Chapter 8

Proteomic Analysis of Redox-Dependent Changes Using Cysteine-Labeling 2D DIGE

Hong-Lin Chan, John Sinclair, and John F. Timms

Abstract

Redox-modification of proteins plays an important role in the regulation of protein function and cellular physiology and in pathological conditions such as oncogenic activation, inhibition of tumor suppression, and ischemia reperfusion injury. This occurs, at least in part, through the reduction or oxidation of cysteine groups in these proteins resulting in the modulation of their activities. Herein, we focus on the development of a pair of cysteine-labeling iodoacetylated cyanine dyes (ICy3/5) for two-dimensional difference gel electrophoresis (2D DIGE) to monitor redox-dependent changes on cysteine residues. The method is applied to a cellular model of human mammary luminal epithelial cells treated with H_2O_2 to induce oxidative stress. Differences in labeling are caused either by differential protein expression or from the loss or gain of reactive thiol groups of cysteines in response to oxidative stress. Proteins displaying differential labeling would then be picked for MS-based identification. In summary, this cysteine-labeling 2D-DIGE approach provides an MS-compatible and reproducible technique for identifying alterations in the expression and redox-modification of free thiol-containing proteins.

Key words: Thiol-reactive cyanine dyes, Two-dimensional difference gel electrophoresis, Redox proteomics, Mass spectrometry

1. Introduction

Two-dimensional gel electrophoresis (2DE) is one of the most widely used proteomic separation methods and has been employed for the analysis of differential protein expression in many different biological sample types (1, 2). However, as most users realize, 2DE and the methods commonly used for in-gel protein visualization are inherently variable and many replicate gels must be run before significant differences in protein expression can be ascribed with accuracy. Moreover, these protein visualization methods often have narrow linear dynamic ranges of detection, making them unsuitable for the analysis of biological samples where protein copy

Rainer Cramer and Reiner Westermeier (eds.), *Difference Gel Electrophoresis (DIGE): Methods and Protocols*, Methods in Molecular Biology, vol. 854, DOI 10.1007/978-1-61779-573-2_8,

numbers vary enormously. A significant improvement in the ability to use gel-based methods for protein quantitation and detection was achieved with the introduction of two-dimensional difference gel electrophoresis (2D DIGE), which enables co-detection of several samples on the same 2DE gel, so avoiding gel-to-gel variation (3–7).

Several chemical moieties have been found to be potential regulators of cellular redox status. One of these, the free thiol group (R-SH) of cysteine residues is a potent nucleophilic agent and can undergo a number of redox-induced modifications under physiological and pathological conditions. Modifications of R-SH include the formation of protein disulfides and mixed disulfides (e.g., with glutathione and free cysteine) and oxidation to the sulfenic (R-SOH), sulfinic (R-SO_2H), and sulfonic (R-SO_3H) acids depending on the oxidative capacity of the oxidant (8). The R-SH group can also be modified by reactive nitrogen species to give the *S*-nitrosylated form (R-SNO), while oxidized forms can be glutathionylated for active secretion from cells or interconverted between forms by various enzyme activities (see Fig. 1).

Numerous studies have combined 2DE with cysteine thiol labeling to study redox-dependent protein changes. Maleimides, iodoacetic acid, iodoacetamide, and other chemicals have been modified with labels (biotin, fluorophores, radionuclides) to study

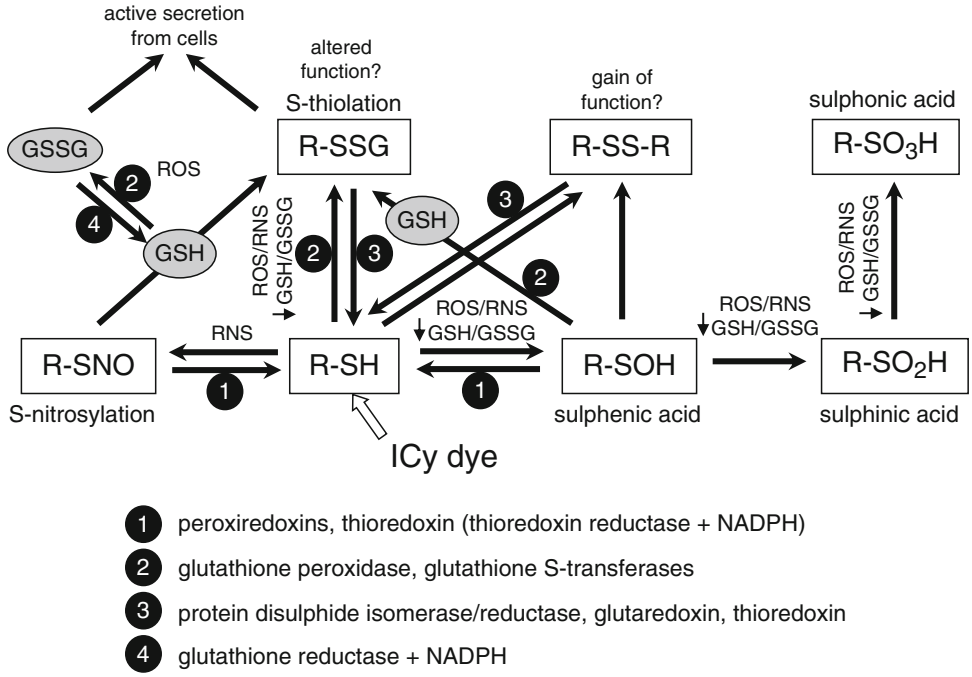

Fig. 1. Mechanisms of thiol-dependent cellular redox regulation. *GSH* reduced glutathione; *GSSG* oxidized glutathione; *ROS* reactive oxygen species; *RNS* reactive nitrogen species.

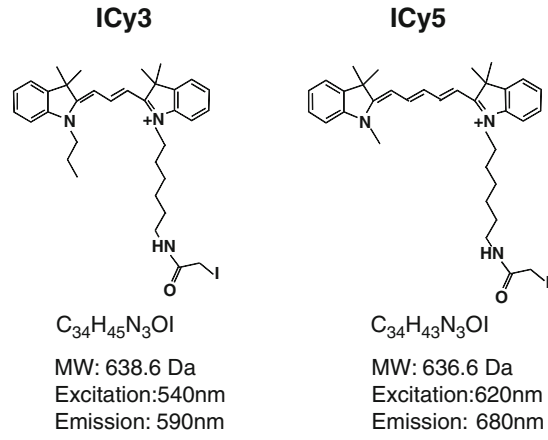

Fig. 2. Structure of the matched ICy3 and ICy5 iodoacetylated cyanine dyes. Chemical formulas, molecular weights, and excitation/emission wavelengths are shown.

changes in the redox status of proteins in 2D gels (9, 10). For example, *N*-(biotinoyl)-*N′*-(iodoacetyl) ethylenediamine was used for detecting selenium metabolite targets with labeled proteins detected using HRP-streptavidin and chemiluminescence (11). In another approach to monitor protein thiol oxidation in cultured cells, reduced thiols were first blocked with *N*-ethylmaleimide, then any oxidized thiols were reduced with dithiothreitol and subsequently labeled with 5-iodoacetamidofluorescein prior to 2DE and fluorescence detection (12). Such labeling approaches, especially using biotinylated derivatives, also allow affinity enrichment of the labeled pool of proteins for improved sensitivity of gel- and MS-based analyses.

A major drawback of these single labeling methods is reduced quantitative accuracy and precision due to the inherent variation of 2DE. To overcome this, we have developed a pair of iodoacetylated cyanine dyes (ICy3/5; see Fig. 2) for cysteine-labeling 2D DIGE to monitor redox-dependent changes on protein thiols and have tested the method in a model cell system of human mammary luminal epithelial cells exposed to H_2O_2 (13) and in plasma preparations disinfected by UVC irradiation (14). It is expected that any differences in labeling are caused by changes in the content of reactive thiol groups. Proteins displaying differential labeling on 2D gels are then picked for identification by MALDI-TOF MS peptide mass fingerprinting or LC-MS/MS with further validation of changes carried out by 1D and 2D immunoblotting. As the outlined protocol directly measures the labeling of free thiols between protein samples, changes in the expression levels of thiol-containing proteins are also expected to give rise to an altered ICy dye signal. For this reason, we recommend running a lysine-labeling experiment with NHS-cyanine dyes in parallel to aid in the discrimination of expression changes vs. redox-dependent changes. A detailed protocol for this is provided elsewhere in this volume.

Modifications of this technique have used BODIPY FL-*N*-(2-aminoethyl) maleimide and BODIPY TMR C_5-maleimide for dual labeling of reduced and oxidized cysteines to monitor changes in the thiol redox state of proteins in cells cultured at different oxygen concentrations (15). Similarly, commercial Cy3 and Cy5 maleimides have been also used for differential redox labeling (16), although the reproducibility and specificity of maleimides for thiol labeling has been called into question (17). It is important to note that the ICy dyes used in the protocol here carry a net charge of +1 and would be expected to cause a shift in the p*I* of labeled proteins on a 2D gel adding complexity to the analysis. This problem has recently been circumvented with the synthesis of a pair of sulfonated iodoacetamido-cyanine dyes which have a net charge of zero (17).

2. Materials

2.1. Cell Culture and Hydrogen Peroxide Treatment

1. Cells: human mammary luminal epithelial cells (HMLECs) (18, 19).
2. RPMI-1640 growth medium: RPMI-1640 medium (with 25 mM HEPES and L-glutamine), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL penicillin-streptomycin (all from Gibco/Invitrogen), 5 μg/mL hydrocortisone and 5 μg/mL insulin.
3. Solution of 0.25% trypsin and 1 mM EDTA (Gibco/Invitrogen).
4. Hydrogen peroxide treatment: 30% hydrogen peroxide solution diluted to 0.5 mM final concentration in growth media.

2.2. Preparation of ICy Dye-Labeled Samples for 2D DIGE

1. Iodoacetyl cyanine dyes 3 and 5 (ICy3 and ICy5) (see Fig. 2 and Note 1): From lyophilized powder (stored at −20°C), reconstitute to 10 mM stock by dissolving in the appropriate volume of anhydrous *N,N*-dimethylformamide (DMF). Keep stock solutions in dark at −20°C.
2. 2D lysis buffer: 8 M urea, 4% (w/v) CHAPS, 1 mM EDTA 10 mM Tris–HCl pH 8.3. To make 100 mL, dissolve 48 g of urea in 50 mL of distilled H_2O. Add 4 g CHAPS, 0.5 g NP-40, 0.1 mL of 1 M EDTA, and 0.67 mL of 1.5 M Tris pH 8.8 solution. This should give a final pH of 8.3. Make up to final volume, aliquot, and store at −20°C. Do not heat (see Notes 2 and 3).
3. DTT solution: 1.3 M DTT in H_2O. To make 10 mL, dissolve 2 g DTT in distilled H_2O and make to 10 mL. Aliquot and store at −20°C. Do not heat.
4. Ampholines/Pharmalyte mix: Mix equal volumes of Ampholines (pH 3.5–10) and Pharmalyte (pH 3–10). Store at 4°C. These broad pH range IPG buffers can be replaced with narrow-range buffers depending on the first dimension pH range.

5. Bromophenol blue: 0.05% (w/v) bromophenol blue in H_2O. To make 10 mL, weigh 5 mg bromophenol blue and make to 10 mL with distilled H_2O. Filter and store at room temperature.

2.3. Preparation of 2D-Gels, Imaging, and Image Analysis

1. Bind-Silane solution: For twelve 24 × 20 cm plates, mix 12 μL of PlusOne Bind-Silane (GE Healthcare), 300 μL glacial acetic acid, 12 mL ethanol and 2.7 mL ddH_2O.
2. PlusOne Repel-Silane solution (GE Healthcare).
3. Immobiline DryStrip pH 3–10 NL gel strips (GE Healthcare).
4. Mineral oil.
5. Equilibration buffer: 6 M urea, 30% (v/v) glycerol, 50 mM Tris–HCl pH 6.8, 2% (w/v) SDS. To make 200 mL, dissolve 72 g urea in 100 mL distilled H_2O. Add 60 mL of 100% glycerol, 10 mL of 1 M Tris pH 6.8 solution, and 4 g SDS. Dissolve all powders and adjust volume to 200 mL with ddH_2O. Aliquot and store at −20°C.
6. Ammonium persulfate (APS): Prepare a 10% solution in ddH_2O and store at 4°C for no more than a month.
7. Gel preparation: 30% acrylamide/bisacrylamide solution, 1.5 M Tris–HCl, pH 8.8, 10% SDS, 10% APS, *N,N,N,N′*-tetramethylethylenediamine (TEMED).
8. Agarose overlay: 0.5% (w/v) low-melting point agarose in SDS-PAGE running buffer. To make 200 mL, melt 1 g of agarose in 200 mL of 1× SDS-PAGE running buffer in a microwave on low heat. Add bromophenol blue solution to give a pale blue color.
9. SDS-PAGE running buffer (10×): Tris-glycine SDS buffer 10× (Severn Biotech LTD). Store at room temperature.
10. Ettan DALT*twelve* Large Vertical Electrophoresis System (GE Healthcare).
11. Typhoon 9400 Variable Mode Imager and ImageQuant software (GE Healthcare).
12. DeCyder software (v7.0) (GE Healthcare).

2.4. Post-Staining and Spot Excision

1. Colloidal CBB fixing solution: 35% (v/v) ethanol, 2% (v/v) phosphoric acid in ddH_2O.
2. Colloidal CBB staining solution: 34% (v/v) methanol, 17% (w/v) ammonium sulfate, and 3% (v/v) phosphoric acid in ddH_2O (see Note 4).
3. Coomassie Blue G-250.
4. Bio-Rad GS-800 scanning densitometer and QuantityOne software (Bio-Rad Laboratories Inc. USA.) or Typhoon 9400 Variable Mode Imager and ImageQuant software (GE Healthcare).
5. Ettan Spot Picker (GE Healthcare).

2.5. In-Gel Digestion

1. 50 and 100% HPLC-grade acetonitrile (ACN). Store at room temperature.
2. 5 mM ammonium bicarbonate (ABC) pH 8.0 in ddH_2O. Store at 4°C or prepare a 100 mM stock, aliquot, and store at –20°C.
3. 10 mM dithiothreitol (DTT) in 5 mM ABC. Prepare fresh.
4. 50 mM iodoacetamide (IAM) in 5 mM ABC. Prepare fresh.
5. 5% trifluoroacetic acid (TFA) in 50% ACN. Prepare fresh.
6. 10 ng/μL sequencing-grade modified trypsin (Promega) in 5 mM ABC pH 8.0. Prepare 500 ng-stocks in buffer provided (50 mM acetic acid). Store at –20°C.

2.6. Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry

1. Matrix solution: saturated aqueous 2,5-dihydroxybenzoic acid (Bruker Daltonics).
2. Peptide external calibration standard (Bruker Daltonics).
3. Ultraflex matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometer (Bruker Daltonics).
4. MTP AnchorChip target (Bruker Daltonics).
5. FlexControl software (Bruker Daltonics).
6. FlexAnalysis software (Bruker Daltonics).

2.7. Validation by Immunoblotting

1. Transfer-Blot tank (Bio-Rad).
2. Transfer buffer: 195 mM glycine, 25 mM Tris–HCl, pH 7.4, 20% (v/v) methanol. Prepare 10× transfer buffer in ddH_2O without methanol and store at 4°C. Dilute to a 1× solution prior to transfer, adding 20% (v/v) methanol.
3. Tris-buffered saline with Tween-20 (TBS-T): 50 mM Tris–HCl pH 8.0, 150 mM NaCl, and 0.1% Tween-20 (Sigma).
4. Polyvinylidene fluoride membrane (PVDF) (Immobilon P, Millipore, Bedford, MA), and 3-MM chromatography paper (Whatman, Maidstone, UK).
5. Blocking buffer: 5% w/v low-fat milk in TBS-T.
6. Enhanced chemiluminescence (ECL) reagents (Perkin-Elmer Life Sciences) and Fuji RX X-ray film (Genetic Research Instrumentation).
7. Primary antibody: appropriate primary antibodies chosen for validation of MALDI-TOF results. Appropriate working concentrations of the primary antibodies should be determined beforehand.
8. Secondary antibody: anti-mouse or anti-rabbit IgG-HRP linked antibodies (GE Healthcare). Prepare freshly at a dilution of 1:5,000 in TBS-T.

3. Methods

3.1. Tissue Culture

1. Culture HMLECs in 15-cm tissue culture dishes in RPMI-1640 growth media at 37°C in a 10%-CO_2-humidified incubator.
2. Split cells approximately 1:5 when confluent. Do not over-split.

3.2. Protein Quantification

1. HMLEC cells at ~80% confluence are washed twice with ice-cold 0.5× PBS and then 1,000 μL of 2D-lysis buffer added per plate. Place dishes immediately on ice.
2. Scrape cells and collect them in labeled tubes.
3. Homogenize by passage through a 25-gauge needle 10 times. Vortex and remove insoluble material by centrifugation (13,000×*g*/10 min/4°C) and transfer supernatant to fresh tubes.
4. Determine protein concentration using the Coomassie Protein Assay Reagent. Make a 5 mg/mL stock of BSA in 2D-lysis buffer and prepare serial dilutions of 0, 0.25, 0.5, 1.0, 2.5, and 5.0 mg/mL to make a standard curve. Use a 96-well flat-bottomed assay plate and make triplicate measurements for the BSA standards and four replicates for the experimental samples. For this, add 2 μL of sample per well and 198 μL of assay reagent and mix without introducing bubbles. Use a plate reader at a wavelength of 595 nm and calculate protein concentrations using the standard curve (see Note 5).

3.3. Cell Treatment and Lysis

1. Add H_2O_2 solution to growth media of HMLEC cells at ~80% confluence to a final concentration of 0.5 mM with gentle swirling or leave untreated. Leave treated cells for 2, 20, or 240 min.
2. Cells are washed twice with ice-cold 0.5× PBS and lysed with 1,000 μL 2D lysis buffer per plate containing ICy3/5 at 80 pmoL/μg protein (see Note 6).
3. Scrape cells and collect them in labeled tubes.
4. Homogenize by passage through a 25-gauge needle 10 times. Vortex and remove insoluble material by centrifugation (13,000×*g*/10 min/4°C) and transfer supernatant to fresh tubes.
5. The ICy3/5-labeled proteins are subsequently incubated on ice in the dark for 1 h and quenched with DTT at a 65 mM final concentration.
6. Mix 150 μg ICy3- and 150 μg ICy5-labeled samples to give 300 μg total protein. Volumes are adjusted to 450 μL with 2D-lysis buffer containing 65 mM DTT and 9 μL carrier Ampholines/Pharmalyte (1:1; v/v) (pH 3–10) and 1 μL of

Table 1
Example of differential labeling, mixing, and gel loading for comparison of thiol reactivity and protein expression under four treatment conditions in triplicate using cysteine-labeling 2D DIGE

	ICy3 (μg pool)	ICy5
Gel 1	150	150 μg condition 1 (0 min H_2O_2)
Gel 2	150	150 μg condition 1 (0 min H_2O_2)
Gel 3	150	150 μg condition 1 (0 min H_2O_2)
Gel 4	150	150 μg condition 2 (2 min H_2O_2)
Gel 5	150	150 μg condition 2 (2 min H_2O_2)
Gel 6	150	150 μg condition 2 (2 min H_2O_2)
Gel 7	150	150 μg condition 3 (20 min H_2O_2)
Gel 8	150	150 μg condition 3 (20 min H_2O_2)
Gel 9	150	150 μg condition 3 (20 min H_2O_2)
Gel 10	150	150 μg condition 4 (240 min H_2O_2)
Gel 11	150	150 μg condition 4 (240 min H_2O_2)
Gel 12	150	150 μg condition 4 (240 min H_2O_2)

bromophenol blue solution added. The mixed samples are allocated appropriately for separation on triplicate 2D-gels as shown in Table 1. This scheme controls for dye bias, although labeling combinations are interchangeable so long as each gel is loaded with samples labeled with distinct dyes. This experiment generates 24 images for matching, cross-comparison, and statistical analysis in the biological variation analysis (BVA) module of the DeCyder software.

7. Rehydrate Immobiline DryStrip pH 3–10 NL gel strips with samples for at least 12 h in the dark at room temperature in a rehydration tray (passive rehydration method). Strips should be covered with mineral oil.

3.4. Preparation of 2D-Gels, Imaging, and Image Analysis

1. For SDS-PAGE casting, an Ettan DALT*twelve* system (24 cm) is employed.
2. Prior to gel casting, treat low-fluorescence glass plates with 1 mL of fresh Bind-Silane solution per plate, wiping over one surface with a lint-free tissue. Leave plates to dry for a minimum of 1.5 h (see Note 7 and Fig. 3).
3. Treat the clean and dry inner surface of the other plate with 1 mL Repel-Silane to ensure easy separation after electrophoresis.

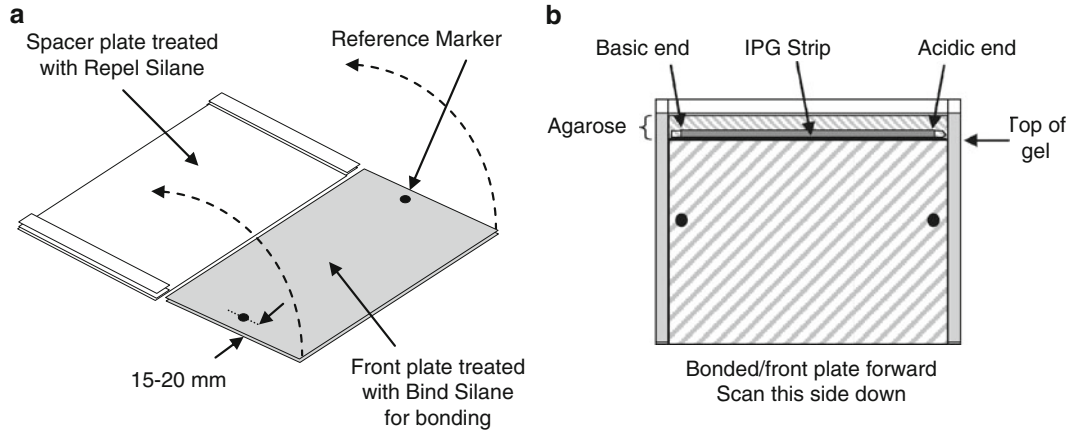

Fig. 3. (**a**) Treatment of plates for bonding and reference marker positioning. (**b**) Casting and loading of second dimension gels based on the Ettan Dalt 24-cm strip format.

Apply Repel-Silane solution to a lint-free tissue and wipe over the surface. Remove excess Repel-Silane by wiping with a clean tissue, rinse with ethanol, then with distilled H_2O. Leave to dry for 10 min (see Notes 7 and 8 and Fig. 3).

4. Stick fluorescent reference markers to the bonded surface of the plates. These should be placed half-way down the plates and 15–20 mm in from each edge. These markers are used as references for determining picking coordinates for automated spot picking using the Ettan Spot Picker (see Fig. 3).
5. Assemble plates in casting chamber according to the manufacturer's guidelines.
6. Prepare 12.5% acrylamide/bisacrylamide resolving gel solution and pour into assembled casting chamber leaving approximately 1.5 cm at the top for the IPG strip (~100 mL resolving gel solution per gel). Overlay carefully with water or butanol. Leave gel to polymerize for at least 4 h.
7. Perform isoelectric focusing (IEF) for a total of 80 kVh in the dark according to the manufacturer's instructions.
8. Equilibrate strips for 15 min in equilibration buffer containing 65 mM DTT for reduction and then 15 min in the same buffer containing 240 mM iodoacetamide for alkylation.
9. Rinse strips in 1× SDS-PAGE running buffer and place onto the top of second dimension gels in melted 0.5% agarose overlay, with the basic end of the strip toward the right hand side when the bonded plate is facing backward.
10. Run second dimension gels until the dye front has completely run off to avoid fluorescence signals from bromophenol blue and free dye. For the Ettan DALT*twelve* system, this can be

achieved by running 12.5% gels for approximately 16 h at 2.2 W per gel.

11. Images are best acquired directly after the 2DE by scanning gels between glass plates using a Typhoon 9400 Imager or a similar device. Ensure that both outer plate surfaces are clean and dry before scanning and that the bonded plate is the lower plate on the scanner bed.
12. Perform an initial low-resolution scan (1,000 μm) for one gel on the Cy3 and Cy5 channels with the photomultiplier tube (PMT) voltages set low (e.g., 500 V). The excitation/emission wavelengths for fluorescence detection using the Typhoon 9400 are 532/580 nm for ICy3 and 633/680 nm for ICy5. An image is then built up by the scanner for each channel and is converted to grayscale pixel values.
13. Using ImageQuant software for the Typhoon 9400, establish maximum pixel values in various user-defined, spot-rich regions of each image, and adjust the PMT voltages for a second low-resolution scan to give similar maximum pixel values (within 10%) on each channel and without saturating the signal from the most intense peaks. Repeat scans may be required until values are within 10% for the two channels (see Note 9).
14. Once set for the first gel, use the same PMT voltages for the whole set of gels scanning at 100-μm resolution. A 24- × 20-cm gel image takes approximately 10 min to acquire per channel and two gels are scanned simultaneously. Images are generated as gel files.
15. Crop overlayed images in ImageQuant and import into DeCyder Batch Analysis software for subsequent BVA analysis, according to the DeCyder software user manual. An example of using ICy to monitor H_2O_2-induced redox-proteome alterations in HMLECs is shown in Fig. 4. A number of proteins showed a rapid increase in labeling (e.g., spots 25 and 26), suggesting generation of new free thiols, for example, via scission of disulfides. On the other hand, a number of proteins showed a rapid decrease in labeling (e.g., spots 24 and 27), implying oxidation of free thiols that would not be labeled with the ICy dyes. In some cases, the change in ICy labeling recovered over time, indicating reversible modification (e.g., spot 27) (see Note 10).

3.5. Post-Staining and Spot Excision

1. After ICy fluorescence image scanning, gels are immersed in fixing solution and incubated overnight with gentle shaking. Fixed and bonded gels can now be stored for several months at 4°C by sealing in plastic bags with 1% (v/v) acetic acid.
2. For post-staining, the colloidal Coomassie Brilliant Blue (CCB) G-250 staining method is modified from that of (20). Fix gels

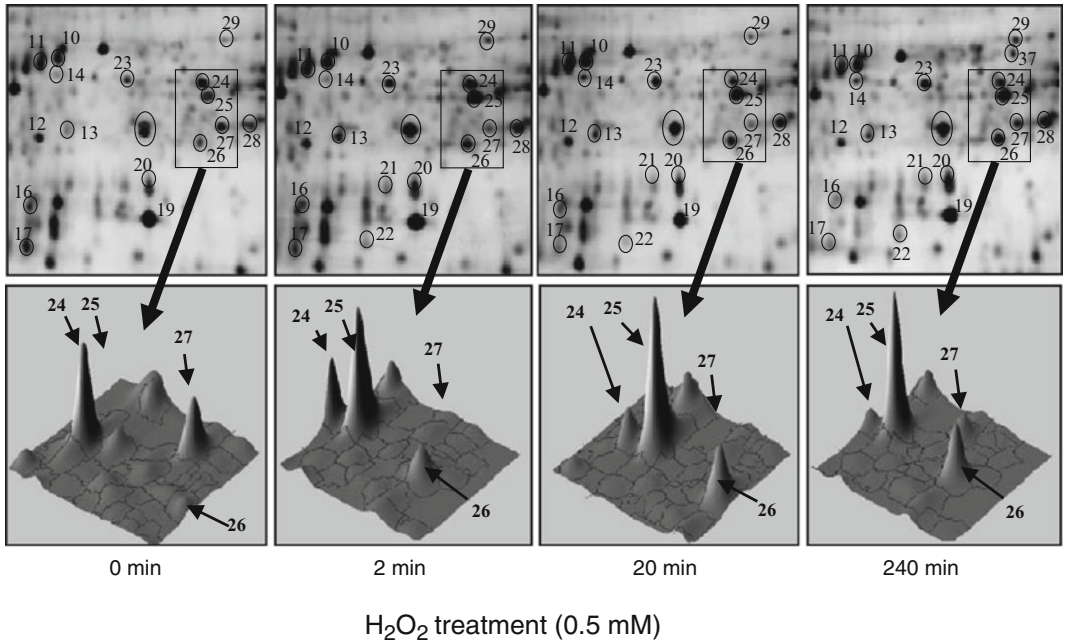

Fig. 4. Multiplex 2D DIGE analysis to monitor oxidant-dependent thiol reactivity in HMLECs. Sections of overlaid 2D DIGE images and 3D fluorescence profiles of HMLEC cell lysates from untreated cells (0 min) and cells treated with 0.5 mM H_2O_2 for 2, 20, and 240 min are shown. Individual lysates were labeled with ICy5 and run on each gel against a standard pool of all samples labeled with ICy3.

in colloidal CBB fixing solution for at least 3 h on a shaking platform. Wash 3 times for 30 min with ddH_2O and incubate in CCB staining solution for 1 h. Add one crushed CCB tablet (250 mg)/500 mL of staining solution (i.e., 0.5 g/L) and leave to stain for 3–5 days. No destaining step is required to visualize proteins. Stained gels can be scanned on the Typhoon 9400 imager using the red laser and no emission filters. Alternatively, the stained images can be scanned on a Bio-Rad GS-800 densitometer (see Note 11).

3. Align post-stained and fluorescence gel images to identify spots of interest for picking. Alignment and spot identification can be carried out by comparing images in DeCyder BVA or using Adobe Photoshop to overlay images.
4. For automated spot picking, process post-stained images in DeCyder BVA software and create a pick list for the spots of interest by comparing with the results of the BVA analysis. To facilitate sample tracking and later data matching with MS results, the post-stained image can be imported and matched within the current experimental BVA workspace. The advantage is that any spot picked according to the post-stained image will have the same master spot number as in the BVA quantitative analysis. Define the positions of the two reference markers in

DeCyder (left then right) and export the pick list coordinate file (.txt) to the spot picker controller. Subsequently, open the imported pick list and align the Ettan Spot Picker with the reference markers according to the manufacturer's instructions. Pick and collect spots in 96-well plates, drain the water, and store at –20°C prior to MS analysis. Alternatively, excision of spots from the post-stained gel can be done manually with a gel-plug cutting pipette. The gel is best submerged under 1–2 mm of distilled water, and picking performed in a dedicated clean area.

3.6. In-Gel Digestion

1. Ideally samples for MS analysis should be prepared in a clean room or other clean area to avoid keratin and other contamination.
2. Shake gel pieces in ddH_2O for 15 min. Replace water with 50% ACN and shake for a further 15 min. Repeat this step 3 times until gel pieces are completely destained (see Note 12).
3. Remove the 50% ACN and dry in a speed vacuum for 15–20 min (see Note 13).
4. Reduce the samples by adding sufficient 10 mM DTT (in 5 mM ABC pH 8.0) to cover the gel pieces and incubating for 45 min at 50°C, with gentle shaking.
5. Remove the DTT solution and alkylate by adding enough 50 mM IAM (in 5 mM ABC pH 8.0) to cover the gel pieces and incubating for 1 h at room temperature in the dark.
6. Remove the IAM solution and wash the gel pieces twice with 50% ACN for 15 min each.
7. Dry the gel pieces in a speed vacuum for approximately 15–20 min (see Note 13).
8. Digest the samples with trypsin. From a 500-ng/μL stock of trypsin in buffer, dilute 100 times (to 5 ng/μL) to provide sufficient volume for all samples (10 μL of trypsin at 50 ng per sample). Allow the trypsin solution to soak into the gel piece and then add sufficient 5 mM ABC pH 8.0 to cover the gel piece. Place samples in an incubator or rocking heater block at 37°C and leave to digest overnight.
9. Briefly spin the samples and collect the supernatant and transfer to new siliconized tubes. Add sufficient 50% ACN/5% TFA to cover the gel pieces and agitate briefly to aid peptide extraction. Remove the supernatant and pool together with the first. Repeat this step twice.
10. Speed vacuum to dryness. Samples can be stored at –20°C or be directly analyzed by MS.
11. Resuspend peptides in 5 μL of 0.1% formic acid by gently shaking prior to MALDI-TOF MS (or ESI MS/MS) analysis.

3.7. Protein Identification by MALDI-TOF MS and Data Analysis

1. 0.5 μL of tryptic digest is mixed with 1 μL of saturated aqueous 2,5-dihydroxybenzoic acid matrix solution and spotted onto a target plate and dried.
2. Mass spectra are acquired on an Ultraflex TOF/TOF mass spectrometer in the reflector mode. The spectrometer is calibrated using the peptide external calibration standard, and internal calibration is carried out using trypsin autolysis peaks at *m/z* 842.51 and *m/z* 2,211.10.
3. Peaks in the mass range of *m/z* 500–5,000 are used to generate a peptide mass fingerprint that is searched against the updated NCBInr database using Mascot Peptide Mass Fingerprint software (Matrix Science, London, UK; http://www.matrixscience.com/search_form_select.html). The following parameters are used for the search: *Homo sapiens* (or relevant taxonomy); tryptic digest with a maximum of one missed cleavage; carbamidomethylation of cysteine; protein N-terminal acetylation, methionine oxidation, and glutamine to pyroglutamate as variable modification; and a mass tolerance of ±50 ppm (see Note 14).
4. A positive identification is accepted based on a significant Mascot MOWSE score ($p < 0.05$), at least 6 peptide masses matching a particular protein, matched peptides covering >20% of the matched protein sequence and general agreement between the observed and theoretical molecular weight on 2D gels.

3.8. Validation by Immunoblotting

1. Separate samples by 1D SDS-PAGE and transfer electrophoretically to PVDF membrane using transfer buffer in a transfer tank (Bio-Rad Transfer-Blot cell). Wet the PVDF membrane in 100% methanol for 1 min, then in transfer buffer for 5 min and place on top of the gel without air bubbles. Sandwich membrane and gel between two pieces of Whatman paper soaked in transfer buffer. Remove air bubbles with a plastic pipette and fix firmly in the tank cassette. Insert the cassette into the transfer tank containing transfer buffer and make sure the membrane is located between the gel and the anode. Connect the tank to an appropriate power supply and transfer at 350 mA for 5 h.
2. Once transfer is complete, remove the "sandwich" from the cassette and disassemble. The colored molecular weight markers should be clearly visible on the membrane if the transfer has worked.
3. Incubate the membrane in 500 mL of 5%low-fat milk in TBS-T for 1 h at room temperature on a rocking platform. The membrane can also be left in blocking buffer over night at 4°C.
4. Discard the blocking buffer and rinse the membrane with TBS-T prior to the addition of a dilution of primary antibody.

5. Incubate with the appropriate primary antibody for 2 h at room temperature or overnight at 4°C on a rocking platform.
6. Remove the primary antibody and wash the membrane 3 times for 15 min in TBS-T on a rocking platform.
7. Incubate with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature on a rocking platform.
8. Remove the secondary antibody and wash the membrane 3 times for 15 min in TBS-T on a rocking platform.
9. Mix the two ECL reagents 1:1 and incubate immediately with the membrane for 1–2 min ensuring full coverage of the membrane.
10. Remove the membrane from the ECL reagents, drain excess fluid, enclose the membrane in Saran wrap, and tape it into an X-ray cassette.
11. In a darkroom, place an X-ray film on the top of the membrane and close the cassette. Leave to expose for an appropriate period to give a reasonable signal that is not saturated.
12. Scan the X-ray film on a Bio-Rad GS-800 densitometer and quantify the intensity with QuantityOne software.

4. Notes

1. The synthesis and purification of the ICy dyes are detailed in (13).
2. Urea decomposes to ammonium cyanate at temperatures above 30°C. The ammonium cyanate subsequently modifies the primary amino groups of proteins via carbamylation resulting in altered p*I*.
3. The lysis buffer should not contain thiourea, which competes with the ICy dyes. Thiourea is often used in 2-DE sample buffers to improve protein solubility.
4. Prepare the staining solution at least 1 h prior to use, considering that it takes the ammonium sulfate a while to dissolve completely.
5. It is recommended that at least four replicate assays are performed for each sample for accurate protein determination. Dilute concentrated samples with lysis buffer if necessary.
6. For ICy labeling, cells are lysed in the presence of dye to limit post-lysis thiol modification. Since ICy dyes interfere with the protein assay, protein concentrations are determined on replica lysates not containing the ICy dyes.

7. Low-fluorescence glass plates are used to reduce background in this experiment.
8. Only one plate in each set should be treated with Bind-Silane; treat the smaller, nonspacer plates if using Ettan DALT 24 cm gel plates. Bonding allows easier handling of gels during scanning, protein staining, storage, and importantly, for robotic spot excision (see Fig. 3).
9. By increasing PMT voltages, it is possible to increase the overall sensitivity of detection while saturating only a few of the signals from the more abundant proteins. This allows analysis of lower-abundance proteins that give reasonable signal-to-noise ratios.
10. In the protocol outlined, we are essentially measuring the change in R-SH reactivity upon oxidative insult. However, the method can be adapted to identify proteins whose thiols have become oxidized by the insult. In this case, the samples are first alkylated (e.g., with *N*-ethylmaleimide or iodoacetamide) to block existing R-SH groups, and then reduced (e.g., with DTT or TCEP) to regenerate R-SH groups from the oxidized forms prior to ICy dye labeling. Both alkylating and reducing agents must be removed after each step, either by protein precipitation or by size-exclusion columns.
11. Gels in containers should be placed on a rocking platform for staining. In addition, containers should be properly sealed to avoid evaporation.
12. The use of siliconized or low-bind tubes avoids protein or peptide loss due to adsorption on the tube walls and also avoids contaminants derived from the tube plastic.
13. Make sure the gel pieces are completely dried.
14. It is also possible to search for the ICy dye modifications and mono-isotopic masses of 512.36 Da $[ICy3\text{-}I+H]^+$ and 510.36 Da $[ICy5\text{-}I+H]^+$ should be added to searches as variable modifications. It should be noted, however, that we have had difficulty observing the modifications in digested gel extracts. We suspect that the poor recovery may be due to the lowered solubility and inefficient extraction of labeled peptides from gel pieces, since model peptides labeled in solution performed well in MALDI-TOF experiments.

References

1. Herbert, B. R., Harry, J. L., Packer, N. H., Gooley, A. A., Pedersen, S. K., and Williams, K. L. (2001) What place for polyacrylamide in proteomics?, *Trends Biotechnol 19*, S3–9.
2. Rabilloud, T. (1994) Two-dimensional electrophoresis of basic proteins with equilibrium isoelectric focusing in carrier ampholyte-pH gradients, *Electrophoresis 15*, 278–282.
3. Gharbi, S., Gaffney, P., Yang, A., Zvelebil, M. J., Cramer, R., Waterfield, M. D., and Timms, J. F. (2002) Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system, *Mol Cell Proteomics 1*, 91–98.
4. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., and Davison, M. (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology, *Proteomics 1*, 377–396.
5. Unlu, M., Morgan, M. E., and Minden, J. S. (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts, *Electrophoresis 18*, 2071–2077.
6. Timms, J. F., and Cramer, R. (2008) Difference gel electrophoresis, *Proteomics 8*, 4886–4897.
7. Lai, T. C., Chou, H. C., Chen, Y. W., Lee, T. R., Chan, H. T., Shen, H. H., Lee, W. T., Lin, S. T., Lu, Y. C., Wu, C. L., and Chan, H. L. (2010) Secretomic and Proteomic Analysis of Potential Breast Cancer Markers by Two-Dimensional Differential Gel Electrophoresis, *J Proteome Res 9*, 1302–1322.
8. Schafer, F. Q., and Buettner, G. R. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, *Free radical biology & medicine 30*, 1191–1212.
9. Eaton, P. (2006) Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures, *Free radical biology & medicine 40*, 1889–1899.
10. Sheehan, D. (2006) Detection of redox-based modification in two-dimensional electrophoresis proteomic separations, *Biochem Biophys Res Commun 349*, 455–462.
11. Lee, J. S., Ma, Y. B., Choi, K. S., Park, S. Y., Baek, S. H., Park, Y. M., Zu, K., Zhang, H., Ip, C., Kim, Y. H., and Park, E. M. (2006) Neural network-based analysis of thiol proteomics data in identifying potential selenium targets, *Preparative biochemistry & biotechnology 36*, 37–64.
12. Cuddihy, S. L., Baty, J. W., Brown, K. K., Winterbourn, C. C., and Hampton, M. B. (2009) Proteomic detection of oxidized and reduced thiol proteins in cultured cells, *Methods Mol Biol 519*, 363–375.
13. Chan, H. L., Gharbi, S., Gaffney, P. R., Cramer, R., Waterfield, M. D., and Timms, J. F. (2005) Proteomic analysis of redox- and ErbB2-dependent changes in mammary luminal epithelial cells using cysteine- and lysine-labelling two-dimensional difference gel electrophoresis, *Proteomics 5*, 2908–2926.
14. Chan, H. L., Gaffney, P. R., Waterfield, M. D., Anderle, H., Peter Matthiessen, H., Schwarz, H. P., Turecek, P. L., and Timms, J. F. (2006) Proteomic analysis of UVC irradiation-induced damage of plasma proteins: Serum amyloid P component as a major target of photolysis, *FEBS Lett 580*, 3229–3236.
15. Lui, J. K., Lipscombe, R., and Arthur, P. G. (2010) Detecting changes in the thiol redox state of proteins following a decrease in oxygen concentration using a dual labeling technique, *J Proteome Res 9*, 383–392.
16. Hurd, T. R., Prime, T. A., Harbour, M. E., Lilley, K. S., and Murphy, M. P. (2007) Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis: implications for mitochondrial redox signaling, *J Biol Chem 282*, 22040–22051.
17. Bruschi, M., Grilli, S., Candiano, G., Fabbroni, S., Della Ciana, L., Petretto, A., Santucci, L., Urbani, A., Gusmano, R., Scolari, F., and Ghiggeri, G. M. (2009) New iodo-acetamido cyanines for labeling cysteine thiol residues. A strategy for evaluating plasma proteins and their oxido-redox status, *Proteomics 9*, 460–469.
18. Harris, R. A., Eichholtz, T. J., Hiles, I. D., Page, M. J., and O'Hare, M. J. (1999) New model of ErbB-2 over-expression in human mammary luminal epithelial cells, *Int J Cancer 80*, 477–484.
19. White, S. L., Gharbi, S., Bertani, M. F., Chan, H. L., Waterfield, M. D., and Timms, J. F. (2004) Cellular responses to ErbB-2 overexpression in human mammary luminal epithelial cells: comparison of mRNA and protein expression, *Br J Cancer 90*, 173–181.
20. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250, *Electrophoresis 9*, 255–262.