Published in final edited form as:

Anal Bioanal Chem. 2011 April ; 400(1): 213–222. doi:10.1007/s00216-011-4725-9.

Semi-automated image analysis: detecting carbonylation in subcellular regions of skeletal muscle

Vratislav Kostal,

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Kiara Levar,

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Mark Swift,

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Erik Skillrud,

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Mark Chapman,

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

LaDora V. Thompson, and

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Edgar A. Arriaga

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis, MN 55455, USA

Edgar A. Arriaga: arriaga@umn.edu

Abstract

The level of carbonylation in skeletal muscle is a marker of oxidative damage associated with disease and aging. While immunofluorescence microscopy is an elegant method to identify

carbonylation sites in muscle cross-sections, imaging analysis is manual, tedious, and time consuming, especially when the goal is to characterize carbonyl contents in subcellular regions. In this paper, we present a semi-automated method for the analysis of carbonylation in subcellular regions of skeletal muscle cross-sections visualized with dual fluorescent immunohistochemistry. Carbonyls were visualized by their reaction with 2,4-dinitrophenylhydrazine (DNPH) followed by immunolabeling with an Alexa488-tagged anti-DNP antibody. Mitochondria were probed with an anti-COXI primary antibody followed by the labeling with an Alexa568-tagged secondary antibody. After imaging, muscle fibers were individually analyzed using a custom-designed, lab-written, computer-aided procedure to measure carbonylation levels in subsarcolemmal and interfibrillar mitochondrial regions, and in the cytoplasmic and extracellular regions. Using this procedure, we were able to decrease the time necessary for the analysis of a single muscle fiber from 45 min to about 1 min. The procedure was tested by four independent analysts and found to be independent on inter-person and intra-person variations. This procedure will help increase highly needed throughput in muscle studies related to ageing, disease, physical performance, and inactivity that use carbonyl levels as markers of oxidative damage.

Keywords

Carbonylation; Fluorescence microscopy; Image analysis; Mitochondria; Aging; Muscle fiber; ImageJ; MatLab

Introduction

There are two different types of mitochondria found in skeletal muscle, which differ in their location within the muscle cell [1]. The subsarcolemmal mitochondria (SSM) are found in clusters beneath the sarcolemmal membrane and are thought to provide energy for the intercellular signaling, ion and substrate transport, and other membrane-associated activities [2]. The intermyofibrillar mitochondria (IFM) represent the majority of skeletal muscle mitochondria. They are found between myofibrils and are believed to provide most of the energy necessary for muscle contraction [3]. Due to these metabolic differences, it is hypothesized that there is a difference in the levels of oxidative damage in the two mitochondrial types [4].

Carbonyls form in the proteins, DNA, and lipids when exposed to oxidative stress [5]. Carbonyl levels have become the most generally used marker for oxidative stress [6–8]. Methods for evaluating oxidative damage based on carbonyls have included spectrophotometric measurements, capillary electrophoresis [9], use of the Oxyblots™ [10,11], and advanced proteomic methods [12,13]. While these techniques are the gold standard to quantify carbonylation levels, they are not compatible with the subcellular analysis of carbonyls, particularly when measuring carbonylation levels among (or between) different types of mitochondria within the same cell.

We have previously developed a method for the quantification of carbonyls within various regions of the muscle cell using fluorescence microscopy [14]. In this method, carbonyls are derivatized with 2-dinitrophenylhydrazine and then fluorescently tagged with an anti-DNP antibody. Similarly, mitochondria are fluorescently tagged with an anti-COXI antibody. The manual image analysis is lengthy, requiring about 45 min per muscle fiber. Here, we present a semi-automated method for the analysis of the carbonylation in immunolabeled skeletal muscle serial cross-sections from the soleus and semimembranosus of Fischer 344 rats. We use custom-designed, home-written procedures in MatLab and ImageJ to analyze the images. Using this method, we decreased the analysis time to about 1 min per individual fiber. This automated method allows for the analysis of more fibers in a short period of time,

which is needed to obtain strong statistical power (significant data or results) for muscle studies related to ageing, disease, physical performance, and inactivity that use carbonyl levels as markers of oxidative damage.

Materials and methods

Reagents

Mouse anti-COXI primary antibody (A6403), Alexa 568-labeled goat anti-mouse secondary antibody (A11031), Alexa 488-labeled rabbit anti-DNP primary antibody (A11097), and the Prolong Gold anti-fade mounting reagent with DAPI (P36935) were obtained from Invitrogen (Carlsbad, CA, USA). Goat serum was purchased from Zymed Laboratories (San Francisco, CA, USA). 2,4-Dinitrophenylhydrazine (DNPH), hydrogen peroxide, and sodium cyanoborohydride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris-buffered saline, pH 7.6 (TBS), was obtained from Bio-Rad. Solutions of 2 M HCl, 0.1 M PBS (pH=6.0), and 50% methanol in TBS buffer were prepared in the lab. Coverslips used were 1.5 mm, 22×22 mm² (Thermo Scientific, Erie Scientific Company, Portsmouth, NH, USA).

Muscle tissue preparation

Serial transverse muscle cross-sections were obtained from young (12 or 13 months) and old (26 or 27 months) Fischer 344 rats. The soleus and semimembranosus muscles were excised and flash-frozen in isopentane, which was cooled in liquid nitrogen. The samples were stored at −80 °C until cut. Serial muscle cross-sections, approximately 10-μm thick, were sliced at −20 °C using a cryostat (Leica Microsystems, Nussloch, Germany) and were placed directly onto uncoated/untreated microscope slides (Fisher Scientific). The samples were stored at −30 °C until brought to room temperature prior to the detection of mitochondria and carbonyls. All cross-sections were taken from the mid-belly of the muscle.

Fluorescence labeling of carbonyls and mitochondria

Carbonyls and mitochondria were labeled according to previously published procedure [14]. Briefly, muscle cross-sections were rehydrated with TBS buffer and permeabilized with 50% methanol in TBS buffer for 15 min. After washing with TBS, samples were treated with 0.1% DNPH in 2 M HCl. After 1 h incubation at room temperature, the DNPH solution was removed, and the DNP reaction products were stabilized by the incubation with 30 mM sodium cyanoborohydride in 0.1 M phosphate buffer (pH 6) for 1 h at room temperature. After another wash with TBS buffer, the samples were blocked with 2% BSA in TBS buffer for 30 min. After blocking, the sample was treated with Alexa 488-conjugated anti-DNP antibody (1:25) in TBS buffer for 30 min in the dark. After three washes with TBS buffer, the sample was incubated with mouse anti-COXI primary antibody (1:250 dilution) for 1 h in the dark. The sample was rinsed three times with TBS buffer and blocked by goat serum for 30 min. After blocking, the cross-section was incubated with Alexa 568-conjugate goat anti-mouse secondary antibody (1:250 dilution) for 1 h in the dark. Finally, the sample was washed three times and covered with a coverslip set with Prolong Gold mounting reagent and cured overnight at room temperature in the dark.

Image collection

Images were collected using an Olympus IX-81 inverted microscope (Melville, NY, USA) equipped with a ×40 objective (N.A.=0.60) or ×60 objective (N.A.=1.43), 120-W mercury lamp as a light source, and a C9100-01 EM-CCD camera (Hamamatsu corp., Bridgewater, NJ, USA). Signals from the Alexa 568-conjugate goat anti-mouse and Alexa 488-conjugated anti-DNP antibodies were obtained using TRITC (ex. 510–560 nm, 565 nm dichroic, em. 570–650 nm; Chroma, Rockingham, VT, USA) and FITC (ex. 460–500 nm, 505 nm

dichroic, em. 510–560 nm, Chroma) filter cubes. Red and green exposure times were set to avoid saturation of the images and were kept the same while acquiring all images. A fluorescent calibration slide (ex. 488 nm, em. 519 nm; Applied Precision, Issaquah, WA, USA) was imaged using the FITC cube to correct for inhomogeneity of the illumination field. All images were collected using SimplePCI 5.3 software (Compix, Cranberry Township, PA, USA) and saved as raw 8-bit TIFF files. Both the red and green images were corrected for uneven illumination by dividing each of the images by the image of the fluorescent calibration slide using the Simple PCI 5.3 and saved as a 24-bit JPEG file without compression and used for the rest of the analysis.

Selection of subcellular regions

Labeling with anti-COXI antibody (red fluorescence) was used to determine the regions containing mitochondria (Fig. 1a) in the muscle cross-section. Mitochondria were further divided into SSM and IFM based on their spatial localization inside the cell. The SSM lay below the fiber membrane, while the IFM can be found within the fiber (internal). Non-fluorescent areas, without anti-COXI, corresponded to non-mitochondrial regions within the cytoplasm of the fiber (CY) or in the extracellular space of the fiber (EX). Two standard deviations of the average intensity above the background in the EX regions were used as a threshold for mitochondrial detection. Carbonyls (green fluorescence) were detected by derivatization using DNPH and then visualizing the DNP by Alexa488-labeled antibody (Fig. 1b). By superimposing both channels (Fig. 1c), carbonyl levels in each subcellular region can be identified and measured.

Computer-aided image analysis

The overall procedure for image processing is outlined in Fig. 2. It consists of two parts: (1) analysis of carbonylation in the EX space written in MatLab and (2) analysis of carbonylation in the intracellular space written in ImageJ.

Figure 3 illustrates the steps of the procedure to identify the EX space. EX was selected from the brightfield image (BF) using the intensity gradient between the dark cellular space and white extracellular space with a Sobel edge filter. The threshold for the filter is set by the analyst (step no. 1, Fig. 2). Once selected, a binary mask of the EX region was automatically created (step no. 2, Fig. 2) and applied to the anti-COXI stained image (red) to obtain the average background level of the anti-COXI staining in the EX space, which is devoid of mitochondria (step no. 4, Fig. 2). This average plus two standard deviations was used as a threshold, (step no. 5, Fig. 2), for the selection of mitochondria within the fiber in subsequent steps. The same mask was also applied to the anti-DNP probed image (green) to measure the intensity of the carbonylation in the EX space (step no. 4, Fig. 2).

The next step was the analysis of carbonyls inside the muscle fibers. Images representing the steps of the procedure in Fig. 2 are shown in Fig. 4. Before analyzing the intracellular region of the fiber, three parameters were identified by the analyst: (a) the fiber to be measured that was outlined in the brightfield image (step no. 6, Figs. 2 and 4a), (b) the thickness of the SSM region that was demarked in the anti-COXI (red) image (step no. 7, Figs. 2 and 4b), and (c) the threshold for the mitochondria selection that was obtained from the EX procedure (step no. 5, Fig. 2). After the input of the values, the procedure ran entirely automated.

The SSM thickness was used to separate the SSM from the intracellular part of the fiber in both images (step nos. 8–10, Figs. 2 and 4b, c). Using the threshold entered in step no. 5, the program identified mitochondria in the SSM region (step no. 12, Fig. 2) creating a mask (Fig. 4e). This mask was then applied to the original red and green channel images (Fig. 4. e,

f, step no. 15, Fig. 2), and the intensity was measured pixel-by-pixel (step no. 20, Fig. 2), along with their standard deviations. Similarly, mitochondria in the intracellular space were selected based on the threshold (step no. 11), creating masks for IFM and CY (Fig. 4h, i, step nos. 13 and 14, Fig. 2). These masks were applied again to the red and green images to determine the mitochondrial content and carbonylation, respectively (Fig. 4j–l, step nos. 22 and 23, Fig. 2). The same masks were further applied to the image produced by dividing the green and red channel intensities, pixel-by-pixel, in order to determine carbonyl content normalized to the mitochondria amount in the SSM and IFM regions (step nos. 16 and 21, and 19 and 24, respectively, Fig. 2).

In summary, the output parameters are SSM Green (carbonyls in SSM region), SSM Red (mitochondria in SSM region), IFM Green (carbonyls in IFM region), IFM Green/Red (carbonyls normalized to mitochondrial content in IFM region), SSM Green/Red (carbonyls normalized to mitochondrial content in SSM region), CY Green (carbonyls in cytosol), and EX Green (carbonyls in extracellular space). These data were combined and represented as bar charts, mean values, and standard deviations. Both procedures outlined in Fig. 2 can be downloaded from our website (<http://www.chem.umn.edu/groups/arriaga/resources.htm>).

Statistical analysis and method validation

Four images representing four different sets of muscle cross-sections (i.e., young and old semimembranosus (old semi, young semi) and young and old soleus (old sol, young sol)) were used to test the reliability of the data analysis procedure. Four cells were analyzed in each image. These images can be downloaded from our website (<http://www.chem.umn.edu/groups/arriaga/resources.htm>). In order to assess inter-person and intra-person variations of the procedure, four independent analysts performed the data analysis on the same set of images. The analysts included three undergraduate researchers and one Ph.D. student.

All ANOVA analyses were done using R Software [15]. For the intra-person repeatability, we used a simple one-way ANOVA design, where our measurement was governed by the factor “Replicate” (three replicates). This was loaded into R Software, and the one-way ANOVA was performed for each measurement. The Pr-value was then used to determine if “Person” was a significant factor in our results.

To assess the inter-person reliability, analysts were compared using interclass correlation coefficients (ICC), a concept used for comparing agreements among ratings [16]. Two different models of the calculation of the ICCs were used. The first model, ICC (2, k), considers the analysts as a subset of a larger population of analysts, while the second model, ICC (3, k), considers the tested analysts as the only analysts of the procedure. The ICC can vary between 0 and 1; values above 0.75 indicates good reliability among analysts. In both models, each cell in the four sets (four cells per set, $n=16$ cells) was measured by all analysts ($k=4$), and the ICCs were calculated using these equations:

$$ICC(2, k) = \frac{BMS - EMS}{BMS + \frac{(RMS - EMS)}{n}} \quad (1)$$

$$ICC(3, k) = \frac{BMS - EMS}{BMS} \quad (2)$$

where n is a number of cells analyzed, and k is the number of analysts. BMS, RMS, and EMS are the “between cells,” “between analysts,” and “error” mean squared values. All these parameters were obtained from the ANOVA analysis. Note that “analysts” are referred as “raters” in other fields.

Results

Method validation

The objective of this work was to develop a semi-automated procedure to increase the throughput of the image analysis used to identify carbonyls in muscle cross-sections. First, we compared the accuracy of the semi-automated method relative to the manual method published previously [14]. We compared eight parameters: SSM Green, SSM Red, IFM Green, IFM Red, CY Green, EX Green, SSM Green/Red ratio, and IFM Green/Red ratio in nine cells randomly selected from four different images of muscle cross-sections. We found that the relative difference among the methods varied between 1.3% and 3.8% per cell. This suggests that the automated method is accurate and provides similar data as those obtained by an experienced scientist who performs the measurements manually.

Next, we compared the required times for manual analysis and the automated procedure. While analyzing a single fiber with the manual method took about 45 min, it took about 1 min using the semi-automated method.

Intra- and inter-person reliability

Ideally, the computer-aided data analysis should be independent from the analyst performing the analysis. However, some parameters were entered manually (c.f., step nos. 1, 5, 6, and 7, Fig. 2), which may cause inter- and intra-person errors.

To obtain the intra-person reliability of the method, each analyst analyzed four cells in a single image in triplicate. The results of the ANOVA analysis of the intra-person variation are shown in Table 1. The Pr values for all measurements are very close to 1, suggesting that the intra-person variation is not a significant factor of the analysis. Also, this means that a single measurement of a particular cell is sufficient to produce reliable data.

The inter-person variation was tested by analyzing four cells in four different images (total of 16 cells) by all four analysts. Figure 5 compares the results of the four analysts. To compare these values statistically, we calculated the interclass correlation coefficients (ICC) for each parameter using two different statistical models, which differs in how the analysts are classified. ICC (2, k) considers the analysts as a sample of a larger population of analysts. This is particularly important for the routine analysis of the method, where the data are collected and analyzed by different analysts. ICC (3, k) considers the testing analysts as the only analysts who will use the analysis. As seen in Table 2, all ICC values for both models exceed 0.75, with the majority exceeding 0.95. The strong ICC values suggest that the semi-automated procedure is reliable and independent of the analyst performing the analysis.

Analysis of carbonylation in muscle cross-sections of Fisher 344 rats

An example of the results obtained by the semi-automated method is shown in Fig. 6. Panel A shows the total carbonylation in subcellular regions of two different muscles obtained from Fischer 344 rats 24 or 12–13 months old. Panel B shows SSM Green/Red and IFM Green/Red (carbonylation normalized to the mitochondrial mass). The entire data set was collected in about 25–30 min.

Discussion

Multiple muscle studies related to ageing, disease, physical performance, and inactivity have used carbonyl levels as markers of oxidative damage [17–19]. These studies would benefit tremendously from the determination of the subcellular localization of carbonyls, which is a surrogate of oxidative damage [6–8]. For instance, it would be important to establish whether different skeletal muscle fiber types in the same muscle have different subcellular patterns of oxidative damage [20].

We previously reported an immunohistochemical approach for quantitation of carbonylation in subcellular regions of skeletal muscle serial cross-sections [14]. This approach provided a clear demarcation of four regions based on immunolabeling of mitochondria in skeletal muscle fibers: the extracellular space, the IFM, the SSM, and the cytosolic non-mitochondrial regions. Since carbonylation does not appear any higher at the boundaries of these regions (i.e., biological membranes), it was assumed that all measurements were representative of each overall region. However, the selection and image processing of such regions was done manually taking on average 45 min to analyze one fiber cross-section. Since many muscle studies would require analyzing many fibers to obtain statistically significant results, the length of the previous manual procedure would detract investigators from assessing subcellular oxidative damage with a sufficient number of samples. In addition, fluorescence quantitation of carbonyl levels used a mercury lamp as excitation source, which proved to be non-homogeneous thereby biasing the quantitative power of the analysis. This report provides practical improvements on quantitative power and throughput.

In order to minimize the bias in the carbonyl measurement caused by the uneven field illumination, a common problem in fluorescence microscopy, each fluorescence image captured is divided, pixel-by-pixel, by an image of a fluorescent slide captured at the same focal plane as the muscle cross-section. Such flat-field adjustment normalizes the variation in the fluorescence intensity among the cells and also enables using the whole image for calculating the average background level of the anti-COXI staining in the EX space, which is devoid of mitochondria (step no. 4, Fig. 2) and the carbonyl levels in the EX region.

The method presented in this paper provides a unique measurement of the carbonyl levels among individual cells and in the subcellular regions. However, in order to obtain an absolute measurement of the carbonyl content, a further validation with a reference method would be necessary. As an example, thin films of gelatin of known concentrations would be deposited onto a microscope slide and processed in a similar fashion as the muscle cross-sections. Subsequently, the levels of carbonyls measured with the fluorescence microscopy (muscle cross-section) would be compared to the absolute carbonyl content in the gelatin samples measured in bulk with the standard method (e.g., spectrofluorometry or capillary electrophoresis).

The semi-automated image analysis described here has the ability to process a cell image at a rate of 1 min per cell with minimal bias. The variable image quality of cross-sections makes automated fiber selection in ImageJ challenging. The tools offered by MatLab were particularly more suitable for the analysis of the EX space compared to ImageJ. Thus, the combined image analysis was a good compromise. The analysis time may be reduced further if one platform was sufficient to carry out all the image-processing steps described in Fig. 2.

The semi-automated image analysis required analyst input, including (1) a threshold for the edge filter to identify the intracellular space, (2) an outline for the fiber cross-section, and (3) the thickness of the SSM region. These are potential sources of intra-person and intra-person variation. Using ANOVA tests, we confirmed that the intra-person reliability did not significantly contribute to the variation of analyzed data, suggesting that the method is

robust with respect to the repeated selection of cells (Table 1). Thus, analyzing cells in a single image only once is sufficient to produce the reliable data. Using ICC values determined that the outcome of the image analysis is consistent among analysts, regardless of their prior training (Table 2 and Fig. 5). The results of the intra- and inter-person variation demonstrate that the results from image analyses done by different analysts can be combined to obtain more power in statistical comparisons. In the future, development of fully automated imaging algorithms could eliminate the remaining potential sources of intra- and inter-person variations. These would also further improve the analysis throughput by allowing batch processing of the collected images.

Conclusions

The semi-automated method for image analysis of carbonylation in subcellular regions of skeletal muscle cross-sections decreases the analysis time for a single fiber from 45 down to 1 min. The results are consistent among analysts and when measurements are repeated by the same analyst multiple times. These advances facilitate the analysis of multiple muscle cross-sections in a short period of time, enhancing skeletal muscles studies on aging, disease, physical performance, and physical inactivity. Particularly, it would be interesting to correlate histological fiber phenotyping with the various carbonylation parameters reported by the semi-automated imaging method reported here.

Acknowledgments

This work was supported by the National Institutes of Health grants R01-AG20866 (EA) and R01-AG17768 (LVT). KB and MS were supported by the University of Minnesota Undergraduate Research Opportunity Program. EA acknowledges support as a Fesler-Lampert Chair in Aging.

References

1. Federico A, Manneschi L, Paolini E. Biochemical difference between intermyofibrillar and subsarcolemmal mitochondria from human-muscle. *J Inherit Metab Dis*. 1987; 10:242–246.
2. Hood DA. Plasticity in skeletal, cardiac, and smoothmuscle—invited review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J Appl Physiol*. 2001; 90(3):1137–1157. [PubMed: 11181630]
3. Palmer JW, Tandler B, Hoppel CL. Biochemical properties of subsarcolemmal and inter-fibrillar mitochondria isolated from rat cardiac-muscle. *J Biol Chem*. 1977; 252(23):8731–8739. [PubMed: 925018]
4. Judge S, Jang YM, Smith A, Selman C, Phillips T, Speakman JR, Hagen T, Leeuwenburgh C. Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Reg I*. 2005; 289(6):R1564–R1572.
5. Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem Res Toxicol*. 1997; 10(5):485–494. [PubMed: 9168245]
6. Bautista J, Mateos-Nevado MD. Immunological detection and quantification of oxidized proteins by labelling with digoxigenin. *Biosci Biotechnol Biochem*. 1998; 62(3):419–423. [PubMed: 9571770]
7. Stadtman ER. Protein oxidation and aging. *Free Radic Res*. 2006; 40(12):1250–1258. [PubMed: 17090414]
8. Stadtman ER. Protein oxidation in aging and age-related diseases. *Ann NY Acad Sci*. 2001; 928:22–38. [PubMed: 11795513]
9. Feng J, Arriaga EA. Quantification of carbonylated proteins in rat skeletal muscle mitochondria using capillary sieving electrophoresis with laser-induced fluorescence detection. *Electrophoresis*. 2008; 29(2):475–482. [PubMed: 18064596]

10. Wang P, Powell SR. Decreased sensitivity associated with an altered formulation of a commercially available kit for detection of protein carbonyls. *Free Radic Biol Med*. 2010; 49(2): 119–121. [PubMed: 20230891]
11. Luo S, Wehr NB. Protein carbonylation: avoiding pitfalls in the 2, 4-dinitrophenylhydrazine assay. *Redox Rep*. 2009; 14(4):159–166. [PubMed: 19695123]
12. Feng J, Xie HW, Meany DL, Thompson LV, Arriaga EA, Griffin TJ. Quantitative proteomic profiling of muscle type-dependent and age-dependent protein carbonylation in rat skeletal muscle mitochondria. *J Gerontol A Biol*. 2008; 63(11):1137–1152.
13. Meany DL, Xie HW, Thompson LV, Arriaga EA, Griffin TJ. Identification of carbonylated proteins from enriched rat skeletal muscle mitochondria using affinity chromatography-stable isotope labeling and tandem mass spectrometry. *Proteomics*. 2007; 7(7):1150–1163. [PubMed: 17390297]
14. Feng J, Navratil M, Thompson LV, Arriaga EA. Estimating relative carbonyl levels in muscle microstructures by fluorescence imaging. *Anal Bioanal Chem*. 2008; 391(7):2591–2598. [PubMed: 18548236]
15. R Development Core Team. Vienna, Austria: R Foundation for Statistical Computing; 2010. R: a language and environment for statistical computing. Available at <http://www.R-project.org>
16. Portney LG, Watkins MP. Foundations of clinical research applications to practice. Appleton & Lange, Stamford. 1993
17. Fagan JM, Ganguly M, Stockman H, Ferland LH, Toner M. Posttranslational modifications of cardiac and skeletal muscle proteins by reactive oxygen species after burn injury in the rat. *Ann Surg*. 1999; 229(1):106–114. [PubMed: 9923807]
18. Fagan JM, Slecicka BG, Sohar I. Quantitation of oxidative damage to tissue proteins. *Int J Biochem Cell Biol*. 1999; 31(7):751–757. [PubMed: 10467731]
19. Barreiro E, de la Puente B, Busquets S, Lopez-Soriano FJ, Gea J, Argiles JM. Both oxidative and nitrosative stress are associated with muscle wasting in tumour-bearing rats. *FEBS Lett*. 2005; 579(7):1646–1652. [PubMed: 15757655]
20. Lustgarten MS, Jang YC, Liu YH, Muller FL, Qi WB, Steinhilber M, Brooks SV, Larkin L, Shimizu T, Shirasawa T, McManus LM, Bhattacharya A, Richardson A, Van Remmen H. Conditional knockout of Mn-SOD targeted to type IIB skeletal muscle fibers increases oxidative stress and is sufficient to alter aerobic exercise capacity. *Am J Physiol Cell Ph*. 2009; 297(6):C1520–C1532.

Fig. 1. Subcellular localization of carbonyls and mitochondria in a muscle cross-section after dual immunolabeling. Mitochondria are visualized by labeling with anti-COXI antibody (**a**, *red*); carbonyls are labeled using the anti-DNP labeling (**b**, *green*). **c** The overlay of both images. Regions: *IFM* interfibrillar mitochondria, *SSM* subsarcolemmal mitochondria, *CY* cytoplasm, *EX* extracellular. Conditions: detailed labeling procedures are described in the “Materials and methods” section, magnification, $\times 60$; *scale bar*, 10 μm

Matlab

Fig. 2.

Flow chart of the semi-automated procedure for image processing. *Red boxes* require user input, *blue boxes* are automated procedures, and *green boxes* are outputs. Abbreviations: *BF* bright-field image, *G* carbonylation image (*green*), *R* mitochondrial image (*red*), *IFM* interfibrillar mitochondria, *SSM* subsarcolemmal mitochondria, *CY* cytoplasm, *EX* extracellular, I_R measurement of intensity in the red image, I_G measurement of intensity in the green image

Fig. 3. Steps in the procedure for extracellular space analysis. **a** Bright field image of the muscle cross-section. **b** Mask of the extracellular space (step no. 2, Fig. 2). **c** Measurement of the carbonyl levels in the extracellular space (step no. 4, Fig. 2). Magnification, $\times 60$; *scale bar*, 50 μm

Fig. 4.

Steps in the procedure for intracellular analysis of carbonylation. **a** Cell outlining in a bright field image (step no. 6, Fig. 2). **b, c** Application of the cell outline to the images visualizing mitochondria (*red*) and carbonylation (*green*), respectively. **d** Selection of the subsarcolemmal region (step no. 8, Fig. 2) using the manually entered SSM thickness (step no. 7, Fig. 2). **e** Selection of SSM in the subsarcolemmal region (step no. 10, Fig. 2) using a threshold obtained from the analysis of the extracellular space (step no. 5, Fig. 2) followed by the measurement of the fluorescence intensity of the mitochondria in the masked region (step no. 20, Fig. 2). **f** Measurement of intensity of carbonyls in the SSM region (step no. 20, Fig. 2) using the mitochondrial mask SSM region selected in **e**. **g** Selection of the cell interior (step no. 9, Fig. 2). Selection of IFM (**h**) and CY (**i**) in the intracellular region (step nos. 13 and 14, Fig. 2) using a threshold obtained from the analysis of the extracellular space (step no. 5, Fig. 2) followed by the measurement of the fluorescence intensity of the mitochondria in both regions (step nos. 22 and 23, Fig. 2). **k** Measurement of intensity of carbonyls in the IFM region (step no. 23, Fig. 2) using the IFM mask obtained from **h**. **l** Measurement of intensity of carbonyls in the CY region (step no. 22, Fig. 2) using the CY mask obtained from **i**. Magnification, $\times 40$; scale bar, 20 μm

Fig. 5. Inter-person reproducibility. Carbonylation levels in IFM, SSM, and CY regions were measured in four cells ($n=4$) by four independent analysts ($k=4$) using the semi-automated software procedure. The results obtained by each analyst (SSM Green, IFM Green, CY Green, SSM Green/Red, and IFM Green/Red) are plotted for each cell measured. The insets show the ICC values calculated for each measurement using the two models: ICC(2, k) and ICC(3, k). The ICC values were calculated using Eqs. 1 and 2, described in the “Materials and methods” section. Tissue sample: young semi

Fig. 6. Levels of carbonylation in distinct subcellular regions. **a** Carbonylation in IFM, SSM, cytoplasmic, and extracellular regions of the muscle cross-section. **b** The carbonylation/mitochondria ratios in SSM and IFM regions. A mitochondria selection threshold was not applied for SSM regions in step no. 10, Fig. 2. *Ysemi* young semimembranosus, *Osemi* old semimembranosus, *Ysol* young soleus, *Osol* old soleus. Ages: young=12- or 13-month-old Fischer 344 rats; old=26-month-old Fischer 344 rats. Four cells of each category (*OSemi*, *OSol*, *YSemi*, and *YSol*) were analyzed once by a single analyst, and their data were combined in this graph. *Error bars* represent one standard deviation

Table 1

Intra-person reproducibility

	<i>df</i>	Sum Sq	Sq	Pr (><i>F</i>)
SSM Red	2	33.30	16.67	0.97
Residuals	45	24,585.10	546.34	
SSM Green	2	4.36	2.18	0.96
Residuals	45	2,374.68	52.77	
IFM Red	2	18.30	9.13	0.99
Residuals	45	29,347.20	652.16	
IFM Green	2	0.40	0.22	1.00
Residuals	45	5,022.60	111.61	
CY Red	2	16.90	8.45	0.96
Residuals	45	10,453.30	232.29	
CY Green	2	73.96	36.98	0.59
Residuals	45	3,086.95	68.60	
SSM G/R	2	0.00	0.00	1.00
Residuals	45	19.18	0.43	
IFM G/R	2	0.00	0.00	0.99
Residuals	45	6.96	0.15	

Each of four independent analysts analyzed the same four cells in one of each muscle cross-section (YSol, OSol, YSemi, and OSemi) three times. The significance of the replicates was tested with one-way ANOVA for all obtained parameters (SSM Red, SSM Green, IFM Red, IFM Green, CY Red, CY Green, SSM Green/Red, and IFM Green/Red). All Pr values were greater than 0.05, suggesting there is no significant difference between the replicates

Table 2

Inter-person reproducibility

	Old semi		Old sol		Young semi		Young sol	
	ICC (2, <i>k</i>)	ICC (3, <i>k</i>)	ICC (2, <i>k</i>)	ICC (3, <i>k</i>)	ICC (2, <i>k</i>)	ICC (3, <i>k</i>)	ICC (2, <i>k</i>)	ICC (3, <i>k</i>)
SSM Green	0.983	0.990	0.991	0.994	0.992	0.992	0.998	0.999
IFM Green	0.999	0.999	0.796	0.912	0.944	0.955	0.996	0.996
CY Green	0.995	0.996	0.999	0.999	0.996	0.995	0.999	0.999
SSM Green/Red	0.999	0.999	0.970	0.980	0.992	0.991	0.955	0.959
IFM Green/Red	0.970	0.970	0.882	0.961	0.952	0.962	0.952	0.995

Sixteen cells were analyzed by four independent analysts ($k=4$), and the results were compared by calculating the intra-class correlation coefficients (ICC) for the each parameter measured (SSM Green, IFM Green, CY Green, SSM Green/Red, and IFM Green/Red). ICC (2,*k*) and ICC (3,*k*) represent coefficients representing a subset of analysts and all the analysts participating in the study, respectively. All ICC values were higher than 0.75 with an average of 0.98 ± 0.04 , suggesting a very good reliability between analysts. Description of the calculations of the ICCs is shown in the “Materials and methods” section