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Rapid and Thiol-Specific High-Throughput Assay for Simultaneous Relative Quantification of Total Thiols, Protein Thiols, and Nonprotein Thiols in Cells

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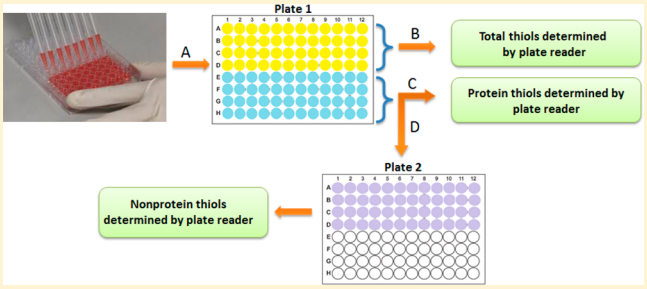
ABSTRACT: Thiol groups in biological molecules play a significant role in various physiological functions and pathological conditions. Thiols are divided into two major groups: protein thiols and nonprotein thiols. Numerous methods have been reported for thiol assays. Most of these methods have been developed for glutathione, the principal nonprotein thiol, despite the fact that cellular protein thiols are more abundant than glutathione. Further, these methods usually involve a process of biological sample preparation followed by a separation method, and they are time-consuming. We reported previously a series of thiol-specific fluorogenic benzofurazan sulfides. These nonfluorescent benzofurazan sulfides react rapidly and specifically with a thiol to form a strong fluorescent thiol adduct. The rapid reaction, thiol-specific and fluorogenic nature of the sulfides successfully yielded an application of one of the sulfides for relative quantitation of total thiols in live cells through fluorescence microscopy. In this work, we employed the same compound to develop the first high-throughput method for simultaneous monitoring of protein thiols, nonprotein thiols, and total thiols in cells in a 96-well plate on a fluorescence microplate reader at λ_{ex} = 430 nm and λ_{em} = 520 nm, respectively. The method is rapid and sensitive, and has been validated by an HPLC thiol assay method. The method can detect thiols with cell concentrations as low as 500 cells/well. We also demonstrated that the method can readily monitor changes in cellular thiol levels. Although the method cannot provide an absolute quantification for thiols because fluorescence intensity of different thiol adducts varies, it provides an accurate measurement of relative quantification, relative to the control. The method will be a valuable tool in thiol-related biomedical/pharmaceutical research.



The thiol functional group (−SH or sulfhydryl) present in biomolecules plays a significant role in various physiological functions and pathological conditions through its nucleophilicity, reduction property, and chelation property.[1] Thiol levels in the biological system are affected by a number of factors[2] and are often used as a parameter to reflect various physiological and pathological states such as aging and neurodegenerative diseases.[3−6] Numerous analytical methods have been reported to measure thiol levels, mostly glutathione (GSH) levels, in the biological system.[7−13] GSH is a three-amino acid peptide and serves as the major antioxidant as well as the major endogenous molecule involved in the detoxification of the biological system.

Thiols in the biological system can be divided into two major groups: protein thiols and nonprotein thiols or small molecule thiols. The nonprotein thiols include GSH, homocysteine, cysteine, and other cysteine-containing low molecular weight peptides with GSH as the principal nonprotein thiol. Although most protein thiols are related to protein structure and function, nonprotein thiols, primarily GSH, serve as the major redox buffer to maintain a reducing cellular environment. Nonprotein thiols protect protein structure and function through prevention of protein thiol oxidation. As a result, nonprotein thiols are more sensitive to changes in cellular oxidative stress. Most analytical methods for thiol determination have been developed to measure GSH. The GSH content is then used to reflect overall thiol status because measurement of protein thiols is more technically challenging, despite the fact that they are more abundant than GSH.[9,14−16] The analytical methods for GSH usually involve a procedure of biological sample preparation, GSH derivatization, and use of a separation technique such as HPLC, capillary electrophoresis (CE), or LC/MS.[7−13] Therefore, these analytical methods tend to be time-consuming. In addition, these methods usually require a relatively large sample volume. A few high-throughput assays for GSH,[17,18] homocysteine and cysteine,[19] cysteinylglycine,[20] and total thiols with a thiol-selective agent, 5-(bromomethyl)fluorescein,[21] have been reported. However, high-throughput methods for simultaneous measurement of protein thiols and nonprotein thiols are lacking.

We recently reported a series of benzofurazan sulfides as thiol-specific fluorogenic agents.[22] These reagents react rapidly

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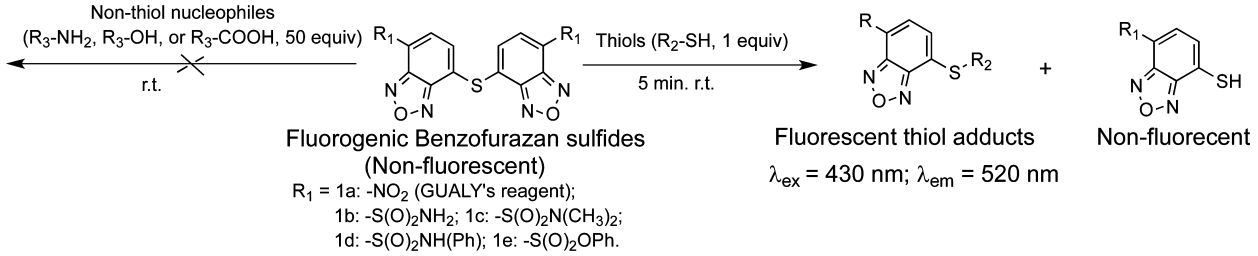
Scheme 1. Thiol-Specific Reaction of the Benzofurazan Sulfides[22]



with a thiol through a thiol-specific thiol−sulfide exchange reaction (Scheme 1). Among these agents, benzofurazan sulfide 1a (Scheme 1) was employed for total thiol imaging in live cells. The reagent is now named GUALY's reagent. As a fluorogenic agent, GUALY's reagent itself exhibits very minimal fluorescence but forms a thiol adduct with strong fluorescence after reacting with a thiol. The formed fluorescent thiol adducts derived from reaction with different thiols (protein thiols and nonprotein thiols) exhibited very similar excitation and emission spectra with λ_{ex} and λ_{em} at 430 and 520 nm, respectively, indicating these two wavelengths are appropriate for detection of various thiols in the biological system.[22] With a 1:1 molar ratio, GUALY's reagent reacts rapidly, within 5 min, with a thiol at ambient temperature while exhibiting no reaction with other biologically relevant nucleophilic functional groups such as $-NH_2$, $-OH$, and $-COOH$ even at a molar ratio of 50:1.[22] The rapid and thiol-specific reaction, the fluorogenic nature of GUALY's reagent, and a large Stokes effect (~100 nm) of the fluorescent thiol adducts has resulted in a successful application of the compound as the first reagent for total thiol imaging and relative quantification in live cells through fluorescence microscopy.[22]

In the present work, we employed GUALY's reagent to develop a high-throughput assay in a 96-well plate for protein thiols, nonprotein thiols, and total thiols in cells. The assay was validated by a reported HPLC thiol assay method. The assay is rapid, convenient, sensitive, and reliable, and provides simultaneous monitoring of changes in protein thiols, nonprotein thiols, and total thiols. It can detect thiols using cell concentrations as low as 500 cells/well, and the fluorescence intensity increased linearly over cell numbers ranging from 500 cells/well to 15 000 cells/well. The assay will be a useful tool in the study of the physiological functions and pathological conditions of protein thiols, nonprotein thiols, and total thiols.

EXPERIMENTAL SECTION

Materials and Solutions. Benzofurazan sulfide 1a (GUALY's reagent) was synthesized according to a literature reported procedure.[22] GUALY's reagent stock solution was prepared as a 1 mM solution in acetonitrile. GUALY's reagent derivatizing solution (0.1 mM) was prepared as a 1:10 (v/v) dilution of the stock solution in phosphate buffer (0.45 M, pH 7.9) containing 2% SDS. The cell lysis solution was a solution of 5% (w/v) sulfosalicylic acid in deionized water containing 0.1% (v/v) Triton X-100.[18] The 3% (w/v) sulfosalicylic acid solution was prepared by dissolving sulfosalicylic acid in deionized water. *N*-Ethylmaleimide (NEM) was prepared as a 5 mM stock solution in deionized water. Stock solutions of GSH (5 mM), cupric chloride (0.7 mM), calcium chloride (341 mM), zinc sulfate (4.3 mM), potassium phosphate (204 mM), magnesium sulfate (91.4 mM), ferrous sulfate (2 mM), and ferric ammonium sulfate (1.2 mM) were prepared in deionized water. All reagents and solvents were purchased from Sigma-Aldrich unless otherwise specified.

Cell Culture. Exponentially growing NCI-H226 cells (human lung cancer) were obtained from the National Cancer Institute and cultured in RPMI 1640 growth medium supplemented with 10% FBS, 100 units/mL penicillin (Mediatech, Inc., Herndon, VA) and 100 μg/mL streptomycin (Mediatech, Inc., Herndon,VA) in a humidified atmosphere containing 5% CO_2 at 37 °C. All cell culture materials were purchased from Atlanta Biologicals (Flowery Branch, GA) unless otherwise specified.

Time Required for Thiol Derivatization by GUALY's Reagent. NCI-H226 cells in RPMI growth medium were plated in a 96-well plate at a density of 15 000 cells/150 μL/well and were allowed to attach in a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h. The medium was removed, and the wells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) before the GUALY's reagent derivatizing solution (100 μL/well) was added. The plate was covered with aluminum foil and shaken at room temperature on a microplate shaker at speed 6 for various time points (1, 2, 3, 4, 5, 10, and 15 min) before the fluorescence intensity was read on a SpectraMax M2 microplate reader using 430 and 520 nm as λ_{ex} and λ_{em}, respectively, with a cutoff wavelength at 495 nm.

Cell Number Requirement and Linearity. NCI-H226 cells were prepared at a concentration of 100 000 cells/mL in RPMI growth medium before dispensing to a 96-well plate at densities varying from 500 cells/150 μL/well to 15 000 cells/150 μL/well. The plate was incubated at 37 °C for 24 h. The medium was removed, and the wells were washed with deionized water twice before the GUALY's reagent derivatizing solution (200 μL/well) was added. The plate was covered with aluminum foil and shaken on a microplate shaker at speed 6 for 5 min at room temperature before fluorescence determination as described above.

High-Throughput Assay of Total Thiols, Protein Thiols, and Nonprotein Thiols in Cells. Cells at a density of 15 000 cells/150 μL/well in a 96-well plate in RPMI growth medium were allowed to attach for 24 h. After 24 h, the medium was discarded, and the attached cells were washed once with DPBS before fresh RPMI growth medium containing different concentrations of NEM was added. The 96-well plate was incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 3 h before the medium was removed, and cells were washed twice with DPBS (150 μL/well). The GUALY's reagent derivatizing solution was added to each well in the top four rows of the plate (100 μL/well) for total thiol determination while the cell lysis solution was added to the bottom four rows (50 μL/well) for nonprotein thiol and protein thiol determination. The plate was covered with

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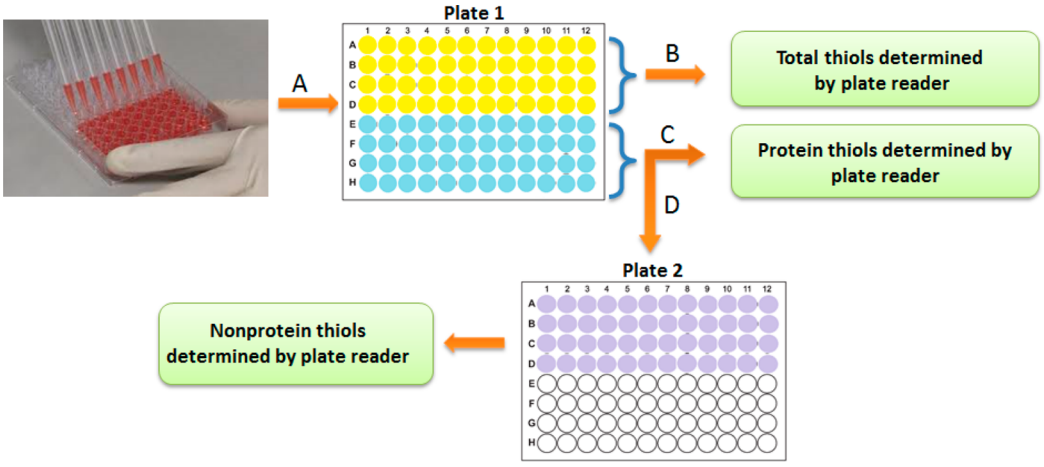

Figure 1. Work flow diagram for the high-throughput assay. (A) Top four rows of plate 1 for total thiol assay while bottom four rows for protein thiol and nonprotein thiol assay. (B) Cells were lysed, dissolved in 2% SDS, and thiols were derivatized by GUALY's reagent followed by fluorescence determination at λ_{ex} = 430 nm and λ_{em} = 520 nm. Cells in the bottom four rows were lysed with the cell lysis solution, centrifuged, and the supernatant was transferred to plate 2 and derivatized by GUALY's reagent for nonprotein thiol determination (D) while the remaining protein residues in the bottom four rows of plate 1 were washed with cell lysis buffer to remove nonprotein thiols, redissolved with 2% SDS, and derivatized by GUALY's reagent before fluorescence determination for protein thiols (C).

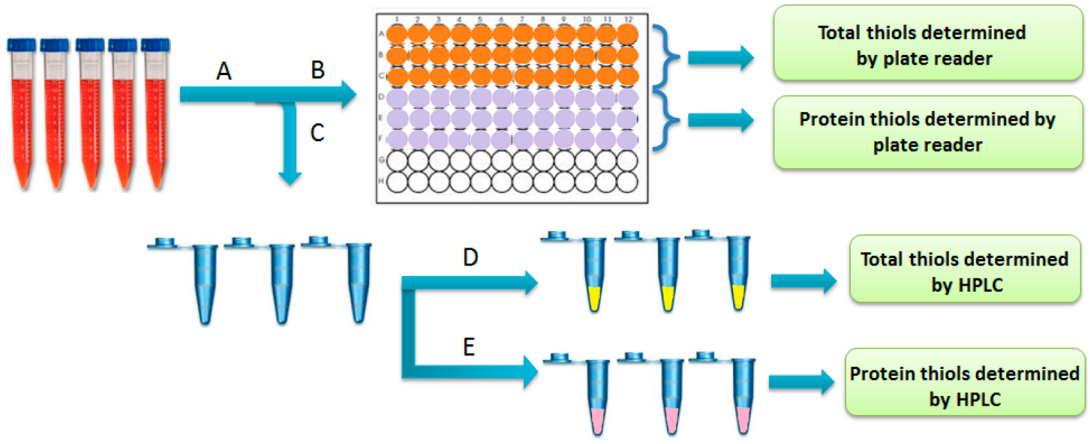

Figure 2. Work flow diagram for validation of the high-throughput assay by HPLC. (A). Cells (1×10^6 cells/10 mL/tube) treated with various concentrations of NEM; (B). 0.6 mL of the 10 mL cell suspension was dispensed at 15 000 cells/100 μL/well to a 96-well plate for 6 wells with three wells for total thiol determination and three wells for protein thiol determination using the high-throughput assay; (C). 9.4 mL of the 10 mL cell suspension was centrifuged. The cell pellets were resuspended in 250 μL of 3% sulfosalicylic acid solution. Out of the 250 μL, 100 μL was used for total thiol assay (D) by HPLC; and 150 μL was used for protein thiol assay by HPLC (E).

aluminum foil and shaken at speed 6 for 10 min. The fluorescence intensity of the top four rows was read as described above. The plate was then centrifuged for 10 min at 4000 rpm (18 g) at 4 °C. The supernatant (~50 μL/well) from the bottom four rows was transferred to another 96-well plate for nonprotein thiol determination. The bottom four rows were washed with cell lysis buffer (50 μL), shaken for 10 min at speed 6, and centrifuged at 4000 rpm (18 g) at 4 °C for 10 min. The supernatant was discarded to remove any residual nonprotein thiols. Each well was added with the GUALY's reagent derivatizing solution (100 μL/well). The fluorescence intensity was obtained as described above. For the nonprotein thiol plate, each well was added with the GUALY's reagent stock solution (20 μL) along with 2% SDS sodium phosphate buffer (0.5 M, pH 7.9, 130 μL). The plate was covered with aluminum foil and shaken for 10 min on a microplate shaker at speed 6 before fluorescence intensity was read as described above. A work flow diagram for the high-throughput assay is presented in Figure 1.

Validation of the High-Throughput Assay by an HPLC Thiol Assay Method. *i. Validation of the High-Throughput Assay for Protein Thiols and Total Thiols.* NCI-H226 cells at a density of 1.5×10^5 cells/mL in 10 mL of RPMI growth medium without FBS were transferred to a 15 mL cell culture tube. Different concentrations of NEM in individual tubes were prepared through the addition of different volumes of the NEM stock solution (5 mM). The tube was vortexed, followed by incubation in a humidified atmosphere containing 5% CO_2 for 3 h at 37 °C with the tube loosely capped. The tube was shaken every hour to avoid settling of the cells. At the end of 3 h, the tube was centrifuged at 2500 rpm for 5 min. The cell pellets were washed with DPBS (2 mL) and centrifuged, and the DPBS was discarded. An additional 10 mL of DPBS was added to each tube to resuspend the cells at a concentration of 1.5×10^5 cells/mL. Out of the 10 mL cell suspension, 0.6 mL was dispensed to a 96-well plate at a density of 15 000 cells/100 μL/well for six wells with three wells for total thiol determination and three wells for protein thiol determination

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as described above. The rest of the cell suspension (approximately 9.4 mL) was centrifuged at 2500 rpm for 5 min to yield cell pellets. To the collected cell pellets were added 250 μL of 3% sulfosalicylic acid solution followed by sonication for 15 min before being transferred to a 1.5 mL Eppendorf tube for protein thiol and total thiol determination using the HPLC assay as described below.

HPLC Assay. The HPLC assay for both total thiols and protein thiols followed a literature reported procedure.[9] Briefly, the 250 μL HPLC sample was divided into two parts with 100 μL for the total thiol assay and 150 μL for the protein thiol assay. The sample for the total thiol assay (100 μL) was first derivatized by 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) as described below. The sample for the protein thiol assay (150 μL) was centrifuged at 14 000 rpm for 5 min. The collected protein pellets were washed once with 3% sulfosalicylic acid (200 μL) followed by addition of 3% sulfosalicylic acid (100 μL) before derivatization by DTNB. For DTNB derivatization, an aliquot of 25 μL from the above samples was transferred to a 1.5 mL microcentrifuge vial containing DTNB (19.8 mg/mL, 17.5 μL), *p*-aminobenzoic acid (10 mg/mL, 25 μL, internal standard), phosphate buffer (0.15 M, pH 7.5, 280 μL), and HCl (10 M, 12.5 μL) with a total volume of 340 μL. The sample was vortexed and centrifuged at 14 000 rpm for 1 min. The total thiols and protein thiols were quantified through HPLC quantification of the released 5-thio-2-nitrobenzoic acid (TNB) as reported.[9] A work flow diagram for validation of the high-throughput assay by HPLC is presented in Figure 2.

ii. Validation of the High-Throughput Assay for Nonprotein Thiols. Validation of the high-throughput assay for nonprotein thiols by HPLC followed the same procedure as that for protein thiols and total thiols except lower NEM concentrations were employed.

Effects of Metal Ions on the Assay. GSH (5 mM, 10 μL) was added to the GUALY's reagent derivatizing solution (~970 μL) followed by addition of a metal stock solution (1.7−24 μL) for a total volume of 1 mL. The solution was covered by aluminum foil and shaken at room temperature for 10 min. The solution was transferred to a 96-well plate at a density of 100 μL/well before the fluorescence intensity was read on a SpectraMax M2 microplate reader using 430 and 520 nm as λ_{ex} and λ_{em} respectively with a cutoff wavelength at 495 nm.

RESULTS AND DISCUSSION

Experimental Conditions for the High-Throughput Assay. GUALY's reagent has been demonstrated to react rapidly and specifically with nonprotein thiols, thiols on BSA, and in cell homogenates.[22] To ensure all thiols in the cell lysate employed in this work (15 000 cells/well) would be completely converted into fluorescent thiol adducts by GUALY's reagent, a study to determine the time required for thiol derivatization was conducted. Cells at a density of 15 000 cells/well in a 96-well plate were treated with the GUALY's reagent derivatizing solution (100 μL/well). The 2% SDS in the GUALY's reagent derivatizing solution has been found to effectively and completely lyse the cells and also dissolve the lysate into a solution, a key for successful measurement of protein thiols. The plate was covered with aluminum foil before the fluorescence intensity was read at different time points over a period of 15 min on a SpectraMax M2 microplate reader. As presented in Figure 3, the fluorescence intensity increased over time for up to 3 min, then remained constant indicating that 3 min were needed to derivatize thiols in the wells and that the fluorescence intensity is stable for at least 15 min. On the basis of the results, 10 min were employed for cellular thiol derivatization for the high-throughput assay.

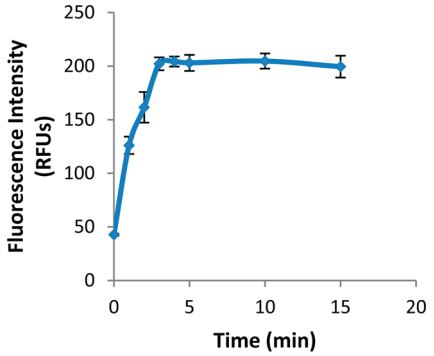

Figure 3. Reaction time determination for cell lysate thiol derivatization by GUALY's reagent. Cells at a density of 15 000 cells/150 μL/well in a 96-well plate were treated with the GUALY's reagent derivatizing solution. The fluorescence intensity was read on a SpectraMax M2 microplate reader using 430 and 520 nm as λ_{ex} and λ_{em} respectively with a cutoff wavelength at 495 nm. The data are expressed as the means ± SD from three wells.

After establishing the thiol derivatization time, we checked the cell detection limit and linearity of the assay. Cells in a 96-well plate ranging from a density of 500 cells/well to 15 000 cells/well were treated with GUALY's reagent in the same manner as presented in the high-throughput assay described below except that NEM treatment was not conducted. Our results demonstrated that the high-throughput assay was able to detect protein thiols, nonprotein thiols, and total thiols with a cell concentration as low as 500 cells/well, and that the detection was linear over cell densities ranging from 500 cells/well to 15 000 cells/well. Figure 4 presents the data derived from nonprotein thiol (A), protein thiol (B), and total thiol (C) determination. Table 1 is a tabular presentation of Figure 4. It shows that the sum of the fluorescence intensity from nonprotein thiols and protein thiols matched well with the fluorescence intensity observed from total thiols—an assurance that all thiols in the cells were completely derivatized by GUALY's reagent.

Detection of Cellular Thiol Changes by the High-Throughput Assay. Next we investigated whether the high-throughput assay could detect changes in cellular total thiols, protein thiols, and nonprotein thiols. NEM was employed to modulate intracellular thiol concentration. NEM blocks thiols through covalent bond formation and is a common tool used to modulate thiol concentration.[23] To ensure enough latitude for thiol concentration modulation by NEM, 15 000 cells/well were employed as the cell density in the experiment. After 24 h of attachment, cells at a density of 15 000 cells/well in a 96-well plate were first treated with various concentrations of NEM. After the treatment, the top four rows of the 96-well plate were used for total thiol determination while the bottom four rows were used for the determination of protein thiols and nonprotein thiols. The GUALY's reagent derivatizing solution was added to wells for total thiol determination before fluorescence intensity determination. Wells for protein thiols and nonprotein thiols were first treated with the cell lysis solution. The lysis solution has been demonstrated to effectively separate protein thiols and nonprotein thiols in attached cells by extracting nonprotein thiols into the

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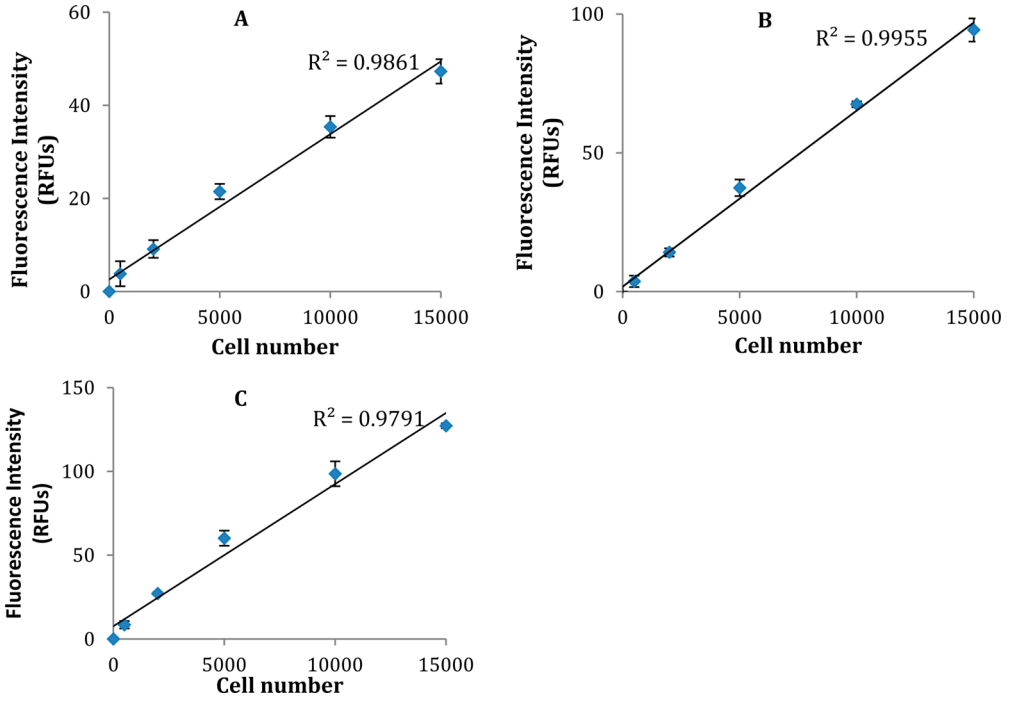

Figure 4. Cell number detection limit and linearity of the high-throughput assay for nonprotein thiols (A), protein thiols (B), and total thiols (C). Attached cells ranging from 500 cells/well to 15 000 cells/well in a 96-well plate were treated with the GUALY's reagent derivatizing solution before fluorescence reading on a SpectraMax M2 microplate reader using 430 and 520 nm as λ_{ex} and λ_{em}, respectively, with a cutoff wavelength at 495 nm. The fluorescence intensity derived from the wells with no cells served as a blank and was subtracted from the fluorescence intensity of each sample. Each data point was an average of fluorescence intensity derived from three wells. The fluorescence intensity is presented as relative fluorescence units (RFUs). The data are expressed as the means ± SD from three wells.

Table 1. Fluorescence Intensity of Nonprotein Thiols, Protein Thiols, and Total Thiols Derived from Different Number of Cells

cell number	0	500	2000	5000	10 000	15 000
observed average fluorescence intensity for nonprotein thiols (RFUs)[a]	0	3.8 ± 3.0	9.1 ± 1.0	21.5 ± 1.2	35.4 ± 1.3	47.3 ± 1.6
observed average fluorescence intensity for protein thiols (RFUs)[a]	0	3.7 ± 1.7	14.1 ± 1.3	37.4 ± 3.0	67.5 ± 1.2	94.3 ± 4.6
observed average fluorescence intensity for total thiols (RFUs)[a]	0	8.5 ± 2.1	27.1 ± 1.8	60.2 ± 3.6	98.6 ± 7.0	127.3 ± 1.5
calculated average fluorescence intensity for total thiols[b] (RFUs)[a]	0	7.5 ± 2.4	23.3 ± 1.1	58.9 ± 2.1	102.9 ± 1.2	141.6 ± 3.1

[a]RFUs: relative fluorescence units, n = three repeat wells from the same 96-well plate. [b]Derived from the equation: $(A + B)/2$. A = observed average fluorescence intensity for nonprotein thiols; B = observed average fluorescence intensity for protein thiols.

Table 2. Determination of Protein Thiols, Nonprotein Thiols, and Total Thiols in Cells Treated with Different Concentrations of NEM

NEM concentration (μM)	0	5	10	20	30	60	200	500
observed nonprotein thiols (% of control)[a]	100% ± 5%	97% ± 4%	80% ± 8%	45% ± 8%	3% ± 4%	3% ± 3%	1% ± 4%	6% ± 1%
observed protein thiols (% of control)[a]	100% ± 5%	103% ± 2%	88% ± 5%	72% ± 8%	43% ± 6%	29% ± 6%	12% ± 5%	7% ± 2%
observed total thiols (% of control)[a]	100% ± 7%	95% ± 5%	81% ± 7%	54% ± 9%	28% ± 5%	21% ± 2%	9% ± 1%	6% ± 5%
calculated total thiols (% of control)[b]	100% ± 5%	100% ± 3%	84% ± 7%	59% ± 8%	23% ± 5%	16% ± 5%	6% ± 5%	6% ± 2%

[a]n = four repeat wells from the same 96-well plate. Data are presented as percentage of a control in which cells were treated with no NEM. Data are from one representative experiment of a triplicate. [b]Derived from the equation: $(A\% + B\%)/2$. A = observed protein thiols; B = observed nonprotein thiols.

supernatant.[18] The supernatant of the bottom four rows was transferred to a different 96-well plate and treated with the GUALY's reagent derivatizing solution for nonprotein thiols determination. The remaining protein precipitates in the bottom four rows were thoroughly washed to remove nonprotein thiols followed by addition of the GUALY's reagent derivatizing solution before protein thiols were determined. Because it has been demonstrated that the sensitivity of the GUALY's reagent to different thiols is different,[22] absolute quantification of thiols would not be possible. The data presented in Table 2 are expressed as the percentage of a control in which cells were treated with no NEM. As shown in Table 2, the high-throughput assay effectively reflected different extents of depletion on protein thiols, nonprotein thiols, and total thiols produced by different concentrations of NEM. The data in Table 2 demonstrated that nonprotein thiols were more

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Table 3. Total Thiols and Protein Thiols Determined by the High-Throughput Assay vs an HPLC Method

NEM concentration (μM)	0	10	30	200
total thiols				
high-throughput method[a]	100% ± 5%	63% ± 3%	41% ± 2%	12% ± 6%
HPLC	100% ± 2%	69% ± 6%	54% ± 3%	20% ± 6%
protein thiols				
high-throughput method[a]	100% ± 2%	89% ± 3%	68% ± 7%	20% ± 3%
HPLC	100%	77%	60%	16%

[a] n = three repeat wells from the same plate. Data are presented as percentage of a control in which cells were treated with no NEM. Data are from one representative experiment of a triplicate.

Table 4. Nonprotein Thiols Determined by the High-Throughput Assay vs an HPLC Method

NEM concentration (μM)	0	4	6	7	8	9	10	15
high-throughput method[a]	100% ± 2%	75% ± 6%	61% ± 6%	59% ± 2%	55% ± 7%	50% ± 1%	30% ± 7%	15% ± 2%
HPLC	100%	81%	63%	54%	52%	46%	22%	7%

[a] n = three repeat wells from the same plate. The data are presented as percentage of the control in which cells were not treated with NEM. The data are from one representative experiment of a triplicate.

Table 5. Effects of Metal Ions on the Assay

	control	$CuCl_2$	$CaCl_2$	$ZnSO_4$	KH_2PO_4	$MgSO_4$	$FeSO_4$	$FeNH_4(SO_4)_2$
metal ion added[b]	0	(7 μM)[24]	(2.5 mM)[25]	(51.7 μM)[24]	(5 mM)[25]	(160 μM)[24]	(17.8 μM)[26]	(17.8 μM)[26]
fluorescence intensity (RFUs)	173 ± 3	176 ± 2	172 ± 3	174 ± 4	172 ± 1	171 ± 2	172 ± 3	171 ± 2

[a] Data are presented as means ± SD of four repeat wells from the same 96-well plate. Data are from one representative experiment of a triplicate. [b] Metal ion concentrations are based on their reported blood plasma concentrations.[24−26]

sensitive to depletion by NEM; all nonprotein thiols were effectively depleted by 30 μM NEM, whereas 500 μM NEM was needed to completely deplete protein thiols (Table 2). Table 2 also demonstrates a close match of the observed total thiols against the calculated total thiols which was calculated based on the equation $(A\% + B\%)/2$ (A: observed protein thiols; B: observed nonprotein thiols).

Validation of the High-Throughput Assay. A reported HPLC method for protein thiols, nonprotein thiols, and total thiols was employed to validate the high-throughput assay.[9] Since more cells are needed with the HPLC method, 1.5×10^6 cells in 10 mL RPMI growth medium without FBS in a 15 mL cell culture tube were used. Removal of FBS in the growth medium prevented cells from attachment. Cells were first treated with various concentrations of NEM for 3 h. The cells were then divided into two parts: 0.6 mL (0.1 mL/well for 6 wells with three wells for total thiol determination and three wells for protein thiol determination) was used for the high-throughput assay as described above, while the rest (9.4 mL) was used for HPLC thiol analysis as described by Chen and co-workers.[9] The initial plan was to validate protein thiols, nonprotein thiols, and total thiols using the same sample. However, the NEM concentrations appropriate to provide a gradient depletion of protein thiols and total thiols were found to be too high for depleting nonprotein thiols. Therefore, nonprotein thiol validation was conducted separately using the same procedure as that for protein thiol and total thiol validation except lower NEM concentrations were used. Table 3 presents a comparison of the data of protein thiols and total thiols obtained through the high-throughput assay versus the data obtained from the HPLC method. A comparison of the data for nonprotein thiols obtained from the high-throughput assay versus the data obtained from the HPLC method is presented in Table 4. As shown in these two tables, the data obtained through the high-throughput assay matched closely with the data obtained through the HPLC method confirming that the high-throughput assay is a valid method for protein thiol, nonprotein thiol, and total thiol determination. The validation also confirmed that GUALY's reagent derivetized all thiols including "buried" thiols in proteins because the HPLC method has been demonstrated to be able to quantify all thiols including "buried" thiols in proteins.[9]

It is noted that the extent of thiol depletion by NEM presented in Table 2 differs from that presented in Tables 3 and 4 (e.g., in Table 2, the percentages of control for the observed nonprotein thiols, protein thiols, and total thiols were 80% ± 8%, 88% ± 5%, and 81% ± 7%, respectively, while in Tables 3 and 4, the observed nonprotein thiols, protein thiols, and total thiols were 30% ± 7%, 89% ± 3%, and 63% ± 3%) despite the same concentration of NEM (10 μM) being used. The difference is likely due to the fact that the data presented in Table 2 was a result of NEM depletion in 15 000 attached cells in a well of a 96-well plate while the data presented in Tables 3 and 4 were the results derived from NEM depletion in 1.5×10^6 cells in 10 mL as a suspension in a 15 mL cell culture tube.

Effects of Metal Ions. Because thiols exhibit excellent affinity for metal ions, the effects of metal ions that are present in the biological system on the assay were investigated. GSH was chosen as a representative thiol. As presented in Table 5, the fluorescence intensity of the thiol adduct formed from GSH and GUALY's reagent remained unchanged in the presence or absence of the metal ions revealing that these metal ions did not interfere with the assay under the experimental condition. The metal ion concentrations chosen for the experiment are reported concentrations of these ions in blood plasma.[24−26]

In summary, we have developed the first high-throughput assay for simultaneous monitoring of protein thiols, nonprotein thiols, and total thiols in cells with a 96-well plate. The method is rapid, sensitive, and reliable, and can detect thiols with cell concentrations as low as 500 cells/well. We also demonstrated

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that this method can readily monitor cellular thiol concentration changes. Although the method cannot provide an absolute quantification for thiols because the fluorescence intensity of different thiol adducts varies, it provides an accurate measurement of relative quantification, a parameter often measured in thiol-related research. In addition to relative quantification of thiols, this method can potentially be used to quantify disulfides upon reduction to thiols by a reducing agent such as $NaBH_4$.[9] The method will be a valuable tool in thiol-related research.

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Notes

The authors declare no competing financial interest.

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