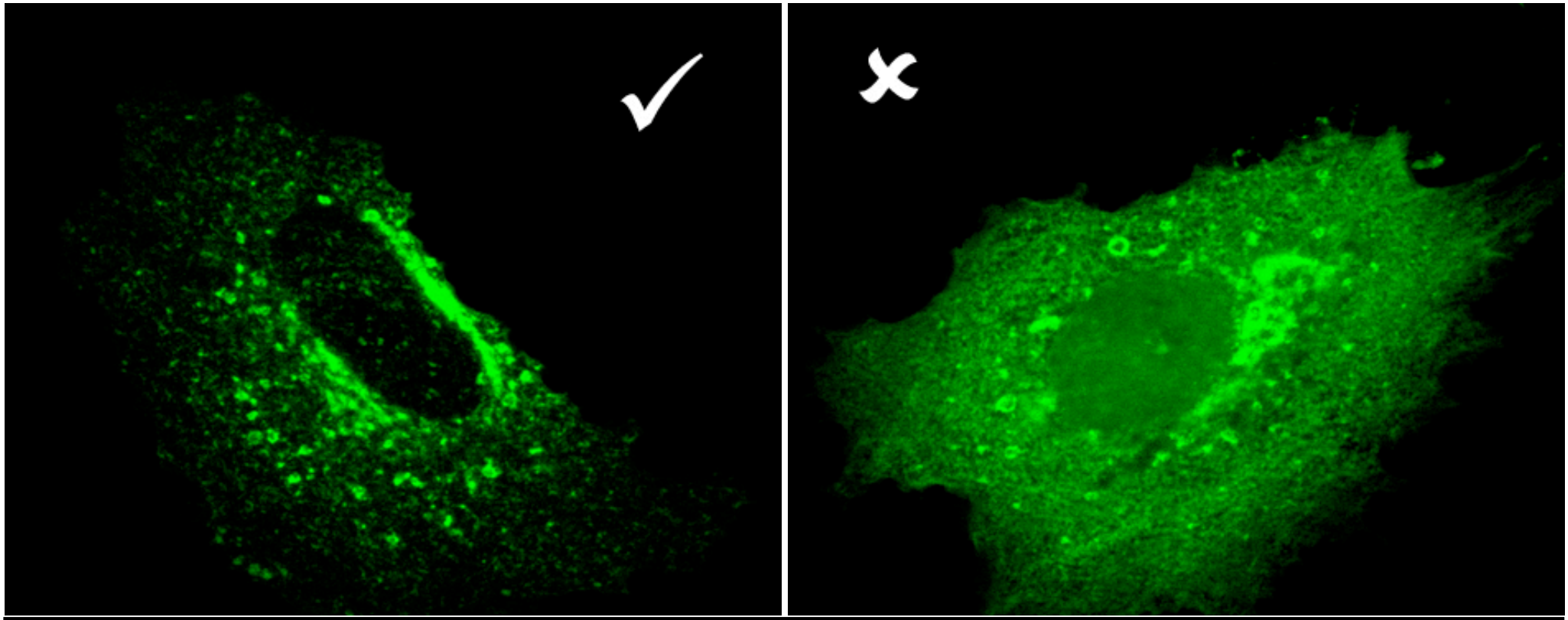


Nonlinear fluorescence–concentration relationships



- Total dye concentration in all three images is the same
- Optimize over a 10 –100 fold range of dye concentration when establishing new protocols

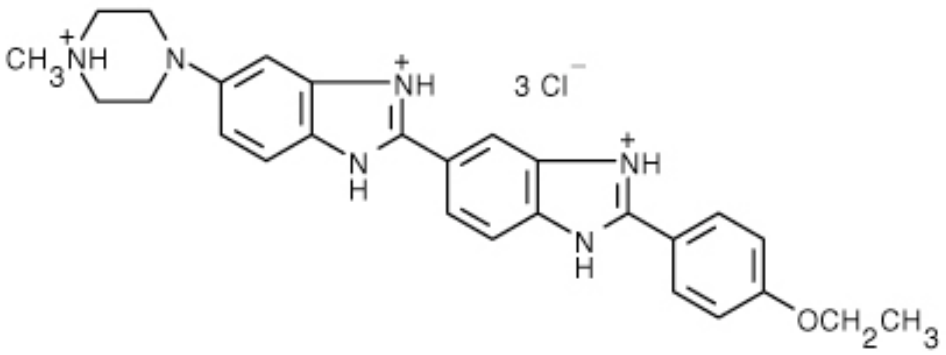
More is not better

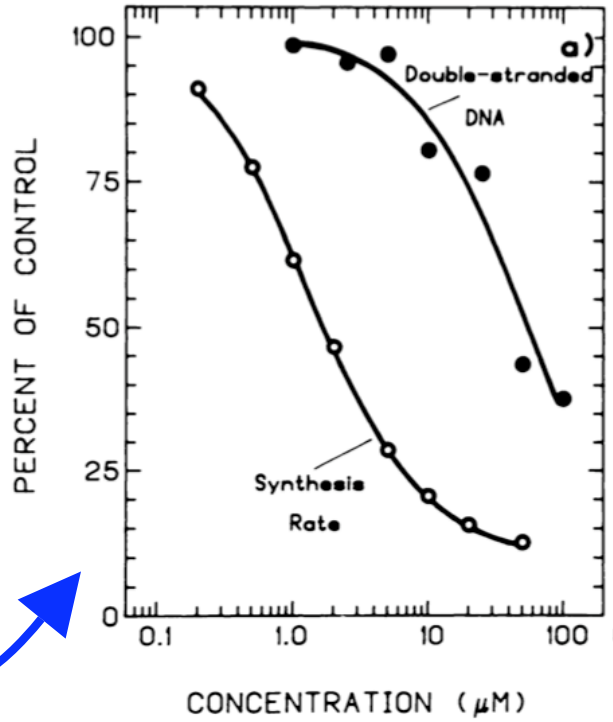


RhoB GTPase is entirely localised to endocytic vesicles, making it a good marker of this cellular compartment. GFP-RhoB localizes correctly at reasonable levels of expression (left image). In grossly-overexpressing cells (right image), GFP-RhoB is seen in the cytoplasm and plasma membrane and endosomal morphology is often disturbed. Images courtesy Harry Mellor, University of Bristol, UK.

- The following deleterious effects are all positively correlated with increased label concentration: phototoxicity, cytotoxicity, nonspecific localization and physiological or structural perturbation.

Sub-lethal perturbation by Hoechst 33342





- Inhibition of DNA synthesis in chinese hamster V79 cells. J Histochem Cytochem (1982) **30**:111–116
- Inhibition of topoisomerase I. Cancer Res (1993) **53**:1332–1337
- 4.4 or 8.8 μM H33342 increases luciferase expression in BC3H-1 myocytes. Arch Pathol Lab Med (2003) **127**:1124–1132
- 15 μM H33342 causes fragmentation of neurites in primary cortical neurons. J Neurochem (2006) **96**:1253–1266
- 10 μM H33342 inhibits PC12 cell differentiationFEBS Letts (2007) **581**:3076–3080
- 10 μM H33342 produces mitochondrial depolarization in HL-60 myelogenous leukemia cells. Ann Clin Lab Sci (2004) **34**:458–466

To wash or not to wash.....that is the question

- Washing is a trade-off: Improved signal:background versus additional protocol step (time) and potential damage to cells resulting from manipulation. **Do** wash with pre-warmed (37°C media) to avoid undue thermal stress.

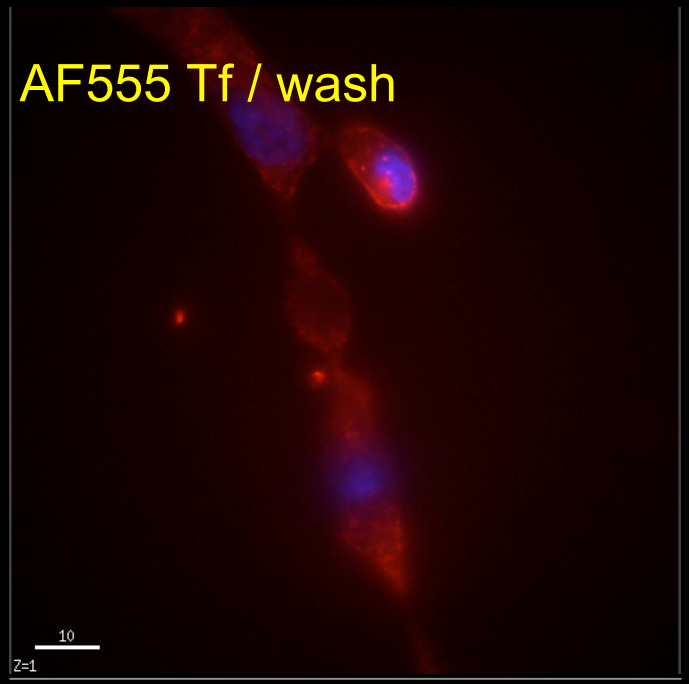
- Labeled proteins (antibodies, transferrins, cholera toxin B etc), lipids (DiI, BODIPY ceramide) and Ca^{2+} indicators (AM form) require a wash step to yield acceptable image contrast.

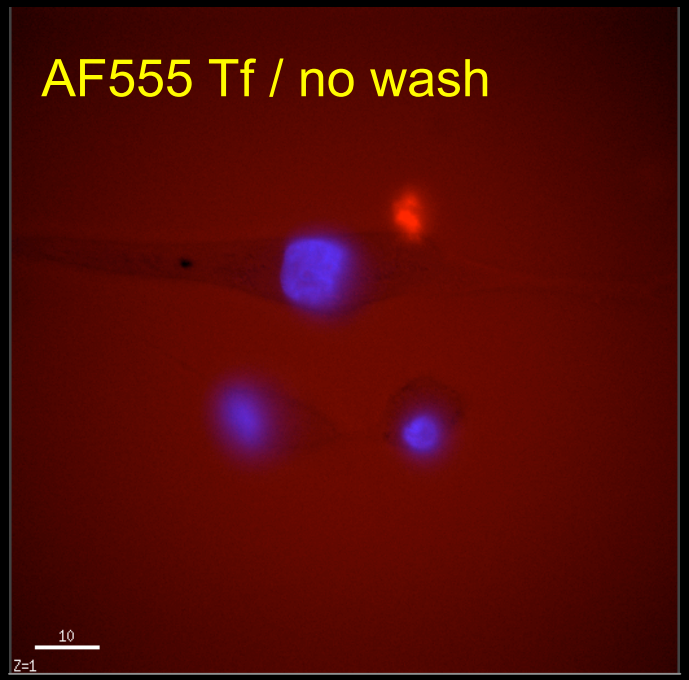
- Fluorogenic probes do generally not need a wash step to remove probe that is not specifically associated with the target (staining solution is "dark"). Fluorogenic probes include most nuclear stains (DAPI, Hoechst 33342, SYTO 16 etc), PED6, FM 1-43 and FM 4-64.

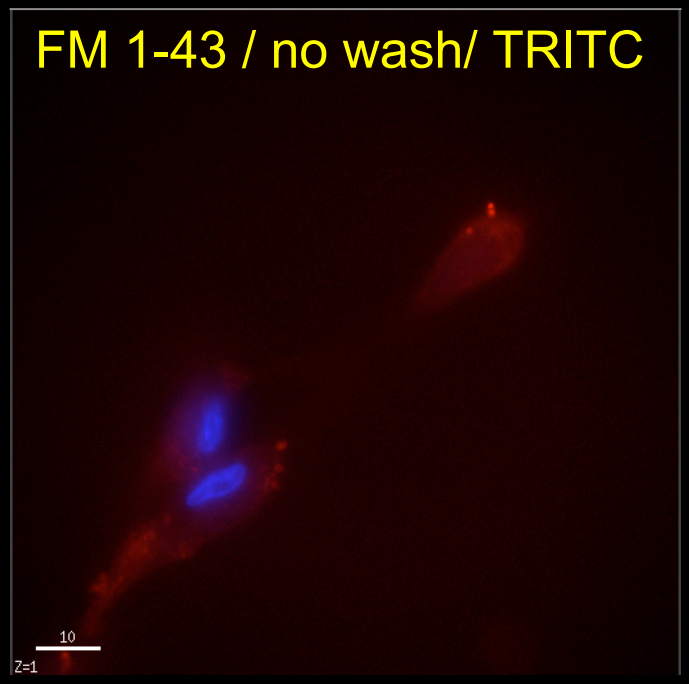
- Some other probes (e.g MitoTracker Red CMXRos), although not fluorogenic, are usually so strongly associated with their target (particularly if you avoid overloading) that there is no background to remove.

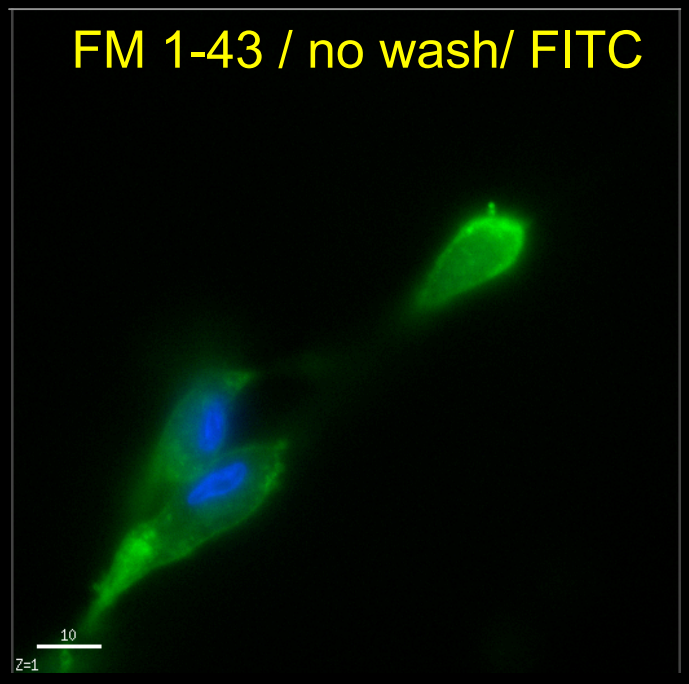
- Some probes must <u>NOT</u> be washed off as their reporting function is based on their extracellular versus intracellular distribution. Examples include propidium iodide (PI) for cell viability and $DiSBAC_2(3)$ for plasma membrane potential.

AF555 Tf/FM 1-43/H33342/HASMC/40X



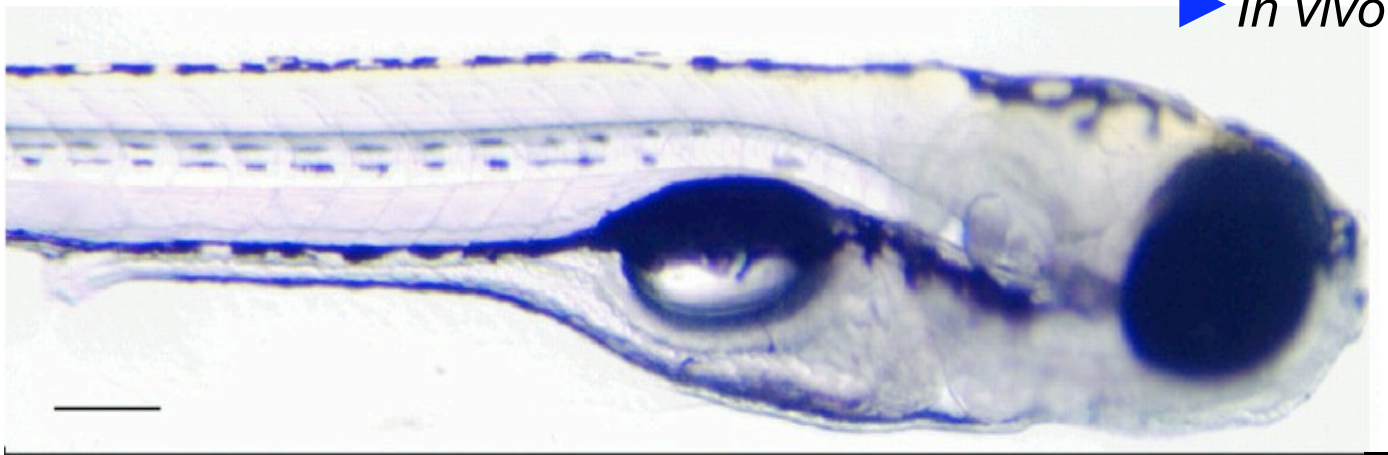




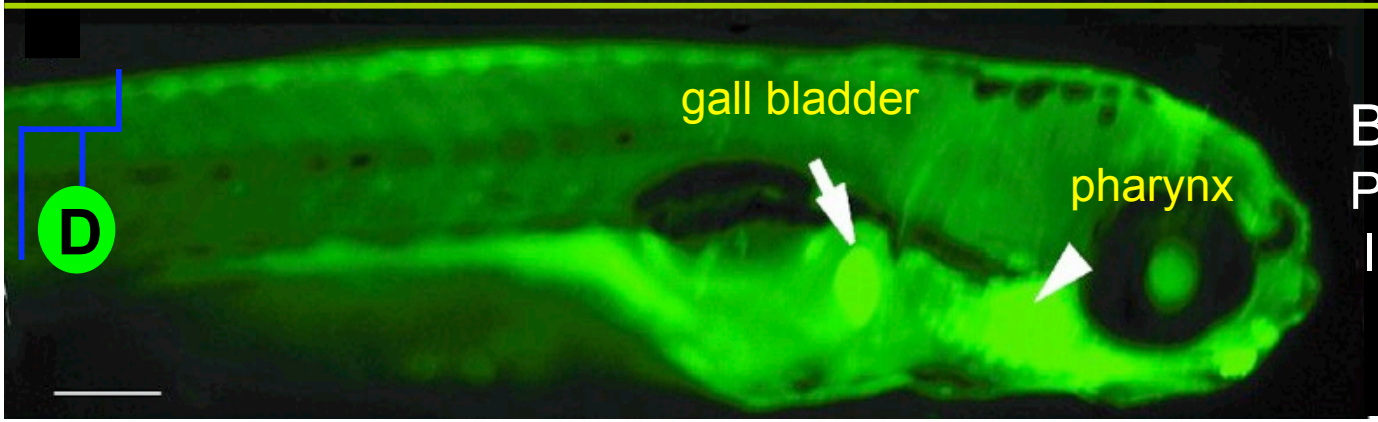


PED6 fluorogenic PLA_2 activity sensor

- *In vivo* imaging of lipid metabolism



PED6
Phospholipase A_2-activity dependent probe

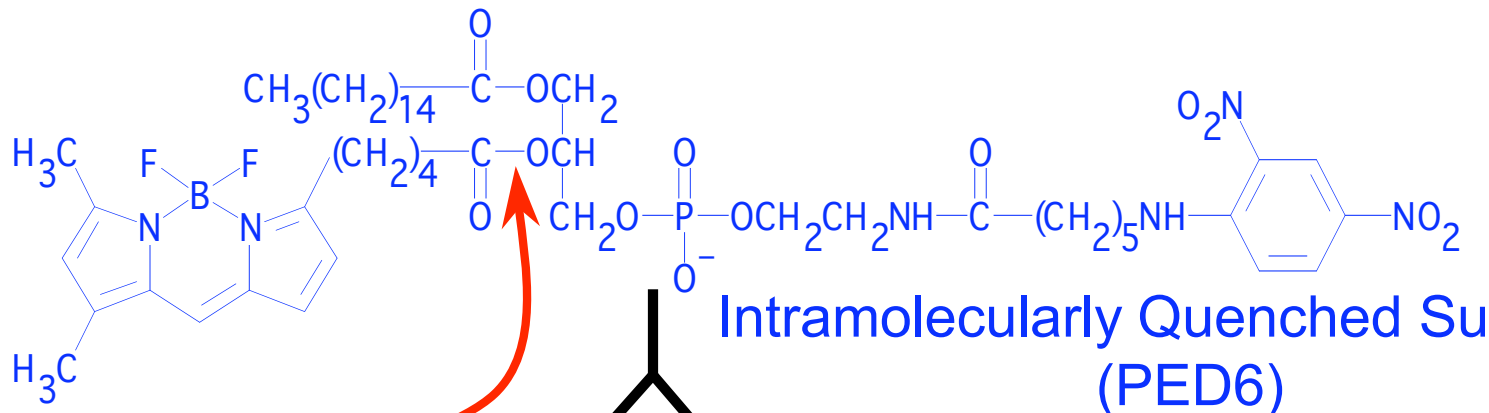


BODIPY PC
Phospholipase-independent lipid marker

Science **292**:1385–1388 (2001)

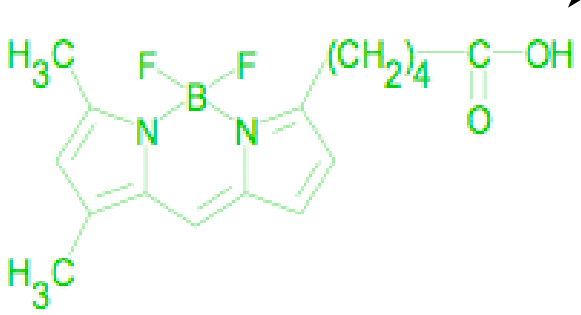
- Washing away unbound dye inside an animal is hard to do!

PED6 phospholipase A_2 substrate

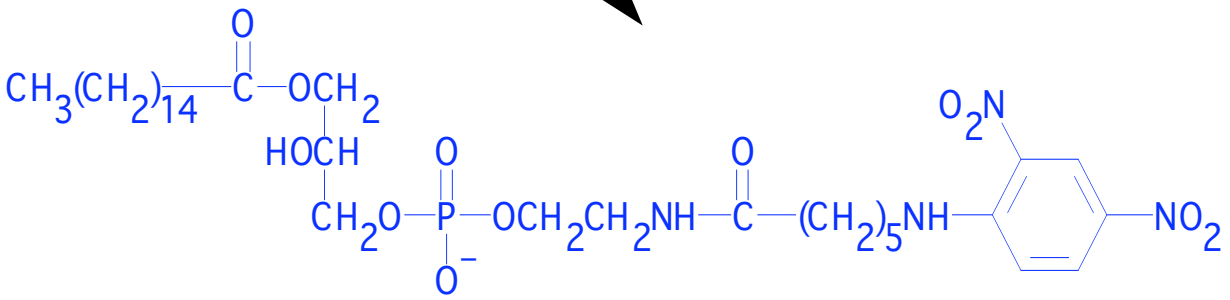


Intramolecularly Quenched Substrate (PED6)

Phospholipase A_2 cleavage



Fluorescent Fatty Acid



Photobleaching the dark side is……

HeLa cells labeled with MitoTracker Red CMXRos: 40X followed by 10X

Photometric Output Factors: Photobleaching

- Irreversible destruction of excited fluorophore
- Proportional to time-integrated excitation intensity
- Avoidance: minimize excitation, maximize detection efficiency, antifade reagents
- Photobleaching and phototoxicity go hand-in-hand. Same reaction, different targets.
- Q_B (photobleaching quantum yield).

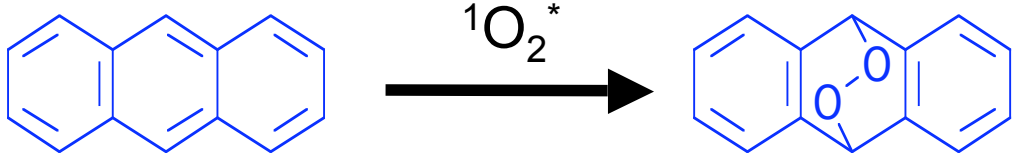
  Q_F/Q_B = number of fluorescence cycles before bleaching. About 30,000 for fluorescein.

Photobleaching Reactions

1 $^1Dye \xrightarrow{1.\ h\nu_{EX}\ 2.\ ISC} {}^3Dye^* + {}^3O_2 \longrightarrow {}^1Dye + {}^1O_2{}^*$

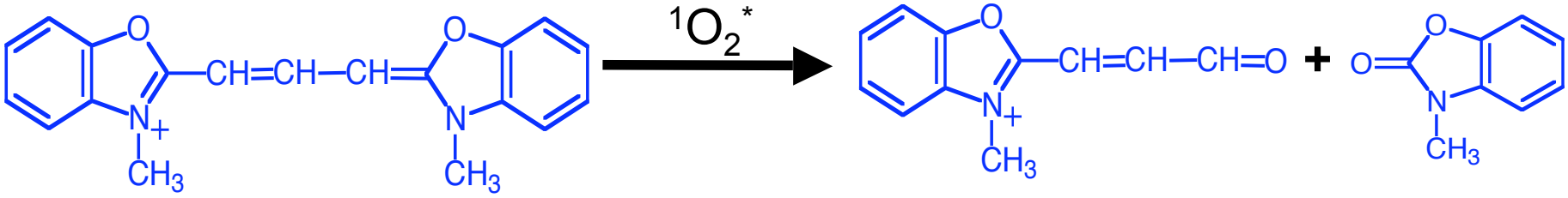
Photosensitized generation of singlet oxygen

2A



Anthracene

2B



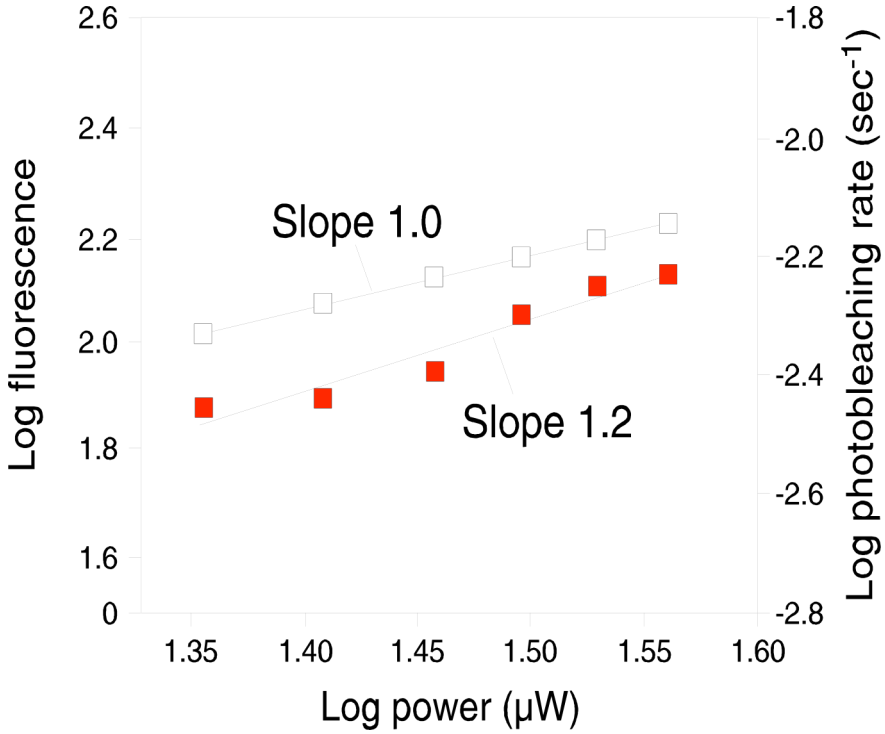
Carbocyanine dye ($DiOC_1(3)$)

Three control variables: 1. light ($h\nu_{EX}$) 2. dye 3. O_2

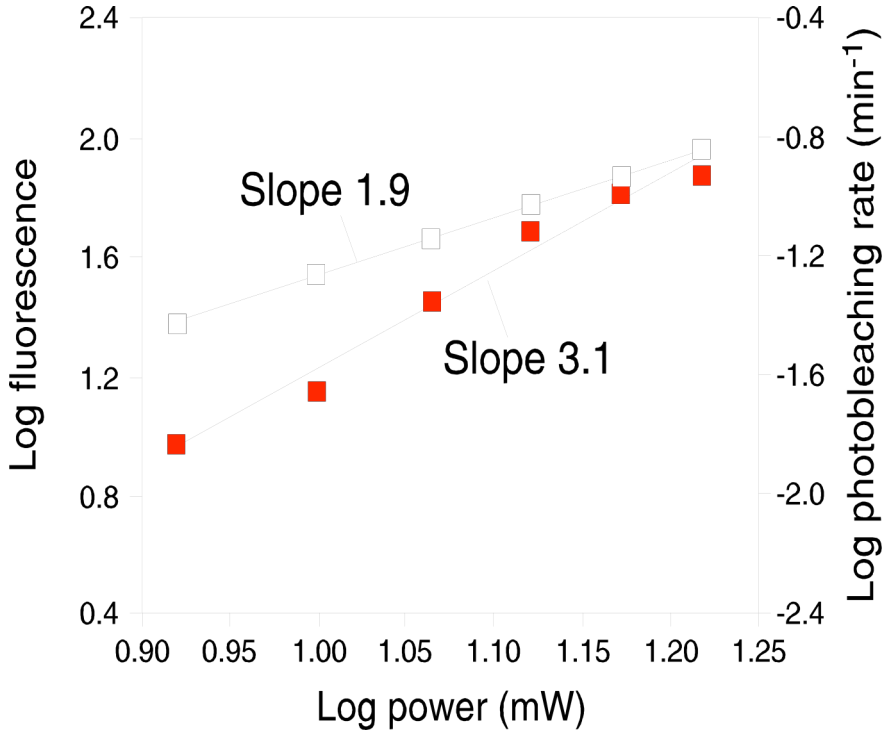
Fluorescein dextran photobleaching under one- and two-photon excitation

Biophys J **78:**2159 (2000)

One-photon



Two-photon



□ Fluorescence intensity ■ Photobleaching rate

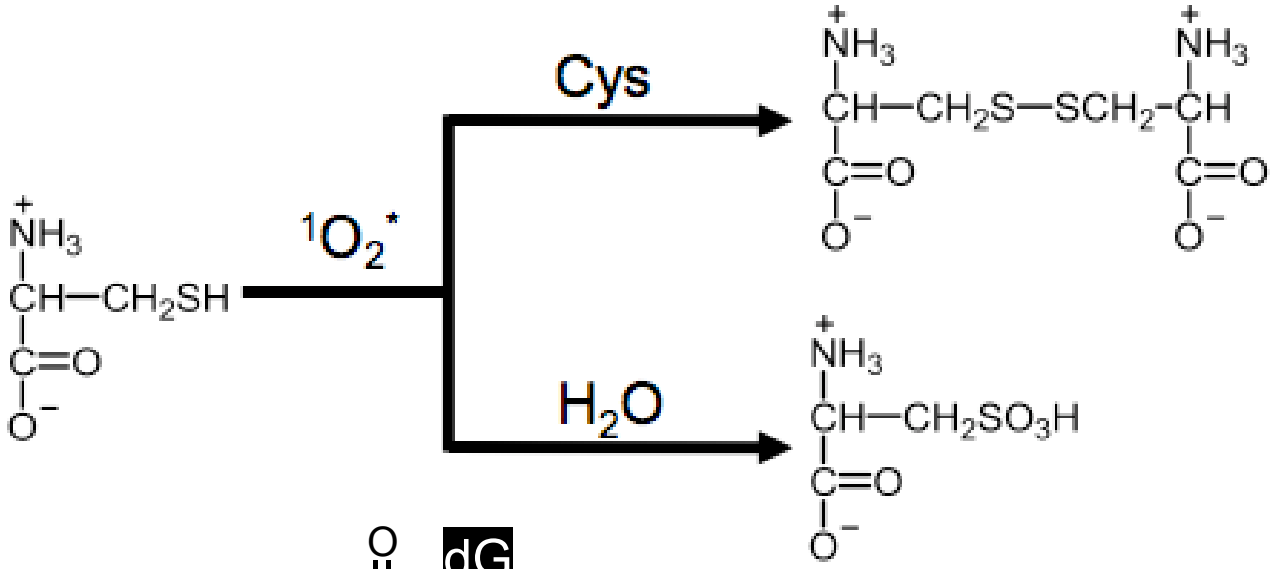
▶ High-speed, low-photodamage nonlinear imaging using passive pulse splitters. Nature Methods (2008) **5:**197–202

Phototoxic Reactions

1 $^1Dye \xrightarrow{1.\ h\nu_{EX}\ 2.\ ISC} {}^3Dye^* + {}^3O_2 \longrightarrow {}^1Dye + {}^1O_2{}^*$

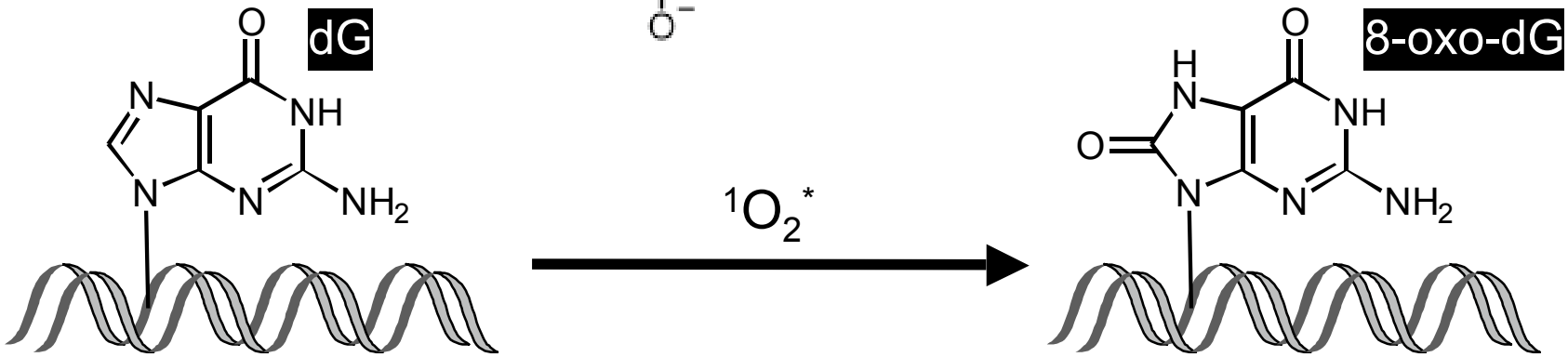
Photosensitized generation of singlet oxygen

2A



8-oxo-dG causes mutations by pairing with adenine during DNA replication, resulting in increased A•T→ C•G transversions

2B



Three control variables: 1. light ($h\nu_{EX}$) 2. dye 3. O_2

Factors influencing phototoxic effects

Factor◆	Trend
Excitation wavelength	Longer wavelengths produce less photodamage
Excitation intensity and duration	Higher power and longer exposure result in more photodamage#.
Cell type/status	Larger cells can sustain a higher phototoxic “burden”. Mitotic cells are particularly susceptible to photodamage.
Culture medium	Riboflavin and tryptophan induce phototoxicity.
Dye concentration	Higher concentrations produce more photodamage
Dye type	Cyanines and halogenated xanthenes (e.g. eosin) are particularly phototoxic.
Dye localization	Calcein and GFP phototoxicity is decreased upon compartmentalization in mitochondria and endoplasmic reticulum respectively.
Antioxidant additives	Ascorbic acid and enzymatic deoxygenation systems reduce photoxicity.

◆Note that some factors are strongly interdependent, e.g. dye localization and dye concentration. #These dependences may by highly nonlinear.

Thank You!

Questions?

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