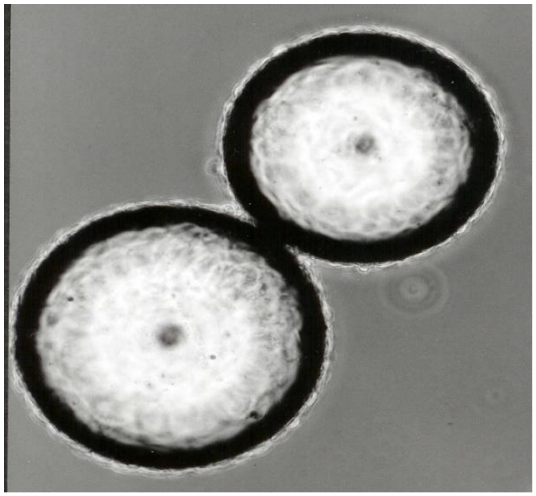
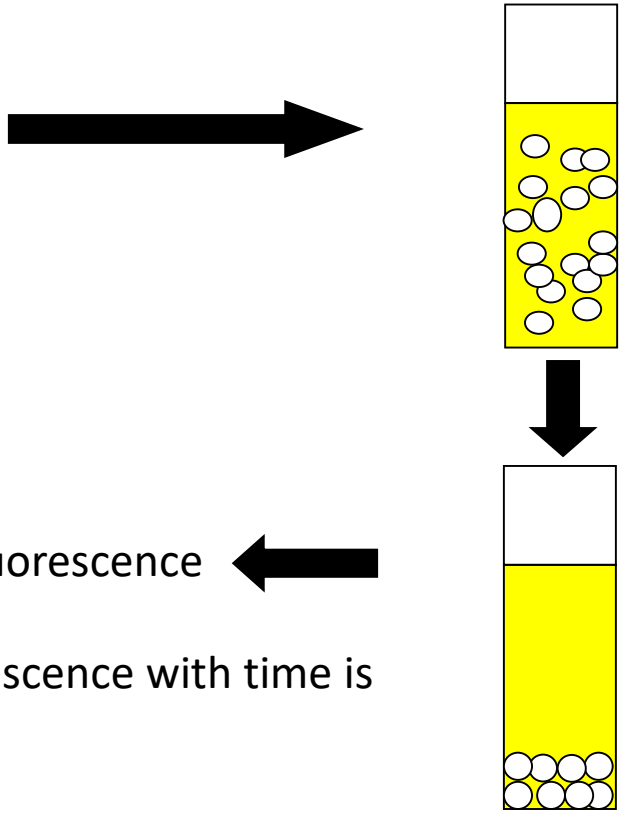
Bovine pulmonary arterial endothelial cells isolated from segment of calf pulmonary Artery were cultured to confluence (monolayer) on Biosilon microcarrier beads (mean diameter 230 μm, 255 cm^2 per gram beads).

2.5 mL assay buffer without/with CDNB (to deplete cell glutathione) and PMR-Cys-FITC probe were mixed with the cells in fluorometric cuvettes. At timed intervals the mixing was halted, allowing the micocarrier beads/cells to fall to the bottom of the cuvette, and buffer fluorescence recorded (Ex490 Em520).





Buffer fluorescence

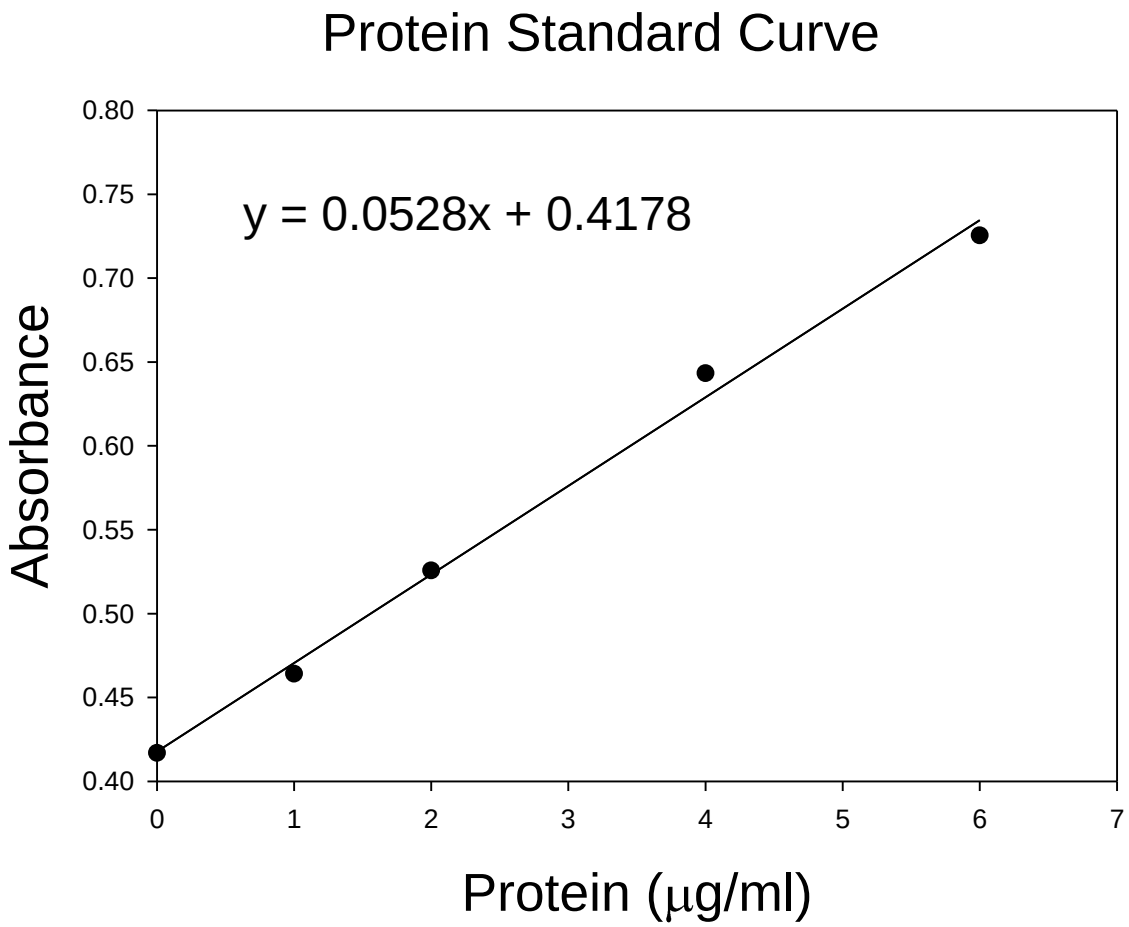
Change in buffer fluorescence with time is Shown in slide 9.

After a the mixing period, the buffer was removed from the cells and assayed for lactate dehydrogenase and glutathione.

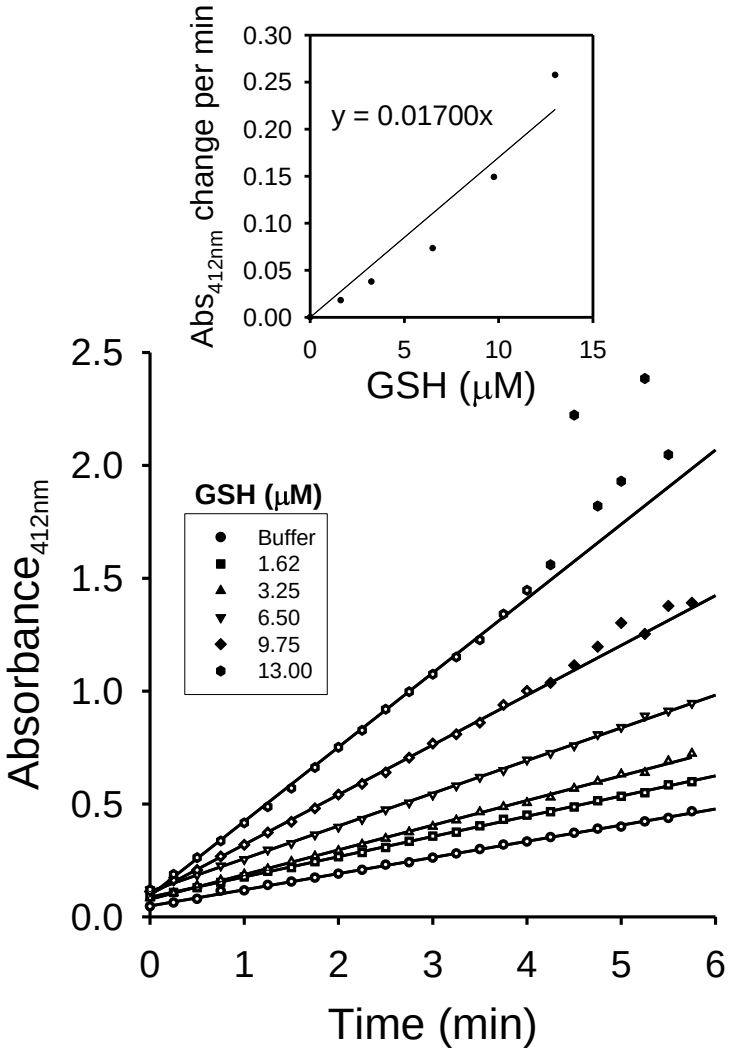
The cells were lysed by sonication on ice, and lysate assayed for protein, lactate dehydrogenase, and glutathione.

Fluorescence of the cell lysate was recorded following sonication and centrifugation (slide 10).

Sample protein determined by the Bradford dye-binding method



Glutathione determinations: Total and cell medium oxidized + reduced glutathione levels Were determined using an enzymatic recycling assay using glutathione reductase and Ellman's reagent (5-5'-dithiobis[2-nitrobenzoic acid]), DTNB). Glutathione reductase reduces GSSG to GSH. DTNB reacts with GSH to form a yellow color chromophore 5-thionitrobenzoic acid (TNB) with an absorbance maxima at 412 nm. GS-TNB is further reduced by GR'ase to GSH and TNB. Enzymatic recycling increases the sensitivity of the assay.

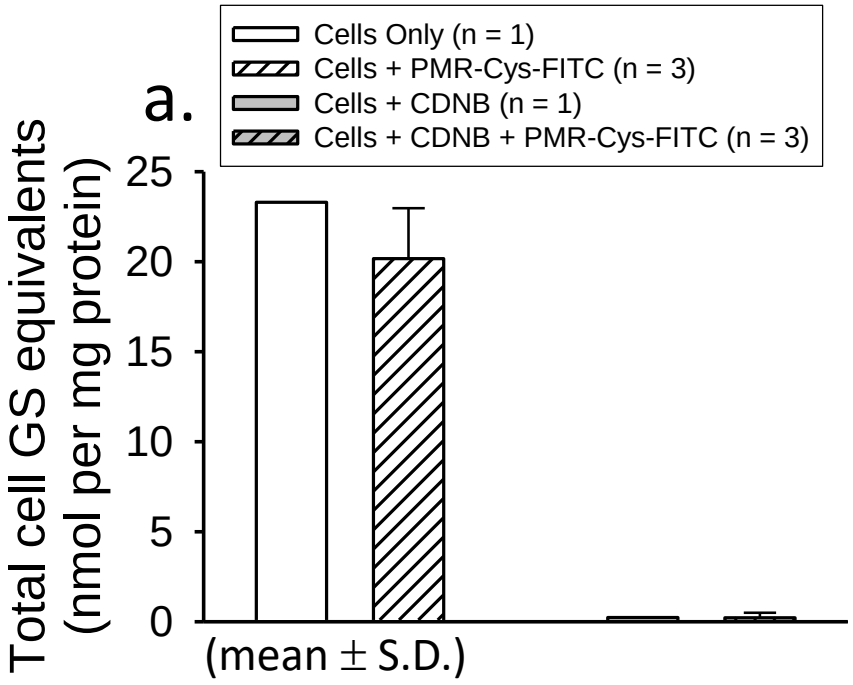


Lactate dehydrogenase was monitored spectrophotometrically at 340nm as the the generation of NADH during the conversion of lactate to pyruvate

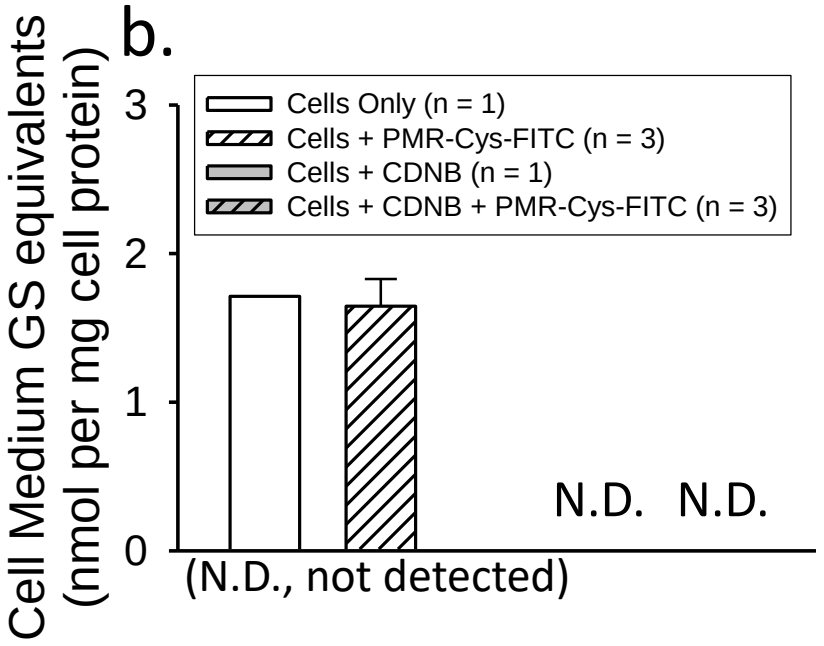
Impact of CDNB and PMR-Cys-FITC treatments on cell glutathione levels:

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

Total Cell GS Equivalents



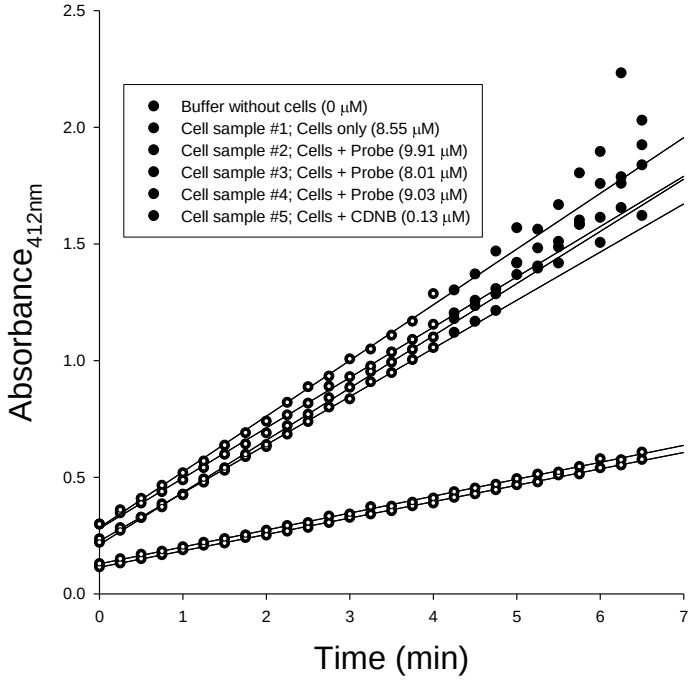
Cell Medium GS Equivalents



Raw glutathione assay data

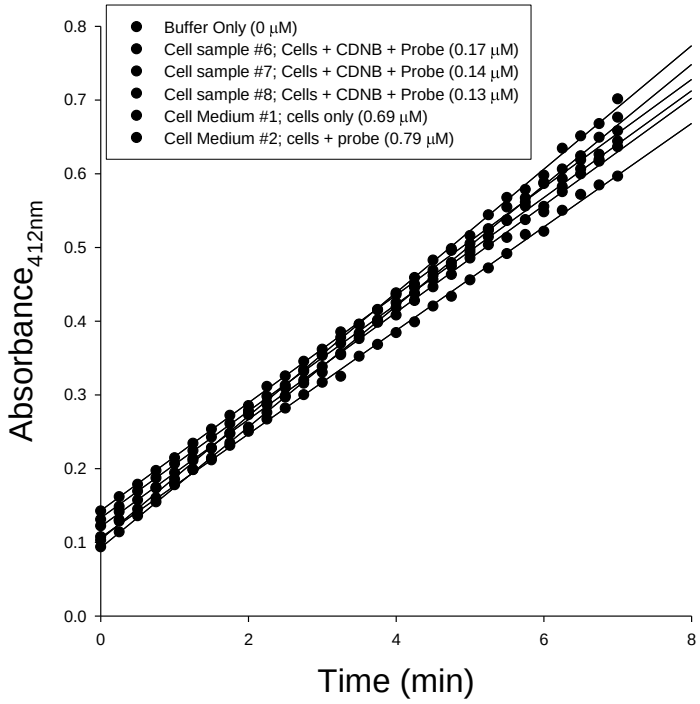
Cell Lysate Total GSH + GSSG

cells lysed in 2.5 mL H/H buffer; 25 μl undiluted lysate used in GSH assay



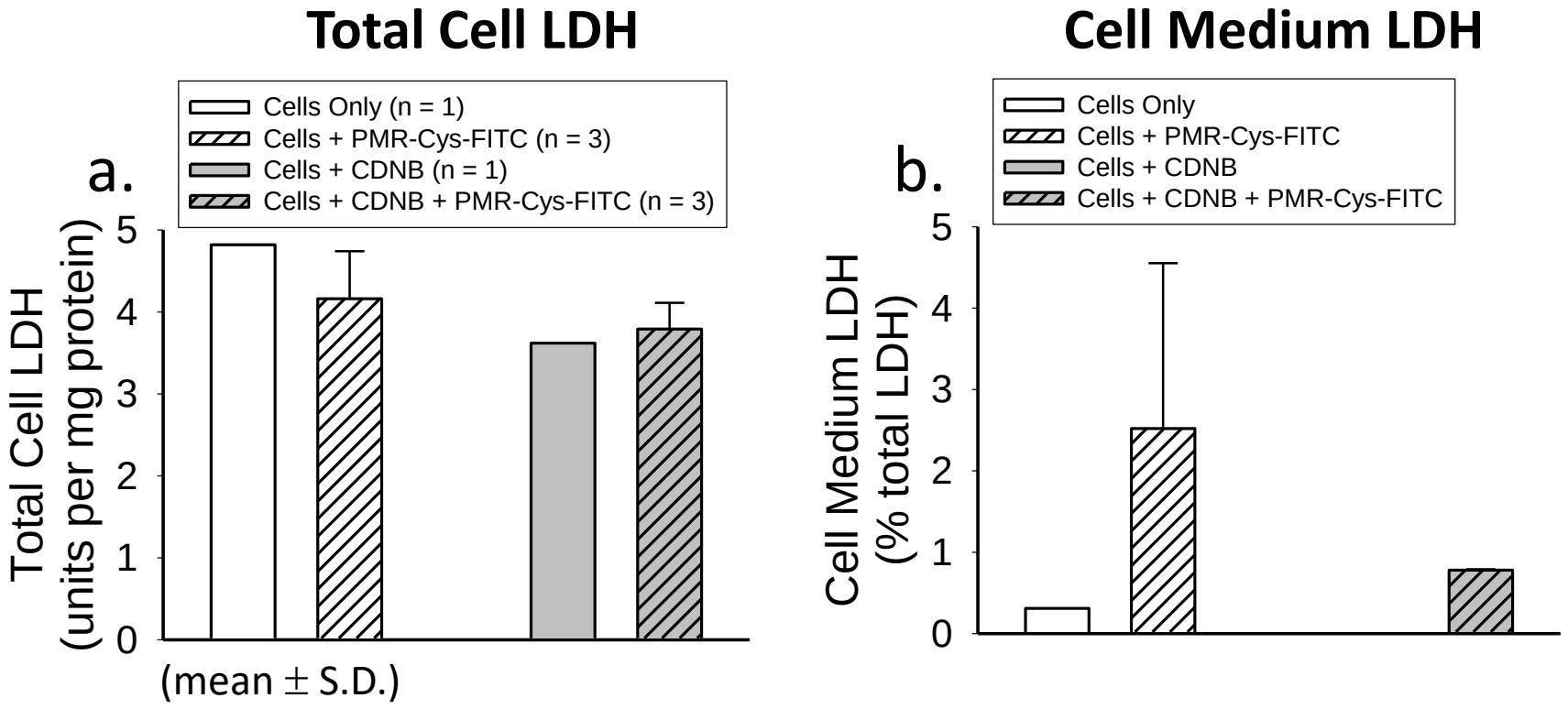
Cell Lysate and Cell Medium Total GSH + GSSG

cell medium volume was 2.5 mL; 25 μl added to GSH assay



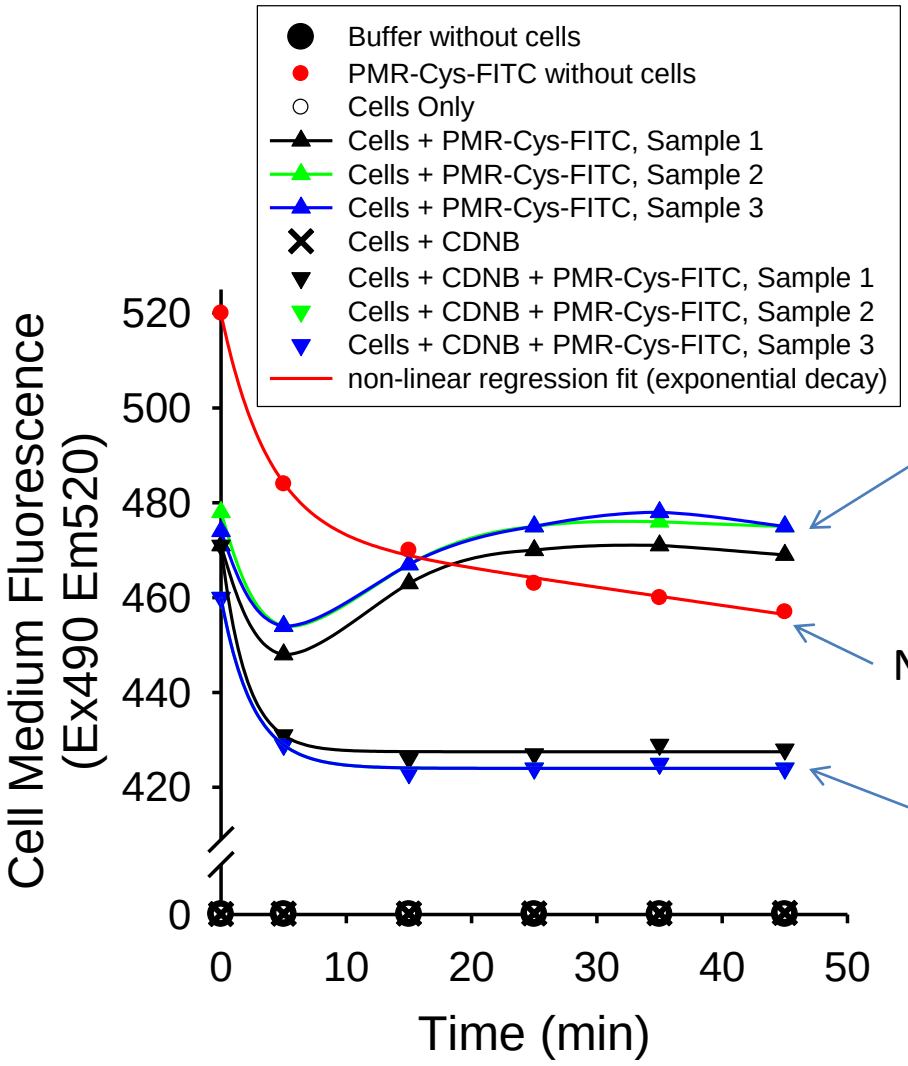
Release of intracellular lactate dehydrogenase to the cell medium: Cytotoxicity measurement

- ***CDNB and PMR-Cys-FITC additions to the cell media were not acutely toxic to the cells over the time course of the experiment, ,measured as LDH leakage into the medium***



Change in cell medium fluorescence with time

2.5 mL assay buffer without and with 0.2 μM PMR-Cys-FITC



Media signal reaches an equilibrium when cells are present, CDNB treated signal different from controls.

Non-specific cuvette binding along with reduced glutathione interaction? Can the reacted probe leave the cell so that the signal can be observed in the medium?

Non-specific cuvette binding?

Non-specific cuvette/cell binding?

Cell lysate fluorescence

There is no obvious difference in the cell lysate fluorescence values between control and CDNB-treated cells.