Published in final edited form as:

Free Radic Biol Med. 2008 October 1; 45(7): 971–981. doi:10.1016/j.freeradbiomed.2008.06.018.

A FRET based method to study protein thiol oxidation in histological preparations

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Abstract

Cysteine residues in proteins have important biological roles. For example, disulfides bonds are important structural elements; additionally, reversible oxidation of thiols to disulfides functions as a molecular switch and constitutes an early response to oxidative damage. Because organs are heterogeneous structures composed of diverse cell types, there is a compelling need for a histological approach to investigate thiol oxidation *in situ* in order to address the role of specific cell types in oxidative imbalance. Here we describe a fluorescence technique – which can be used in association with standard immunological staining procedures – to detect variations in disulfides in histological preparations. Moreover, by monitoring the fluorescence resonance energy transfer (FRET) between a labeled specific primary antibody and the thiol probe described here, this method can detect thiol oxidation in candidate proteins of interest. When applied to an animal model of Parkinson's disease, our technique demonstrated that thiol oxidation occurs selectively in the dopaminergic neurons of the substantia nigra, the same neurons that are lost selectively in the disease. In summary, this technique provides a new, powerful tool to provide further understanding of oxidative imbalance, a phenomenon common to many diseases.

Keywords

fluorescence microscopy; FRET; oxidative stress; cysteine; thiols; disulfides; Parkinson's disease

Thiols are the functional groups in the side chain of the amino acid, cysteine. Depending upon the oxido-reductive (redox) status of the surrounding environment, thiols exist either in the reduced form of free thiols (-SH) or oxidized to disulfides (-S-S-). Disulfides are well known to play an important structural role, contributing to the maintenance of the proper folding in proteins. More recently it was found that the formation of disulfides in proteins also exerts critical regulatory functions: vital mechanisms such as protein import, regulation of signal transduction cascades, regulation of the activity of transcription factors and proper function of the mitochondrial electron transport system rely on disulfide oxidation or reduction [1–4]. Moreover, the formation of disulfides represents an early, reversible response to oxidative

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stress: it precedes higher forms of oxidation, e.g carbonylation as well as the formation of sulfenic or sulfonic acids. Therefore, disulfide formation may play a protective role [5].

Increasing interest in the study of thiols has led to the development of a variety of technical approaches to detect thiols and disulfides. In particular, several spectrophotometry-based protocols were developed to measure thiol levels. More recently, proteomic methods to identify disulfides in proteins have been established [6–8]. However, means to image exact loci of thiol oxidation in cells and tissues are quite limited [9]. Imaging techniques would be particularly useful, since the redox environment differs between cell types and even subcellularly. In fact, the level of free thiols varies greatly in subcellular compartments: whereas the endoplasmic reticulum provides a very oxidizing milieu, the cytosol is highly reducing [10,11]. Also, the intra- and extracellular environments are distinct, with more oxidizing conditions in the extracellular space. Finally, in heterogeneous organs, such as the brain, susceptibility to altered redox potential may differ between cell types because of their different metabolic properties.

Research into neurodegenerative disorders would benefit greatly from an imaging-based technology to evaluate thiol redox state in histological preparations. For example, although it is known that alterations in redox homeostasis contribute to the pathogenesis of disorders like Parkinson's disease, Alzheimer's disease and Huntington's disease, basic questions - such as whether the principal targets of oxidation are neurons or glial cells - remain unanswered [12, 13]. Current spectrophotometric and proteomic techniques rely upon sub-cellular fractionation of total tissue homogenates. As such, they are not suitable to detect differences in the redox state of single cell types in complex tissues.

To elucidate the roles of oxidative stress in neurodegenerative disorders, we developed a new protocol to study thiol oxidation in histological samples. This method can be conveniently combined with classical immunological methods. Therefore – used in association with cell-type specific markers - it provides information about the levels of oxidized thiols in specific cell types. Additionally, by measuring the fluorescence resonance energy transfer (FRET) between fluorescently labeled thiols and a fluorescently labeled specific primary antibody, the method can assess thiol redox state in a protein of interest.

Material and Methods

Reagents

Reagents were purchased from Sigma Aldrich, St. Louis, Mo, unless otherwise specified.

Rotenone treatment and animal care

All animal use followed University of Pittsburgh Institutional Animal Care and Use Committee approved protocols and was in strict accordance with the National Institutes of Health *Guide for the Care and use of Laboratory Animals*. Lewis rats were treated with rotenone as previously described [14].

Primary antibody labeling

The process was carried out using the AlexaFluor 488 antibody labeling kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the lyophilized primary antibody was resuspended in PBS. Sodium bicarbonate buffer (pH 8.5) was added 0.1M final. The antibody solution was incubated with the fluorophore for 1 hour. Excess, unreacted dye was removed with a Micro Bio-Spin chromatography column 6 (Biorad, Hercules, CA).

Assessment of the alkylating efficiency of the thiol probes

SH-SY5Y human neuroblastoma cells were cultured according to the American Type Culture Collection (ATCC) directions. About $2 \cdot 10^6$ cells were used for each thiol probe labeling. Cultured cells were harvested with trypsin and resuspended in PBS. To solubilize the plasma membrane, cells were incubated 10 minutes at room temperature in $5 \mu\text{M}$ digitonin, with gentle shaking. After incubation, permeabilization of the plasma membrane was confirmed counterstaining a fraction of the cells with trypan blue, which will penetrate only in permeabilized cells. In all the experiments, at least 98% of the cells were permeabilized. Free thiols were blocked by incubating the cells with 100 mM N-ethylmaleimide (NEM), 100 mM iodoacetamide (IAM) in Tris HCl 0.1 M, pH 6.8 for 15 minutes. The blocking and the labeling alkylating reactions were carried out at pH 6.8 because the reaction of maleimide with thiols is more selective at a pH slightly lower than physiological pH [15–17]. To remove the excess alkylating agents, cells were washed by spinning them down and resuspending them in PBS three times. Disulfides were reduced with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 25 mM in PBS for 20 minutes at room temperature. Cells were spun down and resuspended in alkylating solutions containing various thiol probes at a concentration of 0.5 mM final. The thiol probes were purchased from Invitrogen and 10mM stock solutions were prepared according to the manufacturer's directions. After 1 hour incubation, cells were spun down and resuspended in SDS-electrophoresis loading buffer (50mM TrisHCl pH6.8, 2% SDS, 0.1% Blue bromophenol, 10% glycerol); the samples were sonicated and heated to denature proteins. Proteins were separated with SDS-electrophoresis according to standard procedures. The gel was imaged with a UV transilluminator (Bio-Doc-IT imaging system, UVP, Upland, CA). To confirm equal protein loading, the gels were counterstained with 0.2% G-250 Coomassie (SERVA, Heidelberg, Germany) dissolved in 35% methanol, 3.5% orthophosphoric acid, 1.3M ammonium sulfate.

Labeling of alpha-synuclein with CPM

10 microgram of alpha-synuclein (Sigma Aldrich, St. Louis, Mo) were resuspended in 50 mM TrisHCl solutions buffered at the indicated pH values. CPM was added 0.5mM final; the reaction was carried out at room temperature for 20 minutes. An aliquot of the reaction (1.5 micrograms of protein) was resolved with SDS electrophoresis according to standard procedures. The gels was processed and imaged as above.

Spectrophotometric analysis

BSA was dissolved in PBS at a final concentration of 10 mg/ml and CPM in Tris HCl (0.1 M, pH 6.8) was added to a final concentration of 0.5 mM. After 20 minutes of incubation at room temperature with gentle shaking, unreacted CPM was removed with gel-filtration (Micro Bio-Spin 6 columns, BIORAD, Hercules, CA). Emission fluorescence spectra were acquired with a RF-5301 PC spectrofluorophotometer (Shimadzu, Columbia, MD). To study FRET between CPM and Alexa-488, an Alexa-488 conjugated IgG (Invitrogen, Carlsbad, CA) was modified with CPM as above. The spectra were acquired as above.

Histological labeling of oxidized thiols

Brains were fixed in 4% paraformaldehyde and processed as described [14]. After cutting with a freezing microtome (Microm, Walldorf, Germany), sections were treated immediately in alkylating buffer (100 mM IAM, 100 mM NEM, TrisHCl, pH6.8) to block free thiols. For cell culture studies, cells were seeded and cultured on coverslips, then fixed in 4% paraformaldehyde, 0.02% Triton-X100 supplemented with 100mM IAM, 100mM NEM to block free thiols. After three washes in PBS – 10 minutes at room temperature – to remove unreacted NEM and IAM, disulfides were reduced with 25 mM TCEP in PBS for 20 minutes.

TCEP was omitted in the control reaction. The samples were labeled with CPM (0.5 mM final) in TrisHCl (0.1 M, pH 6.8) and then washed three times, for 10 minutes, in PBS.

Following the thiol labeling, immunohistochemistry was performed according to standard procedures. Briefly, after a blocking step in 10% serum, samples were incubated over-night with primary antibody (tyrosine hydroxylase, Mab318, Chemicon, Temecula, CA, 1:2000; OX42, MCA 275G AbD Serotec, Oxford, UK, 1:150; GFAP, Mab 3402, Chemicon, Temecula, CA, 1:1000). Fluorescently labeled secondary antibodies were used for detection. For FRET analysis, the thioredoxin antibody (R&D Systems, Minneapolis, MN, 1:500) was directly labeled with AlexaFluor 488, as described above.

Images were acquired using a laser scanning confocal microscope (Fluoview1000, Olympus). The microscope is equipped with a spectral detector technology that provides precise wavelength separation of the emitted light. The following detection ranges were used for each fluorophore: UV probes 440nm-470nm; Alexa488 515nm-535nm; Cy3 560nm-574nm). The acquisition parameters were set in the control reaction performed without TCEP and remained constant for all the consequent analyses. Direct FRET was detected by reading the acceptor emission (FITC derivative conjugated antibody) at 520 nm while exciting the donor (CPM) with the 405 nm laser. Alternatively, FRET was detected by acquiring the donor (CPM) emission before and after photobleaching of the acceptor fluorophore (Alexa488) with the 488nm laser. FRET efficiency (F_{eff}) was calculated by the confocal software (version 1.5) according to the equation $F_{eff} = 1 - (em_{pre}/em_{post})$ - where F_{eff} =FRET efficiency, em_{pre} =donor emission prebleaching, em_{post} =donor emission post bleaching – and expressed in percent ($F_{eff}=1=100\%$). The emission spectra of CPM labeled cells were acquired with the same laser scanning confocal microscope. The microscope software acquires a series of images within a determined range of wavelengths set by the operator, in this case $440nm < \lambda < 610nm$, in windows of 20nm each. The specimens were excited with a 405nm laser.

Analysis of disulfides in cells incubated in redox buffers

Cells were cultured in 96-well plates at a density of $5 \cdot 10^4$ cells per well. Redox buffers were prepared as previously described, with minor modifications [11]. Briefly, different amounts of oxidized and reduced glutathione were combined in 4% paraformaldehyde (0.5 M TrisHCl pH 7.4) and 0.02% Triton X-100 to obtain solutions at the desired redox potential (–150mV: GSH 5mM, GSSG 27.77mM; –180mM: GSH 10mM, GSSG 18.72mM; –210mV: GSH 20mM, GSSG 4.14mM; –240mM: GSH 40mM, GSSG 1.6mM; –270mM: GSH 40mM, GSSG 0.15mM; –300mM: GSH 40mM, GSSG 0.015). Cells were fixed in the redox buffered paraformaldehyde for 1 hour. Thiols were stained as above; nuclei were counterstained with Sytox green 0.5 μ M (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were imaged with a microplate spectrofluorometer (Spectramax Gemini EM, Molecular Devices, Sunnyvale, CA). Thiol levels were expressed as the ratio of CPM staining to Sytox green signal.

Western blot analysis to detect proteins with oxidized cysteines

Cells were lysed in alkylating buffer (0.1M TrisHCl pH6.8, 1%SDS, 100mM IAM, 100mM NEM). The lysate was sonicated on ice to disrupt high molecular weight DNA and incubated 30 minutes at room temperature to allow total alkylation of free cys residues. 200 μ g of alkylated proteins were precipitated with 5 volumes of ice-cold acetone and resuspended in 50 microliters of Tris-SDS buffer (0.1M TrisHCl pH7.4, 1%SDS). Disulfides were reduced by adding TCEP 5 mM final concentration. The samples were incubated 20 minutes at room temperature to allow complete reduction of disulfides to thiols. TCEP was removed with Micro Bio-spin Chromatography columns 6 (BIORAD, Hercules, CA). After the filtration step, the

samples were in about 50 μ L of 10 mM TrisHCl pH 7.4 (volume was adjusted to 50 μ L as necessary). SDS was added to a 1% final concentration.

To label the previously oxidized (and now reduced) cysteines, polyethylene-glycol-conjugated maleimide (molecular weight 10 kDa, SunBio, Orinda, CA) to 50 micrograms of proteins, 1mM final concentration, from a 10mM stock in Tris-SDS buffer. After 1 hour incubation at room temperature, samples were precipitated with ice cold acetone and resuspended in 50 μ L of SDS electrophoresis loading buffer. 10 μ L (about 10 μ g) were used for western blot analysis. The thioredoxin antibody (R&D Systems, Minneapolis, MN) was used 1:1000.

Preparation of affinity resin

Activated thiol-sepharose resin 4B (Sigma, St. Louis, MO, USA) was used in previous studies to purify thiol-containing proteins [18]; the resin extent of binding is 1micromol of thiols per mL of medium. The resin was rehydrated in milliQ-grade distilled water for 30 minutes with gentle shaking at 4°C. After centrifugation (5 minutes, 500 $\times$ g), the sedimented resin was resuspended in 10 volumes of Tris-SDS buffer (TrisHCl 0.1M, pH7.4, SDS 1%) and incubated 5 minutes with gentle shaking at room temperature. This step was repeated three times. The resin was resuspended at a final concentration of 150mg/ml.

Purification of oxidized proteins

Brains were collected and different regions were micro-dissected, separated and homogenized in 0.32 M sucrose containing 30 mM TrisHCl (pH 7.4) and 1 mM EDTA, on ice, using a Teflon glass homogenizer. Samples were spun (900 $\times$ g, 10 min, 4°C) to remove nuclei and debris and the supernatant was spun again (10000 $\times$ g, 15 min, 4°C). The pellet, which represents an enriched mitochondrial fraction, was use for the subsequent analysis. Proteins (0.5 mg) were resuspended in Tris-SDS buffer at the final concentration of 1mg/ml. Proteins were denatured by heating the solution at 70°C for 5 minutes. NEM and IAM were added at the final concentration of 100mM each. The mixture was incubated 30' at 37°C and precipitated with 5 volumes of ice-cold acetone for 1 hour at -80°C. After spinning (25 min, 3200 $\times$ g, 4°C), the pellet was resuspended in 1 ml of Tris-SDS buffer. As a pre-clearing step, the sample was then incubated with the thiol-sepharose medium (300 microliters, about 50 mg of dry resin) for 2 hours at room temperature. After centrifugation (5 minutes, 500 $\times$ g, room temperature), the supernatant was saved and TCEP (25 mM final concentration) was added to reduce cysteines previously engaged in disulfides. The mixture was incubated 15 minutes at room temperature. A control reaction was performed by incubating proteins *without* TCEP. Proteins were then precipitated as above and resuspended in 4 ml of Tris-SDS. Affinity purification was performed by incubating for 2 hours at room temperature, the mixture with 1ml of equilibrated resin (about 150 mg of dry resin). To wash the resin after incubation, the sample was spun for 5 min at 500 $\times$ g, room temperature. The supernatant (unbound) was saved, and the sedimented resin was resuspended in 10 volumes of Tris-SDS, shaken 3 minutes at room temperature, and spun again. A total of 3 washes were performed. Bound proteins were eluted by incubating the resin (5 minutes, shaking, room temperature) with Tris-SDS buffer containing 100 mM DTT. After centrifugation (5 minutes, 500g, RT), the supernatant (eluate) was saved and the sedimented resin was incubated once more with the eluting buffer. After centrifugation, the proteins in the pooled supernatants and in the unbound fraction were precipitated with acetone and used for electrophoresis.

Results

Oxidized disulfides can be modified through a multi-step process, involving the alkylation of free thiols, the reduction of disulfides and the modification of the newly formed free thiols with fluorescent probes (figure 1). In this study, an important new negative control – in which the

reducing step was omitted from the protocol – was included. Under these conditions, the signal detected by the instrument is due to background noise and can be used to set the zero level of the acquisition parameters. Initially, we ran a pilot experiment to determine which fluorescent probe was most effective - under non-denaturing conditions - to label those thiols derived from disulfides. After gentle permeabilization of the plasma membrane, intact cells were labeled with five different probes that differ by thiol-reactive functional group or fluorescent conjugate. The labeling capacity varied greatly, depending upon the functional group and its conjugate. 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), a derivative of maleimide, was found to be the most effective molecule and, therefore, it is the one that was used for subsequent studies (figures 2a, b). Maleimide reacts with thiols through Michael's addition and the reaction has a higher specificity at pH values slightly lower than pH 7.4 [16,17]. To verify this specificity, we assessed the pH-dependence of the ability of CPM to modify residues other than thiols. Incubation of CPM with a protein that does not contain cysteines, alpha-synuclein, results in non-specific labeling at higher pH values. As expected, no signal can be detected when the reaction is performed at pH6.8 (figures 2c, d). Therefore, performing the reactions at pH 6.8 rather than pH 7.4 results in higher specificity for protein thiols. Finally, CPM is more effective at modifying thiol groups than the iodoacetamide derivative IAANS, at pH 6.8 as well as pH 7.4 (figures 2e, f).

In order to assess the ability of CPM to detect variations in disulfides *in situ* as well as to generate a calibration curve, disulfide staining was applied to cells that were fixed in paraformaldehyde solutions at known redox potentials. CPM emission increased linearly over a wide range of redox potentials, from about -270 to -150 mV (figure 3). Importantly, thiol labeling with CPM could detect variations in the levels of oxidized thiols in cells that were treated with tert-butyl-hydroperoxide (tBH), an oxidant commonly used in the study of thiol redox state [11], and then fixed (figure 4a). As well, CPM is compatible with subsequent histological stainings in association with other fluorophores (i.e. FITC and rhodamine derivatives). In fact, the emission spectrum of CPM labeled cells indicates that this probe does not emit significantly at wavelengths longer than 470nm (figure 4b, c); therefore, multiple labeling will not produce non-specific signal due to light leakage between CPM and other dyes.

Next, we applied this method to a model of neurodegenerative disease. Chronic treatment of rats with rotenone to model Parkinson's disease induces oxidative stress and protein carbonylation [14,19-21]. Initial studies – where proteins with oxidized thiols in the form of disulfides were purified by thiol-affinity chromatography - indicated a marked increase in thiol oxidation in mitochondria isolated from the substantia nigra of rotenone treated rats (figure 5). Therefore, we applied our new method to define the particular area of the midbrain, as well as the specific cell type – i.e., neurons or glia – affected by rotenone-induced oxidation. When used in combination with classical immunohistochemical staining for neuronal and glial markers, disulfide labeling indicates that thiol oxidation occurs primarily in dopaminergic neurons of the substantia nigra (figure 6).

We next explored the possibility of using this method to study thiol oxidation specifically in a protein of interest. This issue can be addressed investigating the fluorescence resonance energy transfer (FRET) between two different fluorophores. Because FRET occurs only if the two fluorophores are in close proximity (10–100Å), it can provide information on whether the 2 probes are on the same (or very closely interacting) protein(s) (figure 7a). In a pilot study, we examined the spectral properties of CPM to detect a good FRET partner. A necessary requisite for FRET to occur is that the emission spectrum of the donor molecule – in our case CPM –should overlap with the excitation spectrum of the acceptor. We found that – when conjugated to proteins – FITC or Alexa488 dyes satisfy this prerequisite (figure 7b, red area). To demonstrate that FRET occurs between CPM and Alexa488, if present on the same protein,

we conjugated CPM to an Alexa488-labeled IgG; as expected, excitation of CPM resulted in indirect emission of fluorescence by Alexa488 (figure 7c).

To determine if thiol staining used in combination with classical immunohistochemical staining and FRET could detect oxidation in a protein of interest, we studied oxidation in thioredoxin-1 (trx-1) following treatment of cells with tBH. Initially, we established the experimental conditions under which trx-1 is oxidized. In agreement with previous studies [11,22], we found that low millimolar concentrations of tBH quickly induce the formation of disulfides in trx-1 and that trx-1 has two redox-active thiol couples (figure 8a, b). In this case, the thiols were labeled with maleimide conjugated to 10 kDa PEG and proteins were separated by SDS-PAGE. Thus, for each thiol group labeled with the conjugate, the mobility of the protein shifted by 10 kDa. Therefore, using a homogenate, one can create a “redox-ladder” of a protein of interest under different experimental conditions. When these oxidation conditions were used to study FRET between thiol-bound CPM and a specific Alexa488-conjugated anti-Trx-1 primary antibody, a clear increase in the signal was observed in tBH treated cells, indicating thiol oxidation of Trx-1. As expected, the control reaction, in which the reducing step was omitted, did not produce any measurable signal (figure 8c). These findings were validated when FRET was analyzed through the acceptor photobleaching approach [23] (figure 9). Identical results were obtained when MIANS and FITC – two fluorophores similar to CPM and Alexa488 – were used to label respectively thiols and the antibody (data not shown). As an important final control, betasynuclein – a protein without cysteines in its primary sequence – was studied. Despite a remarkable emission of the Alexa488 conjugated primary antibody, FRET was not detected after tBH treatment (figure 10).

Discussion

Here we described a new method to study disulfides in histological preparations. Our results show that the technique’s sensitivity permits detection of variations in disulfides following treatment with oxidants. Additionally, used in combination with FRET analysis, the technique allows assessment of thiol oxidation in specific proteins of interest.

Although oxidative stress is an area of active investigation in many fields of biomedical research, no tools are available to image thiol oxidation in proteins in intact tissues. There is great advantage to being able to detect thiol oxidation in histological samples. Many organs have a complex composition of cell types and classical biochemical approaches on total tissue homogenates do not allow discrimination of the particular cell type that is affected. Moreover, under pathological conditions, the relative composition of cell types can vary and the measure of levels of oxidation could be biased by the proliferation of a certain cellular population. An example of this scenario is found in neurodegenerative disorders, where neurons tend to die and glia tend to proliferate. When we applied our method to the rotenone model of Parkinson’s disease, which is based on the chronic administration of an inhibitor of mitochondrial respiratory complex I, we were able to show that thiol oxidation occurs specifically in DA neurons of the substantia nigra. Importantly, these are the neurons that are predominantly lost in PD itself; therefore, our data establish a direct correlation between thiol oxidation and DA neuron loss. The detection of high levels of oxidized thiols in DA neurons is consistent with previous reports and is in agreement with the notion that DA neurons are particularly prone to oxidation due to increased hydrogen peroxide production resulting from monoamine oxidase activity [24,25]. Besides its application to studies of the brain, this technique can be applied to other physio-pathological situations – such as cancer, intoxication and infectious disease – in which oxidative imbalance plays a role.

Previous histological methods aimed to detect free thiols instead of disulfides and were based on the use of mercury-orange [26,27]. Although useful, this method has some weaknesses.

First, the chemistry of mercury orange is less specific than the chemistry of maleimides because it reacts with thiols as well as with S-nitroso group (Seville reaction); therefore, the detected signal will be due to the labeling of true thiols – which are a reduced species – as well as the labeling of nitrosocysteines – an oxidized form. The mixed detection of oxidized as well as reduced forms makes the results difficult to interpret. A further weakness of this method derives from the observation that the intracellular environment is a reducing one and thus free thiols prevail over disulfides [28]. In this situation, the sensitivity of the method is greatly reduced if free thiols are labeled in place of disulfides. In fact, because the formation of new disulfides will deplete only a small fraction of the free thiols, the difference in signal will be very small if free thiols are labeled. In contrast, formation of new disulfides will substantially increase the population of disulfides; therefore, labeling of disulfides provides a far more sensitive approach.

A method to label disulfides in cells is described in a recent report [9]. The protocol uses iodoacetamide to block free thiols, DTT to reduce disulfides and iodoacetamide-conjugated fluorescein as a probe. In our method, we used maleimide to block free thiols as it is known to be more efficient in alkylating cysteines [29]. Maleimide is also the functional group in CPM, the probe we found to be more effective in thiol labeling. The modification of thiols is influenced by the pH and we optimized the conditions to minimize non-specific signal, achieving a compromise between specificity and sensitivity. Importantly, CPM is not fluorescent unless conjugated to thiols, a feature that further prevents undesired non-specific noise. In the reducing step, DTT was replaced by TCEP, a reagent that does not interfere with the reaction between maleimide and thiols [30]. Therefore, washing steps in which the samples would be exposed to atmospheric oxygen in the absence of reductants can be omitted. This strategy prevents undesired re-oxidation of thiols and resultant loss in signal. Finally, we provided a new, important negative control reaction, where the reducing agent is omitted. Under these conditions, the signal detected by the instrument will be due to non-specific background. This control reaction is therefore used to set the zero point for the instrument acquisition parameters.

To detect thiol oxidation in a protein of interest, we investigated the proximity of the thiol probe to a labeled antibody specific for Trx-1. Because FRET occurs, the distance between the fluorophores needs to be between 20–80 Å; this distance is comparable to the average size of proteins. However, other key factors as the spectral properties and the orientation of the fluorophores contribute to the occurrence of FRET. Although the fluorophores can be carefully selected to optimize the spectral properties for FRET, the orientation of the fluorophores – once on their target – cannot be controlled easily. It is possible that the location of the epitope recognized by the antibody, its distance from the labeled thiols and the orientation of the antibody after epitope recognition would influence the performance of FRET. Therefore, optimization of the experimental settings using different clones or types of antibodies might be required. In our hands, when studying oxidation of the large huntingtin protein, we found that polyclonal antibodies work better than monoclonal, probably because they constitute a pool of antibodies each recognizing a different epitope and therefore increasing the chances of labeling an optimal epitope for FRET. In agreement with this hypothesis, we observed a great variability between different monoclonal antibodies (data not shown).

In conclusion, we have provided a powerful tool to study protein thiol oxidation in histological preparations. This technique will be useful to provide new insights about the impact of oxidative imbalance in organs and to dissect the roles of different cell types in the control of redox homeostasis.

Abbreviations

FRET, fluorescence resonanace energy transfer; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; MIANS, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; IAANS, 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid; mBBr, monobromobimane; tBH, t-butylhydroperoxide; Trx-1, thioredoxin-1; FITC, Fluorescein isothiocyanate; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride.

Acknowledgements

This work was supported by The Picower Foundation, the American Parkinson Disease Association Advanced Center for Parkinson Research at the University of Pittsburgh and PHS grants U54-ES012068 (JTG) and K99-ES016352 (PGM).

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Figure 2. Identification of the best UV-probe to label thiols in proteins retaining their native conformations

After mild permeabilization of cells with digitonin, protein-thiols were labeled with UV-probes containing different functional groups. All the probes were used 0.5mM final. **(a)** Proteins were separated by SDS electrophoresis and the gel was imaged with a UV transilluminator. CPM (**lane 3**) was the most effective probe and was used for subsequent experiments in this study. **(b)** Coomassie staining of the same gel ensured equal protein loading. **(c)** The reaction between maleimide and thiols is more specific at pH values that are slightly more acidic than the physiological one. When a protein without cysteines, alpha-synuclein, is incubated with CPM, non-specific signal can be detected at basic pH values. **(d)** Coomassie staining of the same gel.

(e) The maleimide derivative CPM is more efficient than the iodoacetamide derivative IAANS at alkylating thiols also independently from the pH. (f) Coomassie staining of the same gel. The images are representative of three independently replicated experiments.

Abbreviations: **CPM**: 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin

MIANS: 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid

IAEDANS: 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid

IAANS: 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid.

disulfide levels in cells fixed in redox buffers

Figure 3. Detection of oxidized thiols in cells fixed at known redox potentials (E_h)

Cells were permeabilized and fixed in paraformaldehyde solutions at known redox potential, as described. Disulfide labeling with CPM (fluorescence emission) increased in a linear fashion as E_h became more oxidized. CPM signal was normalized by the SYTOX green nuclear dye emission, which is proportional to the number of the cells in the well.

Figure 4. Detection of oxidized thiols in cells

(a) Treatment with the pro-oxidant tBH (1 mM) induced a remarkable increase in disulfides (f) compared to vehicle control (e). As expected, no signal was detectable in the negative control (d), where the reducing step of the protocol was omitted. The confocal microscope scanning parameters were set in the control reaction and kept equal for all the following analyses. (b, c) Emission spectrum of CPM labeled cells upon excitation at 405nm. CPM emits significantly only at wavelengths shorter than 470nm (*lower panels*). When images are acquired within an emission range spanning from 440nm to 460nm (b, yellow area in the spectrum), distinct signal is detected (b, *upper panel*). As expected, in the emission range of FITC or Alexa-488 dyes (c, yellow area in the spectrum) no signal is detected (c, *upper panel*). Taken together, these

results show that CPM does not emit directly in the emission range of green dyes and therefore can be used for multiplex stainings.

Figure 5. Chronic exposure to rotenone induces oxidation of thiols in brain proteins

(a) Proteins containing oxidized thiols in the form of disulfides were purified using thiol affinity chromatography. A schematic overview of the strategy is shown. A control reaction was performed by omitting the reducing step (asterisk). In this reaction, no free thiols should be present in the sample and, therefore, no proteins should be purified. (b) Coomassie staining of the eluted proteins. As revealed by the number and the intensity of the bands, a striking increase of oxidized proteins is evident in the substantia nigra from rats that have been exposed to rotenone (*compare lanes 3 and 5*). Because low molecular weight proteins were not detected in the eluate, the gel in the picture – which is representative of the typical result obtained for this kind of experiment – was run for an adequate time to resolve properly the most intense bands. It is not clear whether the lack of low molecular weight proteins was due to depletion of small proteins by the precipitation steps performed in the procedure; in this scenario – in order to study small proteins – the protocol needs to be refined. Alternatively, low molecular proteins might really be under-represented in the eluate; further studies need to be performed to address this important issue. Nevertheless, the gel shows that rotenone treatment induces thiol oxidation in a wide subset of proteins. Protein thiol oxidation is rather specific for the substantia nigra as no major differences are visible in the striatum (*compare lanes 2 and 4*). As expected, there are no eluted proteins in the control reaction (*lane 6*). (c) As a loading control, the flow through was resolved by SDS-PAGE and stained with Coomassie Blue. This

staining also indicates that no major alterations in the protein expression profile are occurring in these samples. The gels shown in the figure are representative of three experiments, which have been independently replicated.

Figure 6. Detection of oxidized cysteines in tissues

Rats were treated chronically with rotenone, a mitochondrial toxin associated with oxidative damage and the development of experimental parkinsonism. Oxidized thiols (blue fluorescence) colocalized extensively with the dopaminergic neuronal marker (TH), but not with the glial marker (GFAP). This is the first evidence that this type of oxidation occurs mainly in neuronal cells.

Figure 7. CPM and Alexa488 are a good FRET pair

(a) For FRET to occur, spectrally compatible fluorophores must be within about 50 Å of each other. In this situation, when the donor fluorophore is excited, some of its energy is transferred to the acceptor fluorophore. As a result, the direct fluorescence of the donor is reduced and fluorescence of the acceptor can be detected. (b) The CPM emission spectrum upon excitation at 380 nm overlaps with the excitation spectrum of Alexa488 (b, red area), a property that renders the two fluorophores a good FRET pair. (c) Emission spectra demonstrating *in vitro* that CPM and Alexa488 make a good FRET pair. When the same goat IgG molecule was labeled with both CPM and Alexa488, excitation of CPM induced emission of Alexa488 due to fluorescence energy transfer (c, gray plot).

Figure 8. Detection of oxidized thioredoxin-1 (trx-1) in cells

(a) Schematic of the procedure used. (b) Treatment of cells with tBH induced thiol oxidation in thioredoxin-1. After exposure of cells to tBH, cells were lysed in the presence of alkylating agents; following reduction with TCEP, free thiols were labeled with 10 kDa PEG conjugated to maleimide. Thus, in this technique, each oxidized thiol increases by 10 kDa the apparent molecular weight of a protein of interest, as analyzed by western blotting. In the case of trx-1, which contains *intramolecular* disulfides, each disulfide reduced yields 2 thiols and therefore increases the apparent MW by 20 kDa. The redox western blot represent the typical result obtained from three independently replicated experiments. (c) Using the same conditions as in (b), thiol oxidation was detected histochemically in fixed cells. After staining the cells for both oxidized thiols and trx-1, direct FRET was detected between CPM and the Alexa488-labeled trx-1 antibody by laser scanning confocal microscopy.

Figure 9. Comparison of direct FRET and acceptor photobleaching

Cells were double labeled for trx-1 (acceptor, pseudo-colored red) and oxidized thiols (-S-S-, donor, pseudo-colored green) at baseline (top row) and after treatment with tBH (bottom row). The Direct FRET image shows the fluorescence resonance energy transfer from the pre-bleach -S-S- signal to the pre-bleach trx-1 channel – Note the marked increase in direct FRET after treatment with tbutyl hydroperoxide. Arrows demarcate the area of photobleaching of the acceptor (red) and the corresponding regions of increased fluorescence of the donor (green). Note that there is a much larger increase in donor fluorescence after tBH treatment, despite the fact that baseline trx-1 fluorescence and the level of bleaching were lower in the tBH-treated cells; this is mirrored in the increased FRET efficiency $[1 - (\text{pre-bleach } F / \text{post-bleach } F)]$. Thus, direct FRET and acceptor photobleaching yield identical results.

Figure 10. A negative control for FRET

Beta-synuclein does not possess any cysteine residues in its primary sequence. As expected, no FRET was detected between oxidized thiols and Alexa488-labeled beta-synuclein antibody. Western blot analysis confirmed the expression of endogenous beta-synuclein in this cell line.