Cell-based analysis of oxidative stress, lipid peroxidation and lipid peroxidation-derived protein modifications using fluorescence microscopy

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ABSTRACT

Oxidative stress plays an important role in the progression of several diseases including inflammation, atherosclerosis, aging and age-related degenerative disorders. Reactive oxygen species damage membrane bound lipids resulting in lipid peroxidation-derived protein modifications. Here, we used three different approaches to measure oxidative stress and lipid peroxidation in cells by fluorescence microscopy. 1) Three new fluorogenic probes, CellROX™ Deep Red, CellROX® Orange and CellROX ® Green Reagents to measure oxidative stress in cells, 2) Premo™ Cellular Redox and H_2O_2 sensors to measure cellular redox potential and hydrogen peroxide respectively, 3) Image-iT® Lipid Peroxidation Kit for a ratiometric determination of lipid peroxidation in live cells and 4) Click-iT® Lipid Peroxidation Imaging Kit, a click chemistry-based approach which utilizes incorporation of an alkyne-modified unsaturated fatty acid analog, linoleamide, into the cellular membranes. The resulting oxidation products, like 9, 12-dioxo-10(E) dodecenoic acid (DODE) can readily modify proteins and these modifications were readily detected in fixed cells by the copper-catalyzed click reaction using fluorescent azides. Using these approaches, we measured oxidative stress and lipid peroxidation caused by several oxidants in cells. Increases in oxidative stress, lipid peroxidation, and protein modifications were assessed by high content imaging and analysis as well as traditional fluorescence microscopy. In the models tested, at least 2-3 fold increases were observed compared to controls was and responses were successfully inhibited by antioxidants. The three strategies described here provide powerful new tools for the assessment of oxidative stress in cells and convey distinct advantages over existing cell-based methods.

INTRODUCTION

Oxidative stress including lipid peroxidation is involved in progression of many human diseases including inflammation, atherosclerosis, aging and age-related degenerative disorders. Lipid peroxidation is the oxidation of lipids, especially unsaturated fatty acids in cellular membranes mediated by oxidative stress in cells . Reactive oxygen species damage membrane bound lipids like linoleic acid to form lipid electrophiles, which can rapidly react with proteins and DNA to form adducts. Here, we describe 3 different strategies to measure oxidative stress, lipid peroxidation and lipid peroxidation-derived protein modifications by traditional fluorescence microscopy and quantitative fluorescence microscopy also known as high content imaging: 1) CellROX™ Reagents for measurement of general cellular oxidative stress; 2) Premo™ Redox and H2O2 sensors to measure cellular redox potential and hydrogen peroxide; 2) A ratiometric lipid peroxidation sensor to measure lipid peroxidation and 3) Click-iT technology based measurement of protein modifications with Click-iT® Linoleamide alkyne.

CellROX® Reagents for oxidative stress measurements

CellROX® Oxidative Stress Reagents are fluorogenic probes designed to reliably measure reactive oxygen species (ROS) in live cells. The cell-permeable reagents are non-fluorescent or very weakly fluorescent while in a reduced state and upon oxidation exhibit strong fluorescence. CellROX® Green Reagent is a DNA dye, and upon oxidation, it binds to DNA, both nuclear and mitochondrial. CellROX® Reagents are supplied as ready to use DMSO solutions. CellROX® Reagents have number of advantages compared to traditional reagents like dichlorodihydrofluoresceins including simplified workflow and the ability to label cells in complete growth medium (fig 1a).

Figure 1a: Simple Workflow of CellROX® Reagents

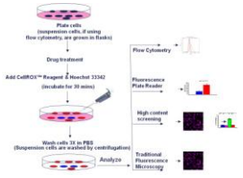


Figure 1b: Comparison of CellROX® reagents with existing probes for ROS measurements

	H2-DCFDA	DHE	CellROX™ Deep Red	CellROX™ Orange	CellROX™ Green
Add in Complete media	No	Yes	Yes	Yes	Yes
Fixable	No	No	Yes	No	Yes
Detergent resistant	No	No	No	No	Yes
Multiplexibility	Yes	No	Yes	Yes	Yes
GFP compatible	No	No	Yes	Yes	No
RFP compatible	Yes	No	Yes	No	Yes
Photostability	*	N/A	***	****	**

Figure 2. Live cell analysis of oxidative stress using CellROX® reagents

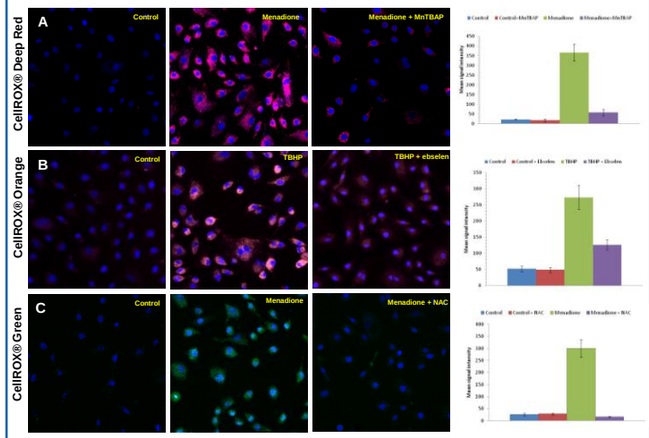


Figure 2A. BPAE cells were treated with or without 100 µM menadione. Superoxide scavenger 100 µM MnTBAP was added to some of the control and menadione-treated wells for the last 30 min of incubation. The cells were stained with CellROX® Deep Red Reagent for 30 mins **2B**. BPAE cells were treated with or without 200 µM tert-butyl hydroperoxide (TBHP). The peroxide scavenger 10 µM ebselen was added to some of the control and TBHP-treated cells. The cells were then stained with CellROX® Orange Reagent and incubated for 30 mins. **2C**. BPAE cells were treated with or without 100 µM menadione. The antioxidant 50 µM N-Acetyl cysteine (NAC) was added to some of the control and menadione-treated wells for the last 30 min of incubation. The cells were then stained with CellROX® Green Reagent The cells were then washed and analyzed on a Thermo Fisher Cellomics ArrayScan® VTI. Reduction of the signal in antioxidant treated cells confirmed indication of oxidative stress by CellROX® reagents

Figure 3. Live cell analysis of hydrogen peroxide using Premo™ Cellular Hydrogen Peroxide Sensor

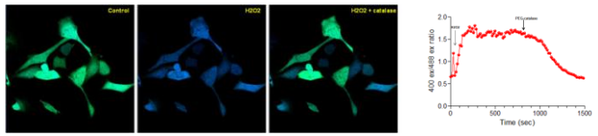


Figure 3: Premo™ Cellular Hydrogen Peroxide Sensor combines the selectivity of a roGFP-Orp1 chimera with the transduction efficiency of BacMam 2.0 technology to measure H2O2 in cells. U2- OS cells were plated on 35 mm dishes at a density of 75, 000 cells and transduced with Premo™ Cellular H2O2 Sensor. After 48 hrs, time lapse imaging was done on Zeiss LSM 710 confocal microscope using 400 nm and 488 nm lasers for excitation with the emission at 515 nm. The images were taken every 15 seconds after addition of 50 µM H2O2 for 5 minutes and then 50 µM PEG-catalase was added to the cells and imaged for another 20 minutes (Fig 3A). Fluorescence intensity values were quantified by making regions of interest on the cells and were used to calculate 400/488 nm excitation ratios. The ratios were plotted against time. The ratios were plotted against time (Fig 3B).

Figure 4. Live cell analysis of redox potential using Premo™ Cellular Redox Sensor

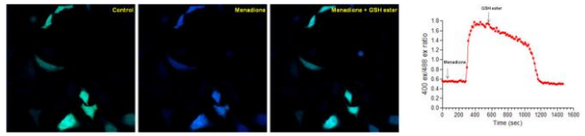


Figure 4: Premo™ Cellular Redox Sensor combines the selectivity of a roGFP-Grx1 chimera with the transduction efficiency of BacMam 2.0 technology to measure redox potential in cells. U2- OS cells were plated on 35 mm dishes (MatTek) at a density of 75, 000 cells and transduced with Premo™ Cellular Redox Sensor. After 48 hrs, time lapse imaging was done on Zeiss LSM 710 confocal microscope using 400 nm and 488 nm lasers for excitation with the emission at 515 nm. The images were taken every 15 seconds after addition of 33µM menadione for 5 minutes and then10 mM GSH ester was added to the cells and imaged for another 10 minutes (Fig 4A). Fluorescence intensity values were quantified by making regions of interest on the cells and were used to calculate 400/488 nm excitation ratios. The ratios were plotted against time (Fig 4B).

Figure 5: Analysis of lipid peroxidation in live cells using Image-iT® Lipid Peroxidation Kit

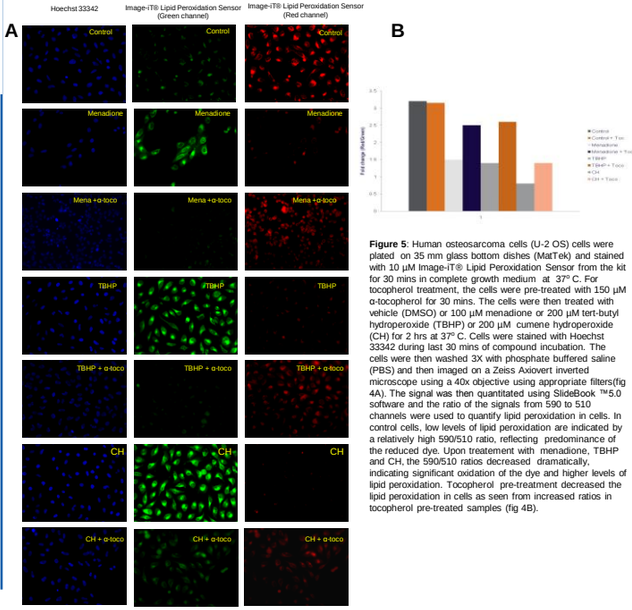
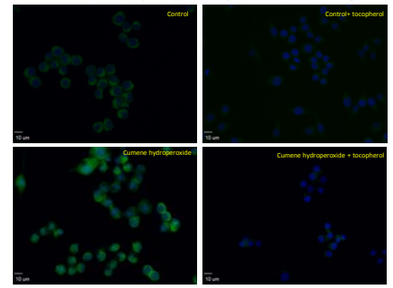


Figure 5: Human osteosarcoma cells (U-2 OS) cells were plated on 35 mm glass bottom dishes (MatTek) and stained with 10 µM Image-iT® Lipid Peroxidation Sensor from the kit for 30 mins in complete growth medium at 37° C. For tocopherol treatment, the cells were pre-treated with 150 µM α-tocopherol for 30 mins. The cells were then treated with vehicle (DMSO) or 100 µM menadione or 200 µM tert-butyl hydroperoxide (TBHP) or 200 µM cumene hydroperoxide (CH) for 2 hrs at 37° C. Cells were stained with Hoechst 33342 during last 30 mins of compound incubation. The cells were then washed 3X with phosphate buffered saline (PBS) and then imaged on a Zeiss Axiovert inverted microscope using a 40x objective using appropriate filters(fig 4A). The signal was then quantitated using SlideBook ™5.0 software and the ratio of the signals from 590 to 510 channels were used to quantify lipid peroxidation in cells. In control cells, low levels of lipid peroxidation are indicated by a relatively high 590/510 ratio, reflecting predominance of the reduced dye. Upon treatement with menadione, TBHP and CH, the 590/510 ratios decreased dramatically, indicating significant oxidation of the dye and higher levels of lipid peroxidation. Tocopherol pre-treatment decreased the lipid peroxidation in cells as seen from increased ratios in tocopherol pre-treated samples (fig 4B).

Figure 6: Cell-based detection of lipid peroxidation-derived protein modifications by fluorescence microscopy

A click chemistry-based method was used to visualize lipid peroxidation-derived protein modifications in cells by quantitative fluorescence microscopy. In this click chemistry-based approach, an alkyne-modified unsaturated fatty acid analog (linoleic acid) was incorporated into the cellular membranes and the products resulting from oxidation, like 4-hydroxy-2-nonenal (HNE) and 9, 12-dioxo-10(E) dodecenoic acid (DODE) can readily modify DNA or proteins. The modified proteins are then detected by a copper-catalyzed click reaction using fluorescent azides.



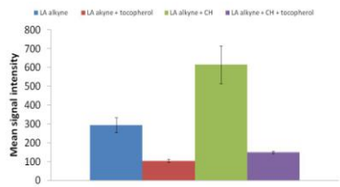


Figure 6: Bovine pulmonary artery endothelial (BPAE) cells were plated on 35 mm glass bottom dishes (MatTek) and incubated in complete growth medium at 37° C. For tocopherol treatment, the cells were pre-treated with 150 µM α-tocopherol for 30 mins. The cells were then treated with vehicle (DMSO) or 100 µM cumene hydroperoxide (CH) followed immediately by addition of 50 µM linoleamide alkyne and incubated for 2 hrs at 37° C. The cells were fixed and permeabilized and click-labeled with 5 µM Alexa Fluor® 488 azide for 30 mins and imaged on a Zeiss Axiovert inverted microscope (figure 7A). The signal intensity was quantitated using SlideBook ™5.0 software. The intensity values were background subtracted and plotted (figure 7B). There is significant increase in signal intensity in CH-treated cells indicating elevated lipid peroxiodation-derived protein modifications. Tocopherol pre-treatment of cells resulted in significant reduction of CH induced protein modifications in cells.

Conclusions

- CellROX™ Reagents are fluorogenic probes to reliably measure oxidative stress in cells with variety of advantages over traditional approaches. Premo™ Cellular Redox and H2O2 sensors respond to low concentrations of oxidants and allow dynamic ratiometric measurments of redox potential and hydrogen peroxide respectively.
- Image-iT® Lipid Peroxidation kit contains a ratiometric probe to measure lipid peroxidation in live cells. Using this probe, we show that menadione, tert-butyl hydroperoxide and cumene hydroperoxide lead to increased lipid peroxidation and α-tocopherol pre-treatment attenuated lipid peroxidation caused by oxidants.
- Click-iT® Lipid Peroxidation Kit contains reagents to detect and quantitate lipid peroxidation derived protein modifications in cells. α-tocopherol pre-treatment attenuated lipid peroxidation-derived protein modifications caused by oxidants.
- CellROX® Reagents, Image-iT® Lipid Peroxidation kit and Click-iT® Lipid Peroxidation kit provide a powerful set of reagents for cell-based analysis of oxidative stress and lipid peroxidation and lipid peroxidation-derived protein modifications.

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