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Practical guide for dynamic monitoring of protein oxidation using genetically encoded ratiometric fluorescent biosensors of methionine sulfoxide

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Abstract

In cells, physiological and pathophysiological conditions may lead to the formation of methionine sulfoxide (MetO). This oxidative modification of methionine exists in the form of two diastereomers, R and S, and may occur in both free amino acid and proteins. MetO is reduced back to methionine by methionine sulfoxide reductases (MSRs). Methionine oxidation was thought to be a nonspecific modification affecting protein functions and methionine availability. However, recent findings suggest that cyclic methionine oxidation and reduction is a posttranslational modification that actively regulates protein function akin to redox regulation by cysteine oxidation and phosphorylation. Methionine oxidation is thus an important mechanism that could play out in various physiological contexts. However, detecting MetO generation and MSR functions remains challenging because of the lack of tools and reagents to detect and quantify this protein modification. We recently developed two genetically encoded diasterospecific fluorescent sensors, MetSOx and MetROx, to dynamically monitor MetO in living cells. Here, we provide a detailed procedure for their use in bacterial and mammalian cells using fluorimetric and fluorescent imaging approaches. This method can be adapted to dynamically monitor methionine oxidation in various cell types and under various conditions.

1. Introduction

Living cells are constantly exposed to oxidants such as reactive oxygen species (ROS), chloramines or peroxynitrites [1,2]. Under physiological conditions, ROS concentrations are controlled and can regulate processes such as proliferation, differentiation and apoptosis [3,4]. However, several conditions can lead to a loss of redox homeostasis upon dysregulation of ROS production or elimination, leading to the accumulation of ROS to detrimental levels [1,4]. ROS can oxidize proteins and virtually all amino acids may be affected. The sulfur-containing amino acids, cysteine and methionine, are prone to oxidation

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at their sulfur atom, and they are the only identified amino acids for which oxidation is reversible [5]. In the case of signal transduction, ROS action generally occurs through oxidation of critical cysteine residues of metabolic enzymes or signaling proteins, such as kinases and transcription factors [1,3]. Numerous enzymatic and non-enzymatic systems exist to reduce oxidized cysteines [4]. Methionine (Met) can also undergo oxidation by the addition of one oxygen atom on the lateral chain leading to the formation of methionine sulfoxide (MetO), which exists as two diastereomers, *R* (Met-*R*-O) and *S* (Met-*S*-O). Oxidation can occur in free amino acid and in protein-based methionine residues, and organisms possess several enzymes of the methionine sulfoxide reductase (MSR) group of protein that reduce the oxidized form [6]. The most widespread of these enzymes are methionine sulfoxide reductases A (MSRA) and B (MSRB), which are found in almost all organisms and are specific for the reduction of the *S* and *R* diastereomers, respectively [7,8]. MSRA can reduce both free and protein-based Met-*S*-O, whereas generally MSRB only reduces Met-*R*-O in proteins [6,9]. It should also be noted that plants possess numerous MSRBs and, among them, some display the capacity to reduce free Met-*R*-O [10,11]. Typical MSRA and MSRB use the reducing power provided by the NADPH-dependent thioredoxin reductase/thioredoxin (Trx) system in a mechanism involving disulfide exchange (*see 2. Theoretical*) [12]. Glutaredoxin and glutathione are also used by some MSRs as the regenerating system [13–17]. Prokaryotes and single-cell eukaryotes possess another enzyme, f*R*MSR, which reduces the free form of the *R*-diastereomer of MetO [18,19]. In bacteria, several enzymes of the dimethyl sulfoxide reductase family containing a molybdenum cofactor can have a MSR activity, particularly, the biotin sulfoxide reductase BisC, which reduces only the free form of the *S*-diastereomer of MetO and was shown to have relevant activity for MetO reduction *in vivo* [20]. Very recently, another molybdoenzyme was identified in the bacterial periplasm and shown to reduce free and protein-based MetO without any stereospecificity, making it a ‘lethal weapon’ for the protection of the bacterial envelop against deleterious protein oxidation [21,22].

Little is known regarding the effect of free Met oxidation in cells, but it was shown that, in the absence of an appropriate reducing enzyme, MetO cannot be used as a source of Met in auxotroph organisms [20,22,23]. In proteins, methionine residues are not equally sensitive to oxidation, but it appears that the surface-exposed ones are more sensitive to oxidation than the buried residues. Moreover, the amino acid environment determines the sensitivity to oxidation and the propensity to form one or the other diastereomer, although no sequence ‘signature’ of Met oxidation could be clearly defined [9,24,25]. Oxidation of Met in proteins can have several consequences (see [26,27] for reviews), and depending on these consequences, oxidized proteins can be classified into four groups [9]: (i) proteins not impaired by Met oxidation, which could fulfill, together with MSRs, an antioxidant function through cyclic oxidation and reduction of Met [28], (ii) proteins damaged by Met oxidation, such as those involved in neurodegenerative diseases [29–31], (iii) unfolded proteins and nascent polypeptides whose protein core Met are susceptible to oxidation thereby affecting their proper folding which has been shown to greatly accelerate their degradation [9], and (iv) proteins whose functions are actively regulated by cyclic Met oxidation/reduction. This class, where the oxidation is targeted and purposeful, is a novel emerging field in biochemistry and cell physiology. Studies in recent years yielded several new pathways

involving redox modification of Met. For example, the oxidation can be a necessary biochemical reaction step in crosslinking collagen IV by peroxidasin in the extracellular matrix [32–34]. In other cases, the protein function is regulated by cyclic Met oxidation/reduction, such as the calcium/calmodulin-dependent protein kinase II [35], the bacterial HypT transcription factor [36], and the actin for which Met oxidation was shown to be catalyzed by MICAL monooxygenases [37–39].

Genetic studies and quantification of MSR activity showed that MSRs are implicated in the protection against oxidative stress in numerous organisms and suggest that MetO accumulation is linked with neurodegenerative diseases and aging [29,40–42]. However, the actual quantification of the MetO content was rarely made, and the data supporting these hypotheses are based on the idea that modification of MSR activity is correlated with the MetO content in cells. However, this does not offer a possibility to quantify the effect and help determine if the observed phenotype results from global protein oxidation or from the oxidation of few specific Met residues in key proteins. Related to this, it is particularly important to note that not all oxidized Met in proteins can be reduced by the MSR system because of the lack of accessibility of MetO [9]. This was shown for the oxidized α-synuclein, which possesses four oxidizable Met, and for which only two can be reduced *in vivo* by the MSR system [29]. To study oxidation and reduction of α-synuclein in cells, the authors used an interesting method which introduced an oxidized ^{15}N-labeled protein into cells and followed its oxidation state by NMR.

Prior to the genetically encoded fluorescent sensors of MetO, for which a practical guide is proposed in this paper, two methods were published for quantification of MetO. The first is an HPLC-based method which consists of a total amino acid quantification from a protein extract [28,43]. This method allowed estimation of the levels of MetO in several tissue types and cells, which were 2–10 % of total Met content, and to show their increase up to 60 % in response to oxidative stress [28,44–46]. The second method is a proteomic approach identified oxidized Met in protein extracts by mass spectrometry [24,47]. This protocol has the advantage to clearly identify the affected Met in protein contexts and report relative abundance of MetO for each protein. Neither HPLC nor proteomic methods required genetic manipulation, but could only be used with protein extracts, which precludes dynamic measurements. Both methods also require harsh treatments of the samples, such as protein acid hydrolysis or trypsin digestion, which could induce non-specific Met oxidation.

The importance of MetO as a redox biomarker in numerous physiological and pathological conditions, the limitations of existing methods and the fact that no known chemicals or antibodies react specifically with MetO, prompted us to develop genetically encoded fluorescent sensors to dynamically monitor MetO formation and reduction in living cells [48]. The two created sensors, MetSOx and MetROx, are derived from yeast MSRA and MSRB, ensuring specificity for the *S* and *R* diastereomers of MetO, respectively. The MetO sensors may be expressed in any cell compartment if an appropriate signal peptide is added. MetSOx and MetROx have two excitation peaks around 420 nm and 500 nm corresponding to the protonated and charged forms of the Tyr residue of their fluorescent moiety, respectively. Upon reaction with MetO, the intensity of each peak is modified, conferring ratiometric behavior which is ideal for this type of sensor as it permits signal normalization

independent of the sensor concentration. MetSOx and MetROx may be used to monitor MetO levels and changes in various cell types using simple fluorimeters or more sophisticated microscope systems under numerous conditions.

2. Theoretical

2.1 The concept: mimic Trx/MSR interaction to create MetO sensors

To create genetically encoded fluorescent sensors of MetO, we took advantage of the known mechanism of MSR reduction by the Trx system. We applied a strategy similar to the one used for the creation of the hydrogen peroxide sensor HyPer [49]. This sensor was made by inserting a circularly permutated yellow fluorescent protein (cpYFP) into the sequence of the H_2O_2 sensitive OxyR transcription activator. Upon reaction with H_2O_2, a disulfide bond is formed between two redox-active cysteines of the OxyR parts, inducing a modification of the structural and spectral properties of the cpYFP moiety (Fig. 1A). For the creation of MetO sensors, we replaced both OxyR parts of HyPer by a MSR and a mutated Trx, in which the resolving cysteine was mutated to serine. We chose yeast MSRA and MSRB, as these are typical 2-Cys MSRs, and their catalytic mechanisms and structure were well characterized [9,50]. Regeneration of MSR activity by Trx proceeds in a three-step mechanism (Fig. 1B): (i) reduction of MetO leads to the formation of a sulfenic acid on the catalytic cysteine; (ii) a resolving cysteine forms an intramolecular disulfide bond with the catalytic cysteine, reducing the sulfenic acid; (iii) the disulfide is reduced by a thioredoxin (Trx) in a process involving a transient intermolecular bond between catalytic cysteine residues of the two partners [6,12]. In our chimera proteins, mutation of the second redox-active cysteine of the Trx allows a stable covalent bond to be formed between the MSR and Trx moieties, thus affecting cpYFP structure (Fig 1C). The structural change induced by the reaction of the sensors with MetO is reflected by the change in the cpYFP's spectral properties.

2.2 Spectral properties of MetSOx and MetROx

MetSOx spectrum shows two peaks of excitation with maxima at 425 nm and 505 nm and a single peak of emission at 510–516 nm. Oxidation of MetSOx by the reaction with MetO induces a substantial increase in emission fluorescence intensity and of the 505 nm excitation peak without change at 425 nm. Thus, the ratio of fluorescence intensity 510/425 nm increases upon reaction with MetO-containing substrates. In the case of MetSOx, the spectrum presents two peaks of excitation with maxima at 410 nm and 500 nm and a single peak of emission at 510–516 nm, and the reaction with MetO lead to an increase in fluorescence intensity at 410 nm and a decrease at 500 nm with an isosbestic point at 447 nm. Thus, the 500/410 nm fluorescence intensity ratio decreases with the oxidation of MetROx [48]. To efficiently monitor MetO oxidation using MetSOx and/or MetROx, the experimental setting should allow to directly measure the fluorescence intensity for the two excitation wavelengths upon emission at ~510 nm, with a fluorimeter, or alternatively, to record fluorescence emission intensity for the two excitation wavelengths (~410 nm and ~500 nm) with a microscope.

2.3 The use of negative controls and pH measurements

Similar to what was shown for HyPer [49] and cpYFP [51], MetSOx and MetROx fluorescence intensity is dependent on pH. Fluorescence intensity increases or decreases with the increase in pH when excited at ~500 nm, or at ~400–425 nm, respectively [48]. Reduced and oxidized forms of MetSOx have pKa of 8.5 and 9.5, respectively, and reduced and oxidized forms of MetROx exhibit pKa of 7.7 and 8.6, respectively. The maximum signal variation is observed at pH ~7–7.5. This requires estimating non-specific changes in fluorescence to accurately measure MetO variation in cells. The mechanism of interaction between the MSR and Trx moieties within the sensors allows preparation of inactive, control versions by replacing the redox-active Cys of the MSR moiety with a non-redox residue, such as serine. Under optimal conditions, the spectra of inactive sensors should correspond to the spectra of fully reduced active MetO sensors and should not show any change upon reaction with MetO. We initially created Cys-to-Ser variants by mutating the MetO-reducing Cys of MSRA (Cys25) in MetSOx and MSRB (Cys129) in MetROx. The C25S form of MetSOx behaves as a fully reduced form of MetSOx and its spectrum does not change upon reaction with MetO, both *in vitro* and in *Escherichia coli* cells, which is expected from the proposed mechanism [48]. In the case of MetROx, we observed that C129S mutation led to a complete loss of spectral modification by MetO, but that the spectrum corresponds to the fully oxidized MetROx. This was somewhat unexpected from the theoretical mechanism and may indicate that in absence of the catalytic Cys of MSRB, the two remaining Cys (Cys69 and Cys417 of the MSRB and Trx moieties, respectively) might form a disulfide bond. We created another inactive version of MetROx by replacing both Cys69 and Cys129 with serine and observed an initial fluorescence ratio corresponding to the reduced sensor, with no reactivity towards MetO (Fig. 2), making this version an appropriate negative control. For accurate MetO measurement, it is necessary to monitor the fluorescence ratio of the active and inactive MetO sensors in parallel experiments. The non-specific change in inactive versions may be used to correct the fluorescence ratio measurement with the active MetO sensors. Another approach would consist of measuring pH changes using another sensor, as it was done with the ‘green’ ADP/ATP PercevalHR and the ‘red’ pH sensor pHRed simultaneously expressed in cells [52,53]. For such controls of pH, the pKa of the sensors of interest should be as close as possible to the pKa of the pH sensors.

2.4 What does the MetO sensors fluorescence ratio really represent?

Created with the MSRs as catalytic parts, the MetO sensors should be able to detect all the MetO which could be reduced by their MSR moiety. Yeast MSRA reduces both free and protein-based forms of Met-*S*-O, whereas yeast MSRB only uses efficiently protein-based Met-*R*-O as substrate [9]. As illustrated by model substrates *in vitro* [9], or for the oxidized α-synuclein in cells [29], not all oxidized Met are substrates of MSRs. Thus, MetSOx is able to detect the formation of Met-*S*-O on free amino acid and accessible Met in proteins, and MetROx should detect accessible Met-*R*-O in proteins. Oxidation of MetO sensors was shown to be reversible in cells, meaning that the disulfide bond formed between the MSR and Trx parts in oxidized sensors is reduced in cells, very likely by the endogenous thioredoxin or glutathione/glutaredoxin systems [48]. Thus, the ratio of fluorescence reflects equilibrium between oxidation resulting in the increase in MetO and reduction, as discussed for the HyPer sensor [54]. In context of dynamic measurement where a stimulus potentially

generates MetO in cells, oxidation of the sensor (i.e. illustrated by the fluorescence ratios increasing and decreasing for MetSOx and MetROx, respectively) is likely due to the increase in MetO content rather than the decrease in reducing capacity. However, when the signal of the sensor reflects reduction (i.e. the fluorescence ratio decrease for MetSOx and increase for MetROx), the cause of this reduction may be due a decrease in MetO content as well as an increase in the reducing capacity. This idea is consistent with transcriptomic and protein translation data showing that genes and proteins encoding Trx and glutaredoxins are overproduced under numerous conditions of oxidative stress [55,56]. It would be of great interest to identify the players responsible for the reduction of the MetO sensors *in vivo* and to precisely determine their kinetics of interaction.

3. Methods

3.1 Monitoring MetO generation in *E. coli* cells with a fluorimeter

This section describes the protocol we used for the dynamic measurement of Met-*S*-O and Met-*R*-O generation in *E. coli* cells in response to sodium hypochlorite using the SpectraMax M5® fluorescence microplate reader [48].

3.1.1 Reagents and biological materials

- *Escherichia coli* SoluBL21™ cells (Gelantis, San Diego, CA)
- Plasmids for expression of MetSOx, MetROx and their inactive version [48]. Plasmids can be obtained upon request from the authors.
- Liquid Lysogeny Broth (LB) media
- LB Agar plates containing 50 μg.ml^{-1} ampicillin
- M9 minimal media
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)
- Ampicillin
- Free L-Methionine-*R,S*-sulfoxide
- Sodium hypochlorite

3.1.2 Equipment

- SpectraMax M5® fluorescence microplate reader (Molecular Devices, Sunnyvale, CA)
- Cornig® Costar Black clear-bottom 96-well plates
- 1-cm path quartz cuvette

3.1.3 Procedure

Cells preparation

1. Transform *E. coli* SoluBL21™ cells using standard procedure [57] and LB Agar ampicillin (50 μg.ml^{-1}) plates with the appropriate expressing plasmid.
2. Inoculate 1 colony in 10 ml LB containing 50 μg.ml^{-1} ampicillin and grow overnight at 37°C under agitation.
3. Inoculate 100 ml LB containing 50 μg.ml^{-1} ampicillin to A_{600} ~0.05 and incubate at 37°C under agitation until A_{600} reaches ~0.3.
4. Induce MetO sensor production by adding 100 μM IPTG and incubate cells for 14 to 20 h at 20°C.
5. Centrifuge 5 or 10 ml at 5,000 g and rinse the cells twice by resuspension/centrifugation in minimal M9 media to remove LB media which could interfere with the fluorescence measurement. Resuspend the cells in M9 media in order to obtain a final A_{600} of ~6 and ~3 for MetSOx and MetROx expressing cells, respectively.
6. Equilibrate the cells at 25°C for 30 min at least under moderately slow agitation. As such, cells are usable for 2h.

Recording fluorescence spectra: To ensure correct expression of the MetO sensor, one must evaluate the fluorescence of the cells by recording the spectra of those expressing the active and the inactive sensors.

1. To monitor Met-*S*-O generation using MetSOx, set up the fluorimeter to record excitation spectra from 380 nm to 510 nm with 530 nm emission and a 530-nm cutoff in a 1-ml cuvette. For monitoring Met-*R*-O generation using MetROx, set up the fluorimeter similarly but the measurement can be done in 200-μl using black clear-bottom 96-wells plates.
2. Record spectra for MetSOx and C25S MetSOx expressing cells. The ratio of fluorescence intensities at 505 nm and 425 nm for C25S MetSOx-expressing cells should be ~1, which corresponds to the fully reduced state of the sensor. For MetSOx-expressing cells, the value should be very close to ~1. A value lower than the one recorded with C25S MetSOx might indicate a non-specific variation of fluorescence intensity, and a higher value indicates that the sensors is oxidized.

   Record spectra for MetROx and C69S/C129S MetROx-expressing cells. The ratio of fluorescence intensities at 500 nm and 410 nm for C69S/C129S MetROx-expressing cells should be ~2 to 2.5, which corresponds to the fully reduced state of the sensor.
3. To test the sensor, incubate the cells with 1 to 2.5 mM free L-Methionine-*R, S*-sulfoxide using concentrated stock solution (typically, 0.1 – 1 M) to

put a small volume (typically, 5 – 20 μl) to avoid cell dilution. Mix and record spectra every minute. Oxidation of the active sensor should be complete after 15 min, and the inactive sensor should not show important changes. It is advised to carefully consider the results of experiments in which variation of the ratio of fluorescence of the inactive sensor exceeds 0.2.

Recording kinetics

1. To dynamically monitor Met-*S*-O generation using MetSOx, set up the fluorimeter to record fluorescence excitation intensities at 425 nm (F_{425}) and 510 nm (F_{510}) with 535 nm emission and a 530-nm cutoff in a 1-ml cuvette. Kinetics time and reads interval are typically set up to 30 min and 10 sec, respectively.
2. For monitoring Met-*R*-O generation using MetROx, set up the fluorimeter to record fluorescence excitation intensities at 410 (F_{410}) nm and 500 (F_{500}) nm with 535 nm emission and a 530-nm cutoff in 200-μl using black clear-bottom 96-wells plates. Kinetics time and reads interval are typically set up to 30 min and 10 sec, respectively.
3. Prior to addition of the reagent, record kinetics for 30 sec to 2 min to ensure signal stability and that the ratio of fluorescence intensity corresponds to the one obtained by spectra recording.
4. If using a 1-ml cuvette to monitor MetO generation, addition of the reagent can be done directly without stopping measurement of fluorescence. Remove the cuvette, add concentrated NaOCl (40 – 100 μM final concentration), mix by returning the capped-cuvette up-and-down and put it back to the fluorimeter. Proceed quickly. If using a 96-well plate, the kinetic should be stopped prior to addition of the reagent. To proceed, record kinetics without the reagent, then stop it after ~2–5 min. On the fluorimeter, set up the software for the next kinetics measurement and open the drawer with the 96-well plate on it. Add concentrated NaOCl (40 – 100 μM final concentration), mix by pipetting up-and-down, close the drawer and start the measurement. Proceed quickly.

3.1.4 Troubleshooting

1. No detected fluorescence. Make sure that protein expression and folding are successful. Bacterial culture could be kept at 4°C for 2 h after protein induction to facilitate sensor folding.
2. Low fluorescence intensity. Make sure that the concentration of cells is sufficient. Use appropriate excitation/emission wavelengths and filters.

3.2 Monitoring MetO changes in mammalian cells by fluorescence microscopy

Here, we provide a protocol for single cell measurements in mammalian cells.

3.2.1 Reagents and materials

- HEK293T/17 cells (ATCC)
- Plasmids for expression of MetSOx, MetROx and their inactive version [48]. Plasmids can be obtained upon request from the authors.
- Dulbecco' s Modified Eagle Medium (D-MEM; Gibco)
- Opti-MEM without phenol red (Gibco) and without serum
- FCS (Gibco)
- Penicillin, Streptomycin (Gibco)
- FuGENE HD transfection reagent (Promega)
- Thirty-five millimeter glass-bottom dishes (MatTek)

3.2.2 Equipment

- A motorized Olympus IX81 inverted microscope equipped with a Hamamatsu Orca ER cooled-CCD camera in conjunction with a zero-drift focus compensation system
- Filters: excitation (420/40 and 500/16), emission (535/30)
- Software: acquisition: MetaMorph software (Universal Imaging, Downingtown, PA), analysis: ImageJ/Fiji.

3.2.3 Procedure

Transfection

1. Seed HEK cells onto 35-mm glass-bottom dishes (10^5 cells/ dish) in D-MEM supplemented with 10% FCS and penicillin/streptomycin. 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 Eppendorf tube (1.5 ml).
3. Mix well 1 μg of sensor coding expression vector in the medium.
4. Add 3 μl of FuGENE HD 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.

Imaging of MetO formation in cells

1. Sixteen to twenty-four h after transfection, examine effectiveness of transfection by checking fluorescence using your cell culture microscope. Use the GFP filter set on the microscope. Since it is a single cell measurement, it is not essential to get all of the cells transfected, but the

more fluorescent cells in a field of view the more information you are able to gather by imaging.

2. 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, and the adequate buffering system (e.g. HEPES) without CO_2 under these conditions. 30 min prior to the experiment, change the medium to Live Cell Imaging Solution (Gibco) supplemented with 5 mM glucose and allow the dish to stay at room temperature.

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, or equivalent 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 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. Make sure to adhere to the following:

    a. Live imaging should be carried out in time series or xyt mode.

    b. Filter set up: For excitation, use 420/40 (channel 1) and 500/16 (channel 2) band-pass excitation filters and 535/30 band-pass emission filter. Please check the fluorescence spectra of sensors and adjust your system setup accordingly. Typically, emission is acquired every 15 to 30 s for 10 – 15 min.

5. Start time series recording.

3.2.4 Troubleshooting

1. No detected fluorescence in mammalian cells. Make sure that the transfection was successful. Control for transfection efficiency by including a GFP control plasmid.

2. Low fluorescence intensity in mammalian cells. Intensity of MetSOx fluorescence is difficult to detect in the mammalian expression system so we did not use it for time series analysis. In the case of MetROx, we did not experience any difficulties detecting fluorescence, however, the transfection conditions and expression time need to be adjusted to different cell types.

3. Photobleaching. Depending on the microscope setup and the length of the experiment, this might be a significant issue. We advise using a wide field (instead of confocal laser scanning) fluorescence microscope setup with high sensitivity detector to record time series. Always record a photobleaching curve prior to starting the actual experiments.

3.3 Data analysis

3.3.1 Calculation of fluorescence intensity ratios

Using a fluorimeter (*e.g. bacterial cells suspensions, section 3.1*): The fluorescent sensor ratio (*R*) is calculated as follows:

For MetSOx: $R_{\mathrm{MetSOx}} = F_{505\ \mathrm{nm}} / F_{425\ \mathrm{nm}}$, where $F_{505\ \mathrm{nm}}$ and $F_{425\ \mathrm{nm}}$ are the fluorescence excitation intensities at 505 nm and 425 nm, respectively.

For MetROx: $R_{\mathrm{MetROx}} = F_{500\ \mathrm{nm}} / F_{410\ \mathrm{nm}}$,, where $F_{500\ \mathrm{nm}}$ and $F_{410\ \mathrm{nm}}$ are the fluorescence excitation intensities at 500 nm and 410 nm, respectively.

Using a microscope (*e.g. single-cell mammalian expression system, section 3.2*): The fluorescence intensity analysis is made using the software ImageJ/Fiji as follows:

1. To analyze the images, 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 ROI on the first cell you would like to measure.
4. Press 't' (or click "*Add*" on the *ROI Manager*) for multiple ROIs. Do not forget to include non-fluorescent ROI-s for background correction. Repeat until all cells are selected.
5. To check all your ROIs, click "*Show All*" on the *ROI Manager*.
6. Use Time series Analyzer to calculate all average intensities in the image stack.
7. The sensor ratio (*R*) is calculated by dividing the background subtracted fluorescence in channel 2 ($F_{500\ \mathrm{nm}}$) by the background subtracted fluorescence in channel 1 ($F_{420\ \mathrm{nm}}$).

3.3.2 Calculation of the oxidized fraction and correction with negative controls

—To calculate oxidized fractions of the sensor use the following equations:

$$\text{For MetSOx:} \quad \text{Oxidized fraction} = (R - R_{\mathrm{Reduced}}) / (R_{\mathrm{Oxidized}} - R_{\mathrm{Reduced}}) \qquad \text{(1a)}$$

where *R*, measured $F_{505\ \mathrm{nm}}/F_{425\ \mathrm{nm}}$ ratio; R_{Reduced}, $F_{505\ \mathrm{nm}}/F_{425\ \mathrm{nm}}$ ratio of fully reduced MetSOx, and R_{Oxidized}, $F_{505\ \mathrm{nm}}/F_{425\ \mathrm{nm}}$ ratio of fully oxidized MetSOx.

$$\text{For MetROx:} \quad \text{Oxidized fraction} = (R_{\text{Reduced}} - R)/(R_{\text{Reduced}} - R_{\text{Oxidized}}) \quad (1b)$$

where R, measured $F_{500\ nm}/F_{410\ nm}$ ratio; $R_{Reduced}$, $F_{500\ nm}/F_{410\ nm}$ ratio of fully reduced MetROx and $R_{Oxidized}$, $F_{500\ nm}/F_{410\ nm}$ ratio of fully oxidized MetROx.

The ratio of the fully reduced sensor can be obtained by adding dithiothreitol, and the ratio of the fully oxidized sensor can be recorded by adding a saturating concentration of free MetO to the cell media. To avoid non-specific changes in the sensor signal, analyze cells expressing the inactive sensors (C25S MetROx or C69S/C129S MetROx), and divide the signal of the active sensor by that obtained with the inactive sensor. Alternatively, the ratio of the inactive sensor could be used as the ratio of the fully reduced sensor in equations 1a and 1b. This allows direct correction of the fluorescence ratio. However, it is possible only if the measured ratio is lower than the one measured for the active sensor in the case of MetSOx and higher than the one measured for the active sensor in the case of MetROx.

4. Conclusions

The sensors we describe in this article are the first tools to investigate MetO metabolism in living cells. This is especially important in the light of the limited availability of tools for characterizing Met oxidation and MetO reduction in any biological context. However, being the first generation MetO sensors, they suffer from some limitations that potential users should be aware of.

1. Since the oxidation of sensors is reversible, the observed differences are affected by the activity and composition of reducing systems and not only by the presence of MetO. We advise taking into consideration the presence and status of reducing systems in the cell in interpreting changes in the fluorescence ratios.
2. Further experiments are needed to provide understanding on how the probes work mechanistically. Future studies may establish the exact role of the cysteines in the MSR and Trx domains in the proposed mechanism. This understanding may provide further details on what fluorescence ratio really reflects and would be able to give us clues about the potential reducing systems responsible for reversibility of the sensors *in vivo*.
3. The pH sensitivity of any cpYFP-containing probe is a major concern when it comes to interpretation of the results, and the MetO probes are no exemption. Circular permutation opens the tertiary structure of the fluorescent protein and exposes the chromophore phenoxy group [51]. Thus, every measurement needs a proper pH control. In case of MetSOx, the control probe (C25S) is an appropriate control since the chromophore's protonation status does not change as a result of the C25S mutation. In the case of MetROx, the double mutant (C69S, C129S) shows a similar fluorescence ratio as the reduced sensor and thereby it can be used as an appropriate control (Fig 2). Alternatively, pH changes can be

followed by another probe using in the red spectrum simultaneously (by pHRed) or in parallel experiments (by SypHer).

If future users of the sensors follow our recommendations and keep these caveats in mind, they will have powerful and unique tools to understand the biological implications of MetO production and reduction with previously unknown temporal and spatial resolution. These sensors combine sensitive and specific detection with ease of use that we hope is evident from this protocol.

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References

1. D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol. 2007; 8:813–824. [PubMed: 17848967]
2. Roos D, Winterbourn CC. Lethal Weapons. Science. 2002; 296:669–671. [PubMed: 11976433]
3. Finkel T. Signal transduction by reactive oxygen species. J. Cell Biol. 2011; 194:7–15. [PubMed: 21746850]
4. Labunskyy VM, Gladyshev VN. Role of reactive oxygen species-mediated signaling in aging. Antioxid. Redox Signal. 2013; 19:1362–1372. [PubMed: 22901002]
5. Davies MJ. Protein oxidation and peroxidation. Biochem. J. 2016; 473:805–825. [PubMed: 27026395]
6. Tarrago L, Gladyshev VN. Recharging oxidative protein repair: catalysis by methionine sulfoxide reductases towards their amino acid, protein, and model substrates. Biochem. Biokhimila. 2012; 77:1097–1107.
7. Grimaud R, Ezraty B, Mitchell JK, Lafitte D, Briand C, Derrick PJ, et al. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J. Biol. Chem. 2001; 276:48915–48920. [PubMed: 11677230]
8. Lowther WT, Weissbach H, Etienne F, Brot N, Matthews BW. The mirrored methionine sulfoxide reductases of Neisseria gonorrhoeae pilB. Nat. Struct. Biol. 2002; 9:348–352. [PubMed: 11938352]
9. Tarrago L, Kaya A, Weerapana E, Marino SM, Gladyshev VN. Methionine sulfoxide reductases preferentially reduce unfolded oxidized proteins and protect cells from oxidative protein unfolding. J. Biol. Chem. 2012; 287:24448–24459. [PubMed: 22628550]
10. Le DT, Tarrago L, Watanabe Y, Kaya A, Lee BC, Tran U, et al. Diversity of plant methionine sulfoxide reductases B and evolution of a form specific for free methionine sulfoxide. PloS One. 2013; 8:e65637. [PubMed: 23776515]
11. Tarrago L, Laugier E, Rey P. Protein-repairing methionine sulfoxide reductases in photosynthetic organisms: gene organization, reduction mechanisms, and physiological roles. Mol. Plant. 2009; 2:202–217. [PubMed: 19825608]
12. Boschi-Muller S, Branlant G. Methionine sulfoxide reductase: chemistry, substrate binding, recycling process and oxidase activity. Bioorganic Chem. 2014; 57:222–230.
13. Couturier J, Vignols F, Jacquot J-P, Rouhier N. Glutathione- and glutaredoxin-dependent reduction of methionine sulfoxide reductase A. FEBS Lett. 2012; 586:3894–3899. [PubMed: 23022439]
14. Tarrago L, Laugier E, Zaffagnini M, Marchand C, Le Maréchal P, Rouhier N, et al. Regeneration mechanisms of Arabidopsis thaliana methionine sulfoxide reductases B by glutaredoxins and thioredoxins. J. Biol. Chem. 2009; 284:18963–18971. [PubMed: 19457862]
15. Vieira Dos Santos C, Laugier E, Tarrago L, Massot V, Issakidis-Bourguet E, Rouhier N, et al. Specificity of thioredoxins and glutaredoxins as electron donors to two distinct classes of

Arabidopsis plastidial methionine sulfoxide reductases B. FEBS Lett. 2007; 581:4371–4376. [PubMed: 17761174]

16. Kim M-J, Jeong J, Jeong J, Hwang KY, Lee K-J, Kim H-Y. Mechanism of 1-Cys type methionine sulfoxide reductase A regeneration by glutaredoxin. Biochem. Biophys. Res. Commun. 2015; 457:567–571. [PubMed: 25600814]
17. Kim H-Y. Glutaredoxin serves as a reductant for methionine sulfoxide reductases with or without resolving cysteine. Acta Biochim. Biophys. Sin. 2012; 44:623–627. [PubMed: 22634633]
18. Lin Z, Johnson LC, Weissbach H, Brot N, Lively MO, Lowther WT. Free methionine-(R)-sulfoxide reductase from Escherichia coli reveals a new GAF domain function. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:9597–9602. [PubMed: 17535911]
19. Le DT, Lee BC, Marino SM, Zhang Y, Fomenko DE, Kaya A, et al. Functional analysis of free methionine-R-sulfoxide reductase from Saccharomyces cerevisiae. J. Biol. Chem. 2009; 284:4354–4364. [PubMed: 19049972]
20. Ezraty B, Bos J, Barras F, Aussel L. Methionine sulfoxide reduction and assimilation in Escherichia coli: new role for the biotin sulfoxide reductase BisC. J. Bacteriol. 2005; 187:231–237. [PubMed: 15601707]
21. Melnyk RA, Youngblut MD, Clark IC, Carlson HK, Wetmore KM, Price MN, et al. Novel mechanism for scavenging of hypochlorite involving a periplasmic methionine-rich Peptide and methionine sulfoxide reductase. mBio. 2015; 6:e00233–e00215. [PubMed: 25968643]
22. Gennaris A, Ezraty B, Henry C, Agrebi R, Vergnes A, Oheix E, et al. Repairing oxidized proteins in the bacterial envelope using respiratory chain electrons. Nature. 2015; 528:409–412. [PubMed: 26641313]
23. Lee BC, Le DT, Gladyshev VN. Mammals Reduce Methionine-S-sulfoxide with MsrA and Are Unable to Reduce Methionine-R-sulfoxide, and This Function Can Be Restored with a Yeast Reductase. J. Biol. Chem. 2008; 283:28361–28369. [PubMed: 18697736]
24. Ghesquiére B, Jonckheere V, Colaert N, Van Durme J, Timmerman E, Goethals M, et al. Redox proteomics of protein-bound methionine oxidation. Mol. Cell. Proteomics MCP. 2011; 10 M110.006866.
25. Aledo JC, Cantón FR, Veredas FJ. Sulphur Atoms from Methionines Interacting with Aromatic Residues Are Less Prone to Oxidation. Sci. Rep. 2015; 5:16955. [PubMed: 26597773]
26. Drazic A, Winter J. The physiological role of reversible methionine oxidation. Biochim. Biophys. Acta BBA - Proteins Proteomics. 2014; 1844:1367–1382. [PubMed: 24418392]
27. Kaya A, Lee BC, Gladyshev VN. Regulation of protein function by reversible methionine oxidation and the role of selenoprotein MsrB1. Antioxid. Redox Signal. 2015; 23:814–822. [PubMed: 26181576]
28. Luo S, Levine RL. Methionine in proteins defends against oxidative stress. FASEB J. 2009; 23:464–472. [PubMed: 18845767]
29. Binolfi A, Limatola A, Verzini S, Kosten J, Theillet F-X, May Rose H, et al. Intracellular repair of oxidation-damaged α-synuclein fails to target C-terminal modification sites. Nat. Commun. 2016; 7:10251. [PubMed: 26807843]
30. Barnham KJ, Ciccotosto GD, Tickler AK, Ali FE, Smith DG, Williamson NA, et al. Neurotoxic, Redox-competent Alzheimer's β-Amyloid Is Released from Lipid Membrane by Methionine Oxidation. J. Biol. Chem. 2003; 278:42959–42965. [PubMed: 12925530]
31. Breydo L, Bocharova OV, Makarava N, Salnikov VV, Anderson M, Baskakov IV. Methionine oxidation interferes with conversion of the prion protein into the fibrillar proteinase K-resistant conformation. Biochemistry (Mosc.). 2005; 44:15534–15543.
32. Péterfi Z, Geiszt M. Peroxidasins: novel players in tissue genesis. Trends Biochem. Sci. 2014; 39:305–307. [PubMed: 24924147]
33. Péterfi Z, Donkó A, Orient A, Sum A, Prókai A, Molnár B, et al. Peroxidasin is secreted and incorporated into the extracellular matrix of myofibroblasts and fibrotic kidney. Am. J. Pathol. 2009; 175:725–735. [PubMed: 19590037]
34. Bhave G, Cummings CF, Vanacore RM, Kumagai-Cresse C, Ero-Tolliver IA, Rafi M, et al. Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis. Nat. Chem. Biol. 2012; 8:784–790. [PubMed: 22842973]

35. Erickson JR, Joiner MA, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell. 2008; 133:462–474. [PubMed: 18455987]
36. Drazic A, Miura H, Peschek J, Le Y, Bach NC, Kriehuber T, et al. Methionine oxidation activates a transcription factor in response to oxidative stress. Proc. Natl. Acad. Sci. U. S. A. 2013; 110:9493–9498. [PubMed: 23690622]
37. Lee BC, Péterfi Z, Hoffmann FW, Moore RE, Kaya A, Avanesov A, et al. MsrB1 and MICALs regulate actin assembly and macrophage function via reversible stereoselective methionine oxidation. Mol. Cell. 2013; 51:397–404. [PubMed: 23911929]
38. Hung R-J, Spaeth CS, Yesilyurt HG, Terman JR. SelR reverses Mical-mediated oxidation of actin to regulate F-actin dynamics. Nat. Cell Biol. 2013; 15:1445–1454. [PubMed: 24212093]
39. Hung R-J, Pak CW, Terman JR. Direct redox regulation of F-actin assembly and disassembly by Mical. Science. 2011; 334:1710–1713. [PubMed: 22116028]
40. Petropoulos I, Friguet B. Maintenance of proteins and aging: the role of oxidized protein repair. Free Radic. Res. 2006; 40:1269–1276. [PubMed: 17090416]
41. Vanhooren V, Navarrete Santos A, Voutetakis K, Petropoulos I, Libert C, Simm A, et al. Protein modification and maintenance systems as biomarkers of ageing. Mech. Ageing Dev. 2015; 151:71–84. [PubMed: 25846863]
42. Stadtman ER. Protein oxidation and aging. Free Radic. Res. 2006; 40:1250–1258. [PubMed: 17090414]
43. Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, et al. Oxidation of either methionine 351 or methionine 358 in alpha 1-antitrypsin causes loss of anti-neutrophil elastase activity. J. Biol. Chem. 2000; 275:27258–27265. [PubMed: 10867014]
44. Stadtman ER, Van Remmen H, Richardson A, Wehr NB, Levine RL. Methionine oxidation and aging. Biochim. Biophys. Acta. 2005; 1703:135–140. [PubMed: 15680221]
45. Laugier E, Tarrago L, Vieira Dos Santos C, Eymery F, Havaux M, Rey P. Arabidopsis thaliana plastidial methionine sulfoxide reductases B, MSRBs, account for most leaf peptide MSR activity and are essential for growth under environmental constraints through a role in the preservation of photosystem antennae. Plant J. Cell Mol. Biol. 2010; 61:271–282.
46. Romero HM, Berlett BS, Jensen PJ, Pell EJ, Tien M. Investigations into the role of the plastidial peptide methionine sulfoxide reductase in response to oxidative stress in Arabidopsis. Plant Physiol. 2004; 136:3784–3794. [PubMed: 15516509]
47. Rosen H, Klebanoff SJ, Wang Y, Brot N, Heinecke JW, Fu X. Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:18686–18691. [PubMed: 19833874]
48. Tarrago L, Peterfi Z, Lee BC, Michel T, Gladyshev VN. Monitoring methionine sulfoxide with stereospecific mechanism-based fluorescent sensors. Nat. Chem. Biol. 2015
49. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, et al. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. Nat. Methods. 2006; 3:281–286. [PubMed: 16554833]
50. Ma X-X, Guo P-C, Shi W-W, Luo M, Tan X-F, Chen Y, et al. Structural plasticity of the thioredoxin recognition site of yeast methionine S-sulfoxide reductase Mxr1. J. Biol. Chem. 2011; 286:13430–13437. [PubMed: 21345799]
51. Schwarzländer M, Wagner S, Ermakova YG, Belousov VV, Radi R, Beckman JS, et al. The “mitoflash” probe cpYFP does not respond to superoxide. Nature. 2014; 514:E12–E14. [PubMed: 25341790]
52. Tantama M, Yellen G. Imaging changes in the cytosolic ATP-to-ADP ratio. Methods Enzymol. 2014; 547:355–371. [PubMed: 25416365]
53. Tantama M, Martínez-François JR, Mongeon R, Yellen G. Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. Nat. Commun. 2013; 4:2550. [PubMed: 24096541]
54. Meyer AJ, Dick TP. Fluorescent Protein-Based Redox Probes. Antioxid. Redox Signal. 2010; 13:621–650. [PubMed: 20088706]

55. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, et al. Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. Mol. Biol. Cell. 2000; 11:4241–4257. [PubMed: 11102521]

56. Gerashchenko MV, Lobanov AV, Gladyshev VN. Genome-wide ribosome profiling reveals complex translational regulation in response to oxidative stress. Proc. Natl. Acad. Sci. 2012; 109:17394–17399. [PubMed: 23045643]

57. Chung CT, Niemela SL, Miller RH. One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. U. S. A. 1989; 86:2172–2175. [PubMed: 2648393]

Highlights

- Oxidation and reduction of methionine play important roles in various biological processes
- MetSOx and MetROx are the first geneticaly encoded sensors to monitor methionine sulfoxide metabolism *in vivo*
- We provide a detailed procedure for the use of these sensors in bacterial and mammalian cells using fluorimetric and fluorescent imaging approaches

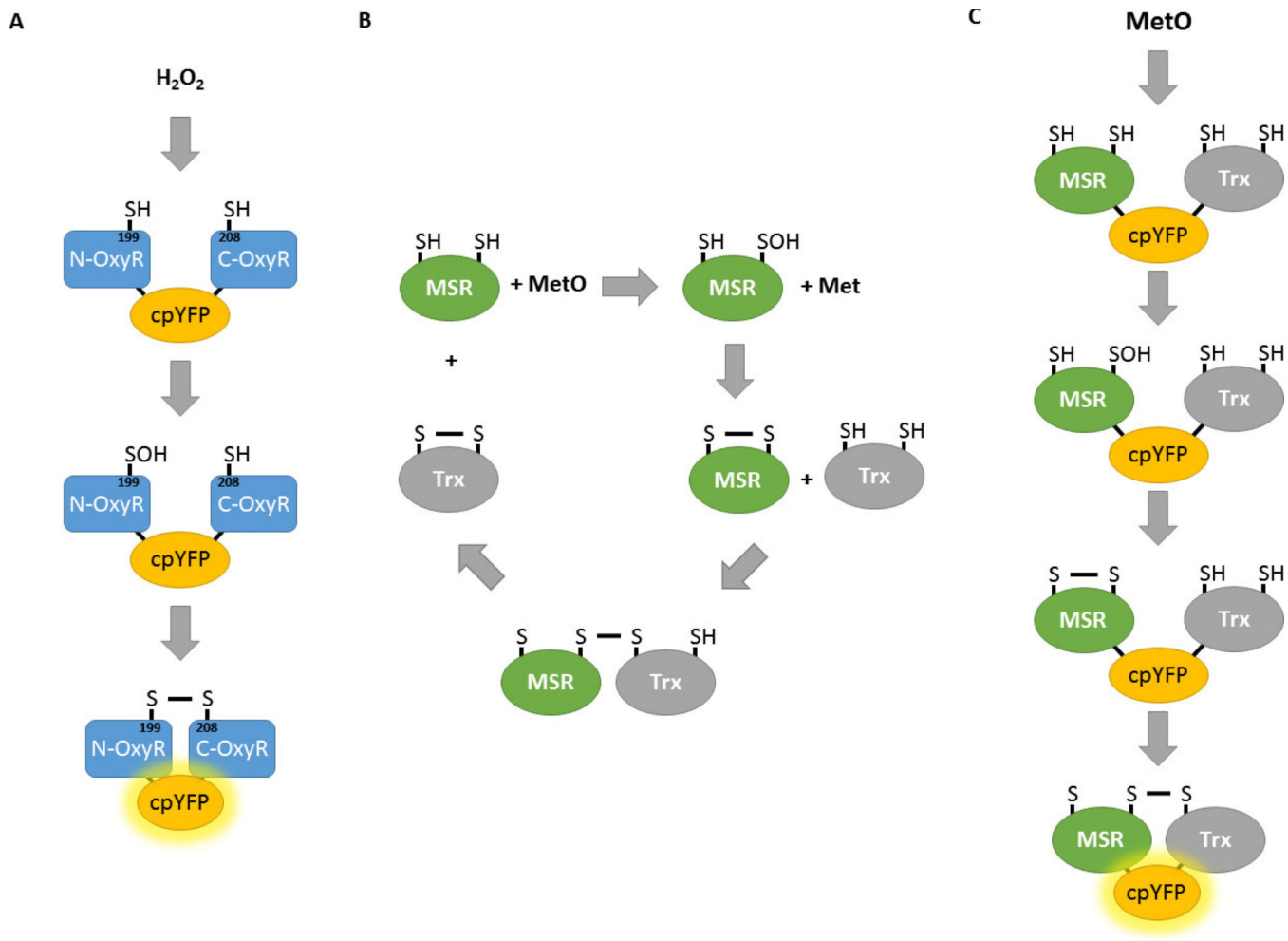

Figure 1. Common features of mechanisms of (A) HyPer sensor, (B) MSR and Trx interaction, and (C) MetO sensors

Both HyPer and MetO sensors are made based on cpYFP.

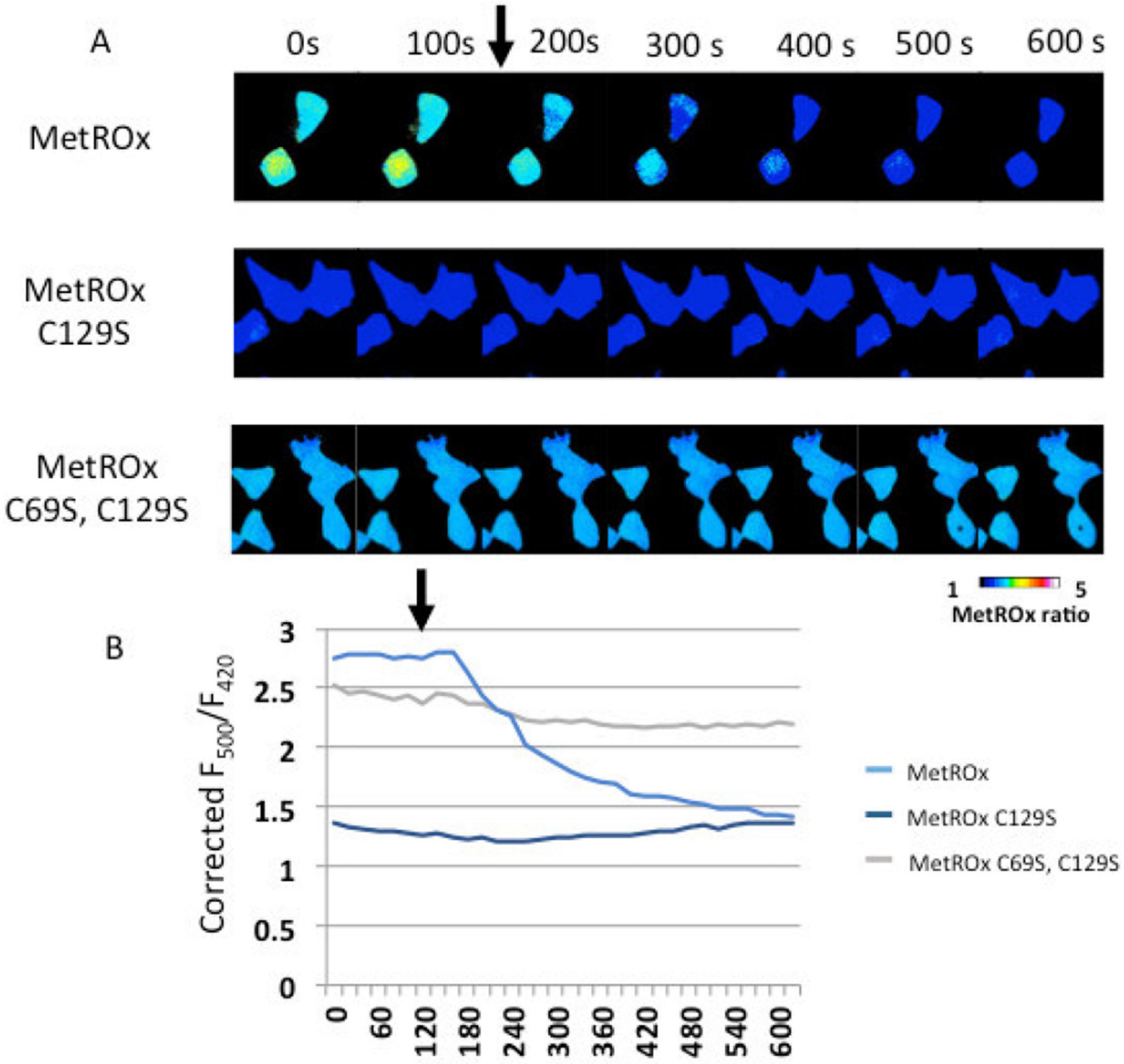

Figure 2. Response of MetROx to 5 mM MetO

(A) Time series of pseudocolored ratio images of MetROx and two controls (MetROxC129S and MetROxC69S, C129S) expressed in cells subjected to 5 mM MetO (arrow). (B) Kinetics of MetROx fluorescence in cell expressing the sensors.