Fluorescent Dithio Probes for Cell Biology:
Monitoring Cellular Thiols

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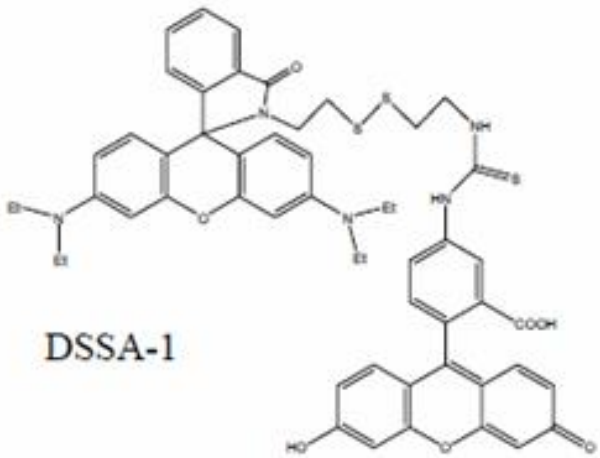
Fluorescent Dithio Probes

Our Current Probe*

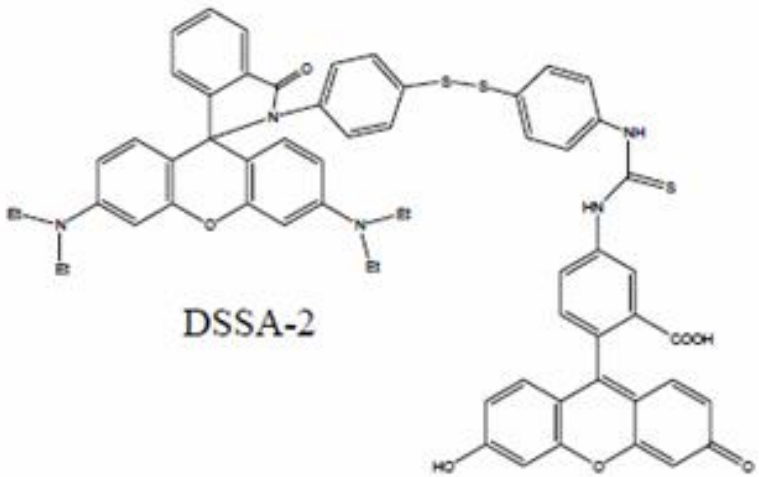
Fluorescein-SS-Rhodamine
Fluorescein-SS-(para-methyl-red)
Fluorescein-SS-coumarin

Thiol redox in cells	Thiol proteomics	Thiol redox
• *total fluorescence; microscopy; cell sorting*	• *identifying PSH and PSSP in cells and gels*	• in NIH 3T3 cells; colocalization studies

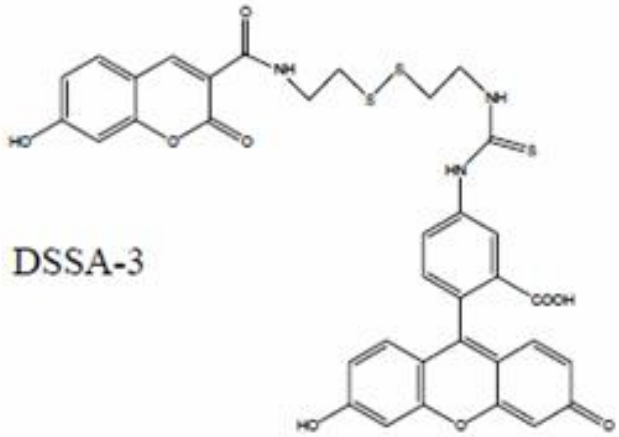
Current Fluorescent Dithio Probes

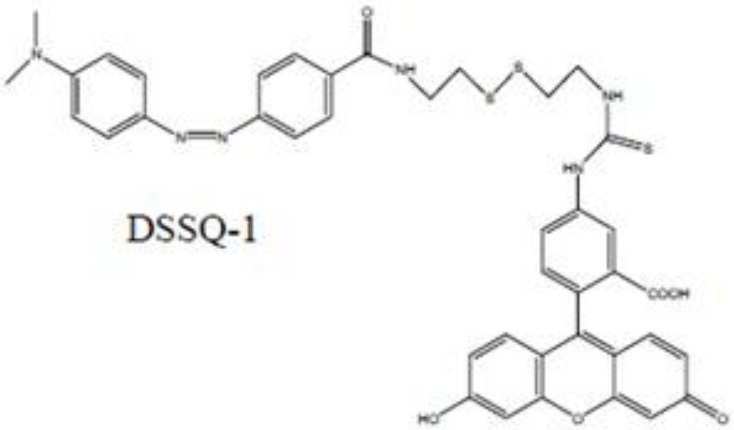


SEMKUR - cell sorting



SEMKUR - quantification

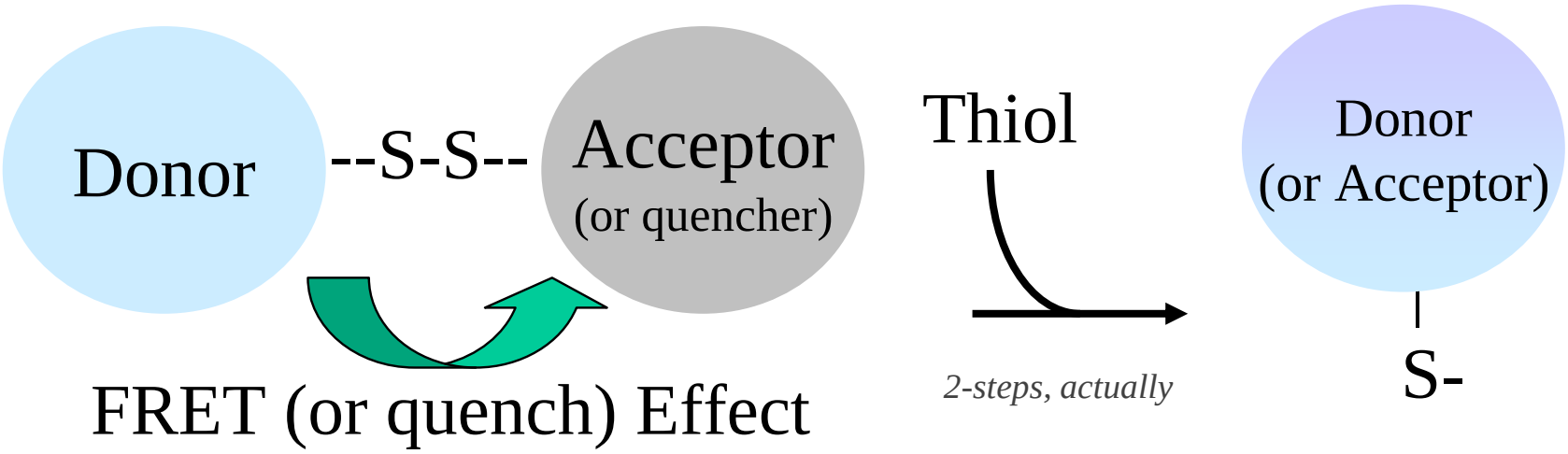




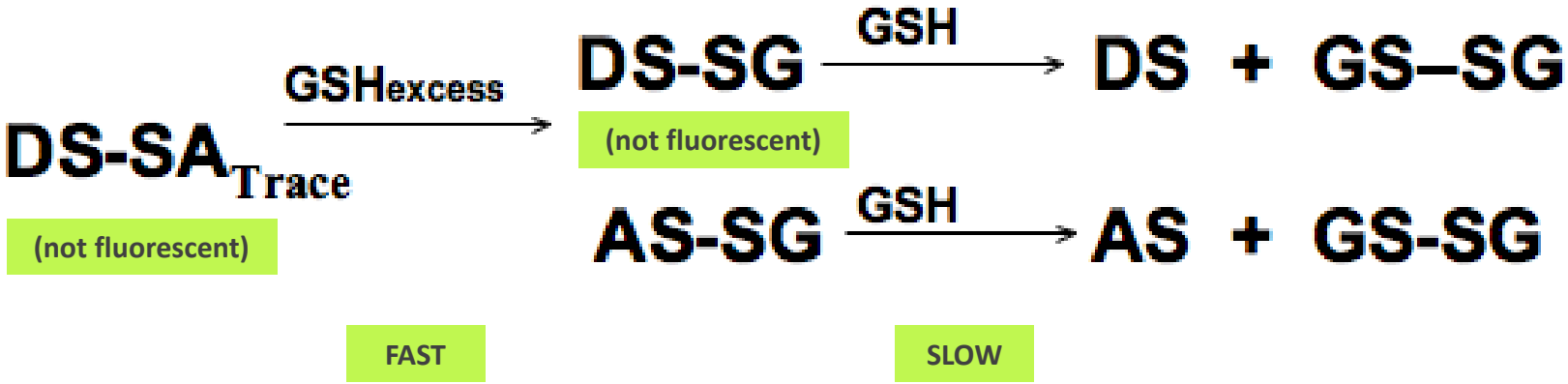
SEMKUR - imaging

Fluorescence-Based Detection of Thiols

Basic design of our probes

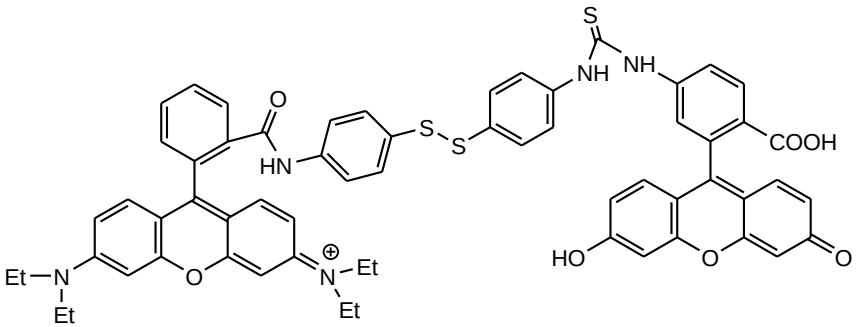


Reduction by the GSH thiol

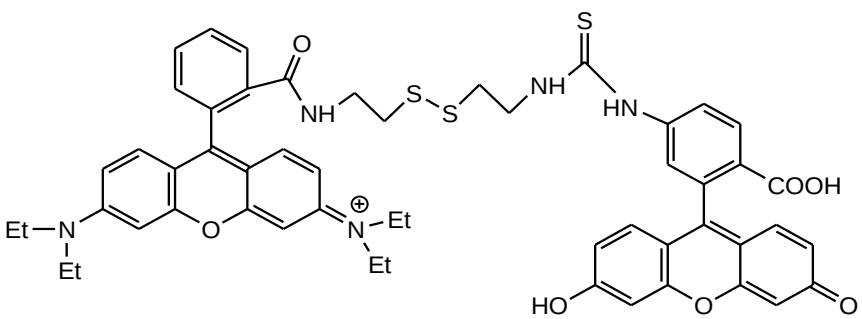


DS	donor fluor
AS	acceptor fluor
GSH	reduced glutathione
GSSG	oxidized glutathione

Initial Fluorescent Dithio Reagents (DSSA)



Diamino phenyl disulphide (DAP) linker with Rhodamine B and FITC ($DSSA_{AR}$)



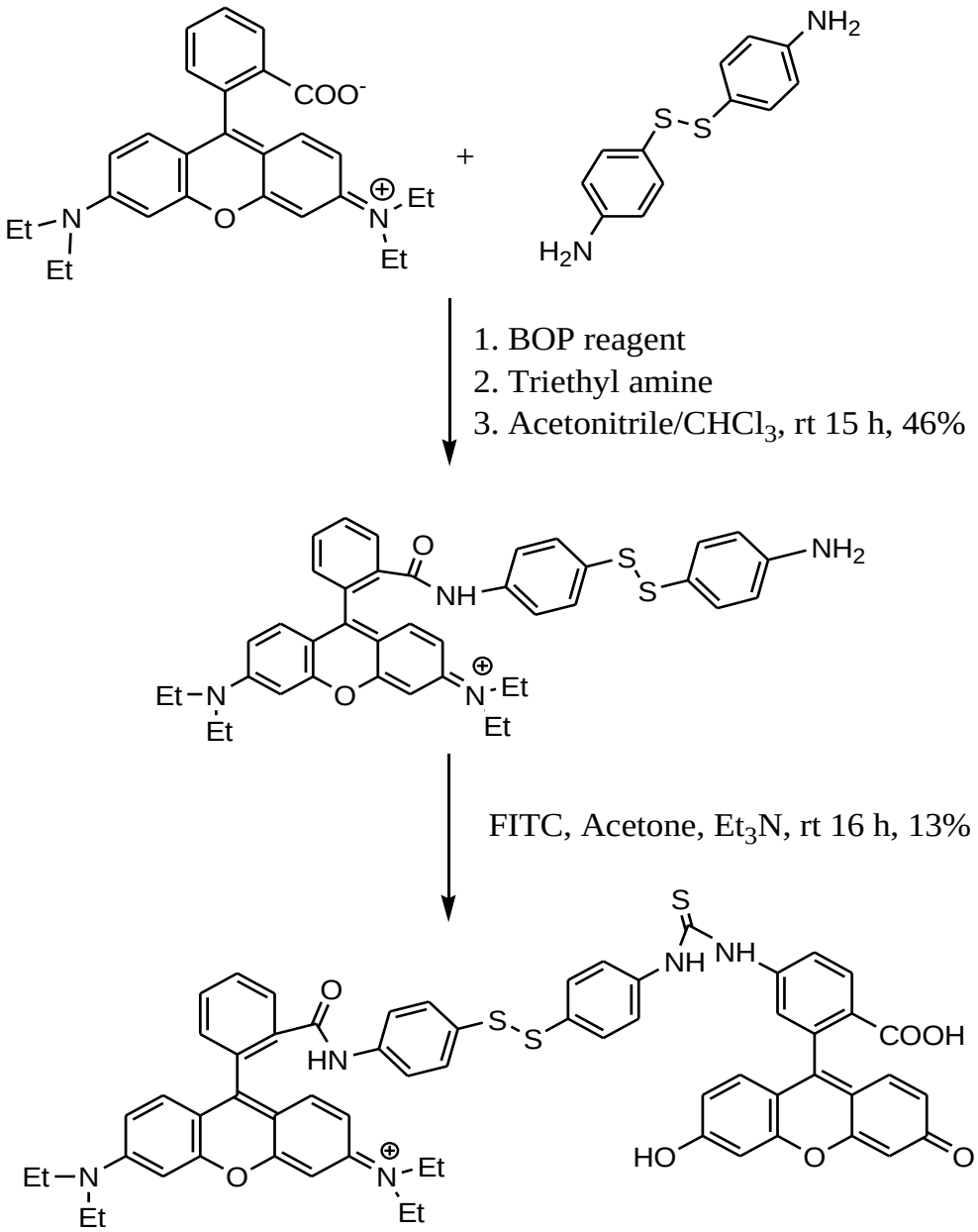
Cystamine with rhodamine B and FITC ($DSSA_{AL}$)

Compound	E°’
DTT	-0.33±0.02 V
Rh-DAP-FITC	-0.63±0.03V
Rh-Cys-FITC	-0.6±0.003 V
TCEP	*-0.29±0.03 V*

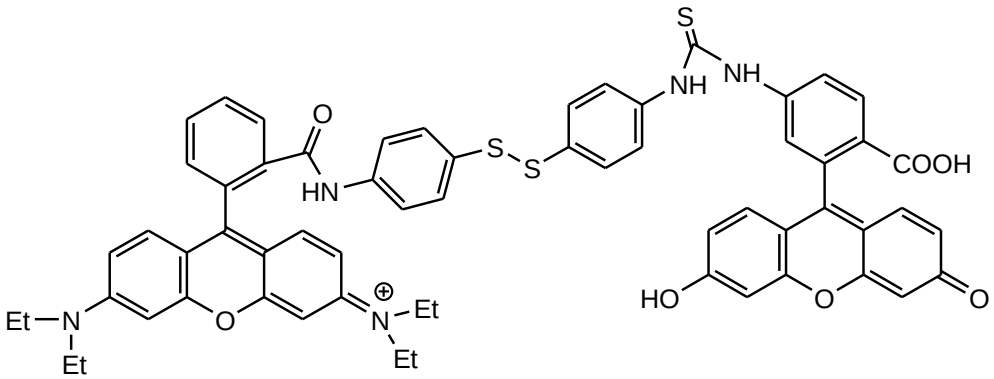
Note: low reduction potential? This can be useful, but why is it so hard to reduce these disulfides?

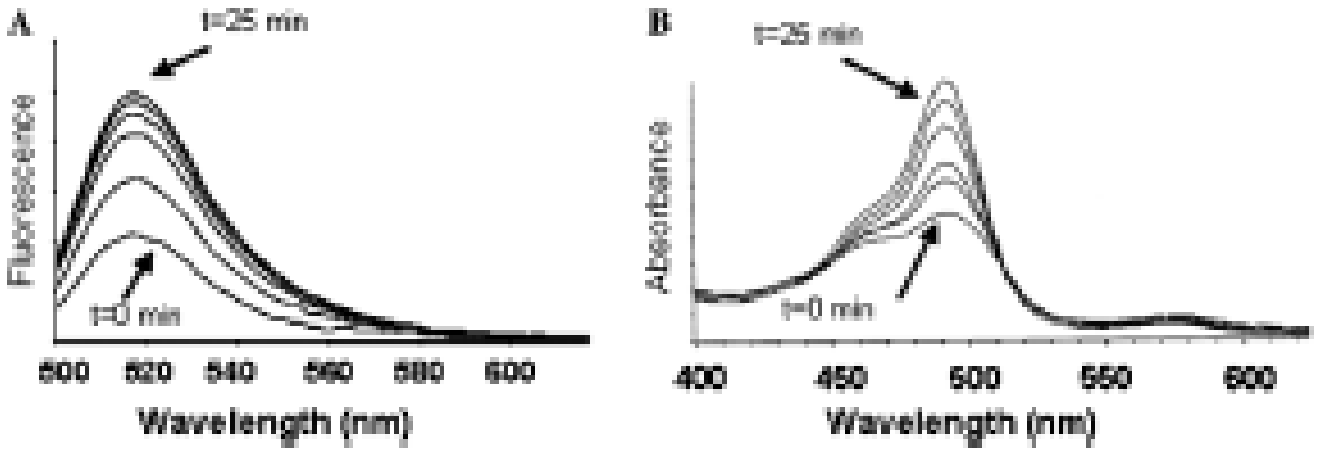
AR = aromatic linker
AL = aliphatic linker

Synthesis Of $DSSA_{AR}$



Reduction of DSSA DTT



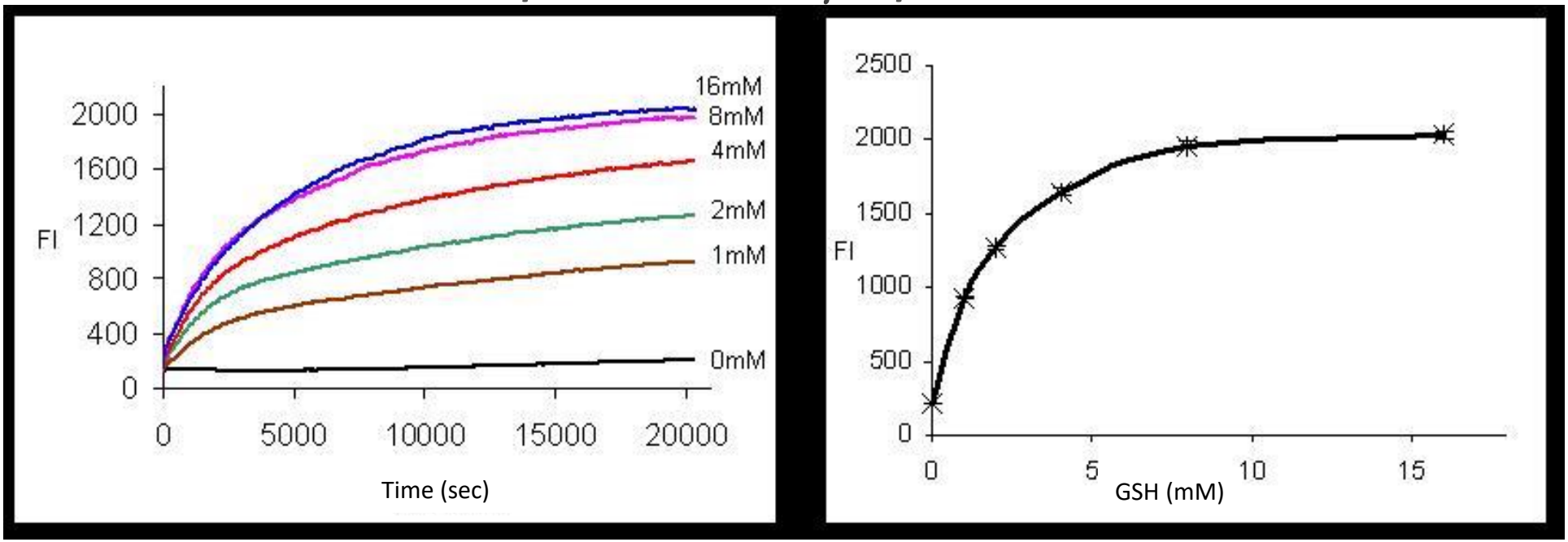


Fluorescence & Absorbance changes for 5 mM probe after 2.5 mM DTT

Absorbance change means there was static as well as dynamic quenching

Thiol (Glutathione, GSH) Detection

Low reduction potential means μM probe detects mM GSH

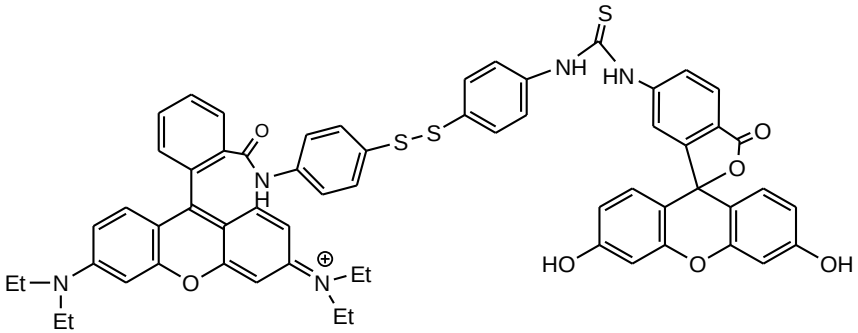


(a) Reaction of 5 μM $DSSA_{AL}$ with different concentrations of GSH (pH 8.1)

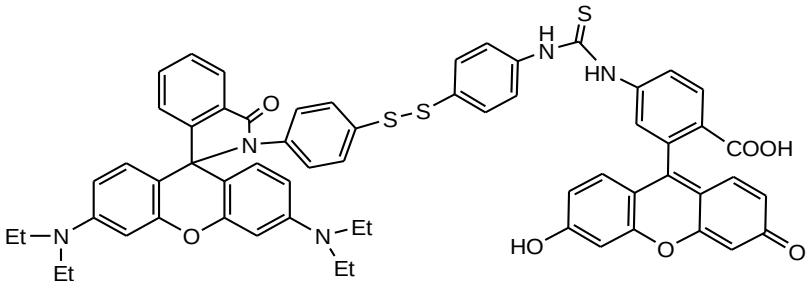
(b) Fluorescence plateau values after equilibrium (after the 2-step reduction)

Pullela, Chiku, Carvan, Sem (2006) *Anal. Biochem,* 352, 265.

Fluorescence Emission *(excitation at 495nm)*



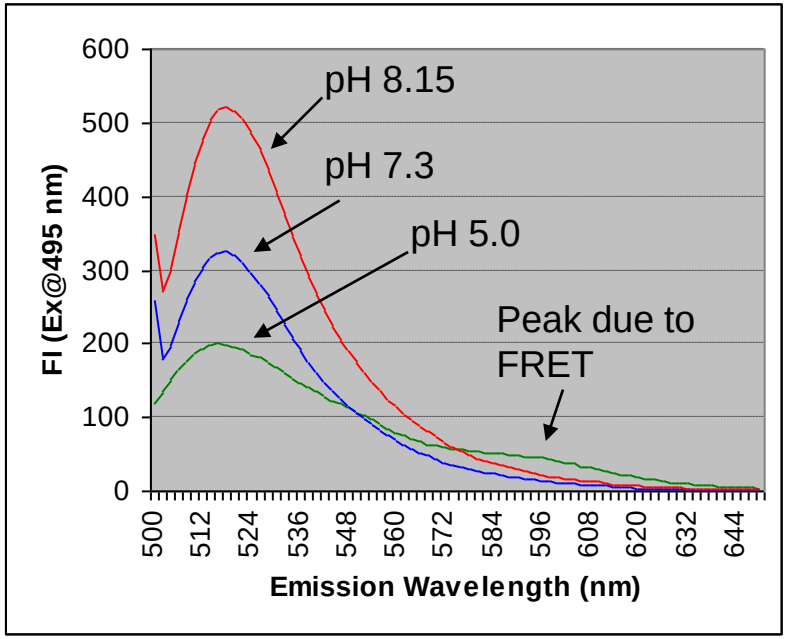
Structure at acidic pH



Structure at basic pH

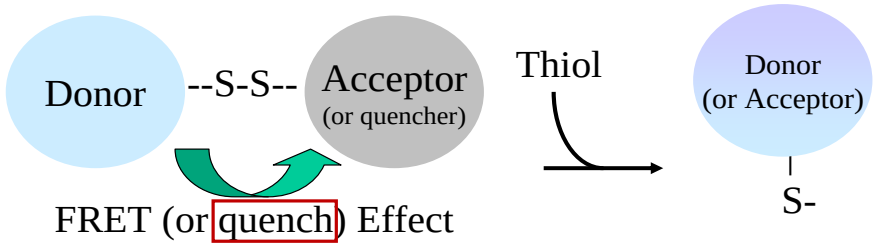
Probe can measure thiols

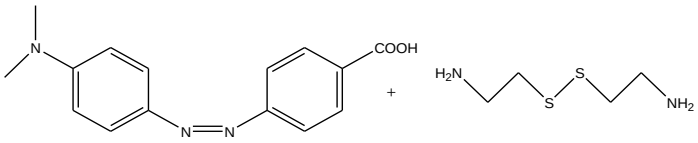
but: pH problem: no FRET*



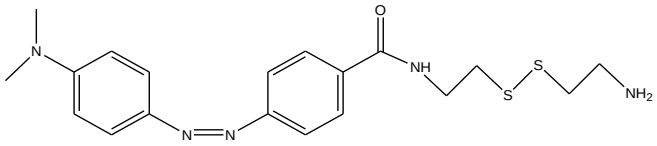
* Problems can be avoided with other D/A pairs: Alexa, Cy, etc.

A True DSSQ Probe (SEMKUR-lM): use a “black-hole” type quencher (PMR = para-methyl red)

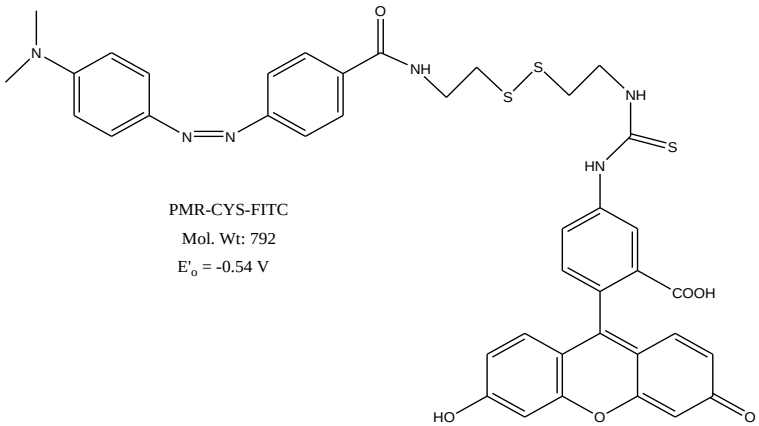




$CH_3CN/CHCl_3$ ↓ 1. BOP reagent 2. Triethyl amine

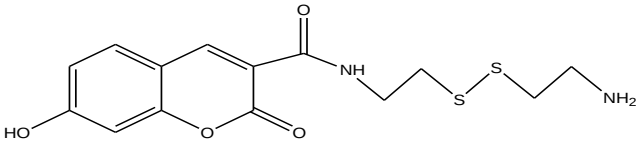


Acetone ↓ 1. FITC 2. Triethyl amine

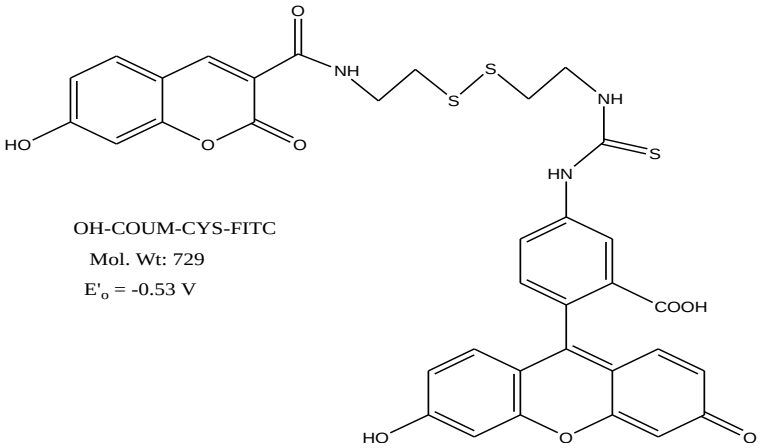


PMR-CYS-FITC
Mol. Wt: 792
E'_o = -0.54 V

$CH_3CN/CHCl_3$ ↓ 1. BOP reagent 2. Triethyl amine

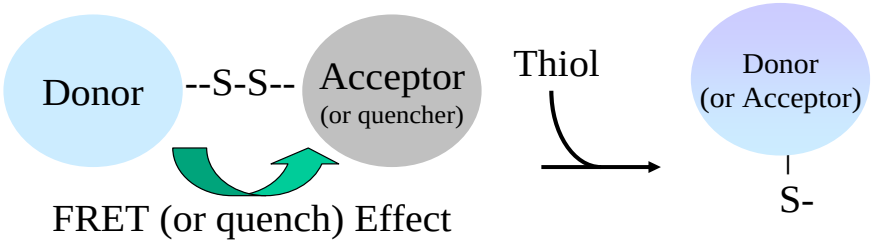


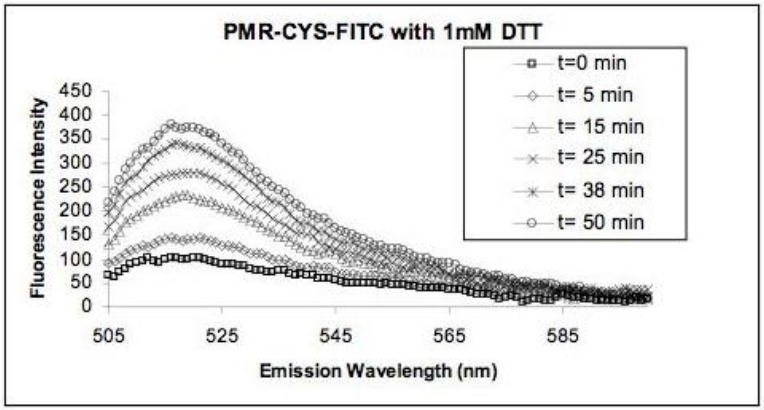
Acetone ↓ 1. FITC 2. Triethyl amine



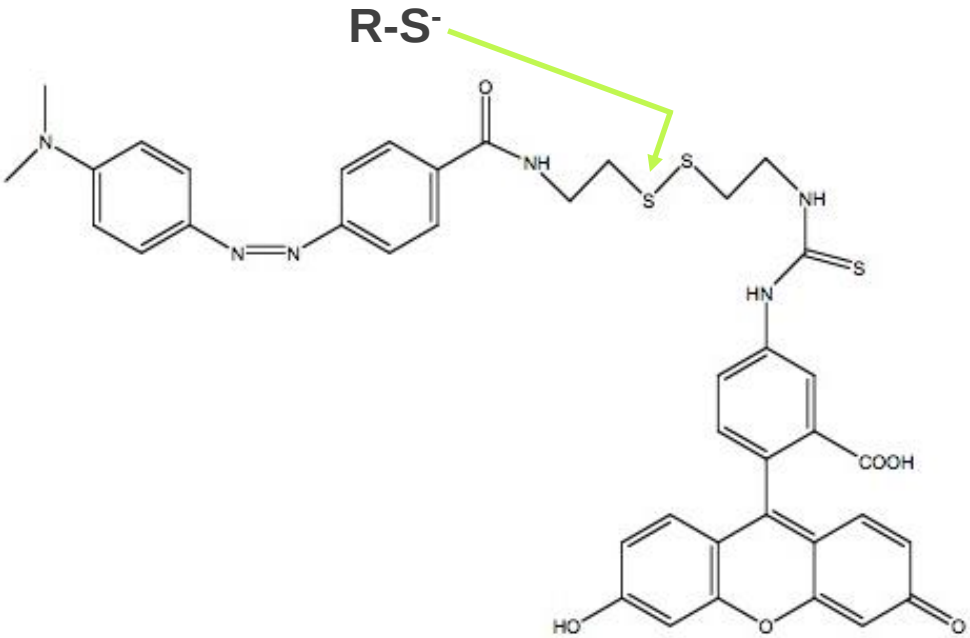
OH-COUM-CYS-FITC
Mol. Wt: 729
E'_o = -0.53 V

Kinetics of PMR-cys-FITC (SEMKUR-IM) Probe Reduction

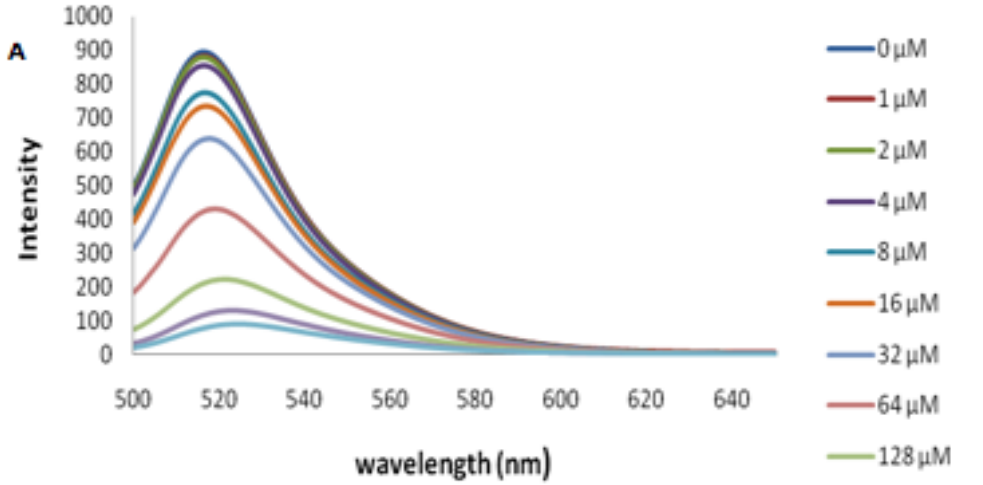


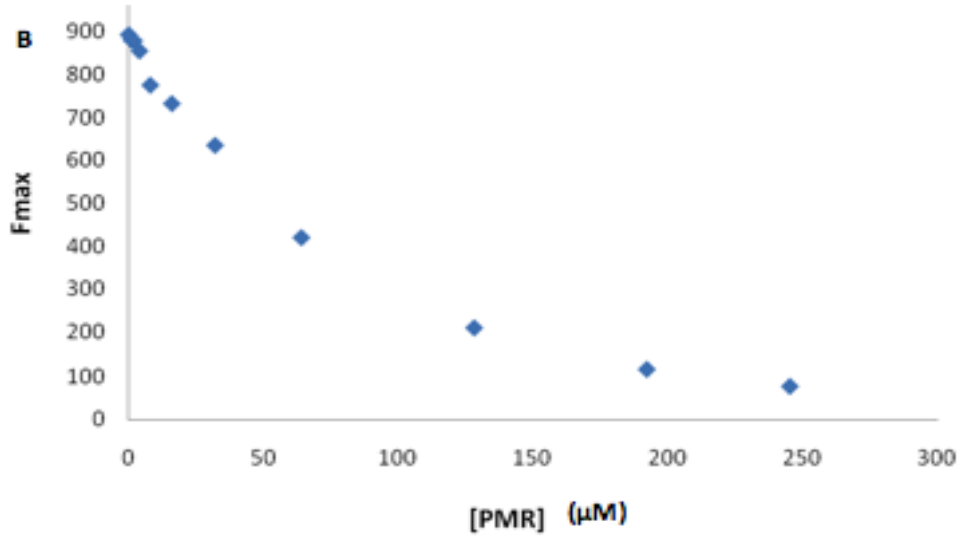


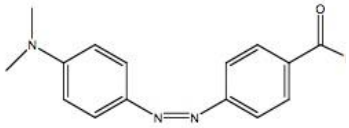
Excitation at 495 nm (fluorescein)



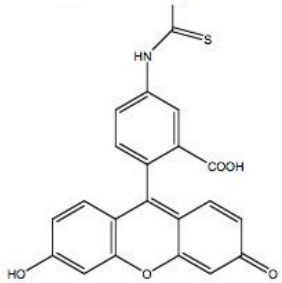
Intermolecular Quenching Effects







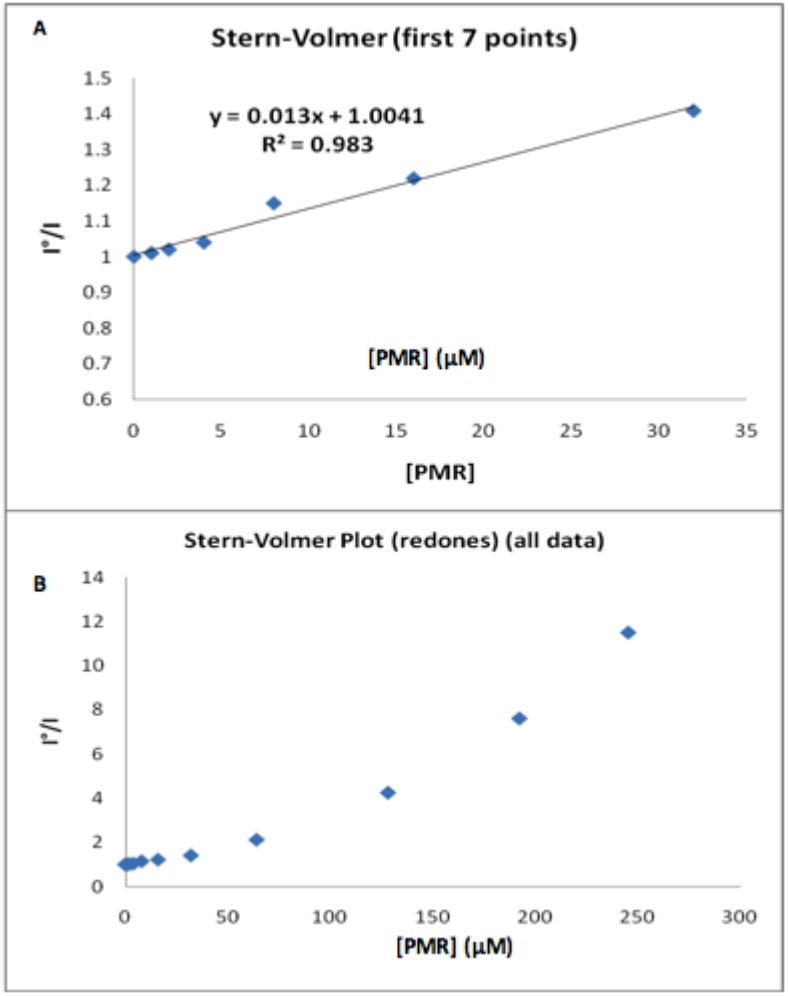
Remove the linker



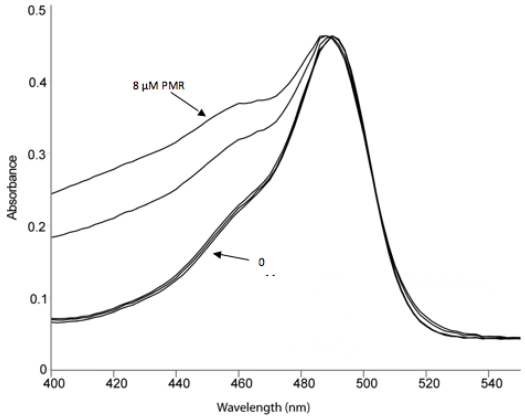
What is the nature of the intermolecular quenching?

Intermolecular Quenching Effects

Loss of linearity at high [PMR] means complex formation occurs (static quenching). Consistent with absorbance spectra.

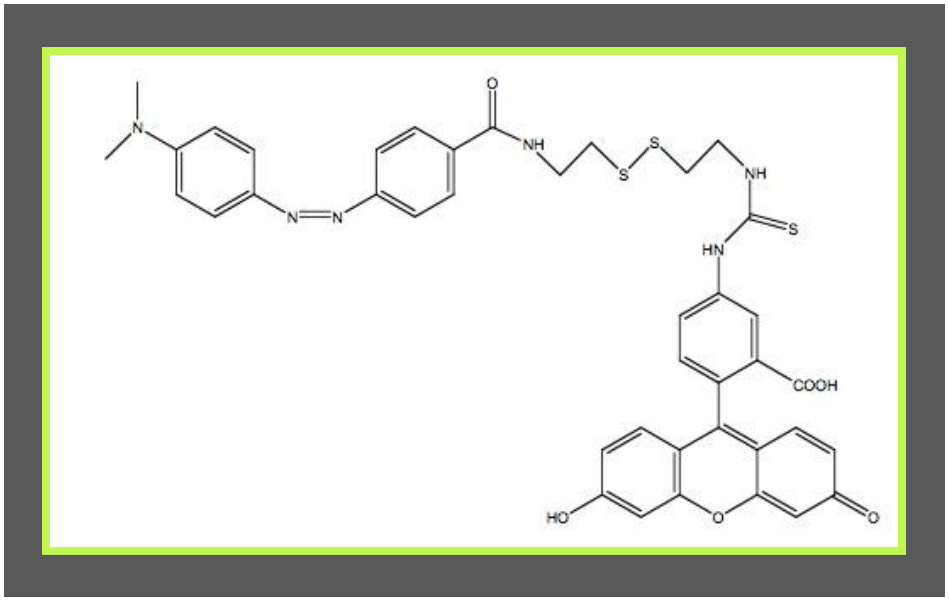


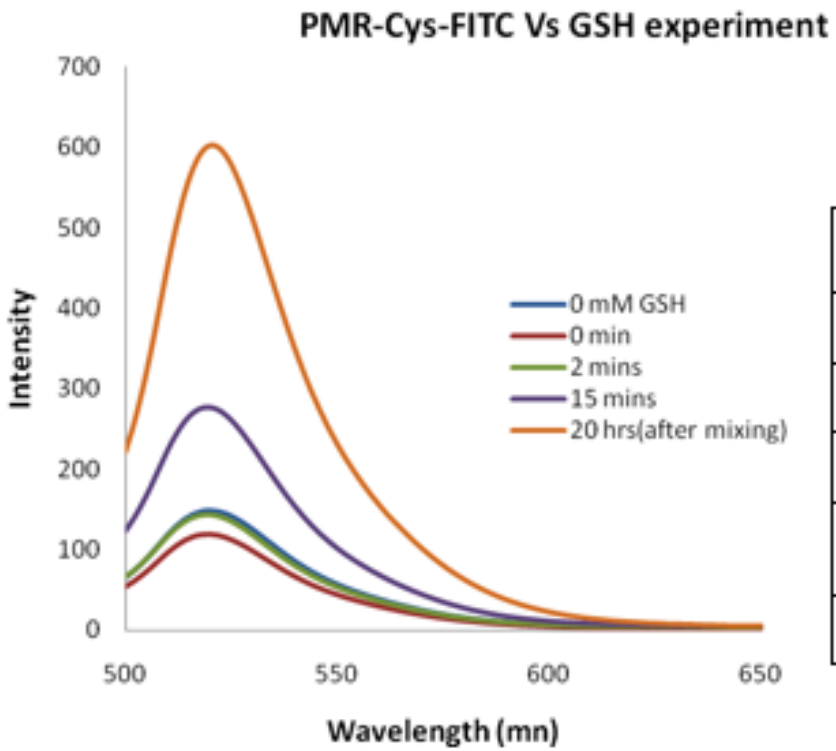
$$\frac{F_0}{F} = 1 + (K_D + K_S)[Q] + (1 + K_S)[Q]^2$$



Intramolecular Quenching Effects:

removed by adding GSH

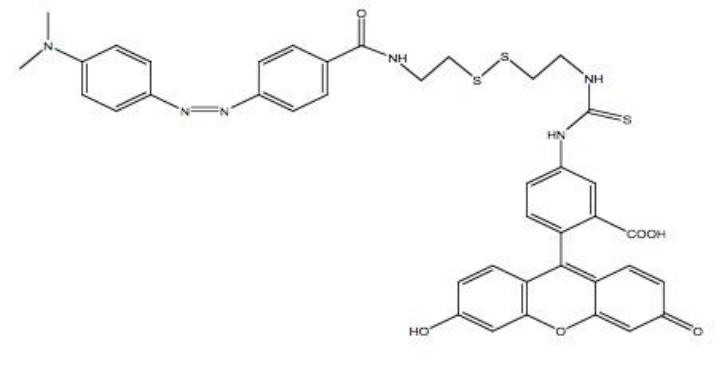




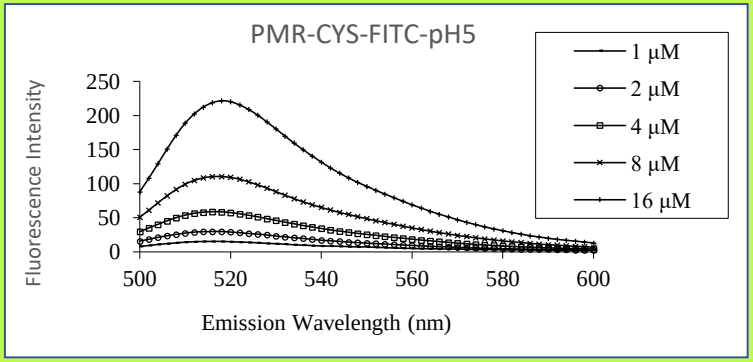
Time	F_{max}	I*/I
0 GSH	149	1
0 min	120	1.24
2 mins	143	1.04
15 mins	276	0.54
20 hrs	602	0.247

"Effective" PMR concentration is 100 μM

Effect of pH and Concentration on DSSQ Probe Fluorescence Emission

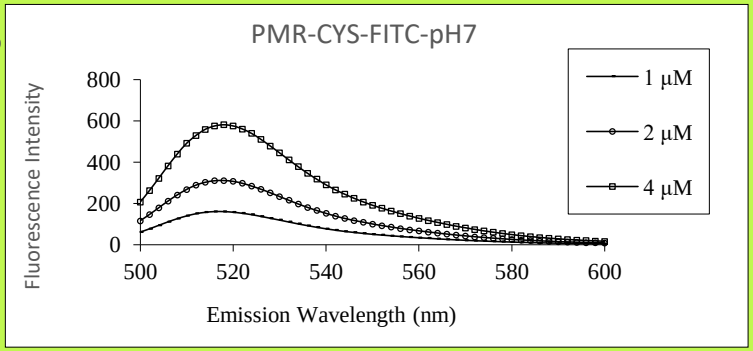


A



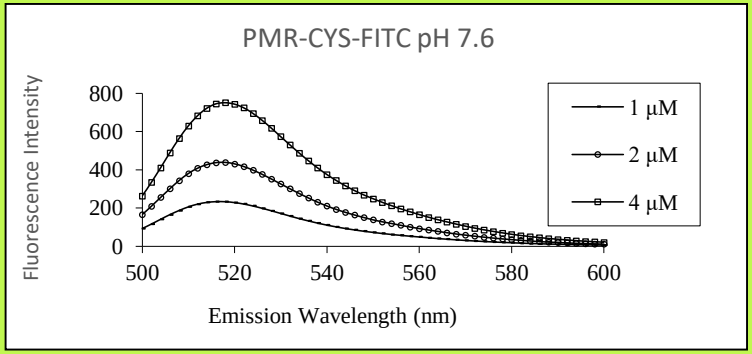
A) 5 µM PMR-CYS-FITC in 100 mM acetate buffer (pH 5)

B



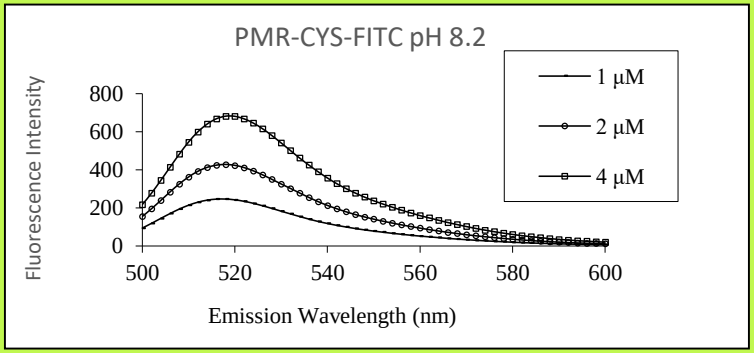
B) 5 µM PMR-CYS-FITC in 100 mM HEPES buffer (pH 7)

C



C) 5 µM PMR-CYS-FITC in 100 mM HEPES buffer (pH 7.6)

D



D) 5 µM PMR-CYS-FITC in 100 mM Tris buffer (pH 8.2)

Excitation at 495 nm

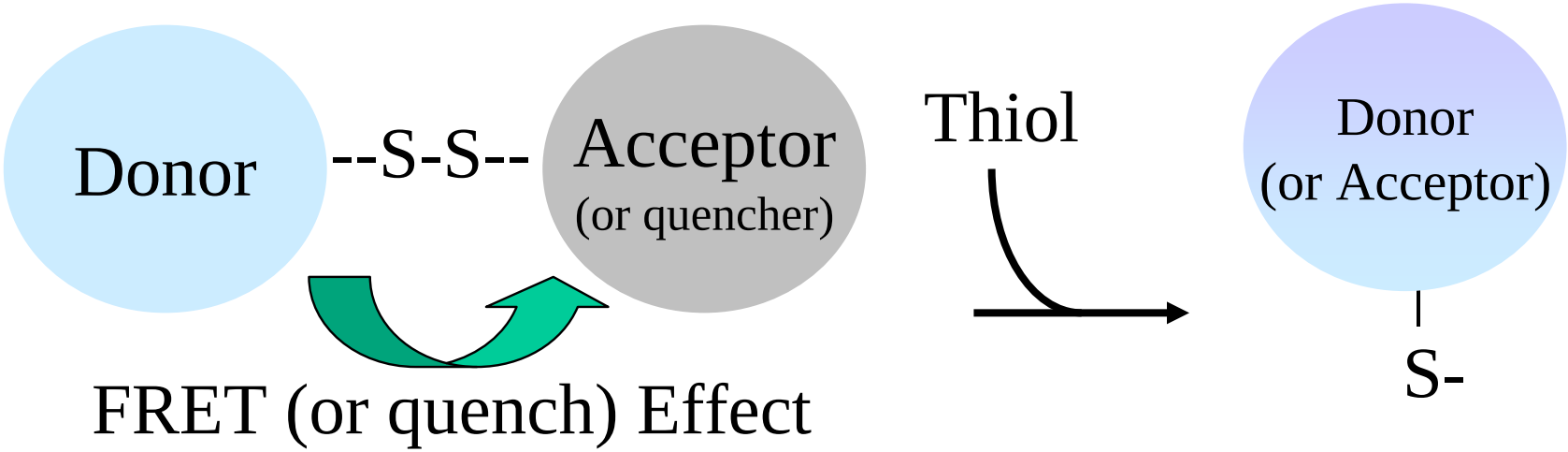
Fluorescence-Based Detection of Thiols

New Reagents : The General Concept

Back to the start:

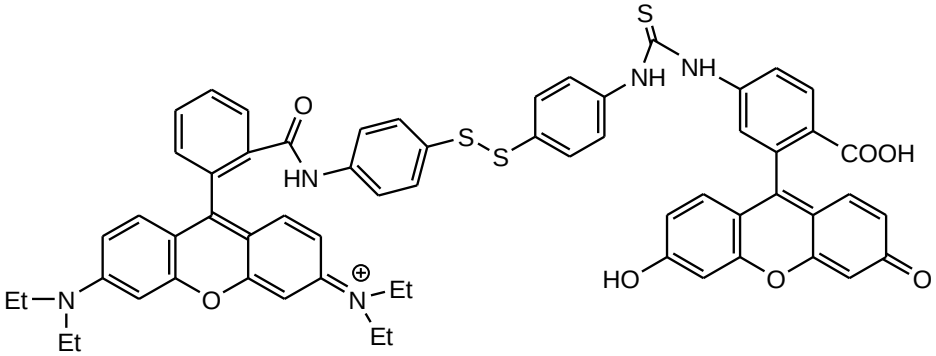
What about quantifying thiols in cells?

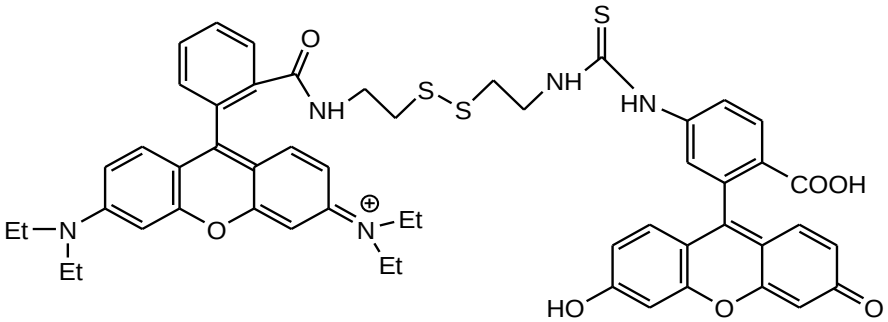
Can't use absolute fluorescence - need to do ratiometric measurements (FRET).

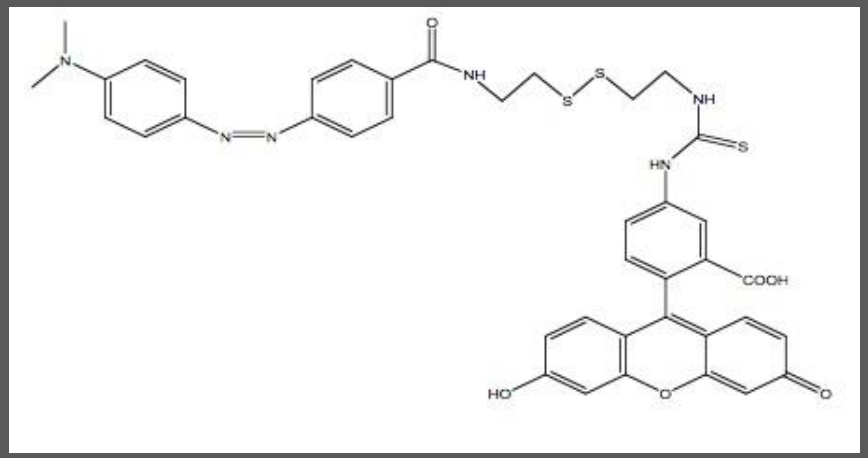


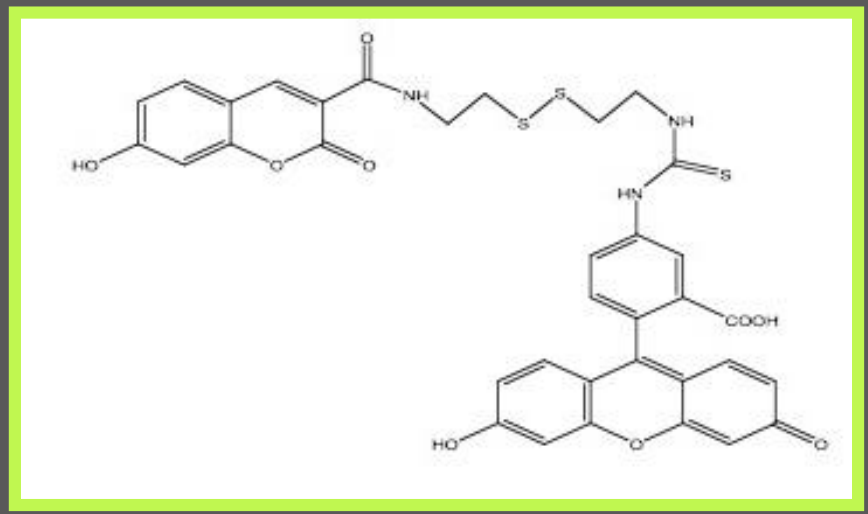
Expanding Our Collection of Probes:

2 NEW PROBES: Quencher & FRET

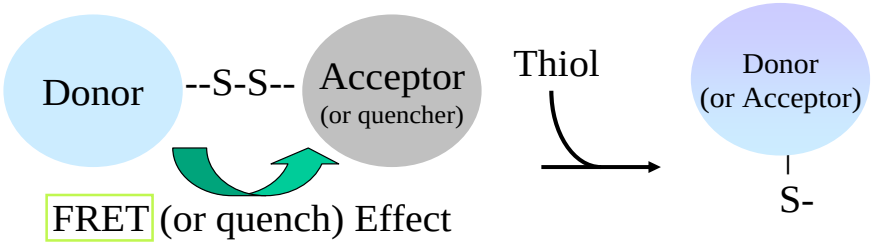


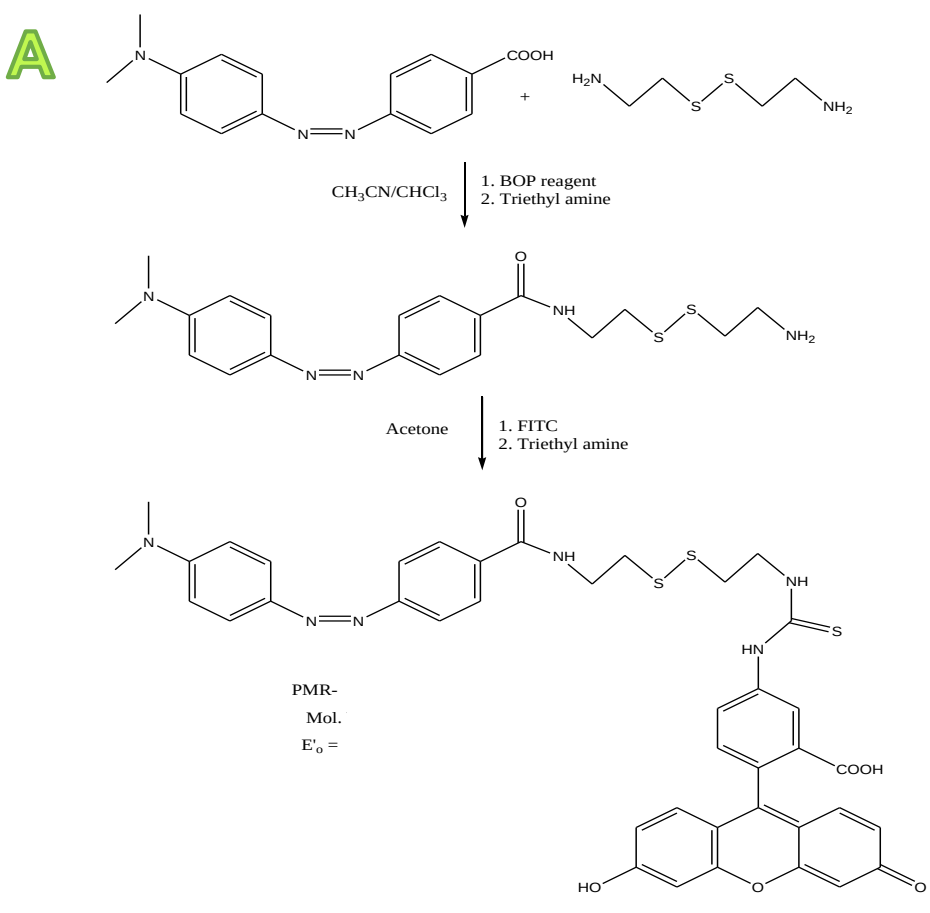


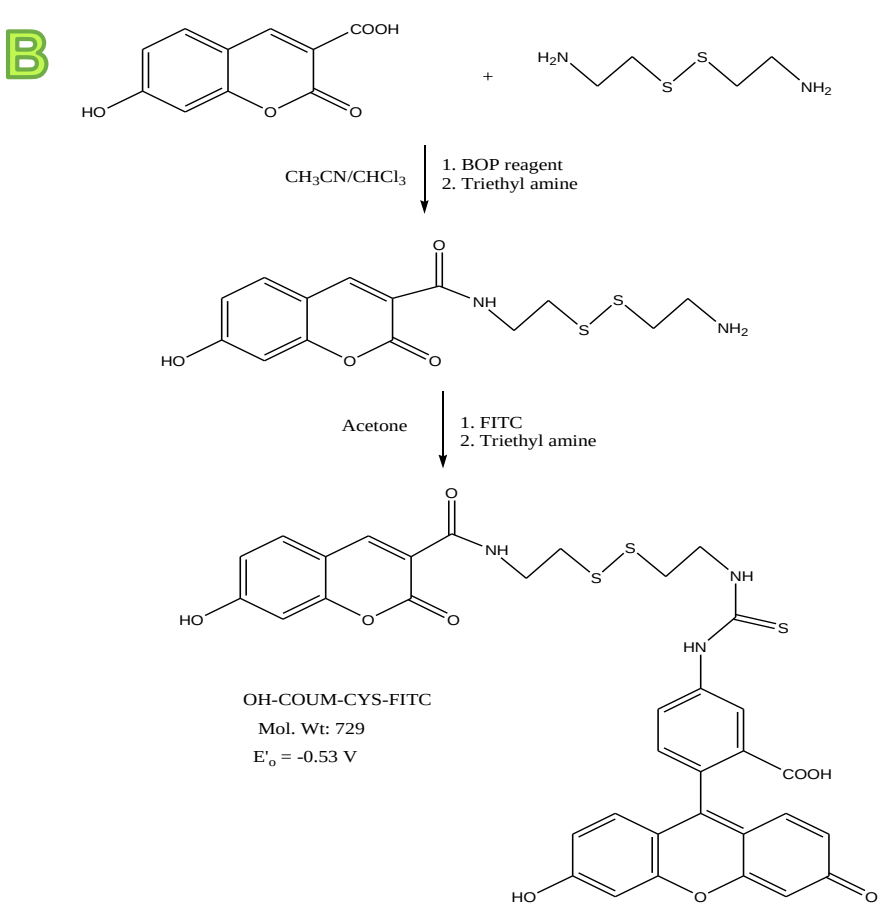




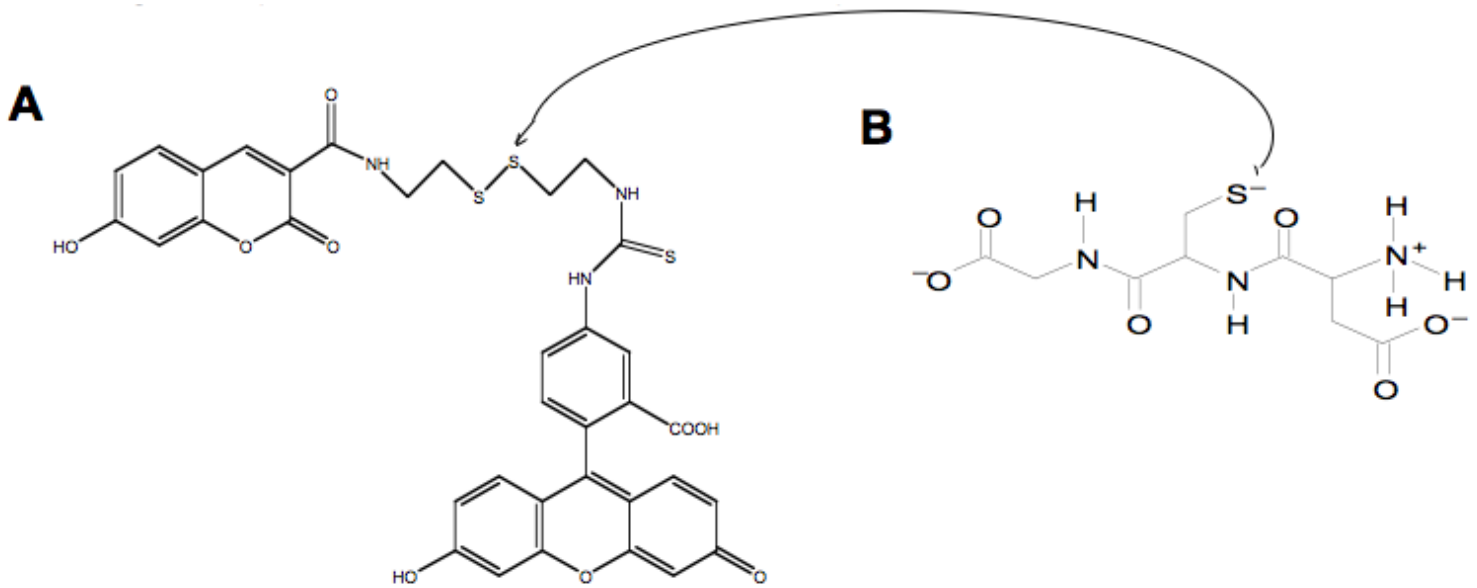
A TRUE DSSA PROBE: Courmarin-cys-FITC



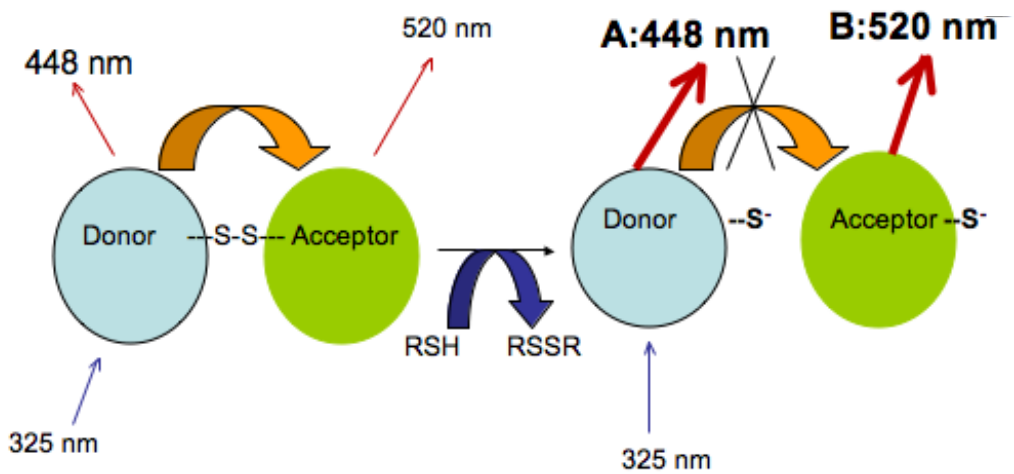




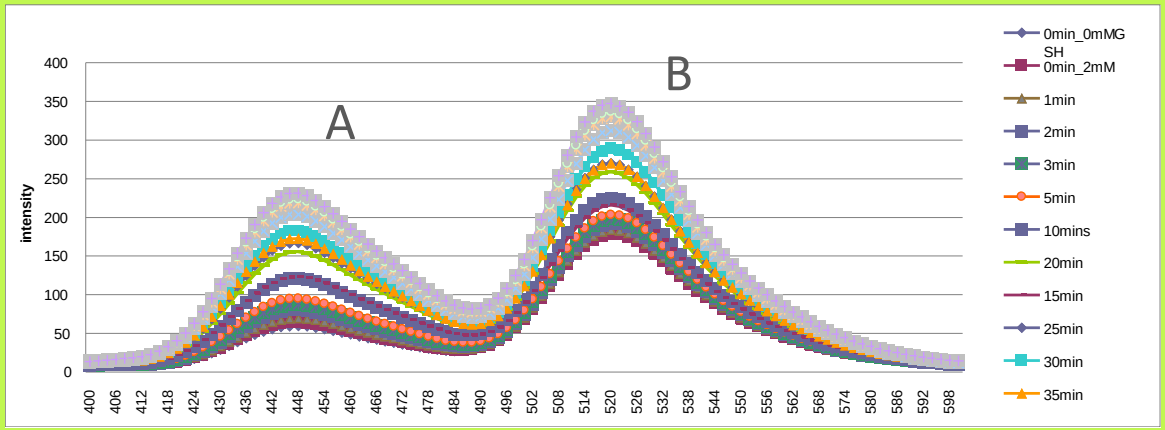
Coumarin-SS-Fluorescein FRET Probes

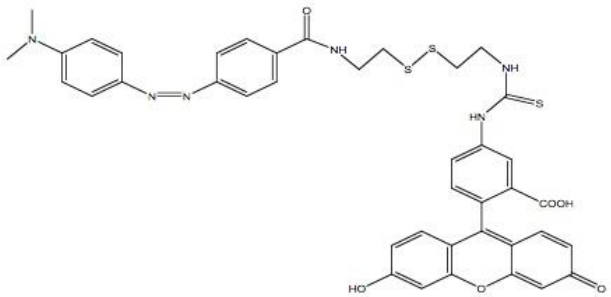


FRET-based thiol detection: DSSA probes

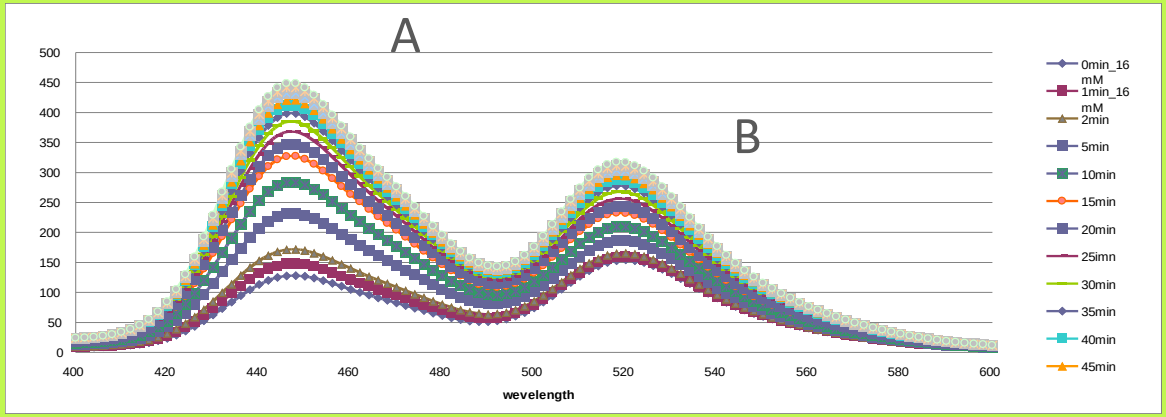


Reduction of HO-coumarin-SS-FITC by 2 mM GSH (Ex. 325nm)





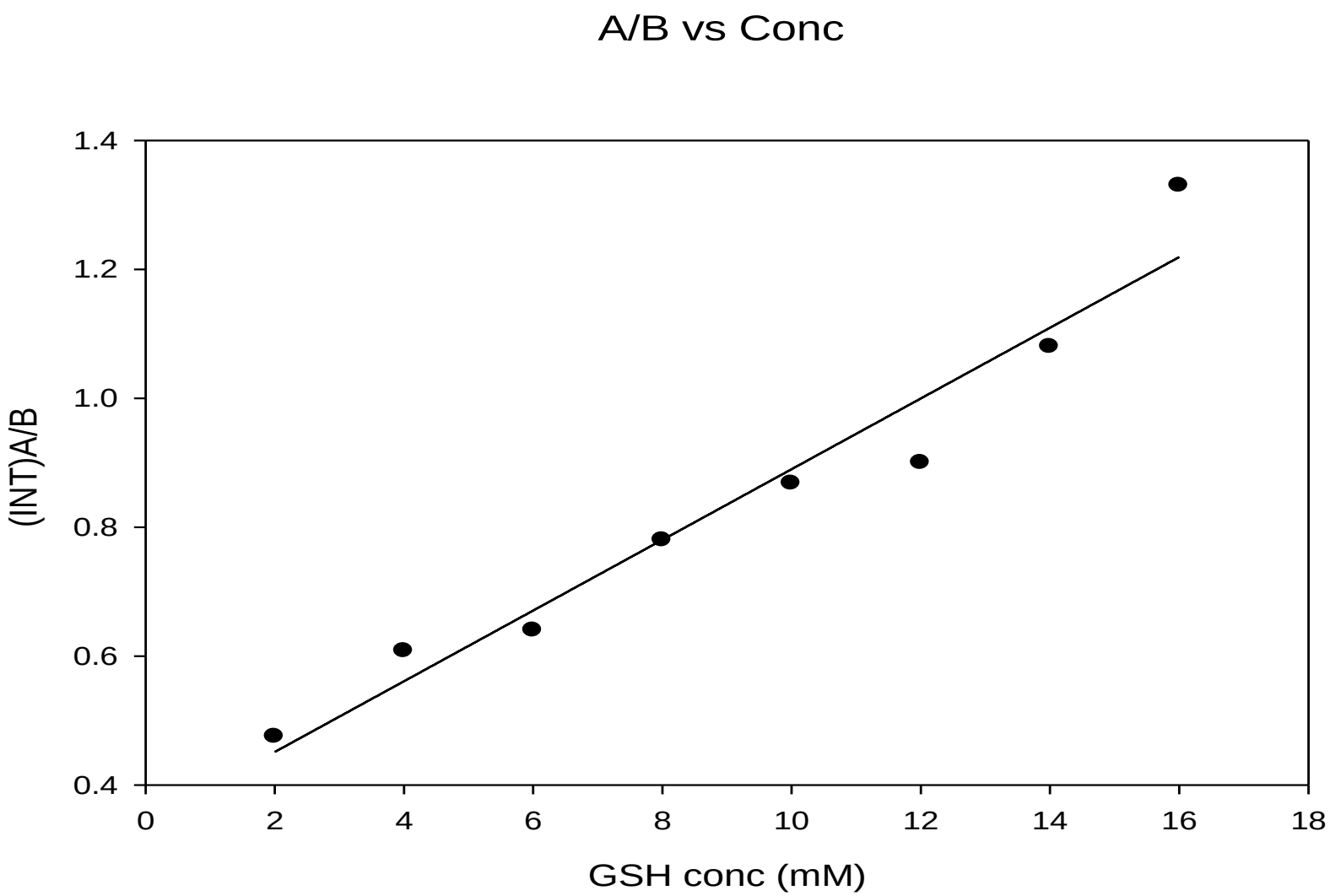
Reduction of HO-coumarin-SS-FITC by 16 mM GSH (Ex. 325nm)



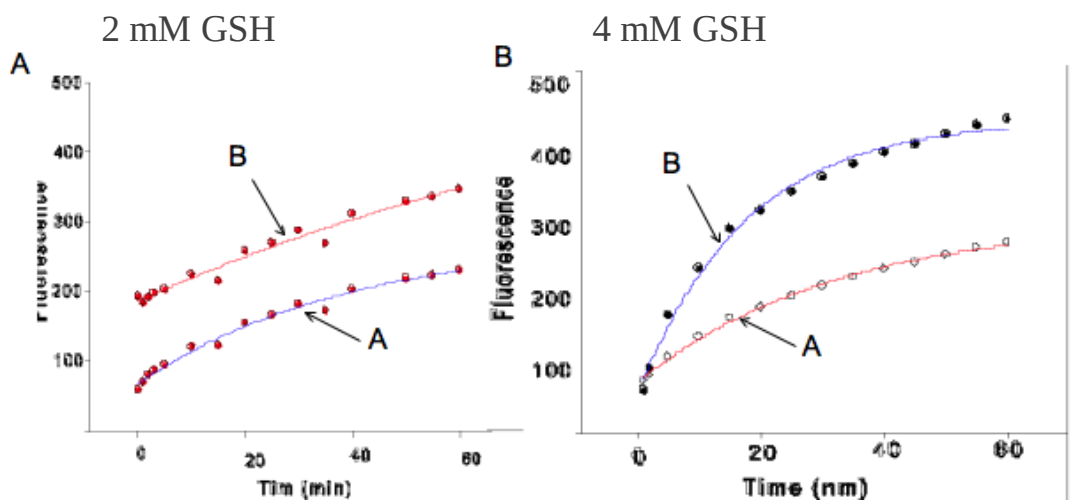
Follow the A/B ration vs. GSH

Follow the A/B ratio vs. GSH:

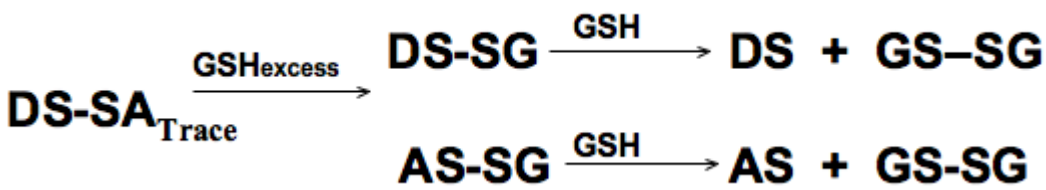
a ratiometric way to get (GSH)



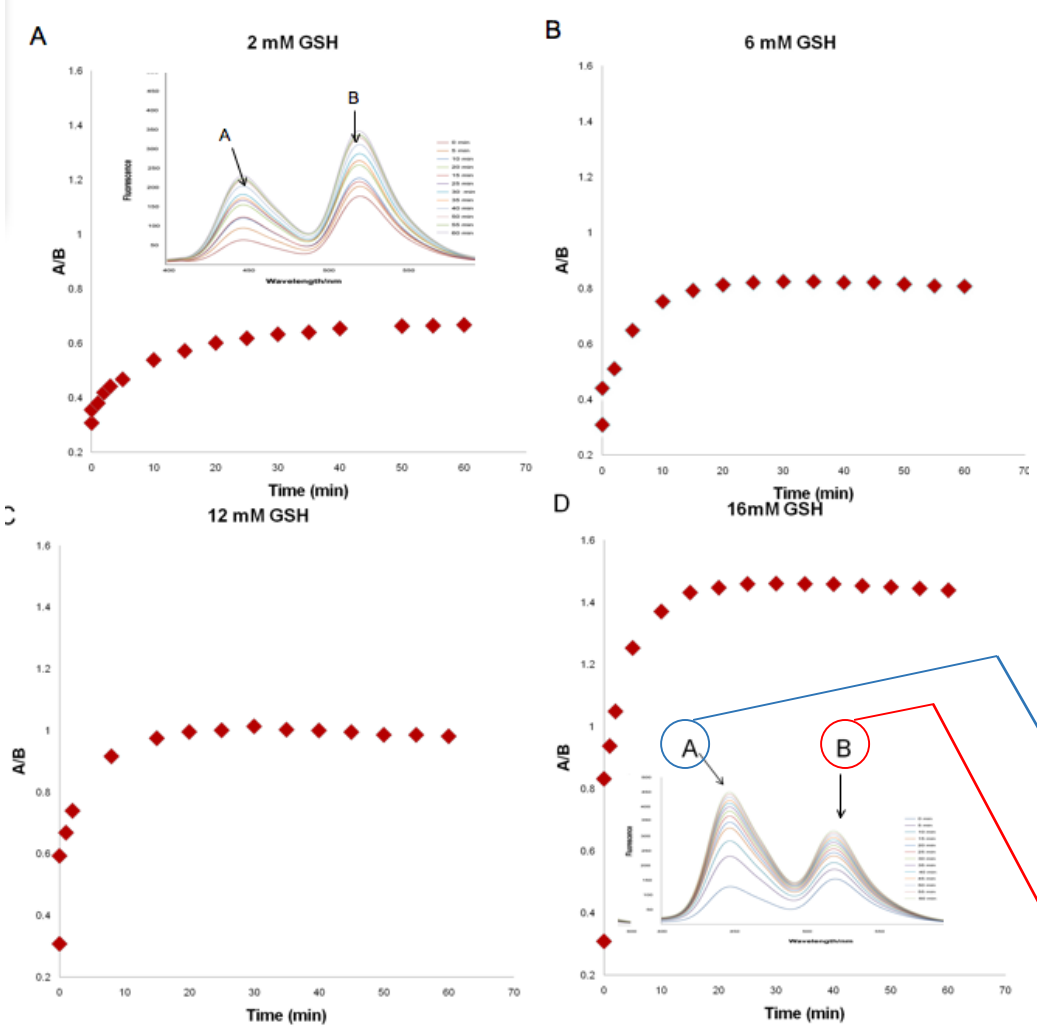
Coumarin-SS-Fluorescein FRET Probes



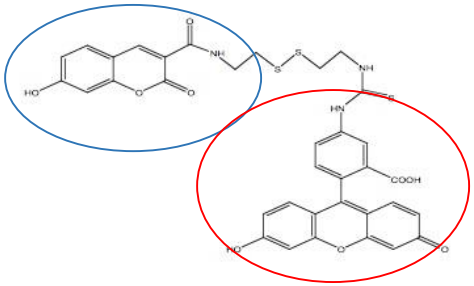
Kinetics of reduction of 5 μM DSSA: emission bands A (440 nm) and B (520 nm)



Coumarin-SS-Fluorescein FRET Probes



A/B is the ratio of the Coumarin/Fluorescein emission bands at Fmax (448 nm and 520 nm)



$$\text{DS-SA}_{\text{Trace}} \xrightarrow{\text{GSHexcess}} \text{DS-SG} \xrightarrow{\text{GSH}} \text{DS} + \text{GS–SG}$$

$$\text{AS-SG} \xrightarrow{\text{GSH}} \text{AS} + \text{GS-SG}$$

Thiol Redox Studies Inside Cells

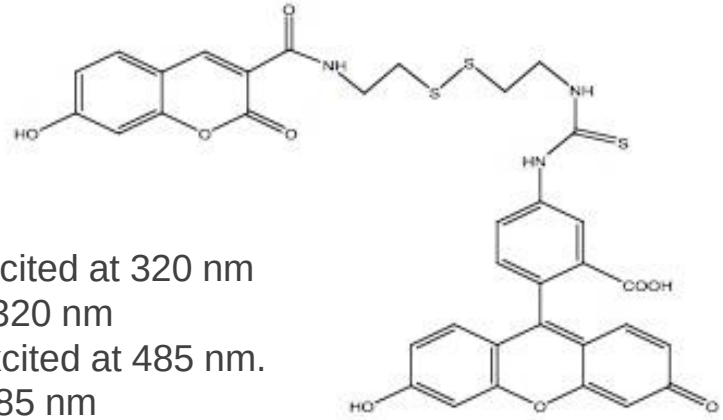
E. Coli:
WT vs. Origami (rich in GSSG)

Bovine endothelial lung cells:
asthma

Rat neuronal cells:
an Alzheimers drug

NIH 3T3 cells
(work done at Aurora)

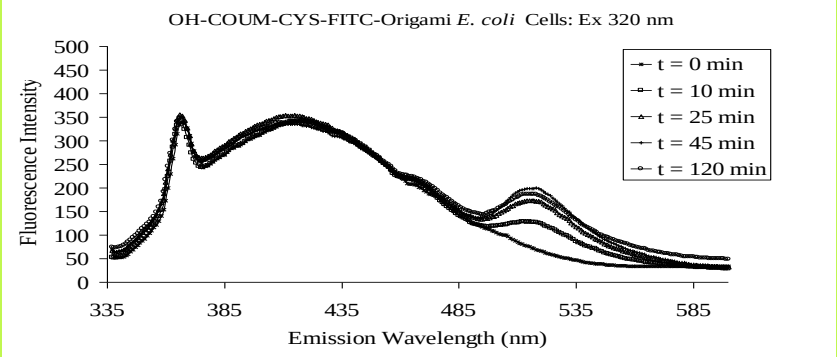
What about inside *E. coli*?



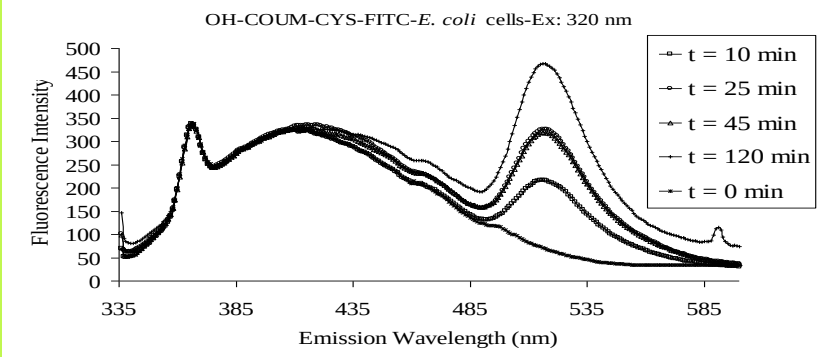
In cell studies of thiol redox state using OH-COUM-CYS-FITC:
A) BL21DE3 Origami *E. coli* cells treated with 10 μM OH-COUM-CYS-FITC-excited at 320 nm
B) BL21DE3 *E. coli* cells treated with 10 μM OH-COUM-CYS-FITC-excited at 320 nm
C) BL21DE3 Origami *E. coli* cells treated with 10 μM OH-COUM-CYS-FITC-excited at 485 nm.
D) BL21DE3 *E. coli* cells treated with 10 μM OH-COUM-CYS-FITC-ecited at 485 nm

Note that Origami E.coli cells are deficient in thioredoxin reductase and glutathione reductase and give less fluorescence compared to BL21DE3 E.coli cells, due to the presence of more thiols in the disulphide form inside Origami cells. Probe incubations were as in previous slide.

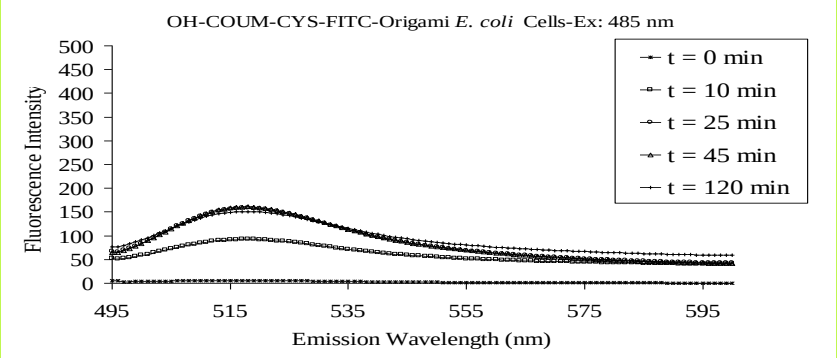
A



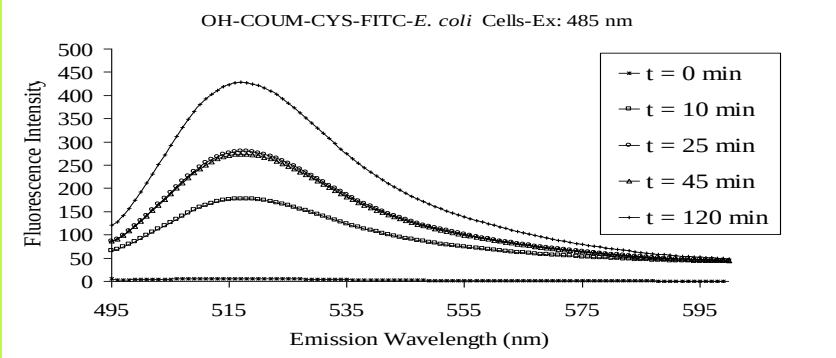
B



C

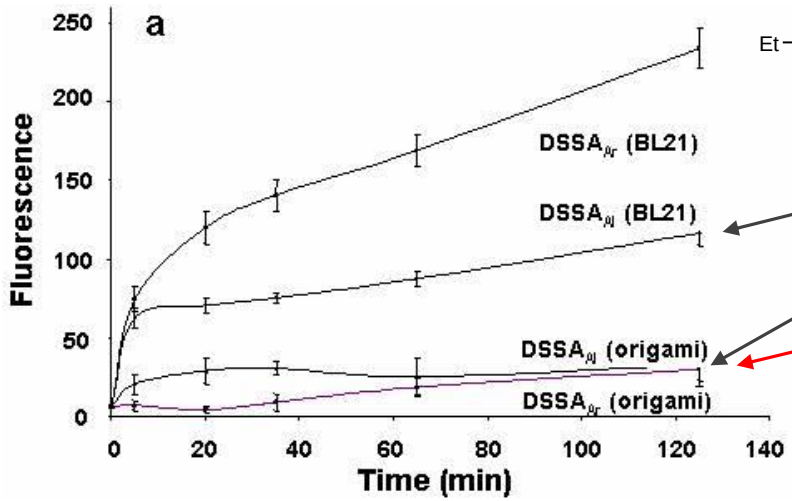


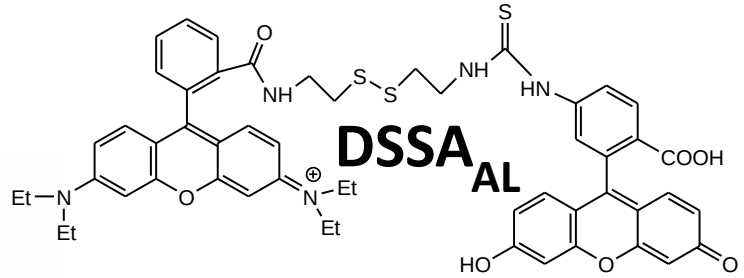
D



Probe is taken up into various cell types

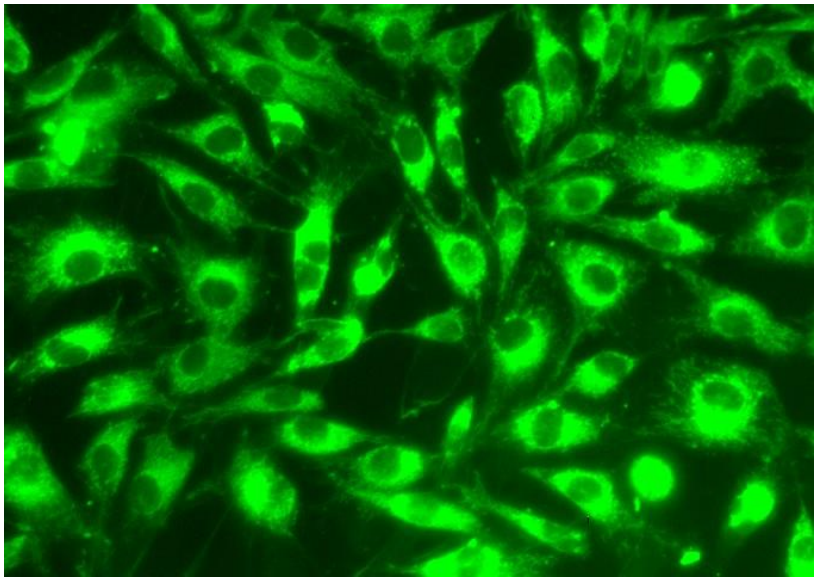
In
E. coli:





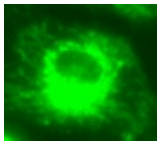
D-SS-glutathione forms, but we know it is not fluorescent

In
bovine
pulmonary
epithelial
cells:



Robert Bongard and Marilyn Merker, MCW (unpublished)

Studies on Bovine Lung Endothelial Cells

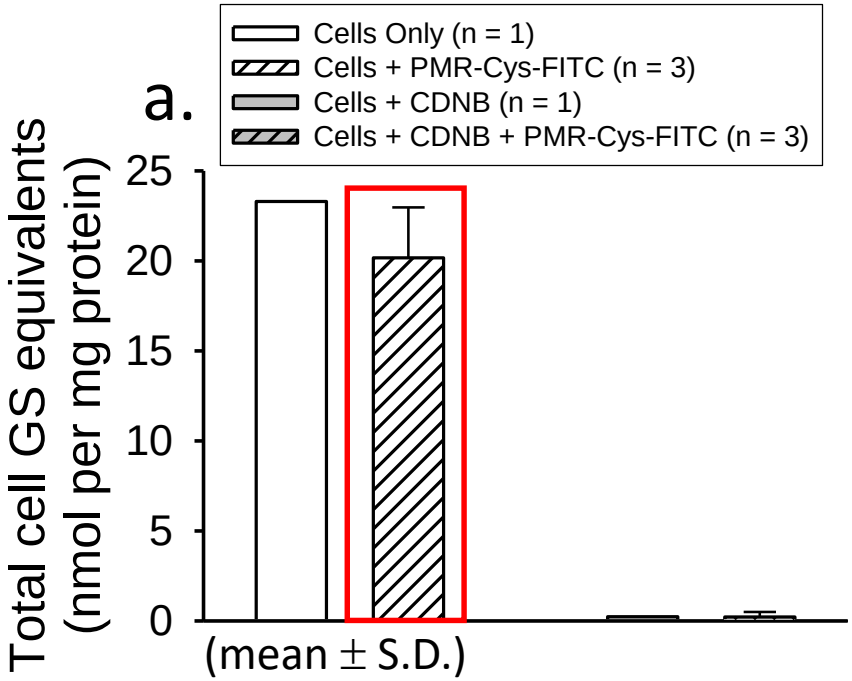


Do the probes affect intracellular levels of glutathione (the component being measured)?	Do the probes cause cell toxicity, as assessed by release of lactate dehydrogenase (LDH) on cell death?
NO	**NO**

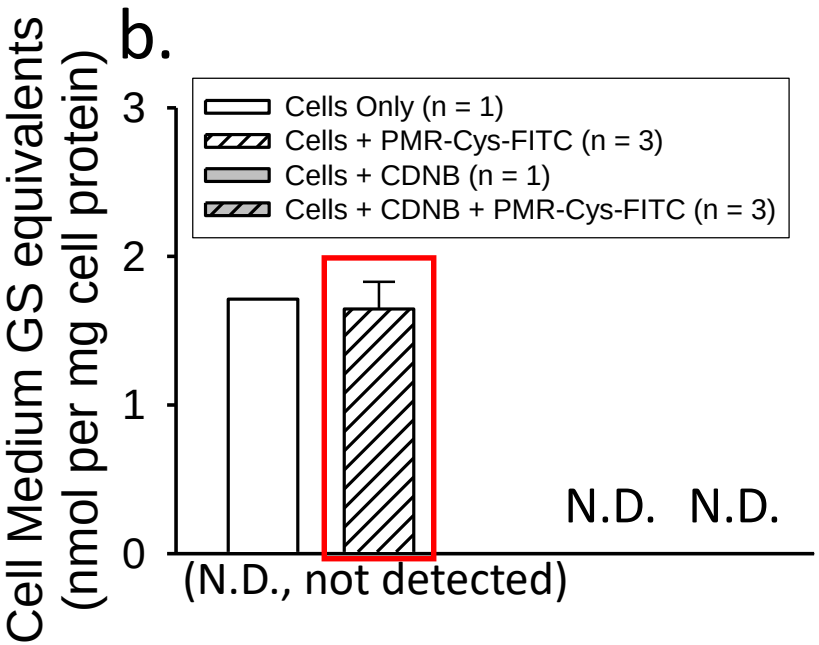
Impact of CDNB and PMR-Cys-FITC treatments on cell *glutathione* levels:

*CDNB (1-chloro-2,4-dinitrobenzene), conjugated to reduced glutathione by glutathione-S- transferases (CDNB-SG), results in decreased intracellular reduced Glutathione (GSH). In contrast, **PMR-Cys-FITC had little impact on cell GSH levels**.*

Total Cell GS Equivalents



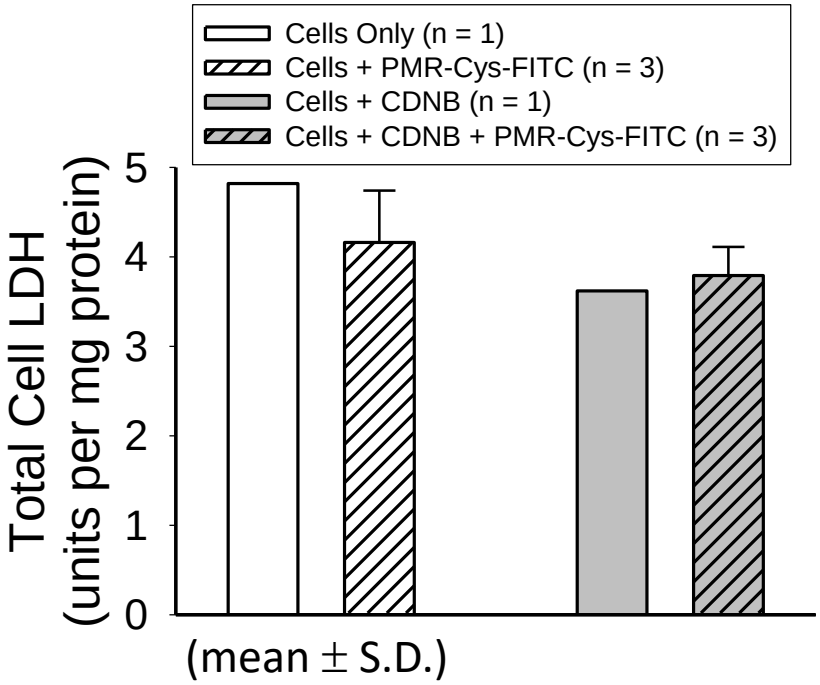
Cell Medium GS Equivalents



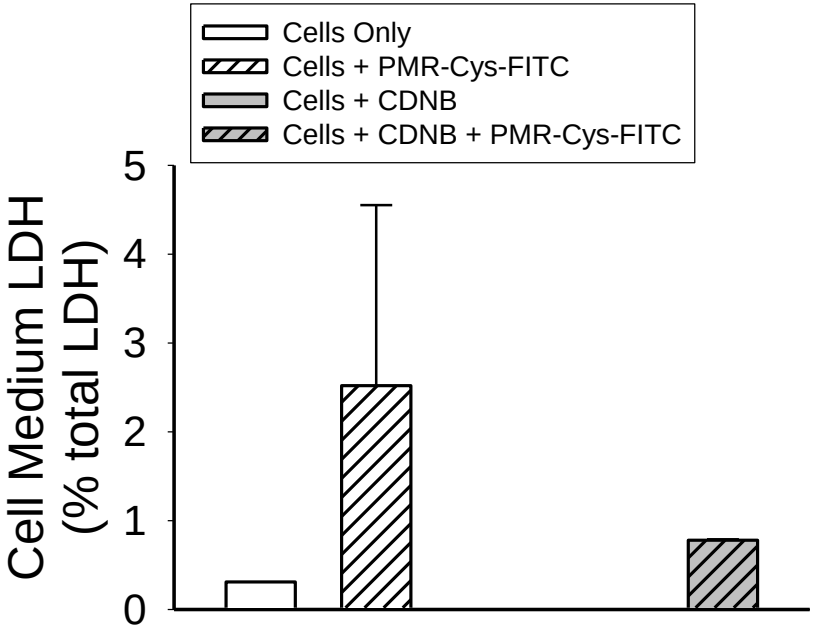
Cytotoxicity measurements – based on LDH release

CDNB and PMR-Cys-FITC additions to the cell media were not acutely toxic to the cells over 1 hr, based on absence of LDH leakage into the medium

A. Total Cell LDH



B. Cell Medium LDH



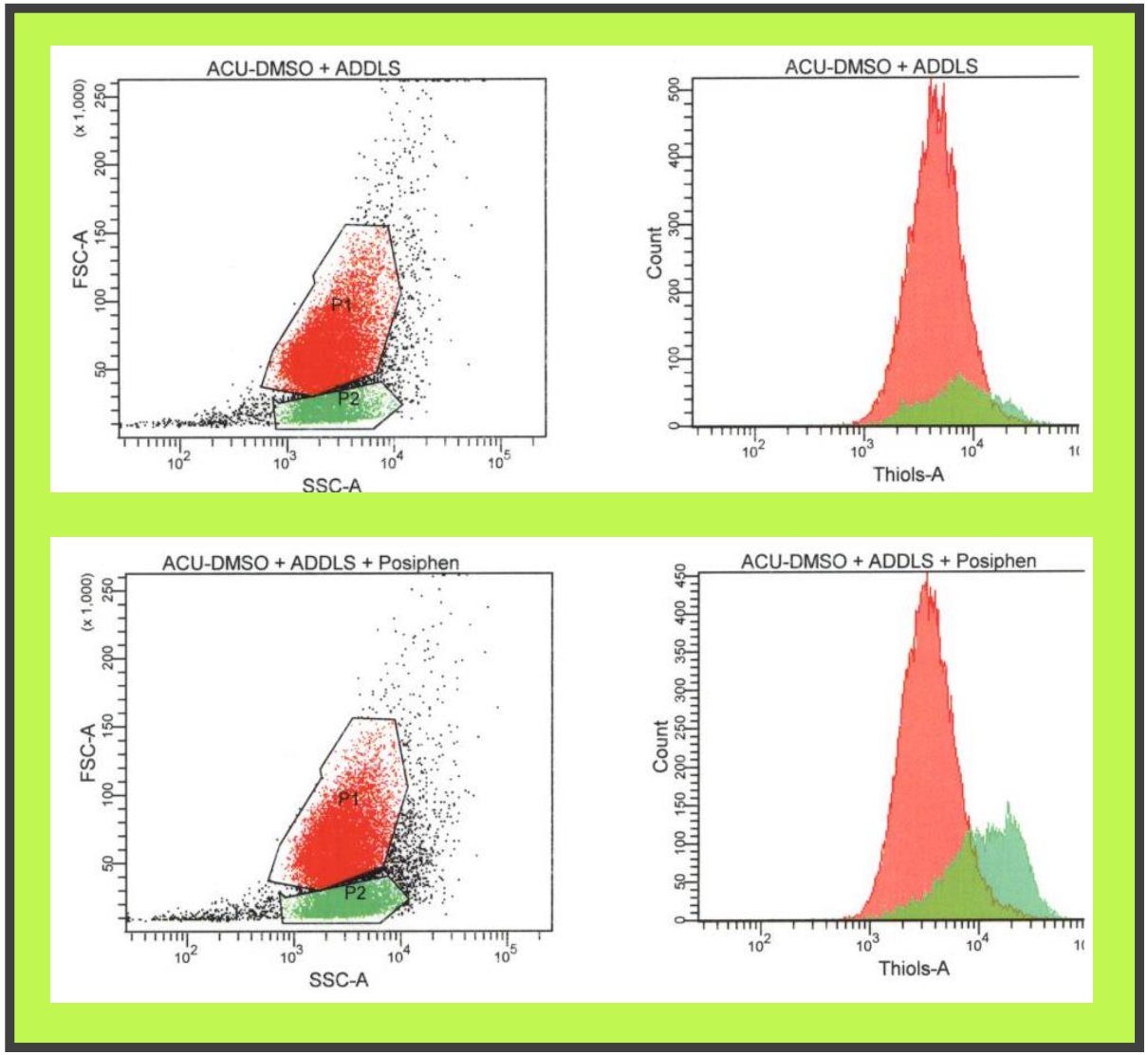
APPLICATIONS OF THE PROBES IN SCREENING FOR NEW DEMENTIA DRUGS

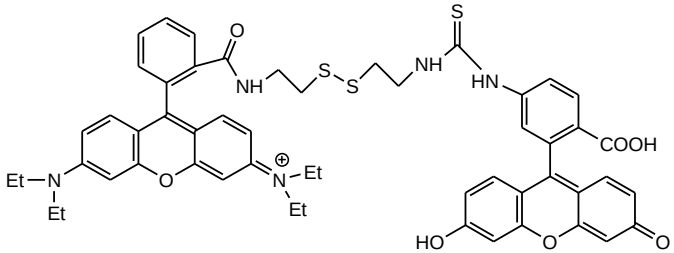
Do the probes permit detection of changes in oxidative stress due to treatment of neuronal cells with a drug lead molecule for Alzheimer's?

Initial study is a cell sorting assay with the drug lead Posiphen

Cell sorting based on thiol labeling:

Alzheimer's neuronal cells + Posiphen





Shift to a more "reduced state" for cells treated with Posiphen

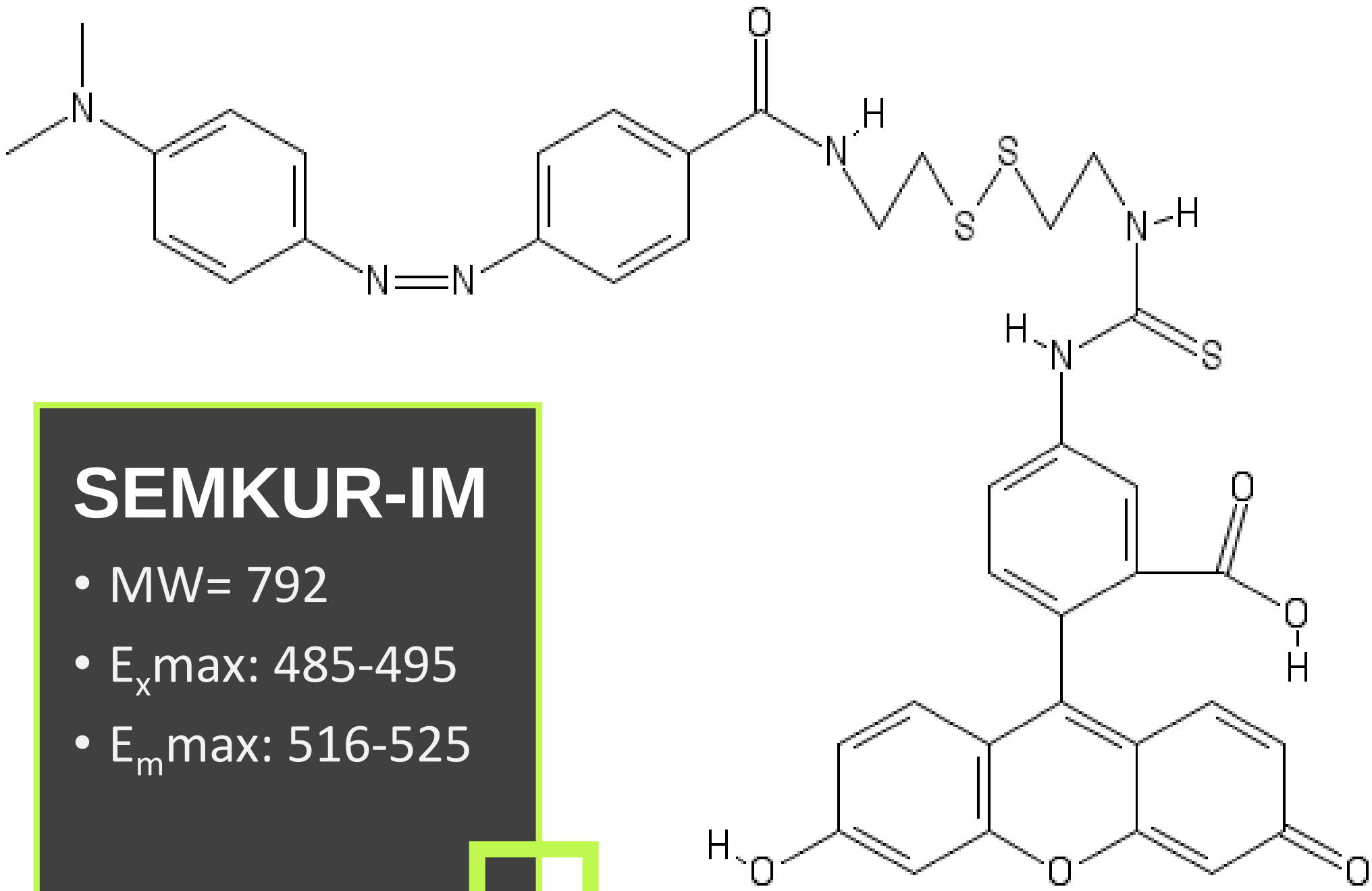
(removal of Alzheimer's associated oxidative stress)

Cells are primary neuron derived from rat hippocampus
Posiphen Dosage= 50 uM at (8hrs).

Data from Tony Giordano, LSU at Shreveport

Biological Study of SEMKUR-IM probe in NIH/3T3 cells

Work done at Aurora



SEMKUR-IM

- MW= 792
- $E_x$max: 485-495
- $E_m$max: 516-525

Co-localization of NIH/3T3 with SEMKUR-IM (20µM) and TMRM (200nM)

METHODS

NIH/3T3 cells were plated on MatTek Dish at 6000cells/mL and incubated w/ CO_2 for 24hours in complete media

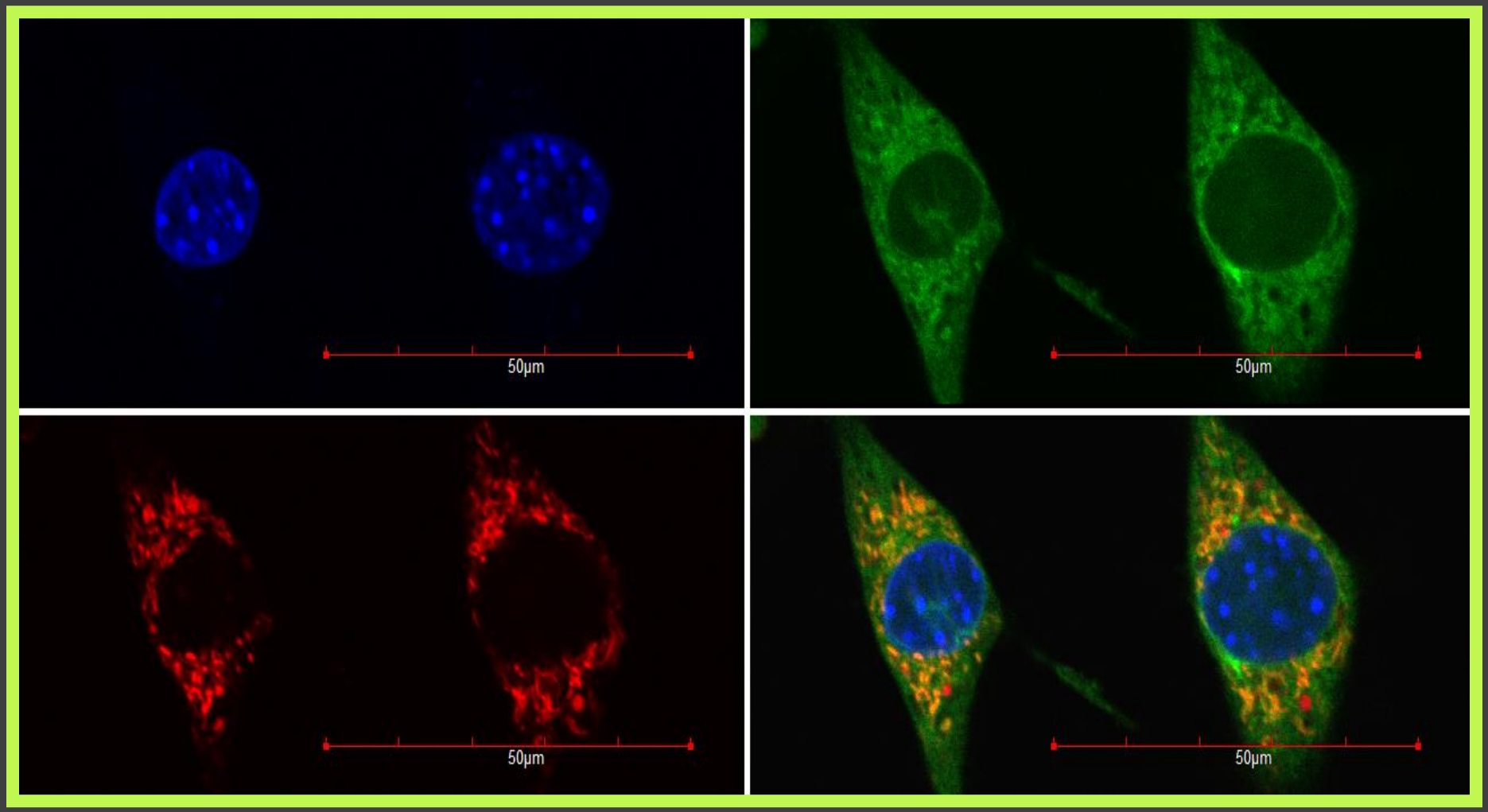
Dish media was washed out and replaced with HBSS plus Hoechst(1µg/ml), TMRM(200nM), and DSSQ1(20µM) then incubated w/ CO_2 for 30min

Dish was washed and imaged using Olympus Confocal and analyzed with Olympus Fluroview

Co-localization of NIH/3T3 with SEMKUR-IM (20µM) and TMRM (200µM)

HOECHST

DSSQ1 (20µM)



TMRM

COMBINED

Co-localization of NIH/3T3 with SEMKUR-IM (20µM) and LysoTracker Red (1µM)

Methods

-NIH/3T3 cells were plated on MatTek Dish at 6000cells/mL and incubated w/ CO_2 for 48hours in complete media

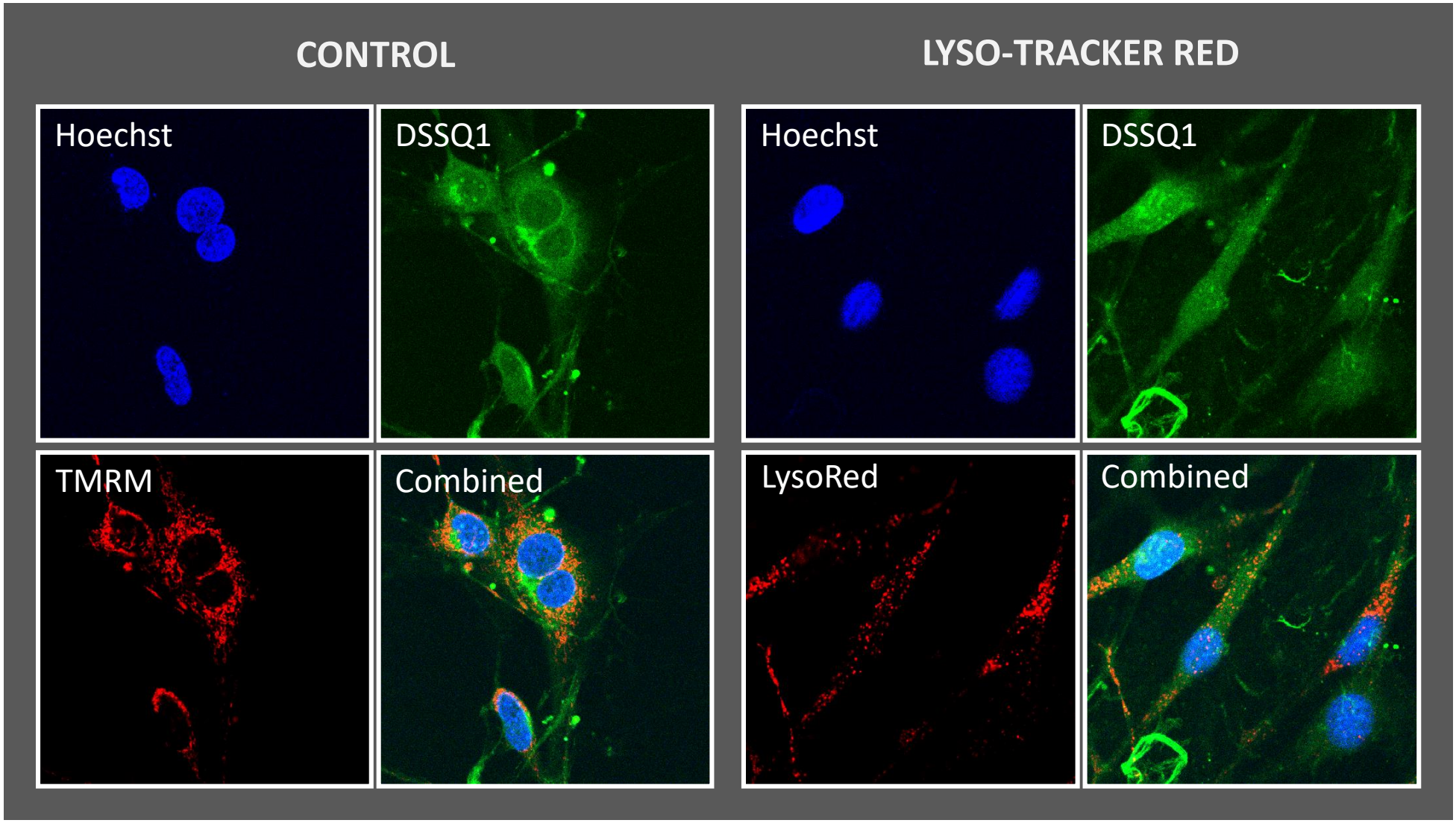
-All dish media was washed out and replaced with HBSS+HEPES (25mM) and incubated for 30min

-Dishes were washed and incubated for 30min with the solutions indicated below

Control	LysoTracker Red
HBSS+HEPES(25mM)	HBSS+HEPES(25mM)
Hoechst(1µg/ml)	Hoechst(1µg/ml)
DSSQ1(20µM)	DSSQ1(20µM)
TMRM (200nM)	LysoTracker Red (1µM)

-Dishes were washed and imaged using Nikon Confocal and analyzed with Nikon NIS Elements

Co-localization of NIH/3T3 with SEMKUR-IM (20µM) and LysoTracker Red (1µM)



Effects of 30 min Pre-incubation of NIH/3T3 cells with N-Acetyl Cysteine (10 mM) on SEMKUR-IM (20µM)

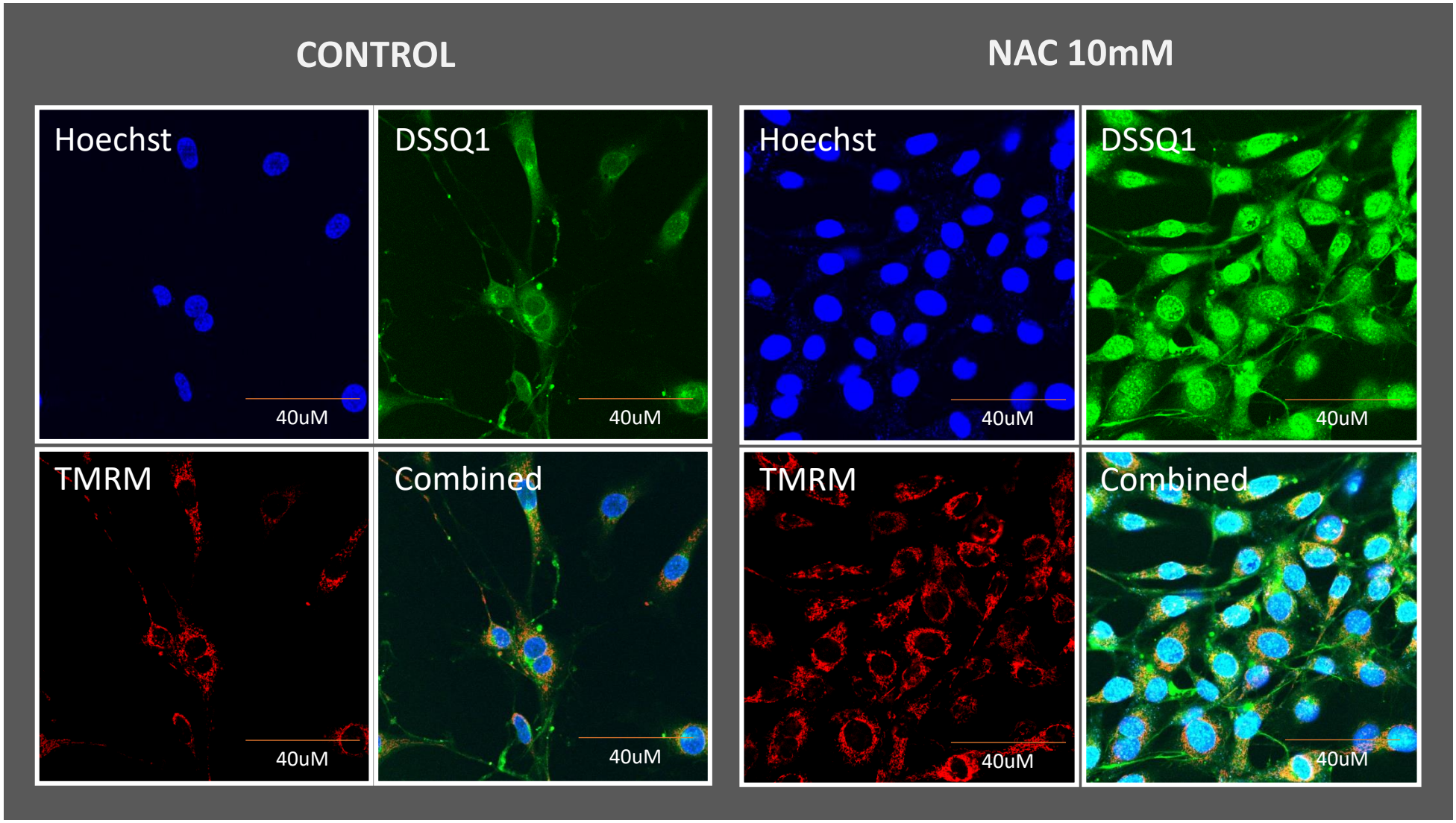
Methods

- NIH/3T3 cells were plated on MatTek Dish at 6000cells/mL and incubated w/ CO_2 for 48hours in complete media
- Dish media was washed out and replaced with the following solutions and incubated for 30min

Control	N-Acetyl Cysteine
HBSS+HEPES(25mM)	HBSS+HEPES(25mM)
Hoechst(1µg/ml)	Hoechst(1µg/ml)
DSSQ1(20µM)	DSSQ1(20µM)
TMRM (200nM)	TMRM (200nM)
	NAC (10mM)

- Dish media was washed out and incubated for 30min with HBSS+HEPES(25mM), Hoechst (1µg/mL), DSSQ1 (20µM), and TMRM (200nM) for 30min
- Dishes were washed out with HBSS+HEPES and imaged using Nikon Confocal and analyzed with Nikon NIS Elements

Effects of 30 min Pre-incubation of NIH/3T3 cells with N-Acetyl Cysteine (10 mM) on SEMKUR-IM (20µM)



SUMMARY

Dithio probes presented here detect reduced vs. oxidized glutathione – even in cells. GSSG produces no fluorescence, but GSH does

The probes have detected thiol redox changes in multiple cellular model systems (both prokaryotic and eukaryotic)

The probes are not toxic to cells, and do not alter cellular GSH levels

We also have a prototype FRET-based probe with two emission bands, that permit ratio metric analysis of thiols. The first such probe.

These dithio probes can be used in fluorescence microscopy and in cell sorting studies.

Applications include a wide range of measurements in cells, such as:

- Drug screening, where changes in oxidative stress (thiol-based) are expected
- QC of protein thiol redox state for in-cell produced proteins (e.g. Mab's)

Fluorescent Dithio Probes for Cell Biology:
Monitoring Cellular Thiols

Jayson Kurfis and Daniel S. Sem

Department of Pharmaceutical Sciences
Vivid Microscopy/ Concordia University