Methods in Free Radical Biology and Medicine

Utilization of fluorescent probes for the quantification and identification of subcellular proteomes and biological processes regulated by lipid peroxidation products

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ABSTRACT

Oxidative modifications to cellular proteins are critical in mediating redox-sensitive processes such as autophagy, the antioxidant response, and apoptosis. The proteins that become modified by reactive species are often compartmentalized to specific organelles or regions of the cell. Here, we detail protocols for identifying the subcellular protein targets of lipid oxidation and for linking protein modifications with biological responses such as autophagy. Fluorophores such as BODIPY-labeled arachidonic acid or BODIPY-conjugated electrophiles can be paired with organelle-specific probes to identify specific biological processes and signaling pathways activated in response to oxidative stress. In particular, we demonstrate "negative" and "positive" labeling methods using BODIPY-tagged reagents for examining oxidative modifications to protein nucleophiles. The protocol describes the use of these probes in slot immunoblotting, quantitative Western blotting, in-gel fluorescence, and confocal microscopy techniques. In particular, the use of the BODIPY fluorophore with organelle- or biological process-specific dyes and chromophores is highlighted. These methods can be used in multiple cell types as well as isolated organelles to interrogate the role of oxidative modifications in regulating biological responses to oxidative stress.

Introduction

Lipid peroxidation is a consequence of oxidative stress and can regulate multiple biological processes [1–3]. Extensive peroxidation of phospholipids can result in the loss of function of critical enzymes involved in ion homeostasis, intermediary metabolism, cell repair, and cell death [4–7]. A primary mechanism through which this occurs is site-specific modification of biomolecules by lipid peroxidation-derived electrophiles [8,9]. Such modifications have the capacity to promote a state of cellular protection or adaptation, or they can have deleterious effects and promote cell death or other maladaptive changes in cell phenotype [1,2,10]. Although modifications to nucleophilic residues of critical signaling proteins, enzymes involved in metabolism, and proteins directing apoptosis have been documented in the literature [8,11–13], it has nevertheless been difficult to determine how such modifications activate or regulate complex biological processes.

The difficulty in defining changes in biological processes caused by lipid peroxidation, in many cases, is due to methodological limitations. Unambiguous detection and quantification of biologically relevant protein modifications have been particularly

difficult for the following reasons: (1) immunological detection of oxidatively modified proteins relies on specific epitopes, which may be biased by surrounding residues; (2) lack of an ability to detect all modifications derived from the broad spectrum of lipid-derived electrophiles formed intracellularly; (3) inadequate sensitivity of detection methods; and (4) inadequate techniques to image the subcellular localization of proteins modified by reactive species. These issues have hindered our ability to understand how the biochemical changes imparted by oxidative protein modifications influence cell physiology.

Fluorophores and chromophores have demonstrated their value in linking biochemistry with changes in multiple cellular processes. Green and red fluorescent proteins, for example, have been used to track protein localization in cell culture experiments [14,15], and they have played critical roles in tracking cell populations in vivo [16,17]. Mitochondria-specific probes have also aided in understanding how mitochondrial dysfunction contributes to injury. The sequestration of most, but not all, of these probes is driven by mitochondrial membrane potential, and this property has been exploited to examine how the loss of mitochondrial function occurs and relates to cell events such as apoptosis (e.g., [18,19]). With respect to the field of oxidative stress, several tagged probes have been developed to interrogate the subcellular location of oxidative stress and to quantify free radical production. Several articles and reviews have been written on such probes, and the authors suggest Refs. [20–26] for information on measuring and quantifying reactive species.

Probes for measuring lipid peroxidation and the impact of lipid-derived electrophiles have also been described. Boron dipyrromethene (BODIPY)[1] dyes as well as fluorophores with similar characteristics have been useful in identifying conditions that favor lipid peroxidation [27]. BODIPY 581/591 undecanoic acid (C11-BODIPY) has been a particularly valuable tool in quantifying lipid oxidation and antioxidant interventions in cultured cells [28,29]. More recently, BODIPY conjugation to lipid peroxidation substrates such as arachidonic acid or to electrophiles themselves has been useful in detecting and identifying proteins susceptible to modification by reactive lipid species and the electrophile response proteome [6,25]. Qualities that make the BODIPY chromophore particularly useful are its relative nonsensitivity to changes in pH, insensitivity to solvent environment, high photostability relative to other fluorophores, high extinction coefficient, lack of ionic charge, and ease of conjugation to other molecules such as unsaturated fatty acids and reactive compounds such as iodoacetamide (IAM) [25,30,31]. BODIPY maintains narrow excitation and emission bandwidths and high fluorescence quantum yield, making it especially useful in tandem with other fluorophores and probes, which can be used collectively to understand how biological processes proceed under conditions of oxidative stress.

Principles

Cysteinyl residues of proteins undergo a variety of modifications. These modifications can have remarkable effects on enzyme activity, and, once formed, they have different stabilities. For example, S-nitrosated, S-glutathio(ny)lated, or sulfenic acid-modified proteins are readily reduced back to their unmodified forms; however, advanced oxy-sulfur acid formation (i.e., sulfinic and sulfonic acids) or addition reactions with electrophilic lipids, such as 4-hydroxynonenal (HNE), result in more stable modifications that can have more prolonged effects on protein function (Fig. 1A). Experimentally, it has been difficult to identify which of
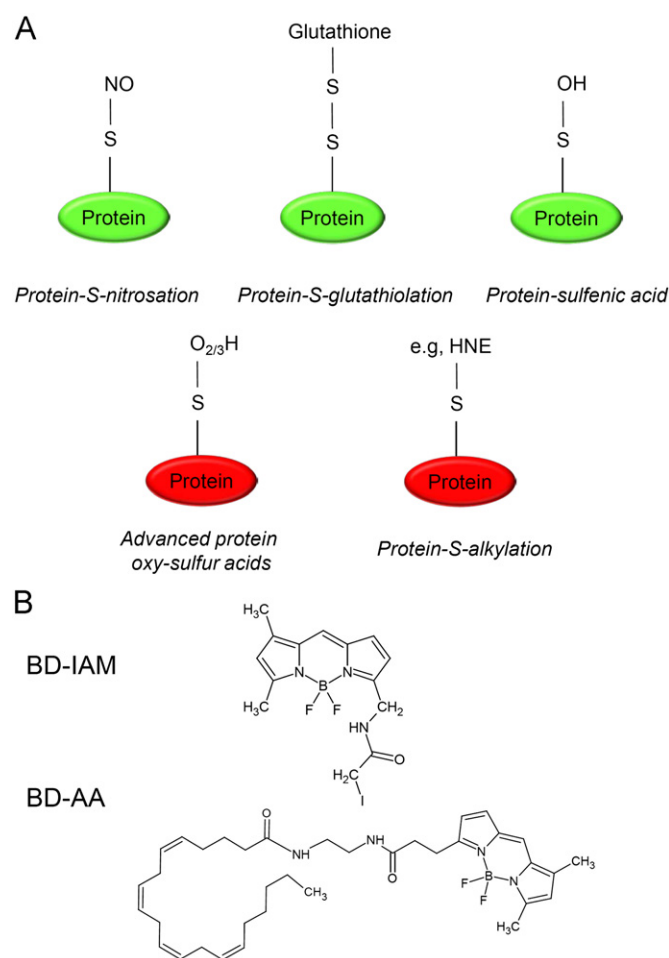

Fig. 1. Common oxidative posttranslational protein modifications and probes that can be used to interrogate their presence in the proteome. (A) Simple diagrams showing the readily reversible protein modifications—S-nitrosation, S-glutathio (ny)lation, and sulfenic acid—and more stable modifications such as sulfinic and sulfonic acids and Michael adducts with lipid electrophiles. (B) Structures of BD–iodoacetamide (BD-IAM) and BODIPY-labeled arachidonic acid (BD-AA), which can be used in indirect and direct protein thiol labeling strategies, respectively.

these modifications contributes to pathology in tissues and cells under conditions of oxidative stress.

We previously described a protocol to conjugate BODIPY to unsaturated lipids to follow the formation of lipid adducts [25]. In addition, we have developed a separate protocol to examine the reactive thiol proteome using BODIPY conjugated with iodoacetamide [31]. In this protocol, we first show how these two methods can be used in concert with available fluorescent indicators of multiple biological processes, with the goal being to better understand how lipid peroxidation products, in particular, modulate the proteome to induce adaptive or maladaptive responses to oxidative stress. The BODIPY probes shown in Fig. 1B can be used in two different labeling strategies. In the negative labeling strategy, cells are exposed to oxidants or insults that promote oxidative stress (Fig. 2). These exposures often lead to mitochondrial damage [6,32], activation of the autophagic program [6,33], endoplasmic reticulum (ER) stress [34], and apoptosis [34,35]. The proteins that could be involved with each of these processes can then be examined by postlabeling with BODIPY–iodoacetamide (BD-IAM), with the result being a decrease in (or negative) labeling under conditions of oxidative stress compared with control conditions. In the second labeling approach, i.e., positive labeling, cells are preloaded with BODIPY-conjugated arachidonic acid (BD-AA) and then subjected to the oxidative insult (Fig. 2). The advantage of this approach is that the lipid peroxidation products will
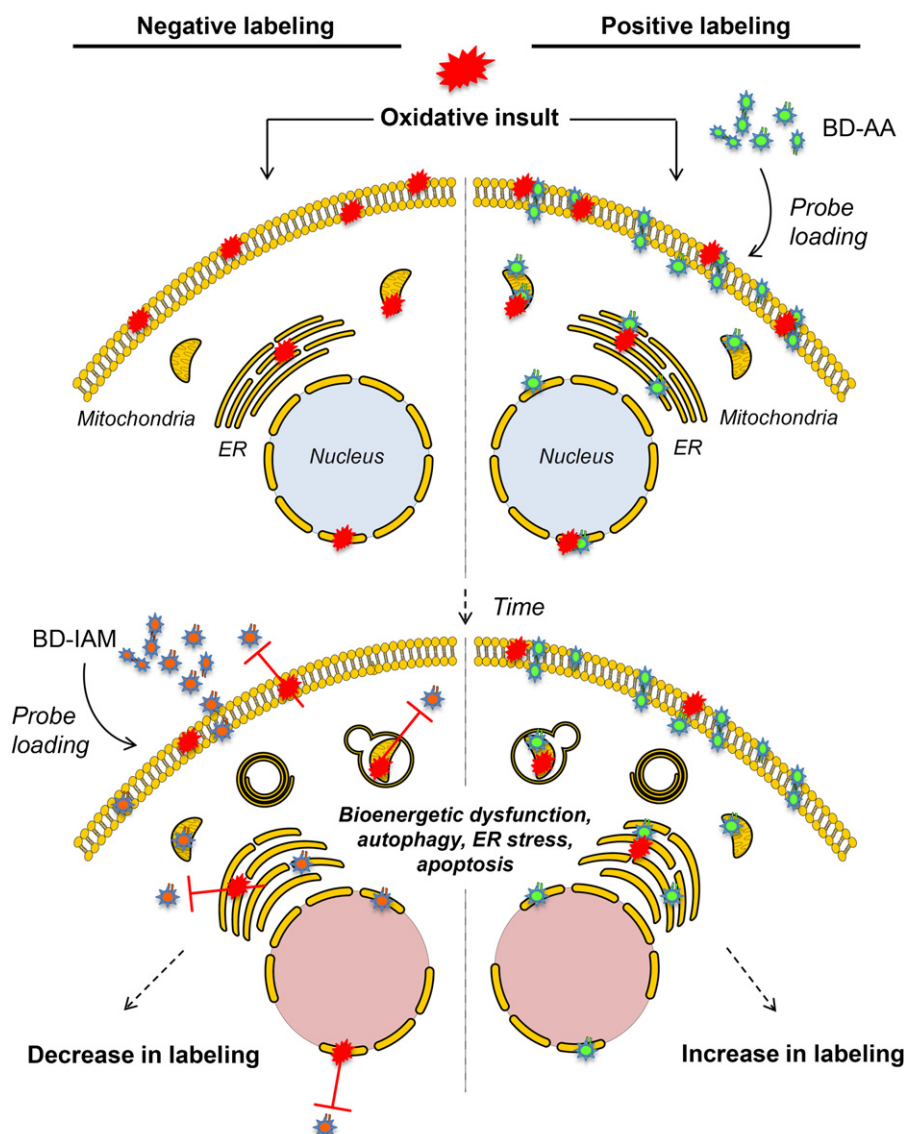
Fig. 2. Illustration of negative labeling and positive labeling strategies for detecting oxidative protein modifications. In negative labeling (left), the cells are first exposed to an oxidative insult such as that induced by hydrogen peroxide, hypoxia–reoxygenation, or exposure to lipid peroxidation products. After the desired amount of time, the cells are exposed to BODIPY-labeled iodoacetamide (BD-IAM). A decrease in the extent of labeling, as detected by fluorescence techniques, indicates an increase in oxidative protein modifications. In positive labeling, the cells can be loaded with BODIPY-labeled arachidonic acid (BD-AA) and then exposed to oxidant (or electrophilic) stress. The proteins modified by BD-labeled reactive lipids generated by lipid peroxidation can then be imaged using fluorescence-based strategies. For both techniques, microscopy may be used to identify the localization of the protein adducts, and protein fractionation may be used to quantify and identify proteins modified by the reactive species.

be generated intracellularly and comprise multiple fluorescently labeled reactive species. Because the BD-AA is uncharged and hydrophobic, it accumulates mostly in hydrophobic compartments and biological membranes, which are primary loci for lipid peroxidation.

Given time with the oxidative stimulus, the labeled probes in both approaches can be followed temporally using fluorescence or confocal microscopy, and the cells can be fractionated to identify products in subcellular compartments. The identification of the proteins in these subproteomes can be facilitated using biochemical separation techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which can be extended to 2D approaches for identifying proteins reactive with oxylipidomes generated endogenously and exogenously. Alternatively, organelles such as the mitochondrion may be isolated and examined using the probes. A major strength of BODIPY probes is their compatibility with multiple organelle-specific probes and oxidative stress dyes, which may be incorporated to examine the localization of proteins modified by lipid peroxidation products and for examining how those modifications

relate with cellular processes induced by oxidative stress, such as autophagy.

Materials

- Arachidonic acid (Calbiochem, No. 181198)
- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Pierce Biotechnology, No. 22980)
- 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine, hydrochloride (BODIPY FL-EDA; Invitrogen, Product No. D2390)
- BODIPY FL C$_1$-IA, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl)–iodoacetamide (BODIPY-IAM; Invitrogen, No. D6003)
- Hoechst 33342 (Enzo Life Sciences, No. ENZ-52401)
- ER-Tracker blue-white (Invitrogen, No. E12353)
- Mito-ID (Enzo Life Sciences, No. ENZ-51018)

- Cell Tracker blue (Invitrogen, No. C2110)
- mCherry-LC3 plasmid
- Ethanol, high-performance liquid chromatography (HPLC) grade
- Acetonitrile, HPLC grade
- Methanol, HPLC grade
- Chloroform, HPLC grade, preserved with 0.75% ethanol
- Acetic acid, glacial, certified ACS plus grade
- Tris base (Fisher, No. BP152-1)
- Triton X-100 (Sigma–Aldrich, No. T-9284)
- Hemin chloride (MP Biomedical, No. 15489-47-1)
- 4-Hydroxynonenal (Cayman Chemical, No. 32100)
- Recombinant aldose reductase (MP Biomedical, No. 199819)
- Dimethyl sulfoxide (DMSO; Sigma–Aldrich, No. D2438)
- Nitrocellulose (Bio-Rad, No. 162-0112)
- 35-mm glass-bottom confocal petri dish (MatTek, No. P35G-0-14-C)
- Chambered coverglass, four-well (Nunc; No. 155383)
- Amber glass vials, 1.5 ml (SUN SRi, No. 200 252)
- Caps with rubber septa (SUN SRi, No. 500 062)
- Borosilicate glass tubes, 16×100 mm (Fisher, No. 14-961-29)
- Bradford protein assay reagent (Bio-Rad, No. 500-0006)
- Lowry DC protein assay reagent (Bio-Rad, No.500-0001)
- 18-Mega-Ω water
- Precast 10.5–14% SDS–PAGE Criterion gels (Bio-Rad, No. 345-9949)
- Anti-rabbit/mouse-Cy5- or Cy3-conjugated antibodies (GE Healthcare, No.PA43002 or PA43010V)

Instruments

- Mass balance with 0.1 mg readability
- Pipettes
- SDS–polyacrylamide gel apparatus
- Slot- or dot-blotting apparatus
- Rocker (tilting shaker)
- Vortex mixer
- Spectrophotometer
- Cuvettes
- HPLC system equipped with ultraviolet/visible spectrophotometer and fraction collector
- Preparative column: Gemini C18 reversed-phase column with 10-μm particle size and dimensions of 250×21.2 mm (Phenomenex, No. 00G-4436-P0)
- Semipreparative column: Luna C18 reversed-phase column with 5-μm particle size and dimensions of 250×10 mm (Phenomenex, No. 00G-4041-N0)
- Confocal microscopes: (1) Nikon A1 line-scanning laser confocal microscope equipped with three solid-state lasers to generate 405-, 488-, and 561-nm excitation lines; (2) Leica DMIRBE microscope with TCS SP laser scanning confocal system, 405-, 488-, and 543-nm lines
- Fluorescence imager: Typhoon (GE Healthcare) or other fluorescence imaging system suitable for gel scanning and equipped with a laser and filter suitable for BODIPY excitation and detection

Protocols

Indirect labeling of the thiol proteome sensitive to lipid peroxidation products

This protocol can be used to examine how an oxidative insult or a particular species of reactive lipid affects the thiol proteome. Shown in Fig. 3 is an example of such an experiment. Here, rat aortic smooth muscle cells were exposed to various concentrations of the lipid peroxidation product HNE for 30 min. The HNE-containing medium was then removed and replaced with fresh medium containing BD-IAM, which is commercially available and readily implemented into experimental protocols. After the desired time, the BD-IAM-containing medium was removed, and the cells were washed to remove any unreacted BD-IAM. These cells may then be examined by fluorescence microscopy. Alternatively, the cells may be lysed and the proteins separated by SDS–PAGE. The modified proteome can then be examined by in-gel fluorescence imaging. The thiol proteome modified by exposure of cells to the reactive species may also be examined by slot-blotting techniques.

In this experiment, the cells treated with HNE showed an apparent concentration-dependent decrease in intracellular BOD-IPY fluorescence, indicating that the HNE exposure resulted in loss of intracellular free thiols, which would otherwise be reactive with the IAM probe (Fig. 3A). To examine total protein–HNE adducts and BD-IAM modifications simultaneously, the cell lysates were deposited onto nitrocellulose membranes, and they were probed with an anti-protein HNE polyclonal antibody; here, the secondary antibody used was a Cy5-labeled anti-rabbit antibody. Slot-immunoblotting for HNE adducts showed very little change at the lower concentrations of HNE exposure; however, the adducts began to appear when the concentration reached the 50–100 μM range (Fig. 3B). Interestingly, BD-IAM modifications to the proteome began to decrease with even the lowest concentration of HNE (Figs. 3B and C). The proteome affected by the oxidative insult may be further assessed by 1D or 2D protein fractionation. Shown in Fig. 3D is an SDS–PAGE in-gel fluorescence image of the thiol proteome affected by exposure of cells to HNE. Below is a step-by-step protocol for performing this type of experiment. This may be performed with any cell type and with multiple types of oxidative insults.

Procedure

1. Cells are seeded in six-well dishes 1–2 days before the experimental protocol is implemented. Seed $\sim$100,000–250,000 cells/well (depending on the proliferation rate of the cell type) in suitable medium such as Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, 5% CO_2 (if bicarbonate-buffered). The cells should be nearly ($>90\%$) confluent to ensure the greatest yield of protein. If cell cycle changes are of particular interest, serum-starve cells for $\sim$24 h before exposure to reactive species. *Note. Analysis by confocal microscopy will require seeding in glass-bottom tissue culture dishes or slides (see Detecting the localization of BD-AA and its derived oxidation products).*
2. Wash the cells twice with sterile phosphate-buffered saline (PBS).
3. Exchange growth medium for DMEM or other suitable medium containing appropriate carbon sources (such as pyruvate, glutamine, glucose). This medium should be phenol red free (for microscopy), and, in most cases, serum should be excluded to prevent reaction of the reactive species (e.g., electrophiles) with proteins such as albumin.
4. Add the desired concentration of reactive species or vehicle. In this experiment, ethanol was the vehicle, and a range of 6.25–100 μM HNE was used.
5. Expose cells to the reactive species for the desired time, e.g., 0.5–4 h, at 37 °C. If bicarbonate is the medium-buffering agent, then perform all incubations at 5% CO_2.
6. During incubation, make a stock BD-IAM solution by solubilizing in DMSO.
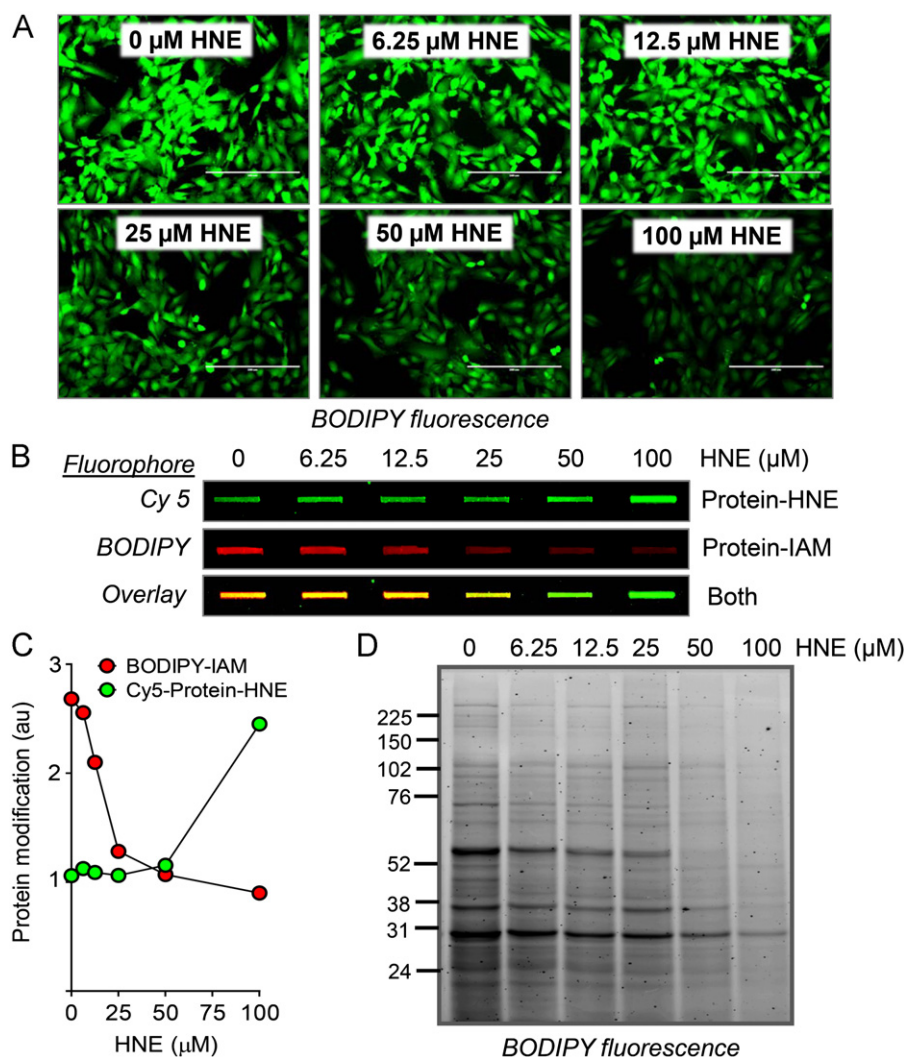
Fig. 3. Negative labeling using 4-hydroxynonenal (HNE) as the model stressor. Examples of various fluorescence labeling detection methods are shown. (A) Rat aortic smooth muscle cells were exposed to HNE (0–100 μM) for 30 min. The HNE-containing medium was then removed and medium containing BD-IAM (15 μM) was added to the cells. After 30 min, the cells were washed and imaged by fluorescence microscopy. (B) The same cells were then lysed, and total protein modification was assessed by slot-blotting. Protein–HNE adducts (green) were detected using a rabbit polyclonal protein–HNE antibody; the secondary antibody was a Cy5-labeled anti-rabbit antibody. The BD-IAM-modified proteins (red) were detected by fluorescence imaging on the membrane. Shown is the overlay of both modifications. (C) Quantification of protein–HNE and BODIPY-IAM adducts from (B). (D) The proteins were then separated by SDS–PAGE, and the BODIPY adducts were imaged by in-gel fluorescence imaging. As shown in all of these examples, a loss of BODIPY signal was indicative of increased protein–HNE adducts and possibly secondary oxidative modifications occurring as a result of the electrophilic insult. au, arbitrary units.

7. Determine BD-IAM concentration by diluting the BD-IAM in methanol, measuring the absorbance at 504 nm, and calculating the stock concentration using an extinction coefficient of $76{,}000 \, M^{-1} \, cm^{-1}$. Perform all measurements under low-light conditions to avoid any photobleaching of the fluorophore. *Keep under low-light conditions from this point forward in the protocol.*

8. Dilute BD-IAM to 20 μM in the appropriate volume of phenol-free medium and incubate the cells in this medium for 0.5 h at 37 °C.

9. Wash the cells twice with PBS.

10. If desired, image cells using a fluorescence or confocal microscope by exciting at 488–505 nm and detecting emission at ~500–550 nm (see Detecting the localization of BD-AA and its derived oxidation products for more on fluorescence imaging). *The excitation and emission maxima for BODIPY FL are shown in* Table 1 *and the spectra are shown in* Fig. 7.

11. To examine protein modifications by slot/dot-blotting or SDS–PAGE, lyse cells in buffer containing 25 mM Hepes, pH 7.0,

1 mM EDTA or DTPA, 1% NP-40, 0.1% SDS, and at least 1 mM *N*-ethylmaleimide (NEM). NEM is preferred if using a detergent-compatible Lowry assay. *Note. It is critical to include either NEM or DTT in the lysis buffer, as this will stop any further reaction of BD-IAM with other cellular nucleophiles. Also, if 2D isoelectric focusing SDS–PAGE will be performed, leave the SDS out of the lysis buffer.*

12. Collect supernatant and perform protein assay.

13. For dot- or slot-blotting, load between 1 and 10 μg of protein into each well of the dot/slot-blot apparatus (generally 1–4 μg is adequate, depending on the sensitivity of the imaging instrument). The general steps for dot/slot blotting are below:

  a. Cut a piece of nitrocellulose to fit apparatus (or use factory precut nitrocellulose membranes).

  b. Soak three precut pieces of filter paper and nitrocellulose in 1× Tris-buffered saline (TBS).

  c. Stack the filter paper and nitrocellulose in the apparatus and seal well. The nitrocellulose should be on top of the filter paper.

Table 1
Common fluorophores used in cell culture studies and their compatibility with BODIPY-FL dyes.

Fluorophore[a]	Staining locale or applications	Ex (nm)	Em (nm)	BODIPY-FL compatibility	Source	Product No.
BODIPY 493/503	Lipid droplets	500	506	N	Invitrogen	D3922
BODIPY FL[b]	–	503	510	–	Invitrogen	D2390
BODIPY-FL-IA	Free thiols	507	510	–	Invitrogen	D6003
BODIPY-C11	Lipid peroxidation sensor	581	591	N	Invitrogen	D3861
Fluorescein	General stain/Tag	460	515	N	Sigma	F245-6
Alexa Fluor 488	General stain/Tag	495	519	N	Invitrogen	A11001
RFP	Tag	563	582	Y	–	–
mCherry[b]	Tag	587	610	Y	–	–
Green fluorescent protein	Tag	493	505	N	–	–
Yellow fluorescent protein	Tag	529	539	N	–	–
Cyan fluorescent protein	Tag	458	489	Y	–	–
DAPI	Nuclear stain	350	470	Y	Invitrogen	D21490
Hoechst 33342[b]	Nuclear stain	343	483	Y	Invitrogen	H1399
Alexa Fluor 350 phalloidin	Cytoskeleton (actin)	346	446	Y	Invitrogen	A22281
Alexa Fluor 594 phalloidin	Cytoskeleton (actin)	593	617	Y	Invitrogen	A12381
Tetramethylrhodamine methyl ester (TMRM)	Mitochondria ($\Delta\Psi$-dependent)	552	577	Y	Invitrogen	T668
TMRM-IA	Mitochondrial thiols ($\Delta\Psi$-dependent)	555	580	Y	Invitrogen	T6006
MitoTracker deep red	Mitochondria ($\Delta\Psi$-dependent)	640	661	Y	Invitrogen	M22425
MitoTracker red	Mitochondria ($\Delta\Psi$-dependent)	578	599	Y	Invitrogen	M7512, M7513
MitoTracker orange	Mitochondria ($\Delta\Psi$-dependent)	554	576	Y	Invitrogen	M7510, M7511
MitoTracker green	Mitochondria ($\Delta\Psi$-dependent)	490	516	N	Invitrogen	M7514
Mito-ID red[b]	Mitochondria ($\Delta\Psi$-independent)	558	690	Y	Enzo	ENZ51007
Nonyl acridine orange	Mitochondria ($\Delta\Psi$-dependent)	495	519	N	Invitrogen	A1372
3,3′-Dihexyloxacarbocyanine iodide	Mitochondria ($\Delta\Psi$-dependent)	482	501	N	Enzo	ENZ52303
CellLight Golgi-RFP	Golgi apparatus	555	584	Y	Invitrogen	C10593
LysoTracker Blue DND2	Lysosomes	373	422	Y	Invitrogen	L7525
LysoTracker Red DND99	Lysosomes	577	590		Invitrogen	L7528
ER Tracker Blue-White DPX[b]	Endoplasmic reticulum	374	430 (640)	Y	Invitrogen	E12353
ER Tracker red	Endoplasmic reticulum	587	615	Y	Invitrogen	E34250
CellTracker Blue CMAC[b]	Whole cell	353	466	Y	Invitrogen	C2110
H₂DCFDA	Oxidant sensor	492	517	N	Invitrogen	D399
Dihydroethidium/hydroethidine[c]	Oxidant sensor	518	606	Y, N	Invitrogen	D23107
MitoSox red[c]	Oxidant sensor	396, 510	580	Y, N	Invitrogen	M36008
CellROX deep red	Oxidant sensor	644	665	Y	Invitrogen	C10422
DAF-FM diacetate	Nitric oxide sensor	495	515	N	Invitrogen	D23844
Monobromobimane	Glutathione sensor	394	490	Y	Invitrogen	M1378
Cy5 (antibody conjugate)[b]	Tag	632	699	Y	GE Healthcare	PA45012
Cy3 (antibody conjugate)	Tag	534	614	N	GE Healthcare	PA43010V

[a] Peak excitation and emission wavelengths often vary depending on the solvent environment.

[b] Fluorophore discussed in this paper.

[c] Dihydroethidium/hydroethidine (DHE)-based dyes, when excited at ~510, may have some overlap with BODIPY FL; however, excitation of the DHE dye near 510 nm may also report ethidium oxidation products other than the 2-OH-Mito-E$^+$, which is formed by direct reaction of the dye with superoxide. The DHE dyes have another excitation peak at 396 nm that seems to primarily excite 2-OH-Mito-E$^+$ (see Zielonka and Kalyanaraman [24] for further details).

d. Add 500 µl 1 × TBS to each well of the apparatus.

e. Pull the TBS through the membrane under vacuum.

f. Add equal amounts of protein to each well of the apparatus. Be sure to dilute the protein in at least 400 µl TBS before adding to well.

g. Pull dry under vacuum.

h. Add 600 µl of 1 × TBS to each well.

i. Pull dry under vacuum.

j. Disassemble apparatus and allow nitrocellulose to dry fully in the dark at room temperature.

k. Block in appropriate blocking buffer for Western blotting or image directly for BD modifications (see below).

14. For gel-based analysis, add protein lysates to Laemmli sample buffer (or to 2D sample buffer if isoelectric focusing is desired). Make sure to include enough DTT or mercaptoethanol to quench all free NEM (if NEM is used in lysis buffer). We use up to 100 mM DTT (final concentration) in our sample buffer.

15. Load 20–40 µg of protein into the wells of an SDS–PAGE gel. Results from Fig. 2D were acquired using a 10.5–14% Bio-Rad Criterion precast SDS–polyacrylamide gel.

16. Image gels on a Typhoon scanner or other suitable imager.

a. For gels in glass plates, first place a single strip of laboratory tape on the edges of the gel plates that contact the platen. This will prevent interference lines that would normally occur at the glass–platen interface.

b. Place the gel plate on the platen and set focal plane height at +3 mm. For BODIPY imaging, the glass plates typical of most common electrophoresis equipment work well; low-fluorescence plates are not required unless other fluorophores will be used. The Criterion plastic cassettes, after being cleaned with deionized water and dried, may be directly placed on the platen and scanned; again, ensure that the focal scan height is set at +3 mm.

c. Alternatively, the gel can be imaged using "platen" for the focal plane setting by removing the gel from the glass or plastic cartridge and directly placing it on the platen on a small amount of deionized water. *Avoid bubble formation between the gel and the platen.*

d. For Typhoon scanning, set the excitation wavelength at 488 nm and use the 520BP40 emission filter. Use at least 200 µm resolution.

Although this protocol and the results in Fig. 3 illustrate the ability to identify thiols modified directly by reactive lipid species, some caveats are worth noting. It is likely that the indirect BD-IAM labeling approach would not be useful in detecting modifications to other nucleophilic side chains such as lysine

and histidine, both of which can be modified by HNE. Furthermore, the immunological approach used to detect aldehyde-modified proteins directly may not be sensitive enough to distinguish relatively lower levels of protein adducts, or the antibodies may react preferentially with only one or a few types of HNE adduct. These shortcomings could negate convergence between immunological labeling strategies and the indirect labeling strategy shown here using BD-IAM. Nevertheless, this technique does allow one to determine the impact of an electrophile or oxidant on the reactive thiol proteome and demonstrates an ability to combine indirect and direct labeling approaches to more fully elucidate the biological impact of oxidative posttranslational protein modifications.

Direct labeling of the electrophile response proteome

Synthesis of BD-AA

Synthesis is performed using a carbodiimide-mediated conjugation reaction. After synthesis, the reaction preparations have a mixture of the original unreacted lipid, the tag, the expected product, and the priming compound, EDC. HPLC with UV–Vis

detectors is then used to purify the tagged lipid. After chromatographic separation, product purity is verified using electrospray ionization mass spectrometry, and the compound is stored in an argon- or nitrogen-purged dark glass vial. For the detailed synthesis and purification protocol, the reader is referred to [25].

Detecting formation of intracellular products of lipid peroxidation products using BD-AA

To directly detect proteins modified by arachidonic acid oxidation products that are formed intracellularly, the BD-AA is incubated with the cells either before or concomitant with the administration of an oxidizing stimulus. It should be noted that BD-AA will not be incorporated into phospholipids because the carboxylate moiety of AA is removed during derivatization with BODIPY. However, its hydrophobicity would probably sequester it primarily to membranes in the vicinity of lipid oxidation processes. As shown in Fig. 3, the BD-AA fluorescence may be viewed using fluorescence microscopy (Fig. 4A) and the proteins modified by lipid peroxidation products may be separated and visualized
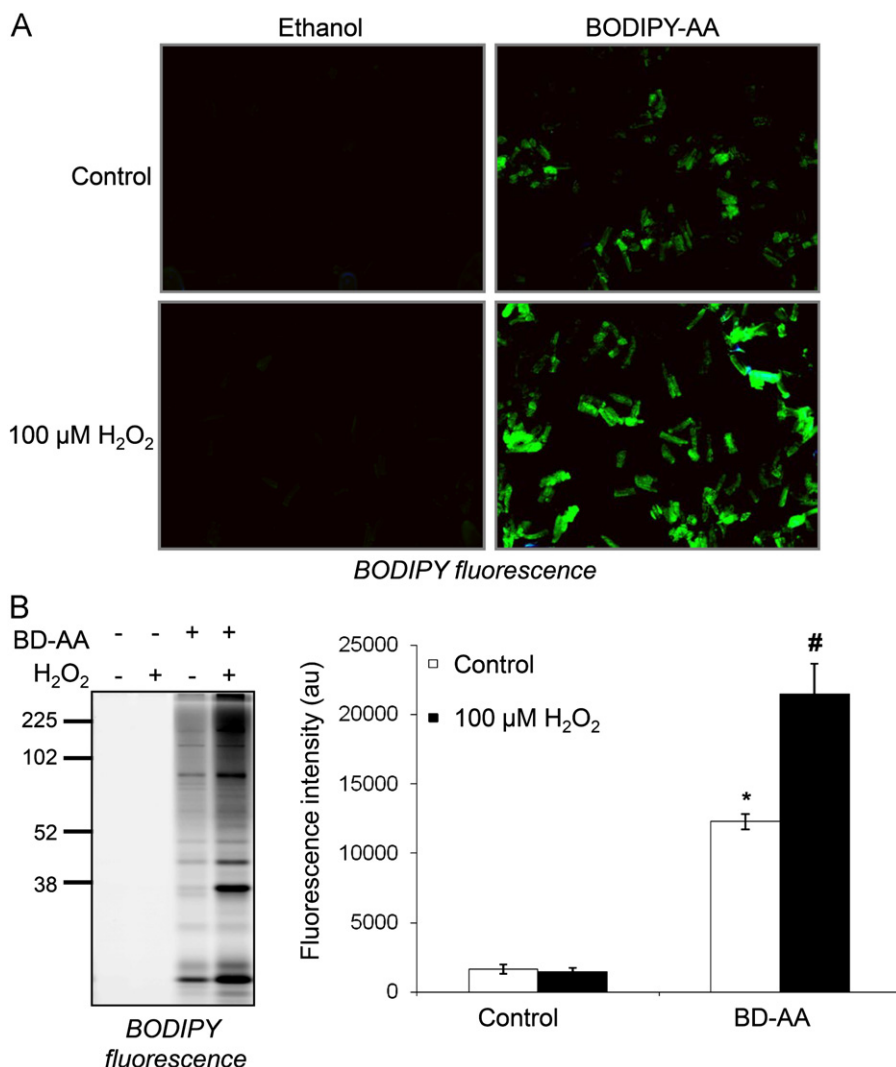

Fig. 4. Positive labeling using a hydrogen peroxide and BODIPY–arachidonic acid (BD-AA) lipid peroxidation system. Fluorescence strategies for detecting protein modifications induced by lipid peroxidation in situ are shown. (A) Adult rat ventricular myocytes were isolated from Sprague–Dawley rats and treated with 10 μM BD-AA or ethanol (vehicle control). The cells were then exposed to vehicle or 100 μM H_2O_2. Cells were fixed on glass slides and imaged for BODIPY fluorescence. Representative images are shown. (B) The myocyte proteins were then separated by SDS–PAGE, and the BODIPY adducts were imaged by in-gel fluorescence imaging. Shown in (A) are representative images at the 2-h time point, (B) shows proteins modified by the 4-h time point, and (C) Quantification of protein modifications shown in panel B. $n=3$ per group; $^*p < 0.05$ vs control without BD-AA, $^\#p < 0.05$ vs BD-AA alone.

by in-gel fluorescence imaging (Figs. 4B and 4C). The following is a detailed protocol for this type of experiment.

Procedure

1. Measure the absorbance spectra of the BD-AA stock using a spectrophotometer. Calculate the concentration using an extinction coefficient of 76 mM^{-1} cm^{-1} at 504 nm.
2. Dilute BD-AA stock in neat ethanol to obtain a working stock of 5 mM.
3. Treat cells with BD-AA to a final concentration of 10 μM. Treat vehicle controls with the same volume of ethanol.
4. Without changing the medium, add treatment (in this case 100 μM H$_2$O$_2$) and incubate the cells for the desired time. *Note. Hydrogen peroxide will work best in cells that contain high levels of heme-containing proteins such as myoglobin. For cell types that do not express myoglobin or adequate levels of heme-containing proteins that facilitate decomposition of unsaturated lipids, hemin at a concentration of ~10–25 μM may be used to induce lipid peroxidation [6,36,37].*
5. For imaging using an epifluorescence microscope, cells in Fig. 4A were washed with PBS to remove medium containing BD-AA, fixed using paraformaldehyde in PBS, and stored in the dark until imaging. Results typical of this protocol are shown in Fig. 4A. *Note. Cells may also be live-imaged.*
6. Alternatively, cells can be lysed in cold lysis buffer and separated via nonreducing SDS–PAGE. Because of the fluorescence of the BODIPY tag, protein bands containing lipid–protein adducts can be directly visualized using a Typhoon imager at an excitation wavelength of 488 nm and the 520BP40 emission filter. An example of results using this method is shown in Fig. 4B. The fluorescence intensity of the bands was quantified using ImageQuant TL software. *Note. We have found that some of the lipid adducts with proteins are reducible with DTT. It is the experimenter's option to use reducing conditions for examining the BD–lipid–protein adducts. Also, any free BODIPY label will travel in the dye front. Therefore, it is important to run the dye front completely off of the gel before imaging.*

Synthesis of an external standard for quantification of protein modifications

The BD-IAM reagent may be used to make an external standard. For this, we have used recombinant aldose reductase (AR) protein. *Note. We have noticed that GAPDH precipitates when conjugated with BD-IAM; therefore, it is recommended that BODIPY-FL-SSE, which will react with amines instead of thiols, be used to make a BD-GAPDH [31]. However, AR remains soluble after conjugation with BD-IAM. Other proteins may also be suitable for making a standard using BD-IAM, but these would require additional validation.* The steps for making BD-AR are given below:

1. First, AR (1 mg/ml) is incubated in 100 mM Tris, pH 8.0, containing 100 mM DTT for 30 min at 37 °C. During this time, equilibrate a Sephadex G25 (PD-10) column with 50 mM potassium phosphate buffer, pH 7.4.
2. The fully reduced AR is loaded into the equilibrated PD-10 column to remove DTT. Collect protein fractions (10 drops per fraction) in glass tubes or Eppendorf tubes. When using this PD-10 column, collect a total of 15 fractions. *Note. The protein-containing fractions usually elute within fractions 3–9.*
3. In six clean, separate tubes, place 100 μl of Bradford reagent. Add 10 μl of your samples from each fraction (i.e., fractions 3–9) to corresponding tubes containing Bradford reagent.
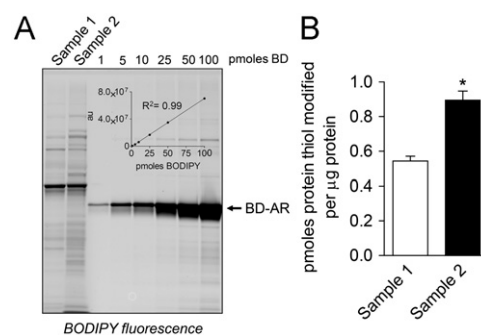

Fig. 5. Example of an external standard constructed using BODIPY–iodoacetamide (BD-IAM). Fluorescence imaging and quantification of samples using an in-gel external standard are shown. (A) A BD-labeled protein standard was constructed by treating prereduced recombinant aldose reductase (AR) with BD-IAM. In this example, the usefulness of the standard is shown in context of samples (isolated mitochondria) that were treated with BD-IAM in a medium at pH 5.0 (sample 1) and in a medium at a pH of 7.2 (sample 2). The mitochondria were then lysed and equal amounts of protein were loaded on SDS–PAGE gels. The BD-AR standard was then loaded alongside the sample lanes and used to construct a standard curve (inset). (B) Quantification of BD-IAM adducts using the external standard. $n=3$ per group; *$p < 0.05$ vs sample 1.

The protein should elute sequentially in two or three tubes. Pool those fractions that turn blue in color.
4. Measure protein concentration by Lowry, Bradford, or other appropriate assay. Calculate the molar concentration of the protein.
5. The protein is then reacted with an equimolar amount of BD-IAM for 30 min at 37 °C in the dark. During this incubation, equilibrate a PD-10 column with phosphate-buffered saline.
6. Pass the reaction mixture through the equilibrated PD-10 column to remove unreacted BD-IAM as in steps 1–3 above.
7. Calculate the protein concentration as in step 4.
8. Calculate the amount of BODIPY bound to the protein spectrophotometrically at 504 nm ($\varepsilon = 76000$ M^{-1} cm^{-1}).
9. Test the standard by running in an SDS–PAGE gel under reducing conditions.

An example of the use of the BD-AR standard is shown in Fig. 5. The dynamic range of this standard is ~10 fmol to 100 pmol. Calculate the extent of protein modification by dividing the picomoles of BODIPY in each lane (acquired using the standard curve) by the amount of protein (in μg) loaded into the respective lane.

Detecting and identifying modifications in isolated organelles

The BD-AA and BD-IAM probes may also be used in isolated organelles such as mitochondria to examine protein susceptibility to modification by lipid peroxidation products or protein thiol reactivity. For this, the organelle should be intact after isolation and exposed to the BD-AA or BD-IAM under the desired conditions (e.g., Fig. 6A). Here, mitochondria were isolated from mouse hearts and loaded with BD-AA. The mitochondria were then placed under conditions of oxidative stress. For example, mitochondria incubated with hemin show an increase in lipid peroxidation-derived protein modifications compared with control mitochondria treated with BD-AA alone (Figs. 6B and C). Antioxidant interventions may also be used to determine specificity of the response; e.g., BHT was included as an antioxidant control in the hemin group (Figs. 6B and C). The lysates may also be used in 2D approaches for identifying proteins modified by reactive lipid species (Fig. 6B, bottom). A general procedure for isolating mitochondria from heart or liver and examining targets of lipid peroxidation is given below.
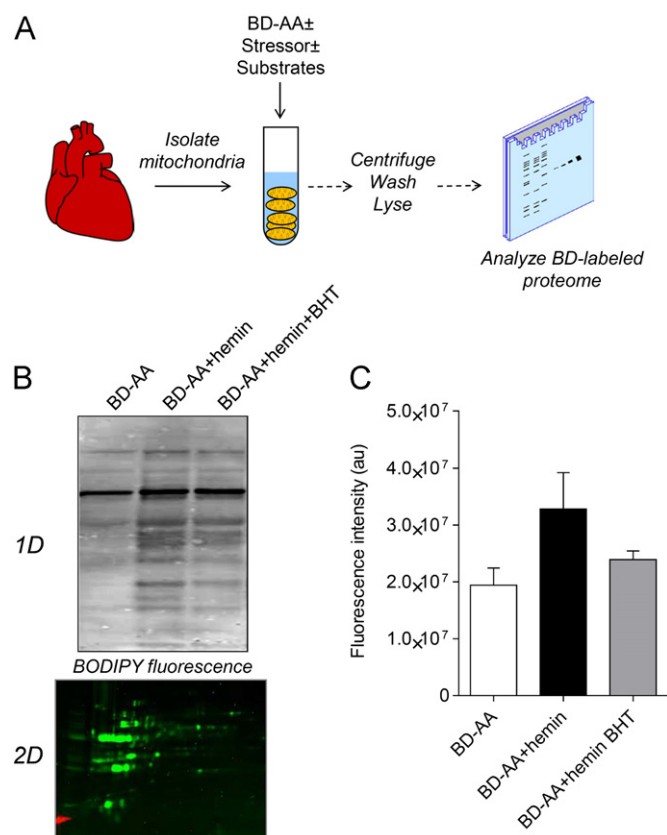
Fig. 6. A positive labeling strategy to identify protein targets of lipid peroxidation in isolated organelles. An example of the use of fluorescence imaging to detect mitochondrial proteins modified by oxidized products of BD-AA is shown. (A) Illustration of the procedure for examining mitochondrial protein targets of lipid peroxidation. Isolated, intact mitochondria derived from rodent hearts may be treated with BD-AA in the absence or presence of oxidative stressors. Additionally, the mitochondria may be placed under various respiratory conditions. The mitochondria are then lysed and the protein targets can be examined using in-gel fluorescence imaging. (B) Isolated mitochondria were treated with BD-AA in the absence or presence of hemin. Lipid peroxidation processes were inhibited in the hemin group using the antioxidant butylated hydroxytoluene (BHT). The image at the bottom shows that such modifications may also be detected in 2D proteomic strategies. (C) Quantification of the groups in (B). $n = 3$ per group.

Procedure

1. After anesthetization, isolate the heart or liver and immediately place it into 10 ml of cold buffer A (containing 220 mM mannitol, 70 mM sucrose, 5 mM Mops, 1 mM EGTA, adjust pH to 7.4 with KOH) in a 15-ml conical tube.
2. Wash the heart 5 × with 10 ml of cold buffer A (until blood is mostly gone). If using Langendorff-perfused hearts, no washes are required.
3. Weigh the heart and then chop finely with scissors in 10 ml of buffer A containing 0.2% defatted BSA and protease inhibitor cocktail (optional). *Note. Use 10 ml buffer to 1 g of tissue.*
4. Homogenize tissue with a tissue tearer, or mince the tissue on ice with a razor blade and use a Teflon-coated Glas-Col homogenizer to homogenize the tissue.
5. Transfer the suspension into a centrifuge tube.
6. Centrifuge at 500–1000g for 10 min.
7. Collect supernatant. Optional: the supernatant may be passed through cheesecloth to filter out any large particles that may dislodge from the pellet.
8. Centrifuge the filtered supernatant at 10,000g for 10 min.
9. Discard the supernatant (or keep as the crude cytosolic fraction if desired).

10. Resuspend the mitochondrial pellet in 5 ml of ice-cold buffer A (without BSA).
11. Centrifuge once again at 10,000g for 10 min.
12. Resuspend the pellet in 0.5–1 ml of buffer A, respiration buffer, or buffer appropriate for the planned assay or experiment. Typical respiration buffer is 120 mM KCl, 25 mM sucrose, 10 mM Hepes, 1 mM MgCl$_2$, and 5 mM KH$_2$PO$_4$, adjust pH to 7.2 with KOH.
13. If assaying respiration, make 0.5 M stocks of glutamate/malate (or pyruvate/malate) and/or succinate and 100 mM ADP preparations. *Remember to always use KOH to adjust pH to 7.2.*
14. *Note. If a highly pure population of mitochondria is desired, resuspend mitochondria in 5 ml of buffer A containing 19% Percoll after step 11. Centrifuge at 14,000 g for 10 min, followed by resuspension of the pellet in the desired buffer.*
15. Measure protein concentration of isolated mitochondria suspension by first lysing 5 µl of mitochondrial suspension in 45 µl of 10% SDS. Use the Lowry DC protein assay reagent to determine protein concentration.
16. Preload a 1 mg/ml solution of intact mitochondria with 20–40 µM BD-AA and incubate at room temperature for 5 min.
17. Add oxidizing stimulus, vortex briefly, and place at 37 °C for the desired time. If using energized mitochondrial preparations (i.e., mitochondria with substrate present), leave the tube tops open and vortex occasionally to help prevent depletion of oxygen in the medium. If assaying under State 3 or uncoupled conditions, decrease the amount of mitochondrial protein to 0.25 mg/ml or less per incubation.
18. Centrifuge mitochondria at 13,000g for 5 min. Aspirate medium and freeze pellet or lyse immediately.
19. Lyse using appropriate buffer containing detergent. A simple lysis buffer that is compatible with both 1D and 2D electrophoresis is 10 mM Hepes (or Tris), pH 7.0, containing 1% NP-40, 0.5% deoxycholate, 1 mM EDTA, protease and phosphatase inhibitor cocktail, and 1 mM NEM. Allow lysates to incubate on ice for 1 h followed by sonication (3 × for 10 s each) on ice.
20. Centrifuge lysates at 13,000g for 10 min and transfer supernatants to fresh tubes.
21. Perform protein assay on lysates and analyze BD-modified proteins as described in the previous sections.

Detecting the localization of BD-AA and its derived oxidation products

One of the major strengths of the BODIPY fluorophore is its narrow excitation and emission bandwidths, which allow it to be used with multiple other fluorophores. Of particular interest are the sites at which lipid-derived reactive species are generated and where they localize under conditions of oxidative stress. Hence, colocalization of BD-AA with specific organelles can be used to indicate primary targets of lipid oxidation products and identify biological processes regulated by oxidized lipids. Here we present a protocol for using BD-AA and BD-IAM in confocal microscopy applications. See Table 1 for a more extensive list of the compatible fluorophores and probes that may be used with BD-AA.

Shown in Fig. 7 are the excitation–emission spectra of BD-AA and Hoechst 33342 (a nuclear dye), Mito-ID red (a non-membrane-potential ($\Delta\Psi$)-dependent mitochondrial dye), ER Tracker blue-white (an endoplasmic reticulum-specific dye), and CellTracker blue (a dye that stains the entire cell). Before each experiment, the excitation and emission spectra should be examined to determine if the stain of choice is compatible with
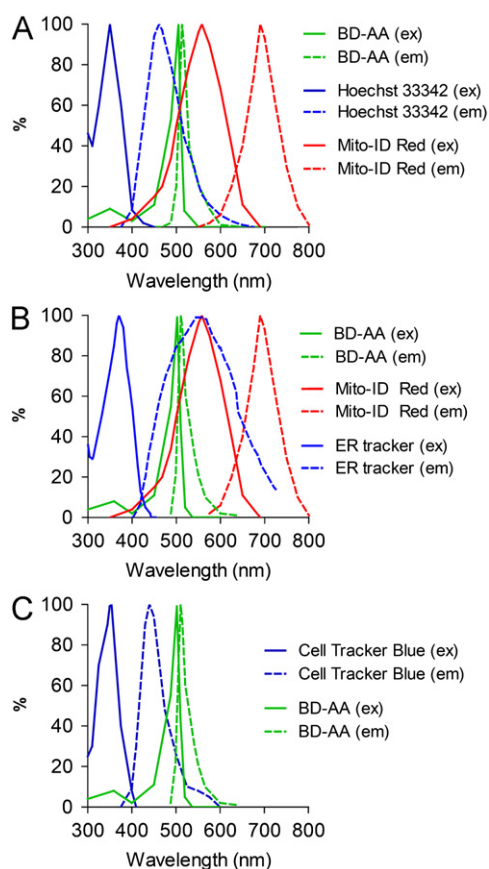
Fig. 7. Excitation/emission spectra of tracking dyes compatible with BD-AA. (A) Spectra for BD-AA, Hoechst 33342 (a nuclear stain), and Mito-ID red (a non-membrane-potential-dependent mitochondrial dye). (B) Spectra for BD-AA, Mito-ID red, and ER Tracker blue-white (an endoplasmic reticulum-specific dye). (C) Spectra for CellTracker blue (a whole-cell dye) and BD-AA.

BODIPY. Applications and tools for spectral comparisons are available from multiple vendors and sites. For example, Molecular Probes (SpectraViewer at http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html or the iPad App), Heliophor, and Zeiss offer guidance for initial screening of compatible dyes.

Shown in Fig. 8 are the confocal images of the above-mentioned dyes used in concert in unstressed cells. Most tracking and organelle dyes minimally affect cell viability, and they can be used for prolonged periods or for time-lapse imaging. Mito-ID red (shown in Figs. 8A and B) is a good choice for examining mitochondrial colocalization because its localization should not be affected by changes in respiratory function. Should the confocal microscope not have differential interference contrast capabilities to show clear boundaries of the cell, a total cell dye such as CellTracker blue may be used (Fig. 8C).

In some experiments, it is useful to transfect cells with reporters such as fluorescently labeled LC3, which forms puncta during times of increased autophagy [38]. The cells can then be stressed with an oxidant or a condition that produces oxidative stress, and the localization of BD-AA can be assessed with respect to fluorescently labeled autophagosomal structures. Shown in Fig. 9 is an example of such an experiment. Here, endothelial cells were transfected with mCherry-LC3 and loaded with BD-AA after 48 h. The spectra of each of these fluorophores are shown in Fig. 9A. The cells were then exposed to vehicle or hemin for 4 h. The localization of the BD-AA with respect to mCherry-LC3 was then assessed by confocal microscopy (Fig. 9B). Hemin has been shown to induce autophagy, which was validated by separate

biochemical approaches [6], and BD-AA localized to the puncta. These data and supporting data from similar experiments (see [6,33] for details) suggest that oxidative stress increases autophagy, which serves to degrade proteins modified by lipid peroxidation products. Below is a detailed protocol for the detection and monitoring of BD-AA under basal and oxidant-stressed conditions, including colabeling with the aforementioned tracking dyes.

Procedure

1. The user should have access to a laser confocal microscope with at least two solid-state lasers that generate substantially different excitation lines. In our studies, we use either a line-scanning Nikon A1 system equipped with a 60× oil immersion objective mounted on a TE-2000E2 inverted microscope as described previously [39,40] or a Leica DMIRBE microscope with similar capabilities [6].
2. Cells should be seeded at ~100,000–250,000 cells per dish (depending on expected proliferation rate) for 1–2 days on 35-mm glass-bottom dishes or on glass coverslips.
3. Cells should then be incubated in phenol-free DMEM with 0.5–10 μM BD-AA at 37 °C for 2 h before addition of any other dyes.
   a. For mCherry-LC3 imaging, cells can be seeded per step 1, then at ~75% confluence, they should be transfected with the desired plasmid.
   b. Carry out transfection per the manufacturer's instructions or in a self-standardized protocol. For the results shown in Fig. 9, the transfection was carried out with 2 μg of plasmid DNA, which was incubated in 150 μl of serum-free DMEM for 5 min at room temperature. The diluted plasmid DNA and Lipofectamine were then mixed gently and incubated for 20 min at room temperature. The DNA–Lipofectamine mixture was added to cells, and, after 6 h, the medium was removed and replaced with DMEM containing 10% FBS. Transfection efficiency was determined by fluorescence microscopy the following day. The cells were then trypsinized and plated at 5×10^4 cells/well in a four-well chamber slide. Confluence was ~70% at the time of imaging. *Note. Optimization of transfection conditions will be necessary depending on cell type and desired confluence.*
   c. Image cells by confocal microscopy. If desired, the experimenter may then determine the effects of oxidative stress on subcellular localization of lipid peroxidation products. An example of this is shown in Fig. 9B. Here, bovine aortic endothelial cells expressing mCherry-LC3 were incubated with 10 μM BD-AA. The cells were then exposed to vehicle (DMSO) or 25 μM hemin for 3 h before imaging. *Note. Transfection efficiency should be optimized for cell type, confluence, and DNA–transfection reagent ratios.*
4. For organelle (Mito-ID, Hoechst, or ER Tracker) and cell tracker dyes, add directly into medium with BD-AA for the final 15–30 min of the incubation period. Agitate diluent into medium to evenly distribute dye.
5. The concentrations of tracking dyes used in Fig. 8 are as follows: ER Tracker was diluted to 500 nM, CellTracker blue was used at 1 μM, Mito-ID was used at 1:10,000 (from manufacturer's stock), and Hoechst 33324 was used at a final concentration of 1 μg/ml.
6. Wash cells 2× in sterile PBS.
7. After imaging, adjust all conditions equally for image quality, brightness, and scale for comparative analysis. *Note. Optimization of dye tracker concentration, labeling times, and other parameters such as buffer pH and medium conditions may be required to improve dye compatibility and image quality.*
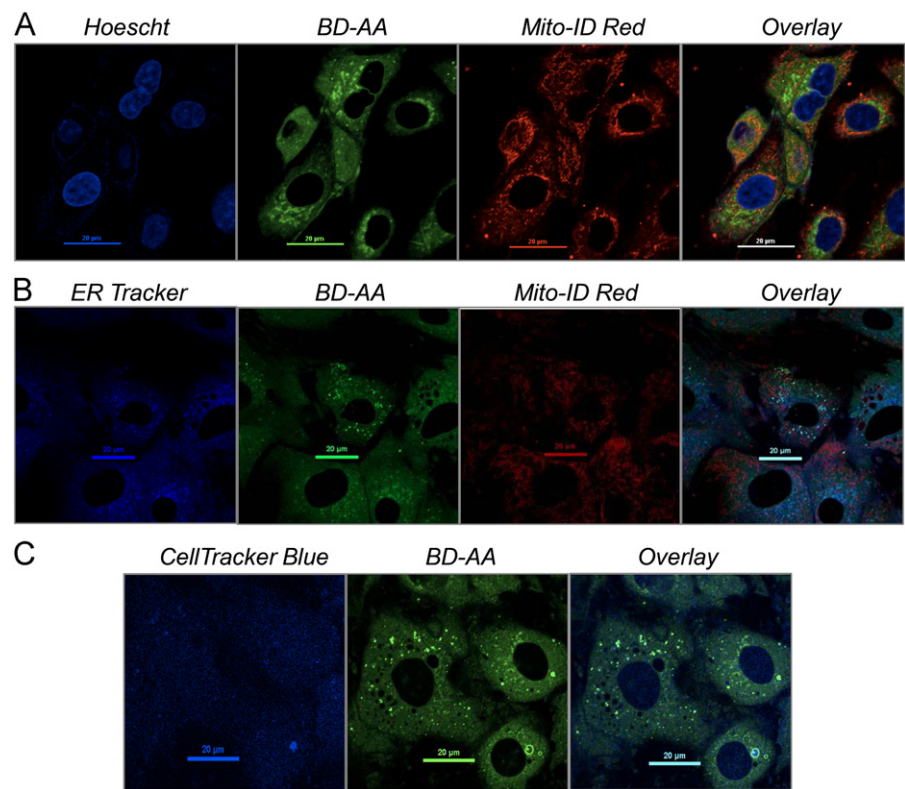
Fig. 8. Using compatible dyes for identifying the localization of BD-AA by confocal microscopy. Confocal fluorescence images of unstressed rat aortic smooth muscle cells in which BD-AA is shown in concert with tracking dyes are shown. (A) Hoechst 33342, BD-AA, and Mito-ID stains; (B) ER Tracker blue-white, BD-AA, and Mito-ID; and (C) CellTracker blue and BD-AA. Table 2 shows the confocal settings and conditions for these images. Additional compatible dyes are found in Table 1.
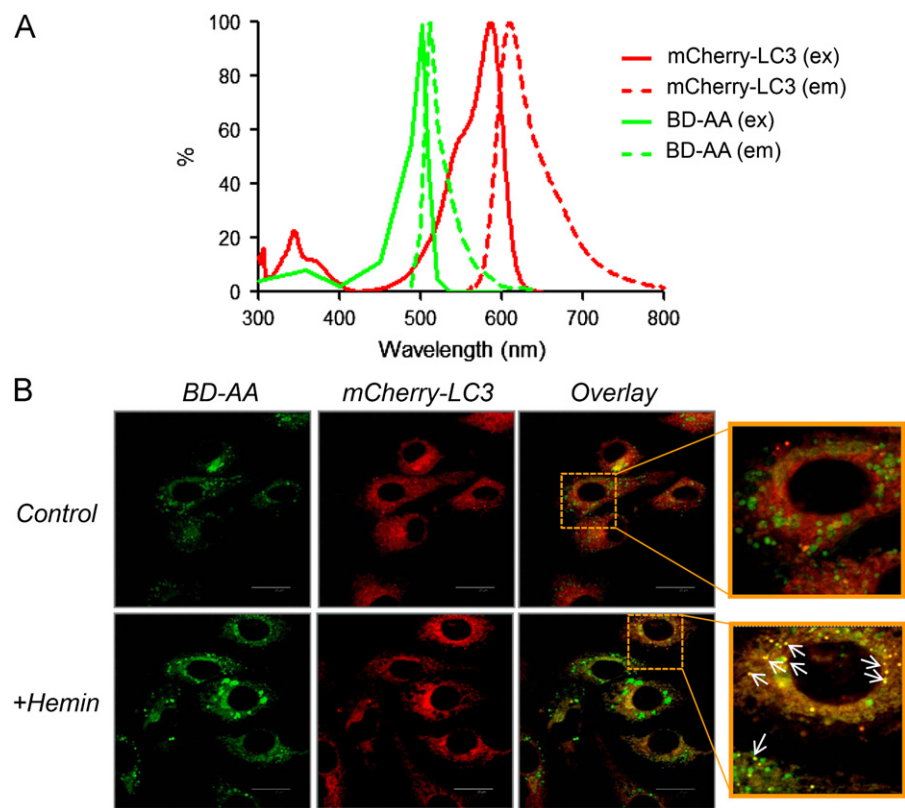

Fig. 9. Example of the use of BD-labeled probes for examining the role of lipid peroxidation and oxidative stress in biological processes such as autophagy. (A) Excitation and emission spectra of mCherry and BD-AA. (B) Representative confocal images of live cells. Cultured bovine aortic endothelial cells were transfected with mCherry-LC3 48 h before labeling with BD-AA. The cells were then exposed to vehicle (DMSO) or hemin (25 μM) for 3 h. The confocal images were acquired after replacing the medium with BD- and hemin-free medium. Arrows indicate apparent colocalization of BD-AA products with mCherry-LC3 puncta. Shown in Table 2 are the confocal settings used for acquiring these images.

Table 2
Details of confocal microscopy protocol for each dye or labeling component that was combined with BD-AA.

Probe/dye	Excitation line (nm)	Emission-BP (nm)	Power	HV
BD-AA	488	525/50	0.3	100
mCherry-LC3	543	630/60	4	90
Hoechst	405	450/50	2.5	100
ER Tracker blue-white	405	450/50	2.5	100
Mito ID red	561	700/75	4	100
CellTracker blue	405	450/50	5	100

BP, band pass; HV, photomultiplier tube voltage.

8. See Table 2 for the details pertaining to each probe/dye and the specific conditions for excitation and emission data collection. For most confocal experiments, images were natively averaged at $2\times$, laser offset was maintained at 0, image size was 1024, pinhole size was 36.1 μm, channel series was ON, and pixel dwell time was 1 μs.

Calculations and expected results

To determine differences in protein modifications (such as that induced by HNE or BD-IAM) after imaging, we utilized Image-Quant TL (Total Lab GE Healthcare) and centered a single band for the entire lane intensity measurements in arbitrary units. Background subtraction is of critical importance; in some cases manual baseline background subtraction may be optimal depending on the intensity of BODIPY bands, lower intensity bands should be manually scrutinized for optimal background removal.

Caveats and other considerations

This protocol describes the use of BD-labeled probes for the detection of proteins modified by reactive lipids species and to determine which reactive protein thiols are most readily oxidized. However, it may be useful, depending on the experiment or intent, to use other types of probes to interrogate the thiol proteome. For instance, a stronger electrophile, such as BODIPY-labeled NEM, may be used to label a wider range of protein thiols and identify changes that IAM may miss [31]. In addition, these protocols could easily be adapted and combined with other fluorophore-labeled probes and protocols to provide further insight into and to quantify potentially important protein thiol modifications. For example, it is possible to extend this protocol not only to detect the overall thiol proteome that is modified but also to begin to discern if those proteins are S-nitrosated, S-glutathiolated, or oxidized. Disulfide-forming probes or probes similar to those used in protein spin labeling studies such as biotinylated glutathione, methyl methanethiosulfonate, or *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide may be used alone or in concert to detect specific protein modifications [31,41,42]. Other specific derivatization agents, e.g., dimedone or dimedone-like compounds [43], or reductants, e.g., ascorbate [44], could be used to detect and discriminate between reversible posttranslational protein modifications such as S-nitrosation and sulfenylation.

A long-standing problem in understanding how oxidative modifications influence protein, cell, and tissue function is the determination of the extent of modification required to elicit a biologically relevant effect. For example, it is possible that 10% of the total pool of a certain enzyme is inhibited by a thiol modification; yet, this may not have a sizeable effect on pathway flux or cell function. Such a modification would readily be detected in positive labeling strategies, in which the detection signal would be well above background. The concern then is that the positive labeling approach could lead an investigator to misinterpret data and place undue importance on a particular modification to a protein or enzyme. In this respect, the indirect labeling approach is advantageous because it can be used to determine the extent of protein thiol modification on a particular protein or subproteome. The indirect approach, however, does require that a fairly substantial proportion of a protein or subproteome is modified to detect a robust difference between groups. Using both indirect and direct approaches can maximize the ability to determine the significance of a particular modification to protein and cell function.

The use of organelle tracker dyes and fluorescent probes should be tested over the range of stimulus concentrations, time points, temperatures, and incubation periods to characterize potential effects attributed to the probe alone compared with those from experimental stimuli. It is particularly important in confocal experiments to control for artifact autofluorescence of the cells themselves. Therefore, in every experiment, it is important to include unstained cells as controls. Moreover, some oxidant stimuli such as hemin are excitable and have fluorescent properties, and these should be included as control samples as well. Empirical determination of the dose of hemin generating minimal autofluorescence or spectral interference with BODIPY or other labeling fluorophores should be conducted before integrating into final protocols.

In the above protocols, we generally use 10–20 μM BD-AA or BD-IAM to determine labeling and adduct formation in vitro or in situ. One consideration would be to determine a dose curve of BODIPY for each condition, especially when considering new electrophilic target proteomes or protocol development. For confocal experiments, it is prudent to optimize the BODIPY probe concentrations and minimize potential artifact labeling in the cell type of interest and (if desired) the oxidant stimulus.

Last, it is important to discuss the reliability of colocalization. The word "colocalization" is used to describe the existence of two or more different molecules in a very close spatial position in the cell [45]. So how close must the two be to truly be colocalized? The phenomenon behind this is quite possibly one of the most misrepresented areas of current basic research, with the most common misconception being that colocalization of antigens equals sharing of their functional characteristics [46]. Therefore, caution and additional biochemical methods are generally required to ensure that the conclusions taken from confocal colocalization studies are accurate. Should more quantitative colocalization studies be required, additional steps should be taken to calculate colocalization coefficients and to perform reliable quantitative colocalization analysis using appropriate software such as CoLocalizer Pro [47].

Acknowledgments

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