

# A New Approach for the Detection of Intracellular Glutathione by Fluorescence Microscopy and Flow Cytometry

Yih-Tai Chen<sup>1</sup>, Robert Aggeler<sup>1</sup>, Jolene Bradford<sup>1</sup>, Yexin Wu<sup>1</sup>, Iain Johnson<sup>1</sup>, Hee-Chol Kang<sup>1</sup>, Kyle Gee<sup>1</sup>, Celia Quijano<sup>2</sup>, Florencia Irgoin<sup>2</sup>, Rafael Raddi<sup>2</sup>, Michael Janes<sup>1</sup>

<sup>1</sup>Invitrogen Corporation • 29851 Willow Creek Road • Eugene, Oregon, 97402 • USA

<sup>2</sup>Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República • Avenida General Flores 2125, CP 11800 • Montevideo • UY

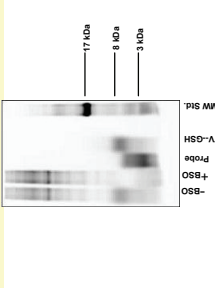
## Abstract

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant low molecular weight thiol in mammalian cells, ranging from 1-10 mM under normal conditions. Along with small molecular weight proteins such as thioredoxin and glutaredoxin, GSH provides critical modulation of the intracellular redox environment. While GSH is oxidized to glutathione disulfide (GS-SG) via GSH-dependent peroxidases in response to oxidants, it is recycled by NADPH-dependent GS-SG reductase.

Sub-cellular detection and localization of GSH is important in understanding the modulation of redox status, the effects of drugs, and the mechanisms of detoxification. Furthermore, differences in GSH levels in response to oxidant stress in subpopulations of cells have been reported, underscoring the importance of detection methods amenable to flow cytometry and automated fluorescence microscopy. Current methods for fluorescence detection of intracellular thiols include monochlorobimane (mBCl) and CellTracker™ Blue (CMAC) dye but these approaches are limited given low fluorescence signals and the requirement for UV excitation, respectively. Antibodies have also been used to detect intracellular GSH but this approach is limited to fixed cells and raises questions regarding access to the nuclear GSH pool.

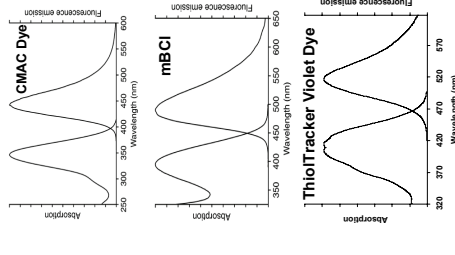
We have recently developed a new fluorescent probe for the detection of intracellular thiols, ThioTracker™ Violet dye (TTV). While this probe may be excited with UV wavelengths of 400 nm, ideal for instruments equipped with violet lasers. The probe is permeable to live cells, survives formaldehyde fixation and detergent extraction, and compared to other probes, is brightly fluorescent upon binding GSH. Along with these attributes, the highly thiol-reactive nature of this probe provides both utility and flexibility in GSH depletion assays. The current study describes a comparison of intracellular GSH detection approaches which demonstrates useful improvements for flow cytometry and fluorescence microscopy that are applicable to studies in oxidative stress and a multitude of other areas in biology wherein redox modulation is important.

## Figure 1 – GSH is a major target of ThioTracker Violet dye



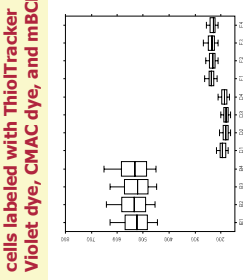
**Figure 1.** U2-OS cells were treated overnight with or without 4 mM buthionine sulfoximine (BSO) before labeling with 10 μM ThioTracker Violet dye in DPBS for 1 hour at 37°C. Cell lysates were analyzed by Western blotting in the presence of protease inhibitor. Lysates were analyzed by NUPAGE (12% Bis-Tris) in MES buffer. As controls, TTV and the GSH conjugate of TTV were run on the same gel. The gel was then scanned with a transilluminator using a SP filter to visualize the fluorescent bands.

## Figure 2 – Spectral properties of CMAC dye, mBCl, and ThioTracker Violet dye



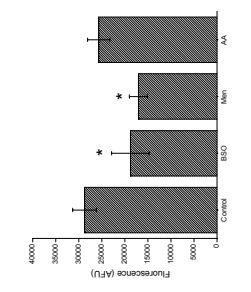
**Figure 2.** Fluorescence absorption and emission spectra for CMAC, mBCl, and TTV obtained by scanning probes in 0.2 M phosphate buffer, pH 7.4. Absorption and emission spectra were obtained by scanning probes in 0.2 M phosphate buffer, pH 7.4. Absorption and emission spectra were obtained by scanning probes in 0.2 M phosphate buffer, pH 7.4. Absorption and emission spectra were obtained by scanning probes in 0.2 M phosphate buffer, pH 7.4.

## Figure 3 – Comparison of fluorescence signal intensity in cells labeled with ThioTracker Violet dye, CMAC dye, and mBCl



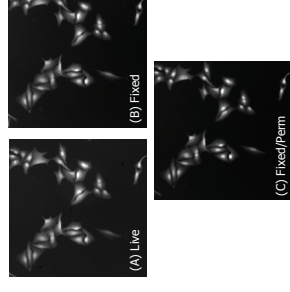
**Figure 3.** U-2 OS cells were incubated with 20 μM TTV in DPBS, CMAC, or mBCl in PBS at 37°C for 30 min. before fixation with 3.7% formaldehyde/PBS for 30 min. RT images were acquired and analyzed with an Arvo-Scyto V1000 microscope equipped with an 10X/0.45 objective and standard filter for DAPI. Fluorescence signal intensities of cytoplasmic area of individual cells were measured and the results were plotted.

## Figure 4 – Detection of intracellular GSH depletion with ThioTracker Violet dye by microplate fluorimetry



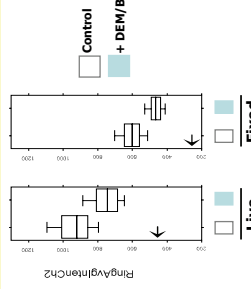
**Figure 4.** BAECS were incubated for 2 hours in media (Control) or with the addition of 10 μM antimycin A (AA), 10 μM Menadione (M) or for 24 hours in 0.2 mM BSO. Cells were then incubated with TTV (10 μM) for 30 min at 37°C and washed. Intracellular thiol levels were assessed by measuring the fluorescence in a microplate reader (λ<sub>ex</sub> = 405 nm, λ<sub>em</sub> = 520 nm).

## Figure 5 – ThioTracker Violet dye is formaldehyde-fixable and survives detergent extraction



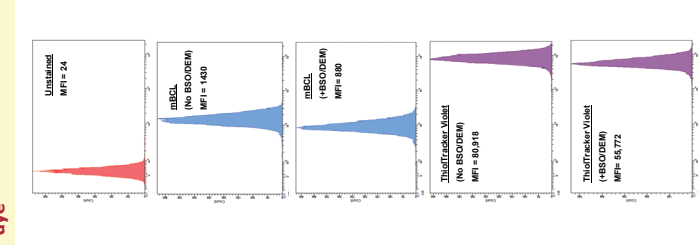
**Figure 5.** U-2 OS cells were incubated with 20 μM TTV in DPBS for 30 min. Cells were then fixed with 4% formaldehyde for 30 min, followed by fixation with 3.7% formaldehyde/PBS for 30 min, at RT, rinsed and imaged (B), followed by extraction with 0.5% Triton X-100/DPBS for 10 min at RT, rinsed and imaged (C). Image acquisition was performed as described in Figure 3.

## Figure 6 – Detection of GSH depletion with ThioTracker Violet dye by microscopy in live and fixed cells



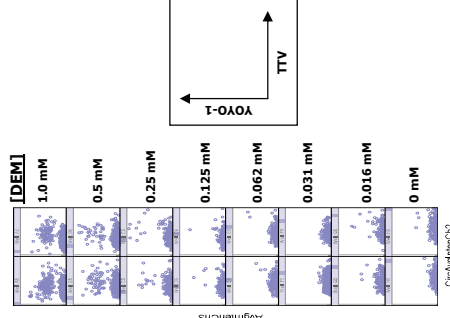
**Figure 6.** U-2 OS cells were treated with DEM (diethyl maleate, 1 mM) & BSO (4 mM) for 2 hours before labeling with TTV (20 μM) for 30 minutes. Imaging was performed before and after fixation as described in Figure 3. Fluorescence signals from the cytoplasmic area of cells were measured. Cell level data were plotted for 16 wells for each sample. Arrowheads show the background signal levels in images.

## Figure 7 – Flow cytometric detection of GSH depletion with ThioTracker Violet dye



**Figure 7.** Jurkat cells were treated with or without 4 mM BSO and 1 mM DEM (diethyl maleate) in normal growth medium for 2 hours. After centrifugation, cells were resuspended in DPBS containing mBCl or TTV (20 μM) and kept at 37°C for 30 min. Cells were centrifuged and resuspended with PBS containing 1% BSA before analysis with a BD FACScan cytometer. Excitation with a 525/50 band pass filter were used to collect 10,000 total cellular events for each sample. Median Fluorescence Intensity (MFI) was measured.

## Figure 8 – ThioTracker Violet dye multiplexed with other fluorescent probes: Toxicity assay with YOYO-1 for plasma membrane integrity



**Figure 8.** Clone 9 rat liver cells on a collagen I coated microplate were treated with DEM (1 mM) in complete growth medium for 2 hours before staining with TTV (20 μM) and YOYO-1 (1 μM) for 30 minutes at 37°C. Cells were then washed, fixed, and imaged as described in Figure 3. The probe signals of TTV and YOYO-1 in the nuclear area of individual cells were measured using standard DAPI and FITC filter sets, respectively. The cell level data were plotted as a scatter plot.

## Conclusions

- ThioTracker-Violet dye is...
  - Highly reactive to thiols with GSH as a major target
  - Excitable with UV and violet light using standard filter sets or lasers
  - Brighter than mBCl and CMAC, enabling intracellular thiol detection by fluorescence microscopy
  - Useful in GSH depletion assays by imaging, flow cytometry, and microplate fluorimetry
  - Amenable to multiplex assays, including cytotoxicity