Chapter 20

Monitoring of Methionine Sulfoxide Content and Methionine Sulfoxide Reductase Activity

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Abstract

The sulfur-containing amino acid methionine (Met) plays critical roles in protein synthesis, methylation, and sulfur metabolism. Both in its free form and in the form of an amino acid residue, it can be oxidized to the *R* and *S* diastereomers of methionine sulfoxide (MetO). Organisms evolved methionine sulfoxide reductases (MSRs) to reduce MetO to Met, with the MSRs type A (MSRA) and type B (MSRB) being specific for the *S* and *R* forms of MetO, respectively. In mammals, the selenoprotein MSRB1 plays an important protein repair function, and its expression is tightly regulated by dietary selenium. In this chapter, we describe a protocol for determining the concentration of protein-based Met-*R*-O and its analysis in HEK293 cells using a genetically encoded ratiometric fluorescent biosensor MetROx. We also describe the procedure for quantifying MSR activities in cell extracts using specific substrates and a reverse phase HPLC-based method.

Key words Fluorescent microscopy, Genetically encoded fluorescent sensors, HPLC, Methionine sulfoxide reductase, Oxidative stress, Protein oxidation

1 Introduction

Living cells are constantly exposed to reactive oxygen species (ROS) and other oxidative insults. Under physiological conditions, ROS levels are tightly controlled, and these species are actively involved in cell signaling. However, abnormally high ROS levels can lead to deleterious oxidation of macromolecules [1]. The amino acid methionine (Met), either as a metabolite or as an amino acid residue, is sensitive to oxidation and is converted by ROS to Met sulfoxide (MetO), which exist in the form of two diastereomers, *rectus* (*R*) (Met-*R*-O) and *sinister* (*S*) (Met-*S*-O). These oxidized forms can be reduced back to Met by specialized enzymes, methionine sulfoxide reductases of type A (MSRA) and type B (MSRB), which are specific for Met-*S*-O and Met-*R*-O, respectively. MSRs use thiol/selenothiol chemistry to reduce their substrates using the reducing power generally provided by the thioredoxin system [2].

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MSRA reduces the free form of Met-*S*-O and the respective oxidized amino acid residue with similar efficiency, whereas MSRB specializes in the reduction of protein-based Met-*R*-O [3]. Bacteria and unicellular eukaryotes also evolved dedicated enzymes for the reduction of the free form of Met-*R*-O, but such enzymes are absent in higher eukaryotes [4, 5]. As a result, mammals cannot efficiently reduce the free Met-*R*-O [6]. Mammalian genomes have a single MSRA and three MSRB genes. MSRB1 is located in the cytosol and nucleus, MSRB2 and MSRB3 are in mitochondria and the endoplasmic reticulum, respectively, whereas alternative first exon splicing allows producing, from a single gene, various MSRA forms that are localized in different cellular compartments [7]. MSRB1 is the only selenocysteine-containing MSR in mammals and was initially identified as selenoprotein R (SelR) and selenoprotein X (SelX) by searching for putative SECIS element structures in EST databases using bioinformatics tools [8, 9]. Knockout mouse models revealed that MSRB1 is a nonessential selenoprotein but it has a major antioxidant function in kidney and liver [10]. Moreover, the expression of MSRB1 is regulated by Se availability and declines as a function of age [10, 11]. In kidney cells, MSRB1 was shown to reactivate the TRPM6 magnesium-channel which can be inactivated by the oxidation of its Met1755 residue [12]. An important function was found in macrophages, in which MSRB1 plays a key role, together with MICAL monooxygenases, in the regulation of actin polymerization through cyclic oxidation and reduction of two key Met residues [13]. As MSRB1 is intricately linked with Se metabolism, studies on Se and selenoproteins may require quantification of Met-*R*-O levels and MSR activity in cells. In this chapter, we describe the procedures that allow determining Met-*R*-O levels and its changes in living cells using the genetically encoded ratiometric fluorescent biosensor MetROx [14, 15]. We also provide protocols for quantification of MSRA, MSRB, and total MSR activities in protein extracts using specific substrates and an HPLC-based procedure.

2 Materials

In all materials and methods described in this article, the terms methionine, methionine sulfoxide, or any of their abbreviations always refer to the L-enantiomers. All solutions should be prepared in ultrapure water (sensitivity of 18 MΩ cm at 25 °C).

2.1 Monitoring Met-R-O Concentration in Living Mammalian Cells

1. HEK293T/17 cells.
2. Plasmids for the expression of MetROx, its inactive version (C129S MetROx or C69S/C129S MetROx) in the appropriate cell compartment, and pEGFP-C3 plasmid (*see* **Note 1**).
3. Dulbecco's Modified Eagle Medium (D-MEM).

4. Opti-MEM without phenol red and without serum.
5. Fetal Bovine Serum (FBS).
6. Penicillin and streptomycin antibiotics solution. Stock solution contains 10,000 U/mL penicillin and 10 mg/mL streptomycin and is diluted 1:100 when used in cultured cells.
7. Transfection reagent (e.g., FuGENE HD from Promega).
8. 35 mm glass-bottom dishes.
9. Live cell imaging solution. Supplement 500 mL of Live Cell Imaging Solution (Life technologies) with 0.45 g glucose, sterilize by filtration through 0.2 μm filter and store at 4 °C.
10. A motorized inverted microscope (e.g., Olympus IX81) equipped with a cooled-CCD camera (e.g., Hamamatsu Orca ER) in conjunction with a zero-drift focus compensation system with the following filters: excitation (420/40 and 500/16), emission (535/30), and the acquisition software (e.g., MetaMorph from Universal Imaging, Downingtown, PA).
11. Dithiothreitol solution. To prepare 1 M solution, add 1.54 g of DTT (MW = 154.25 g/mol, min 98%) to 10 mL of water. Mix well by vortexing. Store solution at −20 °C.
12. L-methionine-*R*,*S*-sulfoxide (MW = 165.21 g/mol, min 99%). To prepare 100 mM solution, add 1.65 g to 100 mL of water. Mix well by vortexing. Store solution at room temperature or below.
13. Software for image analysis (e.g., ImageJ [16] or Fiji [17]).

2.2 Monitoring Methionine Sulfoxide Reductase Activity in Cell Lysates

1. Dabsyl-L-Methionine (Dabsyl-Met), MW = 436.55 g/mol, min 95%.
2. Dimethyl sulfoxide (DMSO), min 99%.
3. Hydrogen peroxide (H_2O_2), 30% (8.8 M).
4. Disposable SEP-PAK® C18 Cartridges (Waters).
5. HPLC solvent B: Acetonitrile HPLC grade.
6. HPLC solvent A: Acetate buffer. To make 29 mM solution, slowly add 1.665 mL of glacial acetic acid (min 99%) to 700 mL water in 1-L graduated cylinder or a glass beaker. Adjust the pH to 4.16 with 5 N NaOH. Adjust the volume to 1000 mL with water in a volumetric flask. Pass through the 0.2 μm filter and degas. Store at room temperature.
7. Tris–HCl buffers. Dissolve 12.1 g of Trizma® base (MW = 121.14 g/mol, min 99%) to 800 mL in a 1-L cylinder or a glass beaker. *Adjust pH at 8.0 with concentrated HCl and adjust the volume to 1000 mL with water in a volumetric flask to obtain a 0.1 M solution.* Filter (0.2 μm). To obtain 30 mM solution, dilute 333 mL of the above solution in 500 mL water

and adjust the volume to 1000 mL with water in a volumetric flask. Filter (0.2 μm) and store at 4 °C or room temperature.

8. For separation of *R*- and *S*-diastereomers of MetO, specific equipment includes: heating stirring plate, heating mantle or oil bath, condenser, glass-sintered funnel, Büchner flask, Schlenck line equipped with high vacuum pump or rotary evaporator, and polarimeter (e.g., Anton Paar Modular Circular Polarimeter 200, $\lambda = 589$ nm) or Nuclear Magnetic Resonance spectrometer (e.g., Bruker Avance III 400 MHz).
9. L-methionine-*R*,*S*-sulfoxide (MW = 165.21 g/mol, min 98%, Sigma-Aldrich).
10. Picric acid crystalline or moistened with water (MW = 229.10 g/mol, min 98%).
11. Acetone (min 99.5%).
12. Triethylamine (MW = 101.19 g/mol, min 99%). To prepare 5 mL of 1 M solution, add 0.70 mL triethylamine using a pipette in a 5 mL volumetric flask, and complete with water to 5 mL. To prepare 50 mL of 0.175 M triethylamine, add 1.23 mL triethylamine in a 50 mL volumetric flask using a pipette and complete with water to 50 mL.
13. 4-*N*,*N*-dimethylaminoazobenzene-4′sulfonyl chloride (dabsyl chloride) (MW = 323.80 g/mol). Dissolve 200 mg of dabsyl chloride in 50 mL acetone to obtain a 4 mg/mL solution.
14. Sodium carbonate buffer. Dissolve 12.88 g of $Na_2CO_3{\cdot}10H_2O$ (MW = 286.14 g/mol) in 50 mL of water. To obtain 0.9 M $NaHCO_3$, pH 9.0 solution, dissolve 3.78 g of $NaHCO_3$ (MW = 84.01 g/mol) in 40 mL of water and adjust slowly the pH to 9.0 with the $Na_2CO_3{\cdot}10H_2O$ solution.
15. Dithiothreitol solution. To prepare 1 M solution, add 1.54 g of DTT (MW = 154.25 g/mol, min 98%) to 10 mL of water. Mix well by vortexing. Store the solution at −20 °C.
16. Reverse-phase High Performance Liquid Chromatography (HPLC) system. The HPLC device must be equipped with a two-solvent mixing pump (e.g., Waters 1525) and UV-visible detectors (e.g., Waters PDA 996 PhotoDiode Array), and optionally with an autosampler (e.g., Waters Autosampler 717 plus). Dabsyl-Met and dabsyl-MetO are detected at 466 nm. The column used is a SunFire™ C18 3.5 μm 3.0 × 50 mm (Waters). The program runs for 8 min and starts with 70% solvent A (acetate buffer) and 30% solvent B (acetonitrile) with a 1 mL/min flow rate. Solvent B percentage increases to 66.7 from 0 to 2 min, then to 100% from 2 to 2.5 min and for a duration of 1 min (3.5 min), then decreases to 30% in 0.2 min (3.7 min) and up to 8 min. Diastereomers of dabsyl-MetO are eluted at 3.5 min and dabsyl-Met is eluted at 4.6 min (*see* **Note 2**).

3 Methods

3.1 Monitoring Met-R-O Concentration in Living Mammalian Cells

3.1.1 Transfection

1. Seed HEK293 cells onto 35 mm glass-bottom dishes (10^5 cells/dish) in D-MEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 μg/mL). Culture the cells for 24 h at 37 °C, 5% CO_2.
2. The next day, add 100 μL serum-free Opti-MEM to a sterile 1.5 mL tube.
3. Mix well 1 μg of the sensor-encoding expression vector in the medium (*see* **Note 3**).
4. Add 3 μL of transfection reagent. Mix rapidly by vortexing, and allow 15 min for the liposome-DNA complex to form.
5. Add the content of the tube to the media covering the cells in the glass-bottom dish. Mix gently.

3.1.2 Imaging

1. 16–24 h after transfection, examine effectiveness of transfection by checking fluorescence using your cell culture microscope. Use the GFP filter set on the microscope (*see* **Note 4**).
2. 30 min prior to the experiment, change the medium to the live cell imaging solution supplemented with 5 mM glucose and allow the dish to stay at room temperature (*see* **Note 5**).
3. Place the dish in the microscope mount, and find a group of transfected cells by using the visual mode of the microscope. Once the cells are in focus, immediately close the lamp shutter to avoid unwanted bleaching of the probe.
4. Switch the microscope to the recording mode and activate the following beam path settings (*see* **Note 6**): for excitation, use 420/40 (channel 1) and 500/16 (channel 2) band-pass excitation filters and 535/30 band-pass emission filter. Live imaging should be carried out in time series or xyt mode.
5. Start time series recording. Typically, emission is acquired every 15–30 s for 10–15 min.
6. Remove 0.1 mL of incubation medium and add chemicals in the same volume of buffer using a regular handhold pipette. To determine the ratio of the fully reduced sensor, prepare dithiothreitol solution in the incubation medium, add it to the cells at 1 mM final concentration and incubate until the fluorescent signal is stable (for 1–5 min). To determine the ratio of the fully oxidized sensor, proceed similarly by adding 10–100 mM L-methionine-*R*,*S*-sulfoxide to the cells and incubate until the fluorescent signal is stable (for 5–10 min). Repeat these assays with the cells expressing the inactive sensor.

3.1.3 Data Analysis

The fluorescence intensity analysis procedure described here is made using the software ImageJ/Fiji.

1. Open the stack in the software. Depending on the software used for the acquisition you might need to install a specific plugin or convert the file to work with the format.
2. Open "*ROI Manager*."
3. Set your first region of interest (ROI) on the first cell you would like to measure. Press "t" (or click "*Add*" on the *ROI Manager*) for multiple ROIs. Include non-fluorescent ROIs for background correction. Repeat until all cells are selected.
4. To check all your ROIs, click "*Show All*" on the *ROI Manager*.
5. Use Time series Analyzer to calculate all average intensities in the image stack.
6. Calculate the sensor fluorescence ratio (R) by dividing the background subtracted fluorescence in channel 2 ($F_{500\ \mathrm{nm}}$) by the background subtracted fluorescence in channel 1 ($F_{420\ \mathrm{nm}}$).
7. Use the following equation to calculate the oxidized fraction of the sensor:

$$\text{Oxidized fraction} = \frac{R_{\text{Reduced}} - R}{\mathrm{R}_{\text{reduced}} - R_{\text{Oxidized}}}$$

   where R is the measured $F_{500\ \mathrm{nm}}/F_{420\ \mathrm{nm}}$ ratio; R_{Reduced} is the $F_{500\ \mathrm{nm}}/F_{420\ \mathrm{nm}}$ ratio of fully reduced MetROx, and R_{Oxidized} is the $F_{500\ \mathrm{nm}}/F_{420\ \mathrm{nm}}$ ratio of fully oxidized MetROx.
8. To avoid nonspecific changes in the sensor signal, analyze cells expressing the inactive sensors (C129S MetROx or C69S/C129S MetROx), and divide the signal of the active sensor by that obtained with the inactive sensor (*see* **Note 7**).

3.2 Monitoring Methionine Sulfoxide Reductase Activity in Protein Extracts

The activity assay measurement is made using MetO derivatized with dabsyl, which is a derivatization reagent that allows detection of amino acids in visible light using a reverse-phase HPLC procedure. We propose two protocols for the preparation of dabsylated MetO substrates. The first is an easy procedure to obtain a racemic mixture of dabsyl-Methionine-*R*,*S*-sulfoxide (dabsyl-Met-*R*,*S*-O), which allows measuring total MSR activity of a protein extract. The second protocol describes the preparation of individual forms of MetO, i.e., dabsyl-Methionine-*R*-sulfoxide (dabsyl-Met-*R*-O) and dabsyl-Methionine-*S*-sulfoxide (dabsyl-Met-*S*-O); this allows measuring MSRB and MSRA activities of a protein extract separately. This protocol consists of the separation of the *R*- and *S*-diastereomers of MetO from a racemic mixture using picric acid precipitation, followed by their derivatization with dabsyl chloride. The

separation of both diastereomers of MetO should be very useful for other applications (e.g., genetic selection and screening [4, 18]). The protocol for measuring MSR activity is similar whichever substrate is used.

3.2.1 Preparation of the Racemic Mixture of Dabsyl-methionine-R,S-sulfoxide

1. In a 50 mL plastic tube, dissolve 25 mg of dabsyl-Met in 11.46 mL DMSO to obtain a 5 mM solution (*see* **Note 8**). Keep 0.5 mL for the HPLC standard curve (*see* Subheading 3.2.3).
2. To oxidized dabsyl-Met to dabsyl-Met-*R*,*S*-O, add 341 μL of 8.8 M (30%) H_2O_2 and 18.7 mL water (to 30 mL final volume). Mix well by vortexing. Protect from light by wrapping the tube with aluminum foil and incubate overnight at room temperature.
3. Take 10 μL, add 30 μL water (1/4 dilution) and check oxidation by HPLC by injecting 10 μL (*see* **Note 9** and Subheading 3.2.3).
4. Centrifuge at 3250 × *g* for 10 min at 25 °C.
5. Eliminate H_2O_2 and DMSO using the disposable SEP-PAK® C18 cartridge. Proceed by using a 5 mL syringe to avoid overpressure. Rinse the cartridge with 5 mL acetonitrile:acetate buffer (80:20 v:v). Equilibrate the cartridge with 5 mL acetonitrile:acetate buffer (5:95 v:v). Load 3 × 5 mL dabsyl-Met-*R*,*S*-O solution. Wash the cartridge with 5 mL acetonitrile:acetate buffer (5:95 v:v). Elute dabsyl-Met-*R*,*S*-O with 2 × 5 mL acetonitrile:acetate buffer (5:95 v:v). Repeat the procedure for the remaining dabsyl-Met-*R*,*S*-O solution. Pool eluted dabsyl-Met-*R*,*S*-O in a 50 mL plastic tube and freeze at −80 °C (*see* **Note 10**).
6. Make holes in a 50 mL plastic tube cap, put it on the 50 mL plastic tube of frozen dabsyl-Met-*R*,*S*-O solution and dry under vacuum. You should obtain a red powder.
7. Resuspend the powder in 0.5 mL 0.1 M Tris–HCl, pH 8.0. Mix very well by repeated vortexing (*see* **Note 11**). Transfer the solution to a 1.5 mL test tube (e.g., Eppendorf). Centrifuge for 10 min at maximum speed (e.g., 14,000 × *g*) on a table-top microcentrifuge. If a small insoluble pellet is formed, transfer the supernatant to a clean tube. Store dabsyl-Met-*R*,*S*-O solution at 4 °C.

3.2.2 Preparation of Dabsyl-methionine-R-sulfoxide and Dabsyl-methionine-S-sulfoxide

1. In a 25 mL round-bottom flask with a ground-glass joint, add Met-*R*,*S*-O (1.333 g, 8.069 mmol), picric acid (1.849 g, 8.071 mmol) as solids and 8.5 mL of water (*see* **Note 12**). Position the flask in an oil bath or a heating mantle standing on a hotplate stirrer apparatus fitted on an elevator. Add a stirrer bar in the flask and adapt a suitable condenser on the top.

2. Bring the mixture to reflux while stirring the suspension. As soon as the mixture refluxes gently, stop the stirrer and watch out for the remaining/undissolved solid at the bottom of the flask. If there is no solid left, proceed directly to **step 3**. Otherwise, add 0.5 mL of water through the condenser, resume stirring/reflux for ~2 min, and watch out for the remaining solid again. Keep adding 0.5 mL aliquots of water until the solid dissolves completely (*see* **Note 13**).
3. Stop the heating and let the orange solution to cool down as slowly as possible (ideally overnight in an oil bath/heating mantle). Yellow crystals start to form rapidly. These are enriched in the picrate salt of Met-*S*-O and contain small traces of Met-*R*-O. In order to maximize the separation, make sure the mixture returns to room temperature (~25 °C) before you proceed further.
4. Filter the suspension on a Büchner mounted with a glass-sintered funnel and wash the solid with 5 mL of cold water twice. Recover the yellow crystals (Met-*S*-O enriched) and the yellow solution (Met-*R*-O enriched). Purify them separately by successive crystallization.
5. Purification of Met-*R*-O. Collect the filtrate solution together with wash solutions. Reduce the volume of solvent to ~3–4 mL using a high-vacuum pump or a rotary evaporator until a small amount of solid starts to precipitate. Add 1.5 mL of water and agitate the flask manually. Filter on a glass-sintered funnel and wash the solid with a minimal amount of water (~2 mL). Dispose of the remaining solid.
6. Repeat **step 5** entirely. Then dry the solution under vacuum to obtain a yellow solid. Weigh the solid (e.g., 1.354 g, 393.3 g/mol, 3.44 mmol).
7. Dispose the solid in a 10 mL Erlenmeyer flask. Add 1.1 equivalent of triethylamine as a concentrated 1 M solution and a stirrer bar (*see* **Note 14**). Stir the solution until complete dissolution, then transfer to a 500 mL Erlenmeyer flask. Add 200 mL acetone portion-wise while stirring.
8. Filter the solid on a sintered funnel and wash with 20 mL acetone twice. Dry the solid under high vacuum to obtain Met-*R*-O > 95% pure as a white solid (e.g., $m = 0.515$ g).
9. Purification of Met-*S*-O. Recrystallize the solid from **step 4** once from water (estimated quantity: 20 mL water for 1.76 g of the picrate salt), and once from methanol (estimated quantity: 11 mL methanol for 1.48 g of the picrate salt) as described in **steps 2–4**. Dry under vacuum and weigh (e.g., 1.345 g, 393.3 g/mol, 3.42 mmol).

10. Dispose the solid in a 200 mL Erlenmeyer flask. Add 1.1 equivalent of triethylamine as a dilute 0.175 M solution and a stirrer bar (*see* **Note 15**). Stir the solution until complete dissolution. Add 200 mL acetone portion-wise while stirring.
11. Filter the solid on a sintered funnel and wash with 20 mL acetone twice. Dry the white solid under high vacuum to obtain pure Met-*S*-O (e.g., $m = 0.533$ g).
12. Check purity of the sample by NMR (^{1}H and/or ^{13}C) or by the measurement of the specific rotation using a polarimeter. Compare with the values from the literature [18–21] (*see* **Note 16**).
13. Derivatize Met-*R*-O and Met-*S*-O with dabsyl chloride. In a 50 mL plastic tube, dissolve 10 mg Met-*R*-O in 12 mL 0.9 M $NaCO_3$, pH 9. Add 24 mL of 4 mg/mL dabsyl chloride in acetone. Mix well by vortexing. Proceed similarly for Met-*S*-O.
14. Incubate at 70 °C for 10 min in water bath. Protect from light by covering the bath with aluminum foil (*see* **Note 17**). Freeze at −80 °C.
15. Make holes in a 50 mL plastic tube cap, put it on the 50 mL plastic tube of frozen dabsyl-Met-*R*-O (or dabsyl-Met-*S*-O) solution and dry under vacuum. You should obtain a red powder.
16. Resuspend the powder in 0.5 mL 0.1 M Tris–HCl, pH 8.0. Mix very well by repeated vortexing (*see* **Note 11**). Transfer the solution to a 1.5 mL test tube. Centrifuge at maximum speed (e.g., 14,000 × *g*) on a table-top microcentrifuge. If a small insoluble pellet is formed, transfer the supernatant to a clean tube. Store dabsyl-Met-*R*-O and dabsyl-Met-*S*-O solutions at 4 °C.

*3.2.3 Determination of Dabsyl-Met-*R,S*-O, Dabsyl-Met-*R*-O, and Dabsyl-Met-*S*-O Concentrations*

1. Prepare a standard curve with the dabsyl-Met solution kept in **step 1**. Add 10–90 µL 30 mM Tris–HCl, pH 8.0 (1/10 dilution) to prepare a solution concentrated at 0.5 nmol/µL. Prepare four dilutions as described in Table 1. Inject 50 µL of each in HPLC to obtain a 0.25, 0.5, 1, and 2 nmol standard curve.
2. Determine dabsyl-Met-*R*,*S*-O concentration using the standard curve made as described in Subheading 3.2.3. The final concentration should be ~80–100 mM. Dilute 10 µL in 990 µL 30 mM Tris–HCl, pH 8.0 (1/100 dilution, final concentration ~0.8–1.0 nmol/µL). Prepare four dilutions similar to those described in Table 1 except that dabsyl-Met-*R*,*S*-O solution volumes should be divided by 2 compared to dabsyl-Met solution volumes and 30 mM Tris–HCl, pH 8 volumes modified accordingly.

Table 1
Examples of dilution used

Final quantity for 50 μL injected (nmol)	0.25	0.5	1	2
0.5 nmol/μL dabsyl-Met solution (μL)	3	6	12	24
30 mM Tris–HCl pH 8 (μL)	97	94	88	76
Acetonitrile (μL)	200	200	200	200

3. Determine dabsyl-Met-*R*-O and dabsyl-Met-*S*-O using the standard curve made as described in Subheading 3.2.3. The final concentration should be ~20–40 mM. Dilute 10 μL in 990 μL 30 mM Tris–HCl, pH 8.0 (1/100 dilution, final concentration ~0.2–0.4 nmol/μL). Prepare four dilutions similar to those described in Table 1 replacing the dabsyl-Met solution with the dabsyl-Met-*S*-O solution. Proceed similarly for the dabsyl-Met-*R*-O solution.
4. Inject 50 μL of each dilution of dabsyl-Met for the standard curve and 50 μL of each dilution of the solution to dose (dabsyl-Met-*R,S*-O; dabsyl-Met-*R*-O or dabsyl-Met-*S*-O) and in HPLC, run the separation program and proceed to the analysis.
5. Using a spreadsheet software (e.g., Microsoft Excel), plot areas of peak of dabsyl-Met for each of the four solutions prepared versus the quantity injected (0.25, 0.5, 1, and 2 nmol). Determine the slope of the linear regression. For each tube of the solution to dose, determine the concentration of dabsyl-MetO and those of dabsyl-Met in mM by applying the following equation:

$$\text{Conc.} = \frac{\text{Area} \times 10}{\text{Slope} \times 50}$$

where Conc. is the concentration (mM), Area is the area of the peak of interest, 10 is the initial dilution factor, Slope is the slope of the standard curve, and 50 is the volume injected in μL. Average the four tubes to obtain the concentration of dabsyl-Met-*R,S*-O; dabsyl-Met-*R*-O or dabsyl-Met-*S*-O solution.

3.2.4 Activity Assays and HPLC Analysis

As it may differ according to the experiment setting, the cell lysis protocol to obtain soluble proteins is not described here. For mammalian cells, you can use your favorite methods (e.g., CelLytic™ M cell lysis reagent (Sigma)) and quantify protein using standard Bradford assay or equivalent.

1. Prepare the substrate dilution. To measure the total MSR activity (MSRA and MSRB), prepare dabsyl-Met-*R*,*S*-O to 5 mM final concentration in 30 mM Tris–HCl, pH 8.0 using an appropriate volume of the concentrated solution. To measure MSRA or MSRB activity, proceed similarly with dabsyl-Met-*S*-O and dabsyl-Met-*R*-O, respectively. It is advised to prepare fresh solutions prior to each experiment. Equilibrate the solution at 37 °C in water bath or in a dry bath with agitation (e.g., Eppendorf ThermoMixer).
2. Prepare the reaction assay. In a 1.5 mL test tube containing the appropriate volume of 30 mM Tris–HCl, pH 8.0, to a final volume of 200 μL, mix 200 μg of protein with 2 μL of 1 M dithiothreitol (20 mM final concentration). Prepare a control tube without protein (*see* **Note 18**). Equilibrate the solution for 5 min at 37 °C. Start the reaction by adding 20 μL of 5 mM dabsyl-Met-*R*,*S*-O for total activity assay, or of individual diastereomer for MSRA or MSRB activity assays (0.5 mM final concentration). Mix well by short-time vortexing and incubate for 30 min at 37 °C (*see* **Note 19**).
3. Stop the reaction by adding 300 μL acetonitrile.
4. Centrifuge at 4 °C for 30 min at 12,000 × *g*.
5. Inject 50 μL of supernatant in HPLC, run the separation program, and proceed to the analysis.
6. Determine the quantity of dabsyl-Met produced and report activity in pmol of dabsyl-Met produced by minute by mg of protein using the following equation:

$$\text{Activity} = \frac{\text{Q Met} \times \frac{\text{Vol.reaction assay}}{\text{Vol.injected}} \times \text{DF}}{\text{Inc.time} \times \text{Q Prot.}}$$

where Q Met is the quantity of dabsyl-Met produced, Vol. reaction assay is a total volume of the reaction (200 μL), Vol injected is the injected volume (50 μL), DF is the dilution factor (200/300 μL), Inc. time is the time of incubation in min (30 min), and Q Prot. is the total quantity of protein by reaction (200 μg).

4 Notes

1. MetROx expression plasmids targeted to cytosol, mitochondria, the endoplasmic reticulum, and nucleus were made by inserting the MetROx chimera gene and an appropriate targeting sequence between the *NheI* and *BamHI* restriction sites of pEGFP-C3 vector. Available plasmids can be obtained upon

Table 2
Available plasmids

Name	Description
pCyto-MetROx	Expression of MetROx in the cytosol of mammalian cells
pCyto-C129S MetROx	Expression of inactive C129S MetROx in the cytosol of mammalian cells
pCyto-C69S/C129S MetROx	Expression of inactive C69S/C129S MetROx in the cytosol of mammalian cells
pMito-MetROx	Expression of MetROx in mitochondria of mammalian cells
pMito-C129S MetROx	Expression of inactive C129S MetROx in mitochondria of mammalian cells
pNucleus-MetROx	Expression of MetROx in the nucleus of mammalian cells
pNucleus-C129S-MetROx	Expression of inactive C129S-MetROx in the nucleus of mammalian cells
pER-MetROx	Expression of MetROx in the ER of mammalian cells
pER-C129S-MetROx	Expression of inactive C129S-MetROx in the ER of mammalian cells

request from the authors and are described in Table 2. These kanamycin-resistant plasmids can be amplified in *Escherichia coli* and purified using standard procedures.

2. The use of the SunFire™ C18 column allows the fast separation of dabsyl-MetO from dabsyl-Met using the described program. Other columns and programs can be used to separate the *R*- and *S*-diastereomers of dabsyl-MetO [22].
3. For a typical measurement, you should use at least one well with the active MetROx sensor and one with the inactive sensor (C129S MetROx or C69S/C129S MetROx).
4. Since it is a single cell measurement, it is not essential to get all of the cells transfected, but the more fluorescent cells are present in a field of view, the more information you are able to gather by imaging. If no fluorescence is detected, make sure that transfection is successful. You may control for transfection efficiency by separately transfecting with the pEGFP-C3 control plasmid. Depending on the microscope setup and the length of the experiment, photobleaching might be an issue. We advise using a wide field (instead of confocal laser scanning) fluorescence microscope setup with high sensitivity detector to record time series and always record a photobleaching curve prior to starting the actual experiments.
5. Short experiments (less than 30 min) can be carried out without CO_2 and temperature control. However, be mindful of the potentially slower kinetics of redox reactions at room temperature. Do not forget to change the bicarbonate-based D-MEM

to a solution with an adequate buffering system (e.g., 10 mM HEPES, pH 7.4) without CO_2 under these conditions.

6. Use equivalent beam path settings depending on the manufacturer of the microscope and the software driving it. Perform several single scans to set the final focus, adjust light power and exposure time. The initial fluorescence intensities in both the channels should be stable. If your system is not equipped with autofocus capabilities, it is important that the microscope focal plane should not change over the experimental period.
7. Alternatively, the ratio of the inactive sensor could be used as the ratio of the fully reduced sensor in the equation. This allows direct correction of the fluorescence ratio. However, it is possible only if the measured ratio is higher than the one measured for the active sensor.
8. Proceed by the successive addition and removal of 1 mL DMSO to the glass bottle to avoid losing the product by weighing. Adjust the volume if starting from a 100 mg-containing glass bottle. Dabsyl-Met solubilized in DMSO can be stored at room temperature or below.
9. Dabsyl-Met-*R*,*S*-O and dabsyl-Met should be eluted at 3.5 min and 4.6 min, respectively. If no oxidation occurred, use a fresh bottle of H_2O_2 solution. Make sure to respect concentrations.
10. The maximum volume of dabsyl-methionine-*R*,*S*-sulfoxide solution to be loaded on the C18 cartridge may vary. To avoid loss of solution, be sure that it does not come out of the column while loading (the solution is red). Adjust the volume accordingly and repeat the procedure. We advise using a new disposable cartridge for each purification. If you prefer to reuse the cartridge, increase the equilibration volume to 3 × 5 mL acetonitrile:acetate buffer (5:95 v:v).
11. We experienced sometimes problems with solubilization, and vortexing might not be sufficient to completely resolubilize the powder. If this is the case, add 5 N NaOH in 5 μL increments and vortex until complete solubilization. Alternatively, solubilization can be made in acetonitrile or 70% ethanol.
12. The quantities of Met-*R,S*-O, picric acid, and water can be slightly adjusted while keeping their ratio constant. However, it must be noted that low-scale recrystallization tends to be less effective, whereas the large-scale ones require particular care in handling.
13. An optimal separation is obtained when the solution is saturated and there is no solid left to interfere with the crystal formation. This means the salts should be *entirely* dissolved in the *minimal* amount of water. If the quantity of salts specified

cannot be dissolved in 15 mL of water, consider checking purity of the starting materials or perform an additional hot-filtration to remove excess solid.

14. The quantity of triethylamine is relative to the amount weighed in **step 6**. In the example given, use 3.8 mL of triethylamine 1 M solution.
15. Similarly to **Note 14**, the volume of triethylamine 0.175 M solution is calculated considering the molar quantity of picrate salt measured in **step 9** (e.g., 21.5 mL).
16. Alternatively, purity could be estimated by reverse phase HPLC as previously described [22].
17. Alternatively, incubation can be done overnight at room temperature. Protect from light by wrapping the tube in aluminum foil.
18. The prepared substrate might be contaminated with dabsyl-Met (generally not more than 5%). Use the control tube to correct the area of the dabsyl-Met formed in a protein-containing reaction.
19. Quantity of protein and incubation time might need to be adjusted for optimal activity measurement. To ensure saturation of MSR with substrate, make sure that sufficient amount of substrate remains at the end of the assay; otherwise, the activity will be underestimated. If no substrate remains, decrease the quantity of protein and/or the incubation time.

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