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Corresponding Author:	Nukhet Aykin-Burns University of Arkansas for Medical Sciences Little Rock, Arkansas UNITED STATES
Corresponding Author's Institution:	University of Arkansas for Medical Sciences
Corresponding Author E-Mail:	NAykinburns@uams.edu
Order of Authors:	Alev Tascioglu Aliyev
	Francesca LoBianco
	Kimberly J. Krager
	Nukhet Aykin-Burns
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TITLE:

Assessment of Cellular Oxidation Using a Subcellular Compartment-Specific Redox-Sensitive Green Fluorescent Protein

AUTHORS AND AFFILIATIONS:

Alev Tascioglu Aliyev[1,2], Francesca LoBianco[1], Kimberly J. Krager[1], Nukhet Aykin-Burns[1]

[1]Division of Radiation Health, Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

[2]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Ege University, Izmir, Turkey

Corresponding Author:

Nukhet Aykin-Burns (NAykinburns@uams.edu)

Email Addresses of Co-Authors:

Alev Tascioglu Aliyev (alev.tascioglu@gmail.com)
Francesca LoBianco (FVLobianco@uams.edu)
Kimberly J. Krager (KJKrager@uams.edu)
Nukhet Aykin-Burns (NAykinburns@uams.edu)

KEYWORDS:

redox sensitive green fluorescent protein (roGFP), cytosolic roGFP, cysteine/cystine ratio, live cell imaging, flow cytometry, oxidation/reduction

SUMMARY:

This protocol describes the assessment of subcellular compartment-specific redox status within the cell. A redox-sensitive fluorescent probe allows convenient ratiometric analysis in intact cells.

ABSTRACT:

Measuring the intracellular oxidation/reduction balance provides an overview of the physiological and/or pathophysiological redox status of an organism. Thiols are especially important for illuminating the redox status of cells via their reduced dithiol and oxidized disulfide ratios. Engineered cysteine-containing fluorescent proteins open a new era for redox-sensitive biosensors. One of them, redox-sensitive green fluorescent protein (roGFP), can easily be introduced into cells with adenoviral transduction, allowing the redox status of subcellular compartments to be evaluated without disrupting cellular processes. Reduced cysteines and oxidized cystines of roGFP have excitation maxima at 488 nm and 405 nm, respectively, with emission at 525 nm. Assessing the ratios of these reduced and oxidized forms allows the convenient calculation of redox balance within the cell. In this method article, immortalized human triple-negative breast cancer cells (MDA-MB-231) were used to assess redox status within the living cell. The protocol steps include MDA-MB-231 cell line transduction with adenovirus to express cytosolic roGFP, treatment with H_2O_2, and assessment of cysteine and cystine ratio with both flow cytometry and fluorescence microscopy.

INTRODUCTION:

Oxidative stress was defined in 1985 by Helmut Sies as "a disturbance in prooxidant–antioxidant balance in favor of the former"[1], and a plethora of research has been conducted to obtain disease-, nutrition-, and aging-specific redox status of organisms[1–3]. Since then, the understanding of oxidative stress has become broader. Testing the hypotheses of using antioxidants against diseases and/or aging has shown that oxidative stress not only causes harm but also has other roles in cells. Furthermore, scientists have shown that free radicals play an important role for signal transduction[2]. All of these studies strengthen the importance of determining the changes in reduction-oxidation (redox) ratio of macromolecules. Enzyme activity, antioxidants and/or oxidants, and oxidation products can be assessed with various methods. Among these, methods that determine thiol oxidation are arguably the most used because they report on the balance between antioxidants and prooxidants in cells, as well as organisms[4]. Specifically, ratios between glutathione (GSH)/glutathione disulfide (GSSG) and/or cysteine (CyS)/cystine (CySS) are used as biomarkers for monitoring the redox status of organisms[2].

Methods used for assaying the balance between prooxidants and antioxidants rely mainly on the levels of reduced/oxidized proteins or small molecules within cells. Western blots and mass spectrometry are used to broadly assess the ratios of reduced/oxidized macromolecules (protein, lipids etc.), and GSH/GSSG ratios can be assessed with spectrophotometry[5]. A common feature of these methods is the physical perturbation of the system by cell lysis and/or tissue homogenization. These analyses also become challenging when it is necessary to measure the oxidation status of different cellular compartments. All of these perturbations cause artifacts in the assay environment.

Redox-sensitive fluorescent proteins opened an advantageous era for evaluating the redox balance without causing a disturbance in the cells[6]. They can target different intracellular compartments, allowing the quantification of compartment-specific activities (e.g., assaying the redox state of mitochondria and the cytosol) to investigate crosstalk between cellular organelles. Yellow fluorescent protein (YFP), green fluorescent protein (GFP), and HyPeR proteins are reviewed by Meyer and colleagues[6]. Among these proteins, redox-sensitive GFP (roGFP) is unique due to different fluorescent readouts of its CyS (ex. 488 nm/em. 525 nm) and CySS (ex. 405 nm/525 nm) residues, which permits ratiometric analysis, unlike other redox-sensitive proteins such as YFP[7,8]. Ratiometric output is valuable because it counterbalances the differences between expression levels, detection sensitivities, and photobleaching[8]. Subcellular compartments of cells (cytosol, mitochondria, nucleus) or different organisms (bacteria as well as mammalian cells) can be targeted by modifying roGFP[7,9,10].

roGFP assays are conducted using fluorescent imaging techniques, especially for real-time visualization experiments. Flow cytometric analyses of roGFPs are also possible for experiments with predetermined time points. The current article describes both the use of fluorescent microscopy and flow cytometry to perform a ratiometric assessment of redox status in mammalian cells overexpressing roGFP (targeted to cytosol) via adenoviral transduction.

PROTOCOL:

NOTE: This protocol was optimized for 70%–80% confluent MDA-MB-231 cells. For other cell lines, the number of cells and multiplicity of infection (MOI) should be reoptimized.

1. Preparation of cells (day 1)

1.1. Maintain MDA-MB-231 cell line in 75 cm^2 flasks with 10 mL of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified atmosphere.

NOTE: DMEM supplemented with 10% FBS, 37 °C, and a 5% CO_2 humidified atmosphere are used for all attachment and treatment incubations throughout the entire protocol.

1.2. Prepare the MDA-MB-231 cells for experiment.

1.2.1. Aspirate the medium within the flask, detach the cells with 2 mL of 0.25% trypsin-EDTA solution for 2 min, and inactivate the trypsin activity with 6 mL of complete medium (DMEM with 10% FBS). Centrifuge the cells at 150 x *g* for 5 min. Aspirate the supernatant and suspend the cells in 5 mL of complete medium.

1.2.2. Mix an equal volume cell suspension and 0.4% trypan blue. Take 10 µL of this mixture and count the cells with the automated cell counter.

NOTE: A Coulter counter or a hemocytometer can also be used for cell counting.

1.2.3. Seed the cells into a 6 well plate for flow cytometry analyses and seed 150,000 cells in 1 mL of medium per well. Wait 16 h for cell attachment.

1.2.4. Seed the cells into a 4 well chamber slide for fluorescent imaging and seed 25,000 cells in 0.5 mL of medium per well. Wait 16 h for cell attachment.

NOTE: Seed control wells in addition to treatment wells. Use one of the control wells to determine cell number (optional: if the attachment period for the cells is shorter than the doubling time, cell number can be assumed to be the same as the seeding density) and the other for a noninfected control (0 MOI).

2. Adenoviral roGFP transduction (day 2 and 3)

CAUTION: Adenoviruses can cause diseases. While transducing the cells, use filtered tips and decontaminate tips, Pasteur pipettes, and microcentrifuge tubes with 10% bleach.

NOTE: This protocol was demonstrated with cytosol-specific roGFP, but other cellular

compartments (e.g., mitochondria or mitochondrial intermembrane space) can be targeted with this same protocol.

2.1. Generate a dose-response curve for the MOI to obtain the highest transduction efficiency by calculating the volume of adenovirus (mL) required for each MOI value for MDA-MB-231 cell line (**Table 1**):

$$x\ mL = MOI \times Cell\ Number \times {}^{1\ mL}/_{PFU}$$

NOTE: Functional titer of each batch of adenoviral stock, which is expressed as plaque forming unit (PFU) per mL, is provided by the company. The optimum MOI for transduction differs between cell types. For most mammalian cells, the optimum MOI range is between 10 and 300. According to the cellular response, MOI values should be recalculated (e.g., MOI range should be reduced if cells have cytotoxic response, or range should be increased if cells have low transduction efficiency).

2.2. Make 1:100 dilution of 6 x 10^{10} PFU/mL adenoviral roGFP solution with cell culture medium (DMEM with 10% FBS) for reliable pipetting.

2.3. Pipette and add 0.0125 mL (12.5 µL), 0.025 mL (25 µL), 0.05 mL (50 µL) of adenoviral roGFP dilution into each well of the 6 well plate in order to transduce the 150,000 cells with 50, 100, and 200 MOI respectively for flow cytometry analysis (**Table 1**).

2.4. Pipette and add 0.0042 mL (4.2 µL) of adenoviral roGFP dilution in the 4-chamber slide wells to transduce 25,000 cells with 100 MOI for fluorescence imaging (**Table 1**).

NOTE: A minimal amount of medium should be used in the wells to ensure the highest interaction between the adenoviral roGFP construct and cells. The serum content of the culture medium may need to be decreased for different cell lines because high levels of serum can negatively affect transduction efficiency in some cell types.

2.5. Incubate cells for 16–24 h under the cell maintenance conditions. The next day (day 3), change the medium to cell culture medium (DMEM with 10% FBS) to allow cell recovery for an additional 24 h. Visualize cells under a microscope to assess their morphology; cells can express roGFP even if they have morphological changes.

NOTE: On day 3, cells should start to express roGFP; therefore, transduction efficiency can be monitored using fluorescence microscopy (filters with ex. 488/em. 525). To obtain consistent assay results, be aware of and document the morphological changes under the phase contrast microscope and observe morphology while evaluating transduction efficiency.

2.6. Construct a dose response curve using the 50, 100 and 200 MOI samples prepared in step 2.3 and their transduction efficiency results obtained from flow cytometry analysis (steps 3.1 and 4.1). Assess optimal transduction efficiency with documentation of morphological changes

(step 2.5) and the dose-response curve of MOI.

NOTE: Although more than 98% of the cell population at 100 MOI and 200 MOI express roGFP (see representative results), 200 MOI group showed substantial changes in cell morphology of MDA-MB-231 cells. Consequently, the most efficacious MOI for MDA-MB-231 cells was determined to be 100 MOI.

2.7. After optimal MOI (here, 100 MOI) was chosen for MDA-MB-21 cell line, conduct experiment with test materials (10 µM H_2O_2 and its vehicle 0.1% deionized water).

2.7.1. Prepare and seed the cells according to section 1. Using the adenoviral transduction volume for 100 MOI calculated in step 2.1, repeat steps 2.2–2.4 for 100 MOI adenoviral transduction of cells. Then incubate the plate and chamber slides according to step 2.5.

3. Acquisition of CyS/CySS balance

3.1. Flow cytometry (day 4)

3.1.1. On day 4, incubate cells from step 2.7.1 with 10 µM H_2O_2 for 1 h.

NOTE: 10 µM H_2O_2 was used as the test substance and 0.1% deionized water was used as vehicle treatment in this protocol. Other oxidizing agents can be used as positive controls here.

3.1.2. Aspirate media from the 6 well plate, replace with 750 µL of 0.25% trypsin-EDTA solution and wait for 2 min for cells to detach. Inactivate trypsin with 2 mL of complete medium (DMEM with 10% FBS) and collect the volume into 15 mL conical tubes.

3.1.3. Centrifuge the tubes at 150 x *g* for 5 min. Discard supernatant and suspend the cells in 500 µL of phosphate-buffered saline (PBS).

3.1.4. Filter the cell suspensions into flow cytometry-compatible tubes using 40 µm mesh. Keep the tubes on ice and away from the light and follow step 4.1 for data analysis.

3.2. Microscopic imaging (day 4)

3.2.1. On day 4, treat cells with 10 µM H_2O_2, acquire images immediately (time point 0) and 1 h after treatment and follow step 4.2 for data analysis.

4. Data analysis

4.1. Flow cytometry quantification

4.1.1. Set flow cytometry method for 3 different analyses: forward scatter (FCS) on x-axis and side scatter (SSC) on y-axis to assess cell size and complexity of cells (SSC can be used for rough

identification of dead and live cells); ex. 488 nm/em. 525 nm (fluorescein isothiocyanate [FITC]) bandpass filter on x-axis and SSC on y-axis to assess CyS-roGFP; ex. 405 nm/em. 525 nm (Brilliant Violet 510 [BV510]) bandpass filter on x-axis and SSC on y-axis to assess CySS-roGFP.

4.1.2. Acquire 0 MOI control sample and visualize cells with FCS and SSC area filters for gating cell population of interest (Gate 1).

4.1.3. Set up the following gatings to minimize background fluorescence for ex. 488 nm/em. 525 nm (Gate 2) and ex. 405 nm/em. 525 nm (Gate 3) bandpass filters with the noninfected (0 MOI) control cells.

4.1.4. Analyze 50, 100, and 200 MOI cells to assess the dose-response curve and later test samples (10 µM H_2O_2 treated cells and vehicle treated cells) with Gates 2 and 3.

4.1.5. Calculate the mean fluorescent intensity ratio between oxidized versus reduced forms of roGFP with the following equation.

$$\frac{Oxidized\ roGFP\ (Thiol\ form)}{Reduced\ roGFP\ (Disulfide\ form)} = \frac{Ex.405\ nm/Em.525\ nm}{Ex.488\ nm/Em.525\ nm}$$

4.2. Image assessment

4.2.1. Use a microscope that contains fluorescence filters for CyS-roGFP and CySS-roGFP (ex. 488 nm/em. 525 nm and ex. 405 nm/em. 525 nm filters, respectively).

4.2.2. In each well of the chamber slide, pick 4 random areas to acquire images, using the 4x objective to visualize larger areas.

NOTE: 20x objective can also be used for image displays.

4.2.3. Open the image with ImageJ software[11]. Apply **Analyze** | **Measure** commands for each image and use the equation in step 4.1.5 to quantify the data.

NOTE: Quantification of the images is ratiometric; therefore, the protocol does not include subtraction of background. However, to be able to compare images, brightness, contrast, and saturation must be the same for each image. Statistical significance was assessed with one-way analysis of variance (ANOVA) and Tukey's post hoc test.

REPRESENTATIVE RESULTS:

The redox state of CyS/CySS is easily assayed with transduced roGFPs. The fluorescent probe quantifies the ratio between the reduced and oxidized forms (excitation wavelengths 488 nm and 405 nm, respectively). Fluorescence data can be obtained by both flow cytometry and microscopy.

A large number of cells can consistently and conveniently be acquired using flow cytometry. The analysis consists of 3 main steps: 1) select the cell population of interest with the FSC area filter (**Figure 1A**); 2) gate the roGFP-expressing cells with ex. 488/em. 525 nm with a selective bandpass filter (**Figure 1B**); and 3) gate the oxidized roGFP-containing cells from the roGFP-expressing cells with ex. 405 nm/em. 525 nm bandpass filter (**Figure 1C**).

Each new cell line should be evaluated for the optimum adenoviral transduction efficiency of roGFPs. Transduction efficiency should be assessed with morphological evaluation of cells and roGFP expression analyses with flow cytometry and/or fluorescent microscopy. This protocol uses flow cytometry to determine the dose-response curve for roGFP analyses and to select the most efficient MOI input (**Figure 2A**–**H**). According to the MOI dose-response curve (**Figure 2I**), 200 MOI gave the highest roGFP expression, but cell morphology was affected, suggesting cytotoxicity. Therefore, the optimum transduction efficiency was determined to be with 100 MOI.

To evaluate the effectiveness of the method, H_2O_2 was used as a positive control for oxidation. One hundred MOI was used for optimum transduction. After the recovery period, cells were treated with 10 µM H_2O_2 for 1 h to obtain the fluorescence ratio via flow cytometry. Oxidized (ex. 405 nm/em. 525nm) and reduced (ex. 488 nm/em. 525 nm) roGFP mean fluorescence intensities were obtained from flow cytometry analyses for vehicle (**Figure 3A**,**B**) and 10 µM H_2O_2 (**Figure 3C**,**D**) treatments. The overlaid histograms represent the shift in the cell numbers of 10 µM H_2O_2 and vehicle treated groups for reduced (**Figure 3E**) and oxidized (**Figure 3F**) roGFP. The ratio between oxidized and reduced roGFP shows that 10 µM H_2O_2 caused a 3-fold increase in oxidation of roGFP compared to vehicle treatment (**Figure 3G**).

Fluorescent imaging of cells was also performed with 10 µM H_2O_2 under the microscope for 1 h. Images were taken under the 4x objective, and representative images were taken under the 20x objective (**Figure 4A**). Fluorescent intensities were evaluated with ImageJ software, and ratios were calculated. A steady state increase in H_2O_2-induced oxidation was detected (**Figure 4B**); incubation with H_2O_2 for 1 h increased the oxidization of roGFP cysteines, which exhibited significant change between vehicle controls.

FIGURE AND TABLE LEGENDS:

Figure 1 : Gating setup for fluorescent intensities of CyS-containing (reduced) roGFP and CySS-containing (oxidized) roGFP residues with non-transduced MDA-MB-231 cells. (**A**) The cell population of interest was selected as Gate 1 with SSC and FSC area filters. (**B**) roGFP-expressing cells were selected according to non-expressing cells as Gate 2 with the ex. 488/em. 525 nm bandpass filter. (**C**) Oxidized (cystine) roGFP-containing cells were gated with the ex. 405 nm/em. 525 nm bandpass filter from the roGFP-expressing population.

Figure 2: MOI dose-response curve assessment with flow cytometry analyses for MDA-MB-231 cell line. (**A**,**B**) Noninfected cells and (**C**,**D**) 50 MOI, (**E**,**F**) 100 MOI, and (**G**,**H**) 200 MOI

roGFP-expressing cell populations acquired for gating setup, respectively. (**I**) Transduced cells were evaluated and plotted as a percentage according to the cell population of interest.

Figure 3: Flow cytometry assessment of CyS/CySS balance in roGFP-transduced MDA-MB-231 cell line. Vehicle-treated cells were evaluated as (**A**) % roGFP-expressing cells, and (**B**) % oxidized roGFP-expressing cells and H_2O_2 treatment were assessed with the same parameters in panels (**C**) and (**D**) respectively. Cell count histograms of vehicle and H_2O_2 treatment were overlaid for (**E**) reduced roGFP ex. 488/em. 525 bandpass filter and (**F**) oxidized roGFP ex. 405/em. 525 bandpass filter. (**G**) Mean fluorescence intensity ratios between oxidized/reduced forms were plotted into a bar graph.

Figure 4: Fluorescent imaging of roGFP-transduced MDA-MB-231. (**A**) Representative images after 1 h treatment with vehicle or H_2O_2. (**B**) Ratios between oxidized/reduced forms were evaluated in 4 randomly chosen areas, and bars represent mean ± standard deviation. Statistical significance between groups indicated as *($p < 0.05$), **($p < 0.01$), or ***($p < 0.005$).

Table 1: Calculation of MOI values.

DISCUSSION:

The thiol/disulfide balance in an organism reflects the redox status of cells. Living organisms have glutathione, cysteine, protein thiols, and low-molecular-weight thiols, all of which are affected by the level of oxidation and echo the redox status of cells[4]. Engineered roGFPs allow the non-disruptive quantification of the thiol/disulfide balance via their CyS residues[7]. The ratiometric property of roGFP provides reliable redox measurements for mammalian cells. roGFP can be easily introduced into cells with transfection methods and/or transduction vectors, but adenoviral transduction has higher efficiency.

The redox status of cells is easily affected by the cellular environment (e.g., confluency of cells and volume of medium). For this protocol, the optimized cell seeding confluency was determined to be 60%–70%, with 15,000 cells per cm^2; on the day of analysis, cells were 70%–80% confluent. However, cell morphology and doubling time differ between cell lines. For this reason, if the researcher intends to use another cell line, cell confluency should be adjusted while acquiring measurements with flow cytometry and/or fluorescence microscopy; this will ensure accurate results based on their experimental design and needs.

roGFPs can be easily introduced to cells with multiple transfection methods and/or transduction vectors. The current protocol uses the cytosol-specific roGFP construct, which is transduced into cells with an adenovirus. Before starting an experiment, it is essential to determine the MOI dose-response for a cell line; this allows the determination of maximum transduction efficiency with minimal cell toxicity in order to design the optimal, reproducible protocol.

The CyS/CySS status of roGFP-transduced cells can be assessed with both fluorescent imaging and flow cytometry. Both analyses have their pros and cons; flow cytometry allows for a larger

cell population to be quickly evaluated, but fluorescence imaging has higher sensitivity to roGFP-specific cells. Furthermore, it also confirms the correct subcellular localization of GFP (e.g., cytosolic versus mitochondrial). Here, both flow cytometry and fluorescent imaging were used by the researchers. Although H_2O_2 is considered a weak oxidant for the roGFP construct[7], the protocol demonstrated that both methods are sensitive enough to detect ratiometric changes between oxidized and reduced forms of roGFP after H_2O_2 treatment.

This roGFP protocol can be used to determine the redox status of different mammalian cell types. To understand menadione-induced cardiomyocyte death in the context of prooxidant/antioxidant balance, Loor and colleagues used both cytosolic and mitochondrial roGFP labelling in cardiomyocytes[12]. roGFP allows the visualization of the redox status of cells while they are alive, and the compartment-specific targeting enables a better understanding of diseases. Esposito and colleagues reviewed the use of roGFP to determine the redox status of cells in neurodegenerative diseases[13]. Because roGFP transduction into different organisms, including plants[14] and bacteria[9], is easily accomplished, monitoring the redox status of both bacterial and host cells during disease states could facilitate innovative treatment approaches. Furthermore, in vivo studies conducted with compartment-specific roGFP in transgenic animals may shed light on the redox status of organisms[15,16].

However, in certain situations, the roGFP biosensor is inadequate for investigating physiologically relevant redox changes[5] and H_2O_2 oxidation[7] within cells. Peroxidase selectivity for H_2O_2 was used to engineer more sensitive probes[6]. Yeast peroxidase ORP1 was linked to roGFP to enable the delicate measurement of H_2O_2-mediated thiol oxidation[17–19]. Likewise, the incorporation of human glutharodexin1 (Grx1) into the roGFP sensor specifically monitors GSH/GSSG equilibrium within the cell compartments[6,18,19].

This easily applicable protocol allows researchers to monitor compartment-specific redox status in intact cells, minimizing artifactual oxidation due to physical stress during cell homogenization. The current protocol demonstrates 2 quantification methods: flow cytometry (beneficial for quick analyses of large cell populations) and fluorescent microscopy (allowing for continuous time-lapse imaging and determining the morphology of the cells).

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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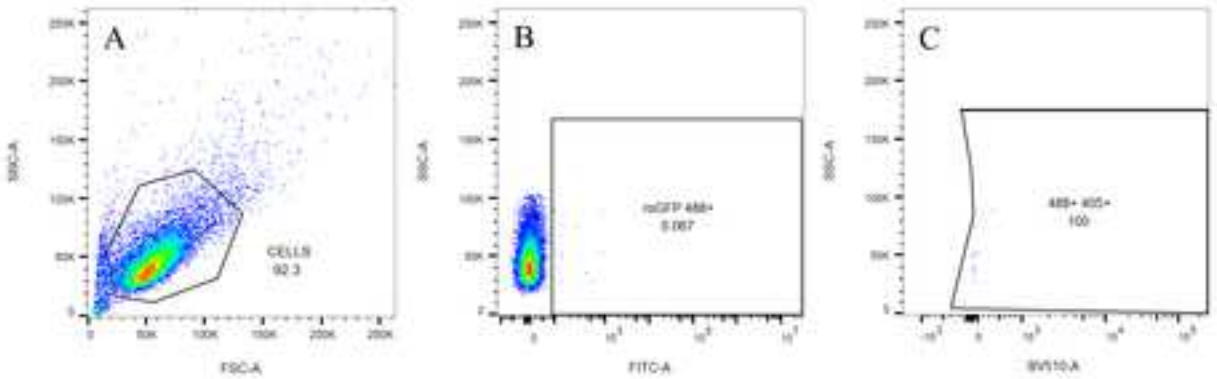

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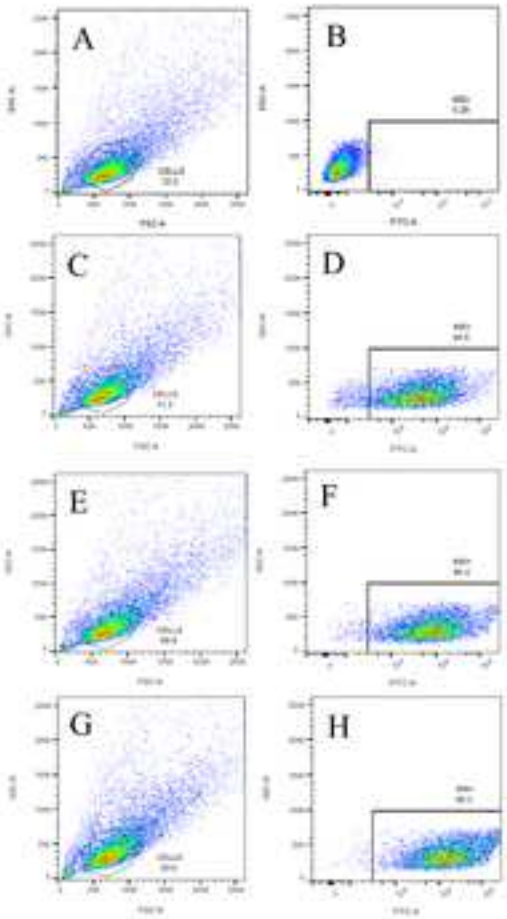

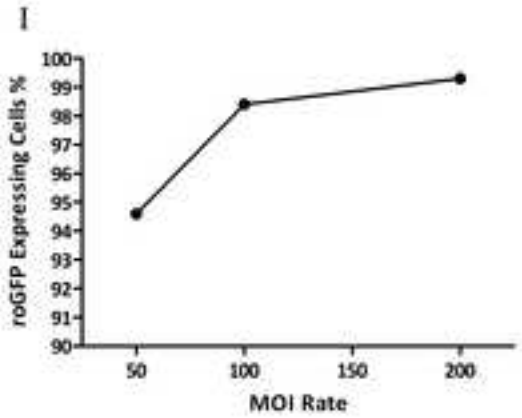

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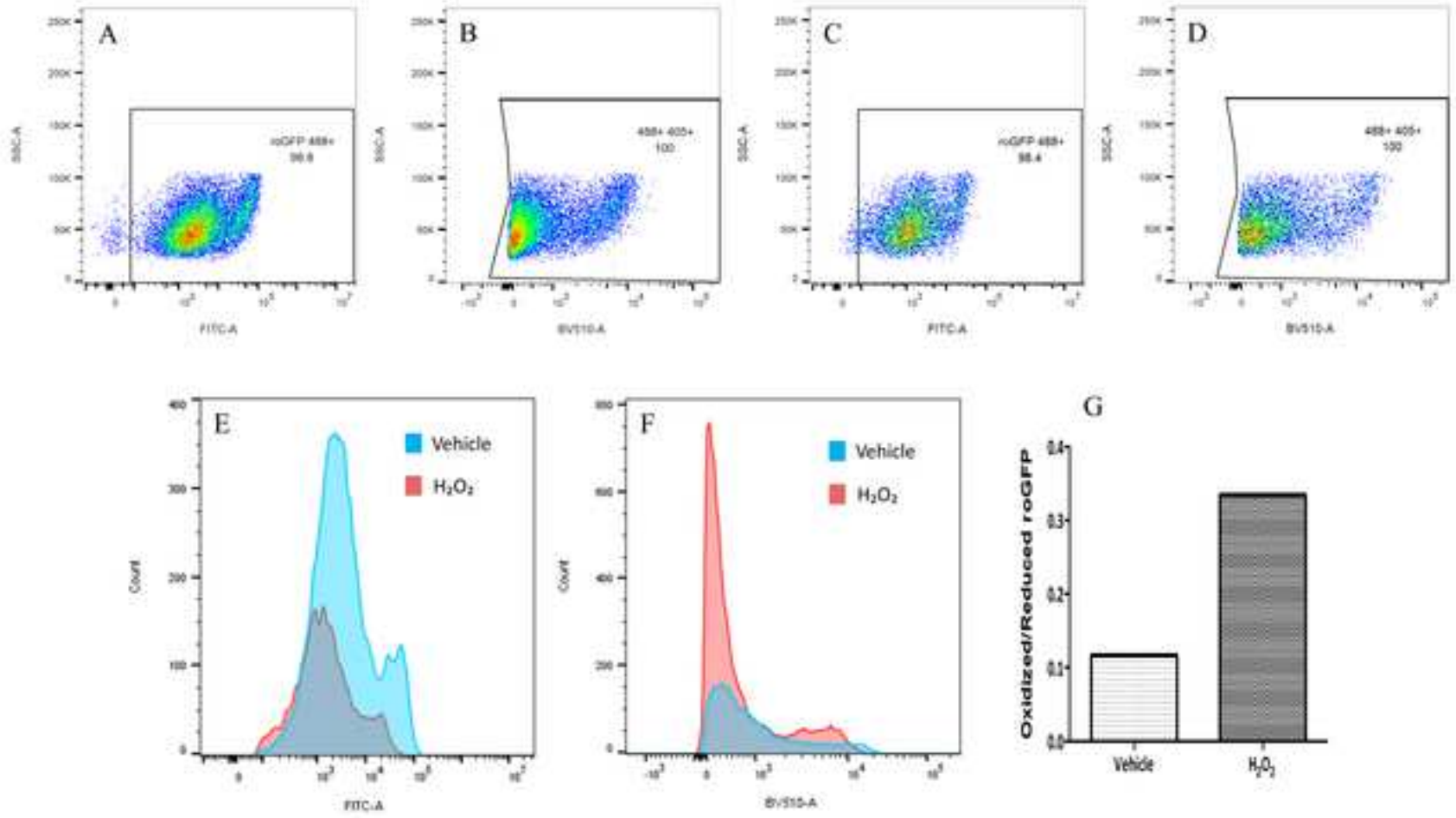

Figure 4

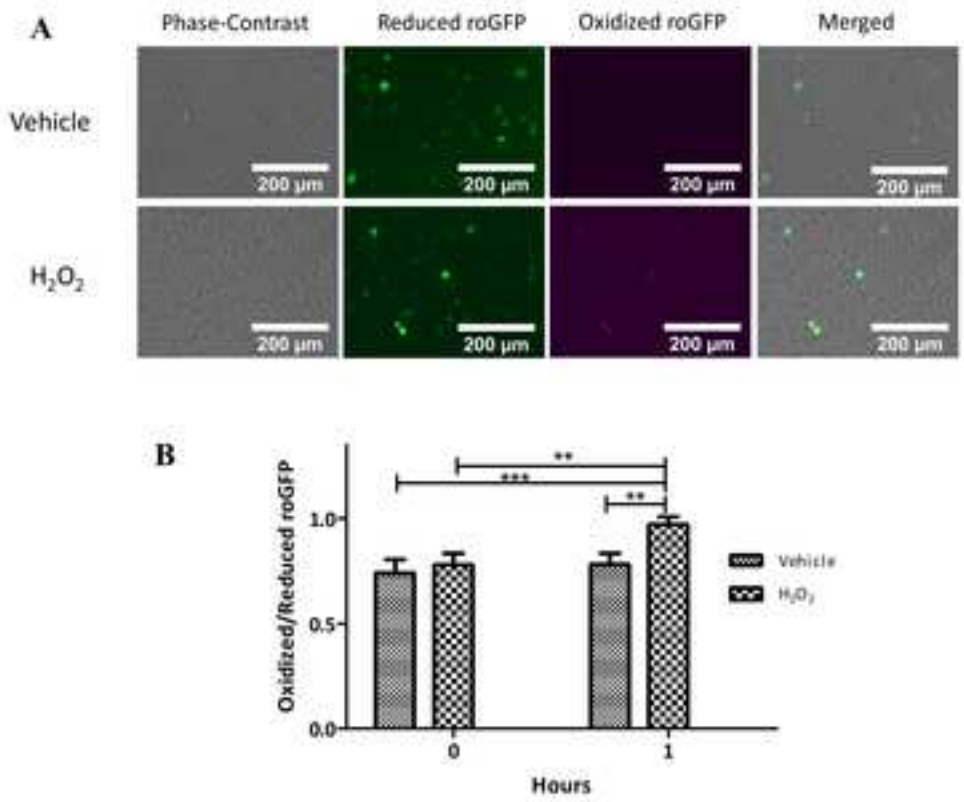

Table 1

Analysis type	Cell number per well	Adenoviral roGFP PFU/mL	1:100 dilution of adenoviral roGFP PFU/mL	MOI	Transduction volume (mL)
Flow cytometry	150,000	6×10^{10}	6×10^{8}	0	0
				50	0.0125
				100	0.025
				200	0.05
Fluorescence microscopy	25,000	6×10^{10}	6×10^{8}	100	0.0042

Table of Materials

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Name of Material	Company	Catalog Number
0.25% Trypsin-EDTA	Gibco by Life Sciences	25200-056
4-well chamber slide	Thermo Scientific	154526
5 ml tubes with cell strainer cap	Falcon	352235
6-well plate	Corning	353046
15 ml conical tubes	MidSci	C15B
75 cm^2 ventilated cap tissue culture flasks	Corning	4306414
Adenoviral cytosol specific roGFP	ViraQuest	VQAd roGFP
Class II, Type A2 Safety Hood Cabinet	Thermo Scientific	1300 Series A2
Countess automated cell counter	Invitrogen	C10227
Countess cell counter chamber slides	Invitrogen	C10283
DMEM	Gibco by Life Sciences	11995-065
FBS	Atlanta Biologicals	S11150
Filtered pipette tips, sterile, 20 µl	Fisherbrand	02-717-161
Filtered pipette tips, sterile, 1000 µl	Fisherbrand	02-717-166
Flow Cytometer	BD Biosciences	LSRFortessa
Fluorescent Microscope	Advanced Microscopy Group (AMG)	Evos FL
Hydrogen Peroxide 30%	Fisher Scientific	H325-100
Light Cube, Custom	Life Sciences	CUB0037
Light Cube, GFP	Thermo Scientific	AMEP4651

MDA-MB-231	American Tissue Culture Collection	HTB-26
Microcentrifuge tubes, 2 ml	Grenier Bio-One	623201
PBS	Gibco by Life Sciences	10010-023
Pipet controller	Drummond	Hood Mate Model 360
Serologycal pipet, 1 ml	Fisherbrand	13-678-11B
Serologycal pipet, 5 ml	Fisherbrand	13-678-11D
Serologycal pipet, 10 ml	Fisherbrand	13-678-11E
Tissue Culture Incubator	Thermo Scientific	HERACell 150i
Trypan blue stain 0.4%	Invitrogen	T10282

	Comments/Description
Cell culture	
Cell seeding material for fluorescent imaging	
Single cell suspension tube for flow cytometry analysis	
Cell seeding material for flow cytometry analysis	
Cell culture	
Cell culture	
roGFP construct kindly provided by Dr. Schumaker	
Cell culture	
Cell counting	
Cell counting	
Cell culture	
Cell culture	
Cell culture	
Cell culture	
Instrument equipped with FITC and BV510 bandpass filters for flow cytometry analyses	
Fluorescent imaging	
Positive control	
Fluorescent imaging of roGFP expressing cells (ex 405 nm)	
Fluorescent imaging of roGFP expressing cells (ex 488 nm)	

Human epithelial breast cancer cell line
Cell culture

Cell culture
Cell culture
Cell culture
Cell culture
Cell culture
CO_2 incubator for cell culture
Cell counting

Re: Manuscript #: JoVE61229

Dear Dr. Nguyen,

We were pleased to hear in your correspondence dated 1/24/2020 that both reviewers felt the manuscript contained important information that could be of interest to the audience of the JoVE61229 pending satisfactory revision. We have now revised our manuscript entitled “Assessment of Cellular Oxidation Using a Subcellular Compartment-Specific Redox-Sensitive Green Fluorescent Protein”, and made the changes suggested by the JoVE editorial office and the reviewers, including the addition of new data testing a lower concentration of H_2O_2 to prevent possible autofluorescence interference from dead cells in our measurements.

Enclosed is a copy of the revised manuscript as well as a detailed response letter addressing each of the reviewers' concerns. Revised text appears as highlighted to be easily distinguished from the original text.

We appreciate the helpful comments provided by both of the reviewers and feel the manuscript has been greatly improved with the inclusion of these changes. We hope that these modifications are satisfactory and that the manuscript can move forward with the production of the video article.

Thank you for your efforts and careful consideration of our work.

Sincerely,

*Nukhet Aykin-Burns, Ph.D.

Associate Professor
Division of Radiation Health
Department of Pharmaceutical Sciences
University of Arkansas for Medical Sciences

*-author to whom correspondence should be addressed.

REBUTTAL:

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
-Manuscript was extensively edited for the English language by the editorial staff of UAMS Office of Scientific Communications. The file containing tracked changes for these spelling and grammar edits can be provided separately if the journal editors request.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
- Table was revised. Supplies, reagents and equipment listed alphabetically.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
- Protocol steps were explained.

4. How are cells counted?
- Cell counting was written under 1.2.

5. 1.2.1/1.2.2/2.3: Incubate as in step 1.1?
- Our incubation conditions for the entire protocol is included as NOTE under 1.1.

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).-
- All personal pronouns were revised/removed.

7. 3.1.2: What concentration of trypsin? Wait how long? What is the media used to stop the reaction?
- Detailed steps for trypsin concentration, incubation time, and media to stop its activity were included under 3.1.2.

8. Please provide all volumes and concentrations used throughout.
- All volumes and concentrations were included in the revised text

9. 3.2.1: How much H2O2?
-Details of H2O2 concentration in 3.2.1. was added to the text.

Dear Reviewer;

Enclosed is a copy of the revised manuscript as well as a detailed response letter addressing each of the reviewers' concerns. Revised text appears as highlighted to be easily distinguished from the original text.

We appreciate the helpful comments provided by the reviewers and feel the manuscript has been greatly improved with the inclusion of these changes. We hope that these modifications are satisfactory and that the manuscript can move forward with the production of the video article.

Thank you for your efforts and careful consideration of our work.

REBUTTAL

Reviewer #1:

Manuscript Summary:
This is a paper that describes how to measure thiol oxidation using roGFP probes through adenoviral transfection.

Major Concerns:

1. This manuscript needs to be more thoroughly proof-read, there are a lot of grammatical mistakes.
-Manuscript was extensively edited for the English language by the editorial staff of UAMS Office of Scientific Communications. The file containing tracked changes for these spelling and grammar edits can be provided separately if the journal editors request.

2. NOTE under #1, the first sentence should be reworded so that it is clearer: Seed control wells in addition to treatment wells.
- Reworded.

3. NOTE under #2, should be reworded so that it is clearer: This protocol was conducted with cytosol specific roGFP, but other compartment (e.g. mitochondria or mitochondrial intermembrane space) can be targeted using this same protocol.
- Reworded.

4. 2.1- List the range of MOI that should be used to determine the most efficacious MOI.
- Range of MOI added as NOTE under #2.1.

5. NOTE under #2.3, the first sentence should be reworded so that it is clearer: On day 3, cells will start to express roGFP; therefore, transduction efficiency can be monitored using florescence microscopy (filters with ex. 488/em. 525).
- Reworded.

6. 2.5- need to state why 100 MOI was chosen, it comes out of the blue with no rationale.

- 100 MOI selection was stated under #2.4

7. 3.1.1- how do you know that 100 uM of H2O2 does not cause substantial cell death 24 hours later and that some of the fluorescence may be due to dead cells?
- We agree with the reviewer 100 µM of H_2O_2 causes substantial cell death which might affect the MFI values due to autofluorescence of the dead cells. In order to respond to the reviewer's concern about that dead cells can cause errors for fluorescent intensity measurements, we repeated our flow cytometry experiment using 1 hour incubation of 10 µM of H_2O_2 and Figure 3 results now reflect this experiment. We also included the overlaid histograms demonstrating the vehicle and H2O2 treated groups' reduced (ex. 488/em. 525) and oxidized (ex. 404/em. 525) fluorescence intensities that we used in the bar graph in Figure 3, hoping this will help readers to visualize the results that is represented in the bar graph.

8. Note under 3.1, it should be added: Other oxidizing agents can be used here as well.
- Added.

9. 3.1.2. should be reworded so that it is clearer: On Day 5, aspirate media from the 6-well plate, place 750 ul of trypsin (please list % of trypsin used) and wait for cells to detach, inactivate trypsin with 2 ml of media and collect the cell containing trypsin-media solution.
- Reworded.

10. 3.2.1, what does 0 time mean? Right before H2O2 treatment, right after treatment? Please clarify this.
- Clarification was made under 3.2.1.

11. 4.1.4, please check, it seems that you should be analyzing your data from Gate 2 and 3, not Gate 1 and 2.
- Corrected.

12. In discussion in line 330- please list these other reengineered biosensors.
- Added

Minor Concerns: None.

Dear Reviewer;

Enclosed is a copy of the revised manuscript as well as a detailed response letter addressing each of the reviewers' concerns. Revised text appears as highlighted to be easily distinguished from the original text.

We appreciate the helpful comments provided by the reviewers and feel the manuscript has been greatly improved with the inclusion of these changes. We hope that these modifications are satisfactory and that the manuscript can move forward with the production of the video article.

Thank you for your efforts and careful consideration of our work.

REBUTTAL

Reviewer #2;
Manuscript Summary:

This work presents interesting results pertaining to evaluate ratiometric assessment of redox status in mammalian cells overexpressing roGFP (targeted to cytosol), using both fluorescent imaging microscopy and the flow cytometric method. The authors show that compartment specific redox status of intact cells can be monitored using engineered roGFPs.

The paper is very well written, with clear structure and careful explanations throughout, enabling others to replicate these techniques if desired. The quality of experimental data is convincing and the conclusions appear to be reliable. I have just a few small comments on the text, which the authors may wish to address:

1. There appears to be an inconsistency of calculating the ratio between reduced versus oxidized forms of fluorescence. At line #200, the calculation based on the ratio of reduced/oxidized, as well as in line #212-214 which referenced the equation in line #200. But Figure 3C & 4B show the ratio of oxidized/reduced.
- We thank the reviewer for catching this inconsistency. The equation is corrected and now shows the calculation of oxidized to reduced ratio.

2. In line #240, add Figure 2 at the end of the sentence "and selection of efficient MOI input"
- Added.

In summary, the paper is presenting solid experimental results backed by convincing and in-depth analysis and I believe that it is a good candidate for being published in Journal of Visualized Experiments.