ThiolTracker™ Violet dye, a new 405 nm excitable fluorescent probe for detecting intracellular thiols in imaging-based assays

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Abstract

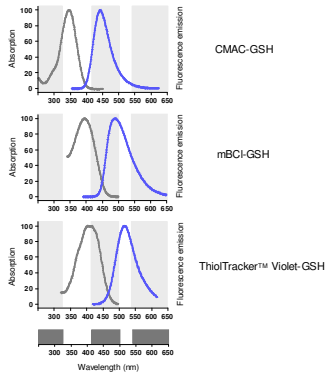
Reduced glutathione represents the majority of intracellular free thiols in mammalian cells. Among the many important physiological and pathological aspects involved, glutathione plays a central role in protecting mammalian cells against damage incurred by free radicals, oxidants, and electrophiles. Currently available fluorescent probes for detecting intracellular thiols in live cells share drawbacks ranging from low fluorescent intensity, species-dependence of labeling, to a requirement of UV excitation. We have developed a new fluorescent, cell-permeable, thiol reactive probe with the following properties: (1) Maximal excitation at 410 nm and maximal emission at 510 nm; (2) Labeled cells can be detected with standard fluorescence microscopy with a filter set for Hoechst 33342 (Ex/Em = 350 nm/461 nm) ; (3) Labeled cells can be detected flow or laser scanning cytometry with 405 nm laser excitation; (4) Signal intensity and cellular staining pattern are well retained after formaldehyde fixation and 0.5% (v/v) Triton® X-100 extraction. In formaldehyde fixed cells viewed with a fluorescence microscope with a standard filter set for Hoechst 33342, the new probe is estimated to be at least 10 times brighter than monochlorobimane (mBCl-glutathione, Ex/Em = 394 nm/490 nm) and at least 2 times brighter than 7-amino-4-chloromethylcoumarine (CMAC, Ex/Em = 353 nm/466 nm, not usable with 405 nm laser based instruments). By using automated microscopy and image analysis, responses of cultured human or murine cells to treatments with agents affecting the intracellular glutathione level, such as buthionine sulfoximine (BSO), diethyl maleate (DEM), various quinone toxicants, were readily quantifiable by labeling cells with this new probe.

Introduction

A new thiol reactive probe, ThiolTracker Violet dye:

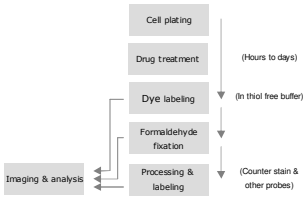
- Cell permeable
- React with thiols through irreversible covalent bond formation (bromomethyl group)
- Excitable with 405 nm laser or Xenon mercury light sources
- Viewable with filter sets for Hoechst 33342

Excitation/Emission Spectra

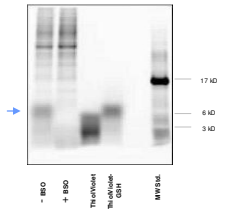


- Light area = excitation & emission bands of a typical filter set for Hoechst 33342 viewing
- CMAC: CellTracker™ Blue CMAC dye (7-amino-4-chloromethylcourmain)
- mBCl: monochlorobimane
- GSH: glutathione
- >> ThiolTracker Violet dye is well excited with 405 nm laser or non-laser based light sources for fluorescence microscopy

Labeling procedure

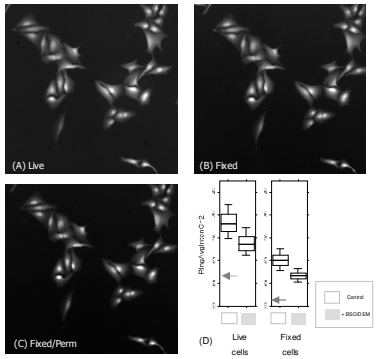


GSH is a major intracellular target of ThiolTracker Violet dye



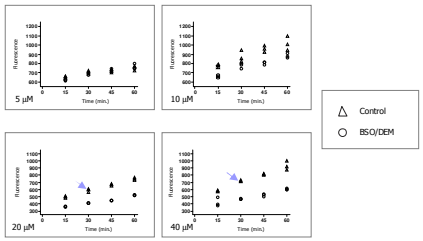
- U-2 OS cells (human, ATCC HTB-96)
- +/- 4 mM buthionine sulfoximine (BSO) overnight
- ThiolTracker Violet dye 40 mM in DPBS for 1 h at 37° C
- Electrophoresis of cell lysates: NuPAGE (12% Bis-Tris) in MES buffer
  - ThiolTracker Violet dye ("ThiolViolet") and "ThiolViolet"-GSH conjugate (position marked by the arrow) on separate lanes for comparison
  - Gel imaging: transilluminator with a SP filter for viewing fluorescence
- ▶ Glutathione is a major intracellular target of ThiolTracker Violet dye

Signal retention of ThiolTracker Violet dye: aldehyde fixation & Triton X-100 treatment



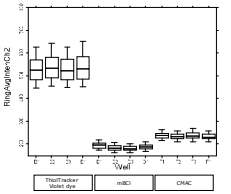
- U-2 OS cells (ATCC HTB-96)
- ThiolTracker Violet dye 20 mM in DPBS at 37° C for 30 min.
- (Imaging and analysis: Arrayscan VTI, 10X/0.3, XF93 filter set for Hoechst 33342)
- rinsed with DPBS and imaged (A);
- after 3.7% formaldehyde/PBS for 30 min. at room temp. (B);
- after 0.5% Triton X-100/PBS for 10 min at room temp. (C);
  - ▶ Retention of the staining pattern: overall nuclear and cytoplasmic stain
- (D) Fluorescent intensity of ThiolTracker Violet dye:
  - BSO 4 mM & DEM 1 mM for 2 hours; ThiolTracker Violet dye 20 µM labeling; Arrayscan VTI for imaging and analysis (10X/0.3; XF93)
  - Each group contained pooled cell-level data from 16 wells
  - Arrows = background signal levels
  - ▶ ~70% signal retention upon aldehyde fixation
  - ▶ Preservation of responses to BSO/DEM treatment upon fixation

Titration: Concentration and labeling time



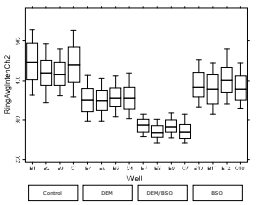
- HepG2 cells (human, ATCC HB-8065)
- Buthionine-sulfoximine (BSO) 4 mM & diethyl maleate (DEM) 1 mM treatment for 3 hours
- ThiolTracker Violet dye labeling:
  - concentrations: 5, 10, 20, or 40 mM
  - labeling time: 15, 30, 45, or 60 min
- Formaldehyde (3.7%) fixation
- Nuclear counter-stain (TO-PRO®-3 dye, 5 µM, for cell identification)
- Image acquisition & analysis: Arrayscan VTI (Thermo Fisher)
  - Three wells for each group; medians of cytplasmic fluorescence intensity of each well is plotted.
- ▶ Based on the results shown above and from other cells lines (not shown), labeling with 20 µM or 40 µM for 30 min. (arrows) is chosen for most experiments.

Comparison: ThiolTracker Violet dye, mBCl, & CMAC

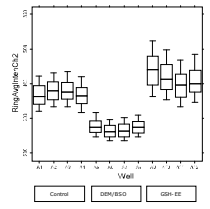


- U-2 OS cells (human, ATCC HTB-96)
- Dye labeling: 20 µM, 30 min. at 37 °C
  - ThiolTracker Violet dye
  - Monochlorobimane (mBCl)
  - CellTracker Blue CMAC dye (CMAC)
- Formaldehyde (3.7%) fixation
- Image acquisition & analysis: Arrayscan VTI (Thermo Fisher)
  - 10X/0.3, XF93 filter set
- Cell-level display
- ▶ Excellent signal with ThiolTracker Violet dye

GSH level modulation detected with ThiolTracker Violet dye



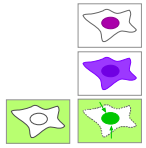
- HepG2 cells (human, ATCC HB-8065)
- Treatments:
  - DEM: dimethyl maleate 1 mM, 3 hours
  - DEM/BSO: DEM 1 mM & buthione-sulfoximine 4 mM, 3 hours
  - BSO: BSO 4 mM, overnight
- ThiolTracker™ Violet 20 µM, 30 min. at 37 °C
- Formaldehyde (3.7%) fixation
- Image acquisition & analysis: Arrayscan VTI
- Cell-level data display of cytoplasmic staining intensity
- ▶ Decreased ThiolTracker Violet dye signals with glutathione reducing treatments



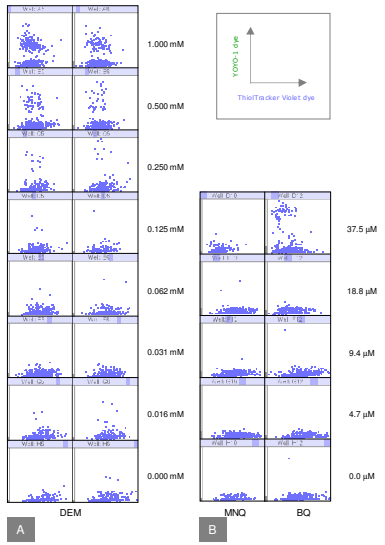
- U-2 OS cells (ATCC HTB-96)
- Treatments:
  - DEM/BSO: DEM, 1 mM & BSO, 4 mM, 2 hours
  - GSH-EE: glutathione ethyl ester, 10 mM, 2 hours
- ThiolTracker Violet dye 20 µM, 30 min. at 37 °C
- Formaldehyde (3.7%) fixation
- Image acquisition & analysis: Arrayscan VTI
- Cell-level data display of cytoplasmic staining intensity
- ▶ Elevated ThiolTracker Violet dye signals with GSH loading

Multiplexing with YOYO®-1 dye, a probe for plasma membrane integrity

Assay principle



Dye	Ex/Em	Function
TO-PRO-3 dye	642/661	Cell identification
ThiolTracker Violet dye	410/510	Thiol content
YOYO-1 dye	491/509	Plasma membrane integrity



- Clone 9 liver cells (rat, ATCC CRL-1439)
- Treatments for 2 hours:
  - 2-methyl-1,4-napthoquinone (MNQ)
  - p-benzoquinone (BQ)
  - diethyl maleate (DEM)
- ThiolTracker Violet dye, 40 µM, & YOYO-1 dye, 1 µM in DPBS for 30 min. at 37 °C
- Formaldehyde (3.7%) fixation
- Nuclear stain: TO-PRO-3 dye, 5 µM (for cell identification)
- Image acquisition & analysis: Arrayscan VTI (Therom-Fisher)
- Data exploration & display: Panmo (The Chi-Square Works, Inc.)
  - Cell-level data display: scatter plot trellis, one graph per well
    - X-axis: cytoplasmic stain, ThiolTracker Violet dye (for thiol content)
    - Y-axis: nuclear stain – YOYO-1 dye (for plasma membrane integrity)
  - (A) Concentration step gradient of diethyl maleate (DEM)
  - (B) Concentration step gradient of MNQ and BQ
- ▶ At high concentrations of toxicants, note the reduced thiol content (left shift of data points) and increase in number of cells with compromised plasma membrane (up shift of data points).

Conclusions

A new thiol reactive probe, ThiolTracker Violet dye:

- much brighter than mBCl and CMAC, two commonly used probes in studying intracellular GSH;
- excellent staining in both human and murine cells;
- suitable for use with 405 nm laser based instrument or traditional fluorescence microscopes; and
- can be used in combination with other probes.