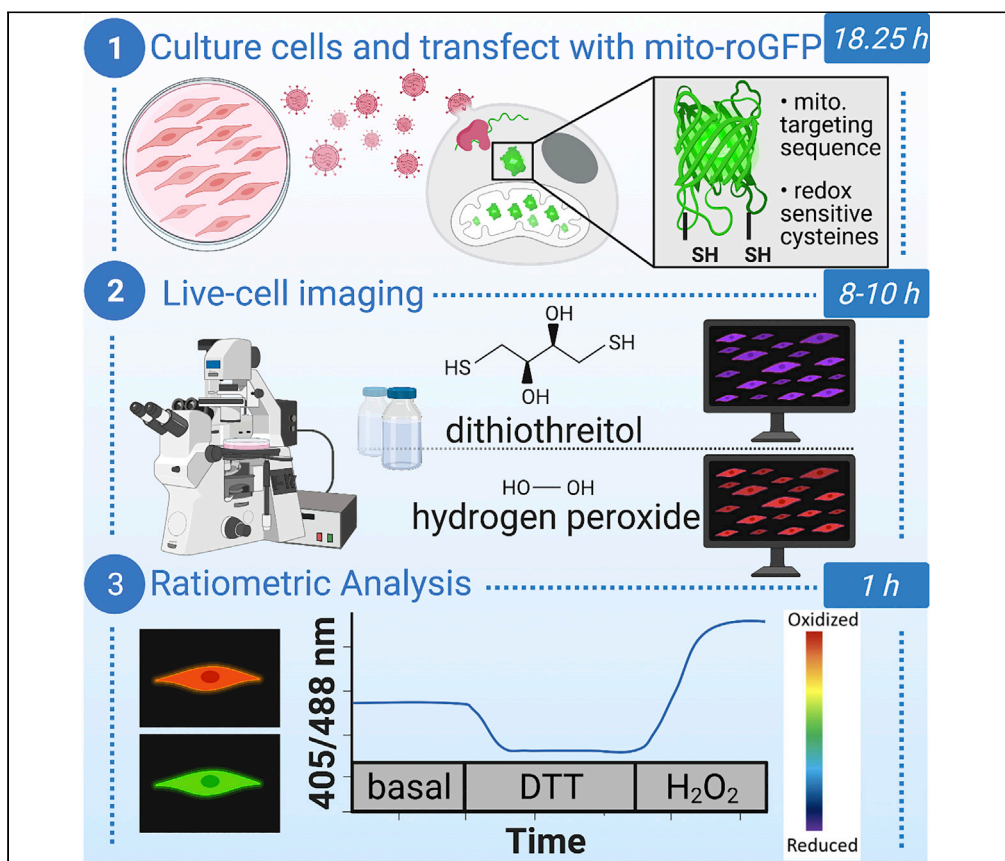


Protocol

Optimization of mito-roGFP protocol to measure mitochondrial oxidative status in human coronary artery endothelial cells



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Highlights
An optimized
protocol for
measuring
mitochondrial
oxidative status of
primary HCAEC

Use of mito-roGFP
adenovirus
transduction

Live imaging using
reducing and
oxidizing factors

Applicable to other
types of murine and
human cells

Reactive oxygen species (ROS) are implicated in endothelial dysfunction and cardiovascular disease. Endothelial cells (ECs) produce most ATP through glycolysis rather than oxidative phosphorylation; thus mitochondrial ROS production is lower than in other cell types. This makes quantification of changes in EC mitochondrial oxidative status challenging. Here, we present an optimized protocol using mitochondrial-targeted adenovirus-based redox sensor for ratiometric quantification of specific changes in mitochondrial ROS in live human coronary artery EC.

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Protocol

Optimization of mito-roGFP protocol to measure mitochondrial oxidative status in human coronary artery endothelial cells

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SUMMARY

Reactive oxygen species (ROS) are implicated in endothelial dysfunction and cardiovascular disease. Endothelial cells (ECs) produce most ATP through glycolysis rather than oxidative phosphorylation; thus mitochondrial ROS production is lower than in other cell types. This makes quantification of changes in EC mitochondrial oxidative status challenging. Here, we present an optimized protocol using mitochondrial-targeted adenovirus-based redox sensor for ratiometric quantification of specific changes in mitochondrial ROS in live human coronary artery EC.

For complete details on the use and execution of this protocol, please refer to Waypa et al. (2010); Liao et al. (2020); Gao et al. (2021).

BEFORE YOU BEGIN

This protocol describes adenoviral transduction, live-cell imaging, and quantification of mitochondrial oxidative status in primary culture of human coronary artery endothelial cells (HCAEC), using a mitochondrial-targeted and redox sensitive fluorescent marker known as mito-roGFP. The protocol requires the administration of dithiothreitol and hydrogen peroxide during live-cell imaging to measure a time-lapse of maximum reduction and oxidation, respectively, and obtain a ratiometric scale to thereby calculate percent of oxidation. This protocol has also been validated with primary culture of mouse heart endothelial cells. While this protocol is designed to study mitochondrial oxidative status of endothelial cells (EC), it can also be adapted for use with other cell types.

Impaired NO bioavailability in ECs leads to endothelial dysfunction and the development of cardiovascular disease, including hypertension, diabetes, and atherosclerosis (Indik, Goldman and Gaballa, 2001; Chen et al., 2005; Feng et al., 2010; Gimbrone and García-Cardeña, 2016; Shafique et al., 2017; Zimmer et al., 2021). In endothelial dysfunction, there is an excessive formation of reactive oxygen species (ROS), which leads to oxidative and nitrosative damage that contribute to the onset and progression of cardiovascular disease (Chen et al., 2005; Shafique et al., 2017). Thus, limiting oxidative stress has long been sought as an approach to restore endothelial and cardiac function in humans with cardiovascular complications. However, global antioxidant therapies have interestingly failed to improve outcomes in clinical settings (Bjelakovic et al., 2007; Bjelakovic, Nikolova and Gluud, 2013). These findings necessitated the study of subcellular ROS, particularly of mitochondrial ROS, to restore mitochondrial function (Brown et al., 2017; Shafique et al., 2017; Kiyuna et al., 2018; Hamedani et al., 2020).

Several fluorescent-labeled probes, such as Mito-SOX, have been developed to allow for mitochondrial ROS measurement (Cheng et al., 2018). However, as EC produce most (more than 80%) of their ATP via glycolysis rather than mitochondrial oxidative phosphorylation, the resulting low levels of



mitochondrial ROS pose a challenge in the reproducibility of results using such probes in ECs (De Bock et al., 2013; Fitzgerald, Soro-Arnaiz and De Bock, 2018). The redox sensor mito-roGFP is advantageous as it allows for specific measurement of mitochondrial ROS. Mito-roGFP is a redox-sensitive, ratiometric fluorescent protein sensor targeted to the mitochondrial matrix (Waypa et al., 2010). Unlike other sensors, the fluorescence excitation spectrum of roGFP is strongly and reversibly dependent on the redox state of the introduced cysteines (Hanson et al., 2004; Waypa et al., 2010). Furthermore, mito-roGFP has the greater advantage of allowing for live cell imaging and time-lapse measurements of cells' response to diverse stimuli, e.g., hypoxia (refer to the third table under the materials and equipment section for more details). A number of research groups have studied redox state using mito-roGFP and optimized the protocol for their cell type of interest, which is summarized in the second table under the materials and equipment section. The different reagents and their amounts support that different cell types have different redox capacities, not only at the global level but the subcellular level as well. This understanding requires the use of cell-specific protocols and, to our knowledge, there is no established protocol for using mito-roGFP in primary HCAEC. This article aims to outline the materials and methods for using mito-roGFP to measure the mitochondrial redox state of HCAEC, which will help study and translation of new therapeutics for cardiovascular disease.

Before you begin, please find a list of the specific reagents, labware and equipment used to validate this protocol in the [key resources table](#).

Isolation and amplification of Mito-roGFP

⌚ Timing: 4 weeks prior to transduction

1. The mito-roGFP adenovirus is provided as a plasmid in bacteria as agar stab. Please follow steps for bacterial culture and plasmid DNA isolation, as per manufacturer instructions, available at: <https://www.addgene.org/recipient-instructions/myplasmid/#:~:text=Bacterial%20Stabs,spreading%20it%20on%20the%20surface>. After DNA isolation, we suggest following the adenovirus amplification and storage protocol from abm, starting at step 2, available at: <https://www.abmgood.com/pub/media/catalog/category/docs/a/4/A4-Ad-amplification-protocol.pdf>.

Culture of HCAEC

⌚ Timing: 1 week prior to transduction

2. HCAECs should be used between passages 4 and 7. Cells stored at passage 3, for example, should be thawed and cultured at least 1 week prior to the estimated date of transduction with replication-deficient mito-roGFP adenovirus.
 - a. Prepare and aliquot all reagents (DPBS, growth media, trypsin and trypsin neutralizing solution).

Note: Growth media is prepared by adding all reagents of the EGM-2 MV Bulletkit (1 × Bottle FBS, 25.00 mL; 1 × Natural Cap Vial with Hydrocortisone, 0.20 mL; 1 × Gray Cap Vial with hFGF-B, 2.00 mL; 1 × White Cap Vial with VEGF, 0.50 mL; 1 × Yellow Cap Vial with R3-IGF-1, 0.50 mL; 1 × Blue Cap Vial with Ascorbic Acid, 0.50 mL; 1 × Green Cap Vial with hEGF, 0.50 mL; 1 × Red Cap Vial with GA-1000, 0.50 mL) with the EBM-2 Basal Medium (500 ml). The Bulletkit and medium are included in the [key resources table](#) as one item, listed as EGMTM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKitTM. After preparation, the EGMTM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKitTM should be stored at 4°C degrees for no longer than 30 days.

- b. Pipette 10 mL of pre-warmed growth media into a 10 cm culture plate. Thaw HCAEC vial by carefully dipping the bottom of the vial in a 37°C degree water bath until a little chunk of ice is left in the middle of the vial.
 - i. Then, transfer the HCAECs from the vial to the plate, making sure that the cells are evenly distributed. Incubate HCAECs for 16 h in a cell culture incubator (37°C degree, 5% CO₂, 85%–95% humidity).
 - ii. Change the growth media after the 16 h incubation described in step 2.b.i. Subsequently, the media should be changed on alternate days, and cells should be 80% confluent at the day of splitting for the assay.

△ **CRITICAL:** All procedures for HCAEC culture must be carried out in a class II biological safety cabinet, to avoid contamination. Visualize your cells under the microscope on alternate days to confirm that they are healthy and free of contaminants before proceeding to the assay.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1X DPBS	Thermo Fisher Scientific	Cat# 14190144
Dithiothreitol (DTT)	Bio-Rad	Cat# 1610611
3% Hydrogen Peroxide (H ₂ O ₂)	Target	Cat# A-15115908
Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific	Cat# 14025076
0.1% Gelatin Solution	Millipore sigma	Cat# ES-006-B
EGM™ -2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™	Lonza	Cat# CC-3202
0.025% Trypsin/EDTA solution (for HCAEC)	Lonza	Cat# CC-5012
Trypsin Neutralizing solution (TNS, for HCAEC)	Lonza	Cat# CC-5002
TrypLE™ Express Enzyme (1X), phenol red (Trypsin for MHEC)	Thermo Fisher Scientific	Cat# 12605010
Clorox liquid bleach	Thomas Scientific	Cat# 158-30966
Deposited data		
Original data for ND files	Mendeley Data	https://doi.org/10.17632/n5s62h3zcc.1
Experimental models: Cell lines		
Human: Primary Coronary Artery Endothelial Cells; Normal, Human (HCAEC)	ATCC	Cat# PCS-100-020™
Recombinant DNA		
Plasmid: Matrix-roGFP (Mito-roGFP adenovirus)	(Waypa et al., 2010)	Addgene Cat# 49437
Software and algorithms		
NIS Elements AR software	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
Microsoft Excel	Microsoft Corporation	https://www.microsoft.com/en-us/microsoft-365/excel
Graphpad Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Nikon Eclipse TE-2000-U microscope	Nikon	N/A
Custom filter set Nikon TE2000 Mounted consisting of AT 405/30x; ET520/40m; AT440dc	CHROMA	N/A - custom-made filter
Desktop computer	DELL	N/A
488 nanometers FITC filter	Nikon	N/A
QImaging RETIGA EXi FAST 1394 microscope camera	TELEDYNE photometrics	https://www.photometrics.com/wp-content/uploads/2020/03/Retiga-EXi-Datasheet.pdf

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CFI Plan Fluor ELWD 40X/0.60 objective	Nikon	N/A
μ-Dish 35 mm, high Glass Bottom	ibidi	Cat# 81158
10 mL Serological Pipettes	USA Scientific	Cat# 1071-0810
TipOne 200 μL Pipette Tips	USA Scientific	Cat# 1111-0706
TipOne 1000 μL Pipette Tips	USA Scientific	Cat# 1111-2721
Eppendorf Easytip 3 pipette filler	Fisher Scientific	Cat# 12-654-105
1000 μL Pipette	Fisher Scientific	Cat# 13-690-032
100 μL Pipette	Fisher Scientific	Cat# 13-690-029
Hemocytometer - Neubauer Bright Line, Double-Counting Chamber	Thomas Scientific	Cat# 1193U54
Cover Slips for Hemocytometer, Glass, 20 × 26 mm, 0.35 mm thickness	Thomas Scientific	Cat# 1159D73

MATERIALS AND EQUIPMENT

Redox Agents

Reagent	Final concentration*	Amount
HBSS	n/a	652.5 μL
DTT (100 μM)	40 μM	400 μL
3% H ₂ O ₂ (880 mM)	38 μM	47.5 μL
Total	n/a	1,100 μL

HBSS, Hank's balanced salt solution; DTT, dithiothreitol.

*Value reflects concentration relative to the total volume at the time the reagent is added in each μ-dish during live-cell imaging. Please see [step-by-step method details](#) for further instructions.

Note: Prepare 100 μM DTT stock solution by measuring 0.0154 grams of DTT/mL of DPBS 1X, vortex until dissolved (DTT is toxic, use face mask).

⚠ **CRITICAL:** DTT and H₂O₂ fall under the category of chemical hazards, please review their respective safety data sheets and take precautions by wearing a mask in addition to lab coat and gloves when handling these agents. Please ensure proper disposal of these reagents into designated chemical waste containers. Do not attempt to add bleach into materials that had contact with DTT, H₂O₂ or TBH. DTT must be prepared immediately before imaging. Do not store diluted DTT. The above table is only for information of final molarity, please follow dilution for these reagents as described in the [step-by-step method details](#). This protocol requires use of an inverted microscope with minimal objective lens of 40× (400× amplification), a high-resolution camera, a custom-built filter set and a software capable of acquiring time-lapse frames within 15 seconds interval for up to one hour, as listed in the [key resources table](#).

Reference values for concentration and incubation time of DTT and tBH/ H₂O₂ in various cell types.

Cell type	Reduction			Oxidation			Reference
	Agent	Conc.	Time	Agent	Conc.	Time	
rat PASCs, SASMCs	DTT	1 mM	15 min.	tBH	1 mM	15 min.	(Waypa et al., 2010)
Embryonic chick cardiomyocytes	DTT	1 mM	10 min.	tBH	1 mM	10+ min.	(Loor et al., 2011)
NHLFs	DTT	1 mM	30 min	H ₂ O ₂	1 mM	30 min	(Jain et al., 2013)
yeast cells	DTT	5, 10 mM	20 min	H ₂ O ₂	5, 10 mM	20 min	(Vevea et al., 2013)
HUVEC	DTT	1 mM	10 min.	H ₂ O ₂	500 μM	20 min	(Kim et al., 2017)

PASCs, pulmonary arterial smooth muscle cells; SASMCs, systemic arterial smooth muscle cells; NHLFs, normal human lung fibroblasts; HUVEC, human umbilical vein endothelial cells; DTT, dithiothreitol; tBH, tert-butyl hydroperoxide.

Reference treatments with concentration and incubation time to reduce or induce mitochondrial oxidation in various cell types.

Cell type	Condition/Agent	Concentration	Incubation time	Reference
PASMCs, SASMCs	Hypoxic Media	1.5% O ₂	30 min.	(Waypa et al., 2010)
Embryonic chick cardiomyocytes	Ischemic Media	NaCl 108.9 mmol/L, KCl 8 mmol/L, NaHCO ₃ 21.4 mmol/L, MgSO ₄ 0.76 mmol/L, NaH ₂ PO ₄ 1 mmol/L, CaCl ₂ 1.21 mmol/L) bubbled with 20% CO ₂ /0% O ₂ /80% N ₂ (pH 6.8).	20 min.	(Loor et al., 2011)
NHLFs	TGF-β1 antimycin A MVE or TPP with TGF-β1	5 ng/mL 1 μg/mL 1 μM	1 h. 1 h. 1 h pretreatment	(Jain et al., 2013)
H9c2 cells	DNCB NAC	100 μM 4 mM	Up to 30 min. Up to 120 min	(Zhang et al., 2012)
ECV304	Apricot Melanoidins	6, 12 μg/mL	6 h.	(Cossu et al., 2012)
HUVEC	VEGF	50 ng/mL	20 min.	(Kim et al., 2017)
rat PECs	Hyperoxia	>95% O ₂	24, 48 h.	(Ma et al., 2018)

Alternatives: Although we found that endothelial cells did not present a consistent response to addition of tert-Butyl hydroperoxide (tBH), other studies have reported successful measurements in other cell lines using tBH as the oxidizing agent, in place of H₂O₂.

PASMCs, pulmonary arterial smooth muscle cells; SASMCS systemic arterial smooth muscle cells; CMs, cardiomyocytes; NHLFs, normal human lung fibroblasts; H9c2, embryonic rat heart cells; ECV304, spontaneously transformed cell line derived from a HUVEC culture; HUVEC, human umbilical vein endothelial cells; PECs, pulmonary endothelial cells; TGF-β1, transforming growth factor β1; MVE, MitoVitE; TPP, triphenylphosphonium; DNCB, thioredoxin reductase inhibitor 1-chloro-2,4-dinitrobenzene; VEGF, vascular endothelial growth factor.

STEP-BY-STEP METHOD DETAILS

The entire protocol requires three days. We have presented the methods in three subsections below starting from day one through day three. We suggest performing a trial run to optimize or troubleshoot any issues prior to your actual experiment.

Day one: Splitting the HCAECs

⌚ Timing: 1.5 h

This initial step consists of splitting the cells and plating them into dishes that will serve for imaging in step 3.

1. Plate 30,000 cells per 35 mm μ-Dish
 - a. Coat each 35 mm μ-Dish with 1 mL of 0.1% gelatin, and incubate for 30 min to 1 h in the incubator. Gelatin will still be liquid after incubation with a thin, invisible coating formed at the bottom. Remove the excess gelatin from the plate and then proceed to the next step.

Note: We recommend preparing at least 3–4 dishes per experimental group (please consider that live imaging can take up to an hour per dish).

Note: If gelatin does not dry or over-dries, then the cells will not attach properly resulting in potential loss of culture.

- b. Wash the cells with DPBS (10 mL), trypsinize (2 mL/10 cm plate, incubate for 2 min, then visualize that cells have detached), inactivate trypsin (6 mL of TNS/ 10 cm plate), centrifuge (800 × g, 5 min), resuspend in 3 mL of growth media and estimate the cell density using the hemocytometer or an automated cell counter.

- c. Remove excess gelatin from the μ -dishes, add 1 mL of growth media containing a volume of cell suspension equivalent to 30,000 cells.

▮▮ Pause point: Incubate cells in a cell culture incubator (37°C degree, 5% CO₂, 85%–95% humidity) for 16 hours.

Note: We recommend continuing subculturing the HCAECs. For that, plate 1 mL of cell suspension in addition to 9 mL of growth media into a 10 cm plate.

Day two: Mito-roGFP transduction

⌚ Timing: 45 min

This step describes the transduction of cells with Mito-roGFP replication-deficient adenovirus.

2. To transduce the cells with replication-deficient adenovirus, replace the media with fresh media containing 5 μ L of mito-roGFP suspension per mL; add 1 mL to each dish.

▮▮ Pause point: Incubate cells in a cell culture incubator (37°C degree, 5% CO₂, 85%–95% humidity) for 16 hours.

Note: Always work in a biosafety level II cabinet while handling Mito-roGFP. Discard all tips containing Mito-roGFP in a waste container with 10% bleach solution. In addition, please follow all requirements of your institution's biosafety committee.

Note: The amount of Mito-roGFP may need to be optimized based on the concentration of adenovirus obtained with amplification and according to how the cells will respond. For this reason, we recommend to either test the dose of 5 plaque-forming units per cell, as suggested by Waypa et al (Waypa et al., 2010) or perform a titration to find a dose at which the cells will express enough mito-roGFP. This can be done by visualizing the fluorescence emission of the cells. Choose the dose that results in a high number of fluorescent ECs (for further information, please refer to problem 1 under the heading "Troubleshooting").

Day three: live-cell imaging of HCAEC transduced with Mito-roGFP

⌚ Timing: 8–10 h

This major step consists of live imaging of the HCAEC and production of data for analysis. Please review the [key resources table](#) for information about the inverted microscope, objective lens, camera and NIS Elements software used in this step.

3. Change the media prior to starting the imaging procedure with fresh media. This step is required to remove any adenovirus that has not entered a cell and avoid any accidental exposure of the investigator to mito-roGFP, which is a biohazard level 2 adenovirus, during imaging procedure, and also to provide a more adequate cellular environment for imaging. Discard all tips and media containing Mito-roGFP in a waste container with 10% bleach solution.

Optional: You may add pre-treatments to the cells. Please see the third table under [materials and equipment](#) for reference treatments based on published literature.

Note: Unlike HCAEC, mouse ECs may need a total of 48–72 hours under culture to achieve a good fluorescence level of Mito-roGFP prior to starting the assay. In this case, change the

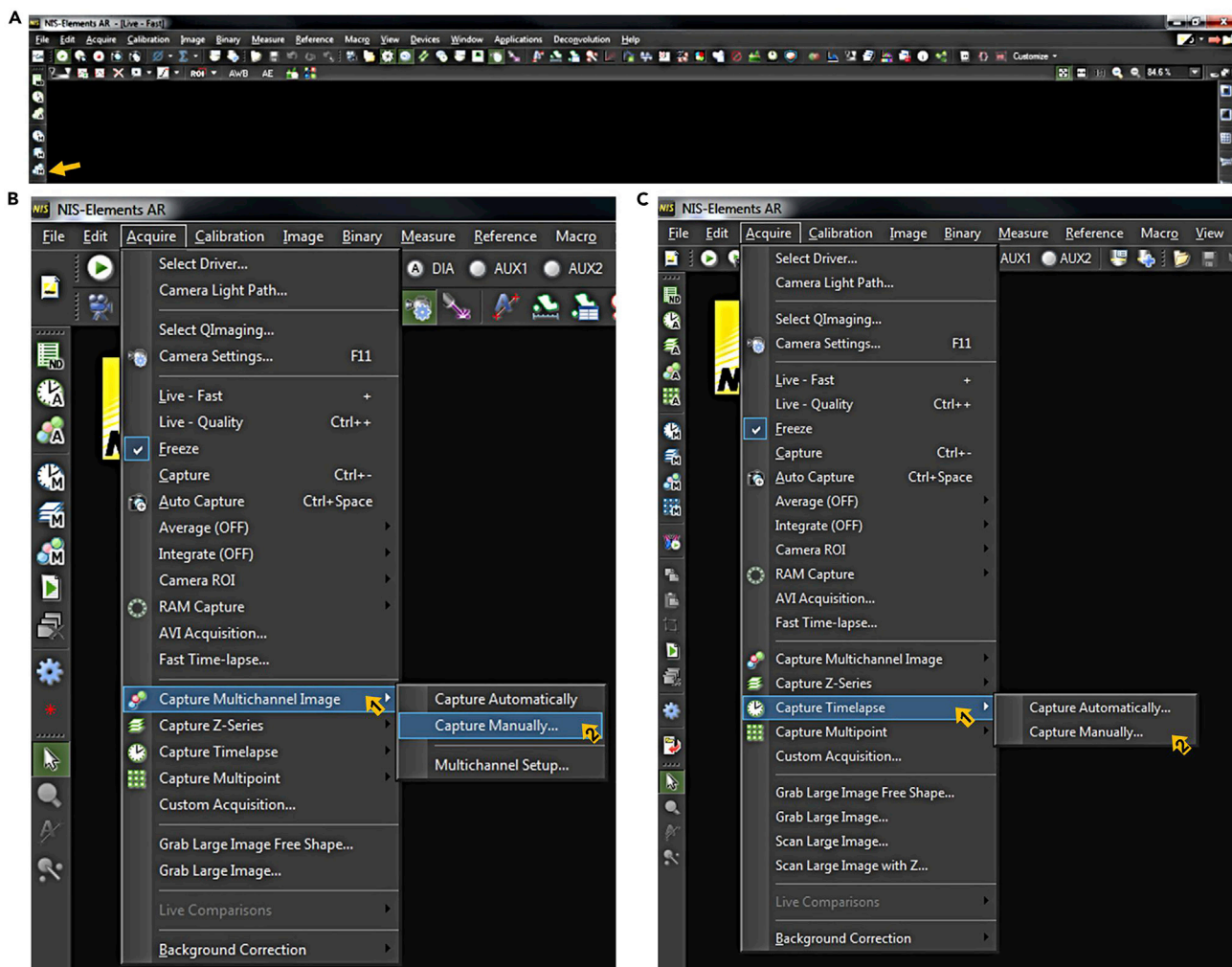


Figure 1. Defining channels, exposure, and time schedule for imaging

Screenshots of step 7a in NIS elements (A) (B) (C). Note that panel (A) is a shortcut method to capture a multichannel image displayed in panel (B).

media again on the next day (for further information, please refer to problem 1 under the heading “Troubleshooting”).

4. Prepare solutions for the assay
 - a. Prepare 100 mM DTT stock solution as described in [materials and equipment](#) section; 400 μL per sample is needed.
 - b. Aliquot 3% H_2O_2 ; 47.5 μL per sample is needed.
 - c. Label 3 tubes per sample:
 - i. 1 (pipet 515 μL of HBSS)
 - ii. 2 (pipet 100 μL of HBSS)
 - iii. 3 (pipet 52.5 μL of HBSS)
 - iv. Keep HBSS tubes in 37°C water bath until ready to use. Keep DTT and H_2O_2 aliquots in the microscope room.
5. Turn ON microscope and all its accessories (fluorescence light, camera) at least 20 min before imaging. The following experiment setup is described based on our experience with NIS Elements Imaging software, but it can be adapted to other programs that accept the same settings (provided below).

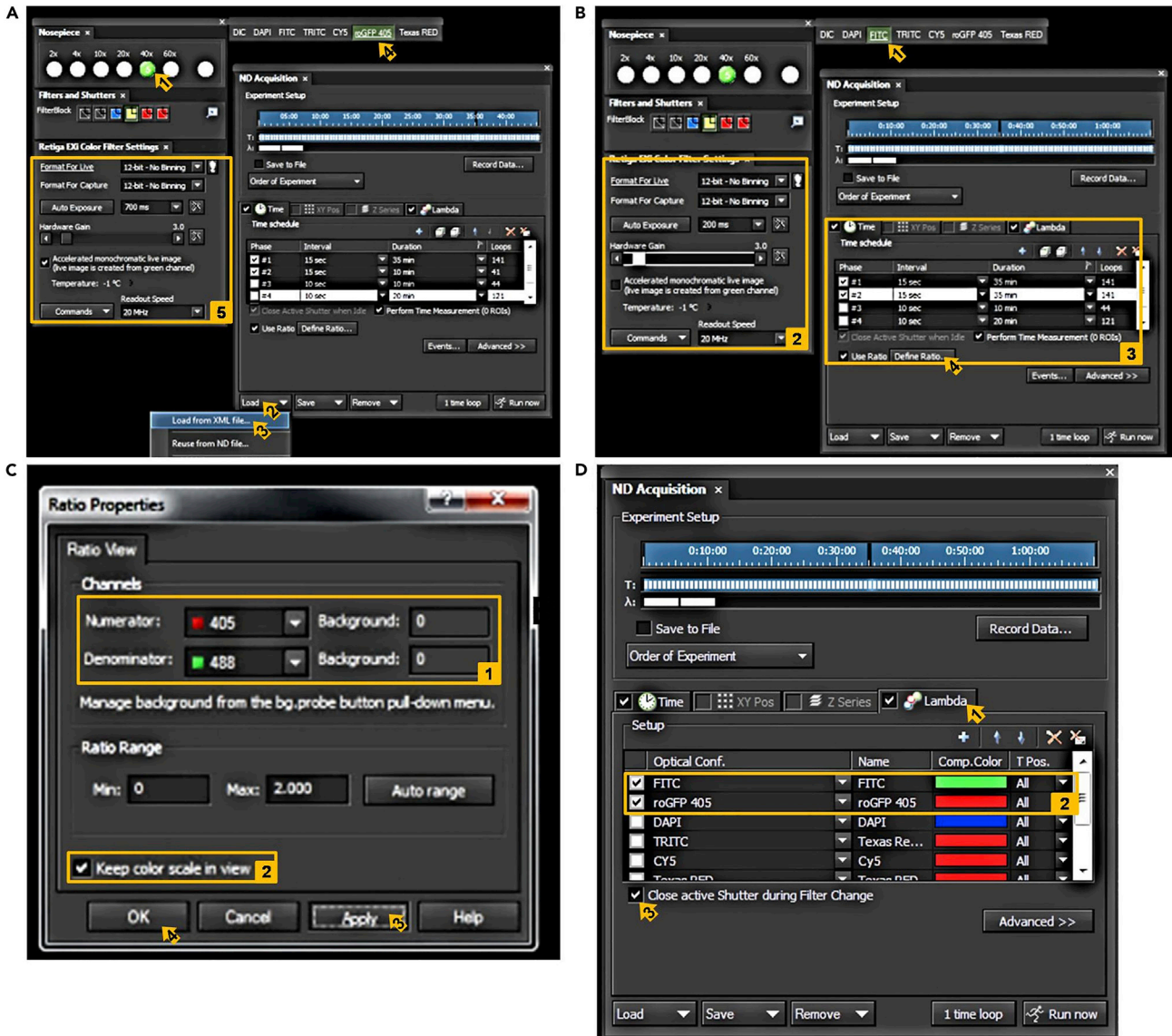


Figure 2. Defining ratio properties for acquisition

Screenshots of step 7a to load mito-roGFP template file (A) or to manually input settings as described in steps 7a-c (B), 7d (C), and 7e (D) in NIS elements

6. Setup experimental conditions in NIS Elements

- a. Open the imaging program. Click on the live multichannel button, as indicated by yellow arrow in Figure 1A, or follow steps shown in Figure 1B. Next, follow the steps shown in Figure 1C. A new window named "ND Acquisition" will open. Select 40x at the nosepiece window (Figure 2A, step 1). At the ND Acquisition panel, click "Load" and select "Load from XML file...", as shown in Figure 2A, steps 2 and 3. Select the file named as "Mito-roGFP TEMPLATE.xml" (available for download in the Mendeley dataset). After loading the template, check all settings as described below to confirm that the data was successfully loaded.

Note: If you do not wish to use the provided template or there is an issue to load it, simply follow all consecutive steps described below and save your own template at the end of the experiment, as described in step 10.

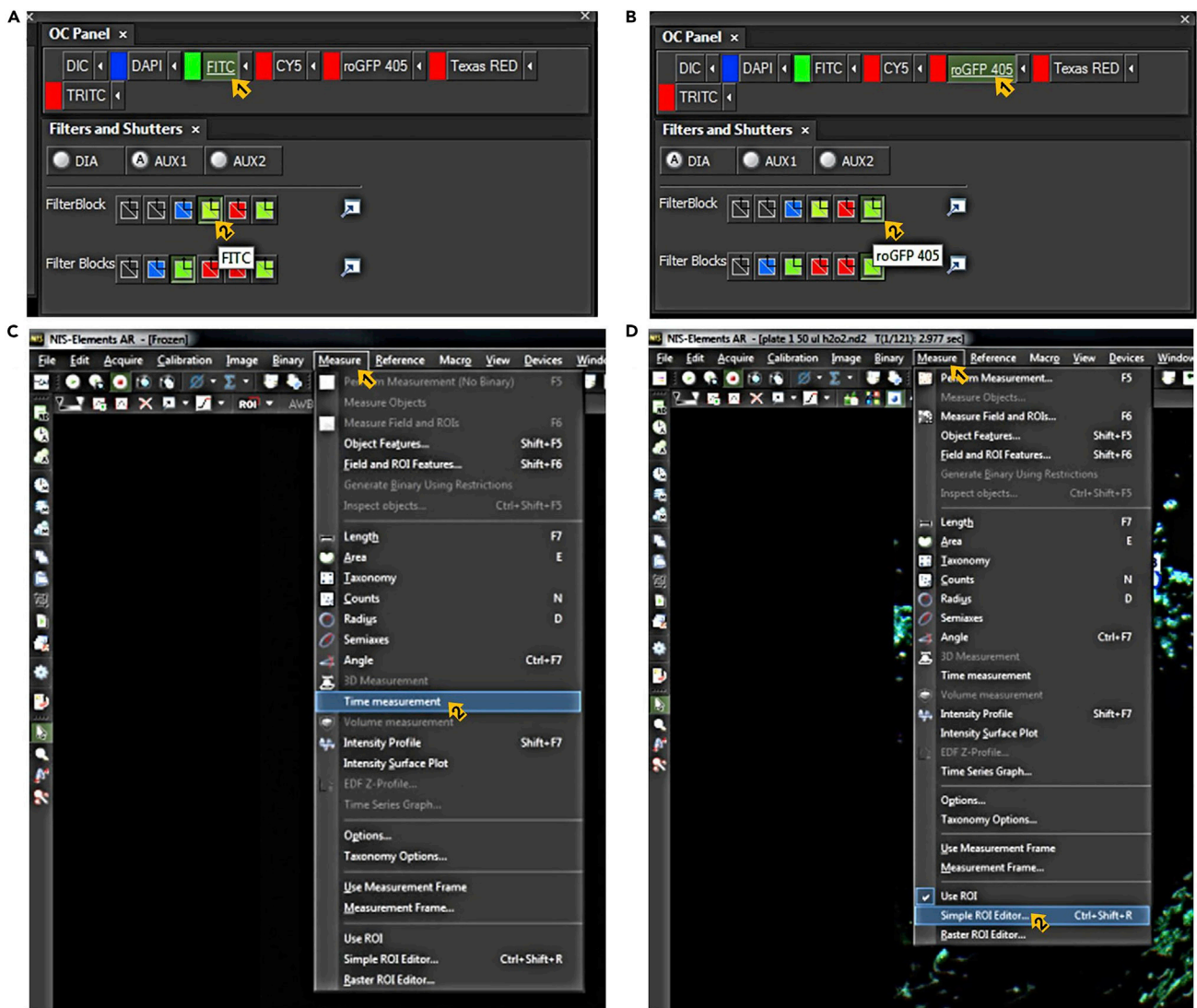


Figure 3. Setting up time measurement
Screenshots of steps 7e (A) (B) (C) and 7g (D) in NIS elements.

- b. Start by selecting the mito-roGFP (405 nm) channel (Figure 2A, step 4). Then, input all data exactly as shown in the yellow square of Figure 2A, step 5. Next, select FITC (488 nm) channel (Figure 2B, step 1). Input settings exactly as indicated in yellow square (Figure 2A, step 2). If setting the experiment for HCAEC, change the exposure value to 1 s, and to 200 milliseconds for Mito-roGFP and FITC channels, respectively. Note that exposure and gain may need to be later optimized for both 488 and 405 channels, according to your experimental conditions.
- c. Confirm intervals and time for measurement (15 s interval, first loop 35 min, second loop 35 min). Check boxes and edit all fields exactly as indicated in the yellow box in Figure 2B, step 3.
- d. Next, click on the “Define ratio...” button (Figure 2B, step 4). A new, small window will open (Figure 2C). Change the numerator to 405 nm and the denominator to 488 nm as indicated in square 1 of Figure 2C. Next, check the box “keep color scale in view”, as shown in square 2 of Figure 2C. Then, apply changes and close the ratio properties window (Figure 2C, 3-4).

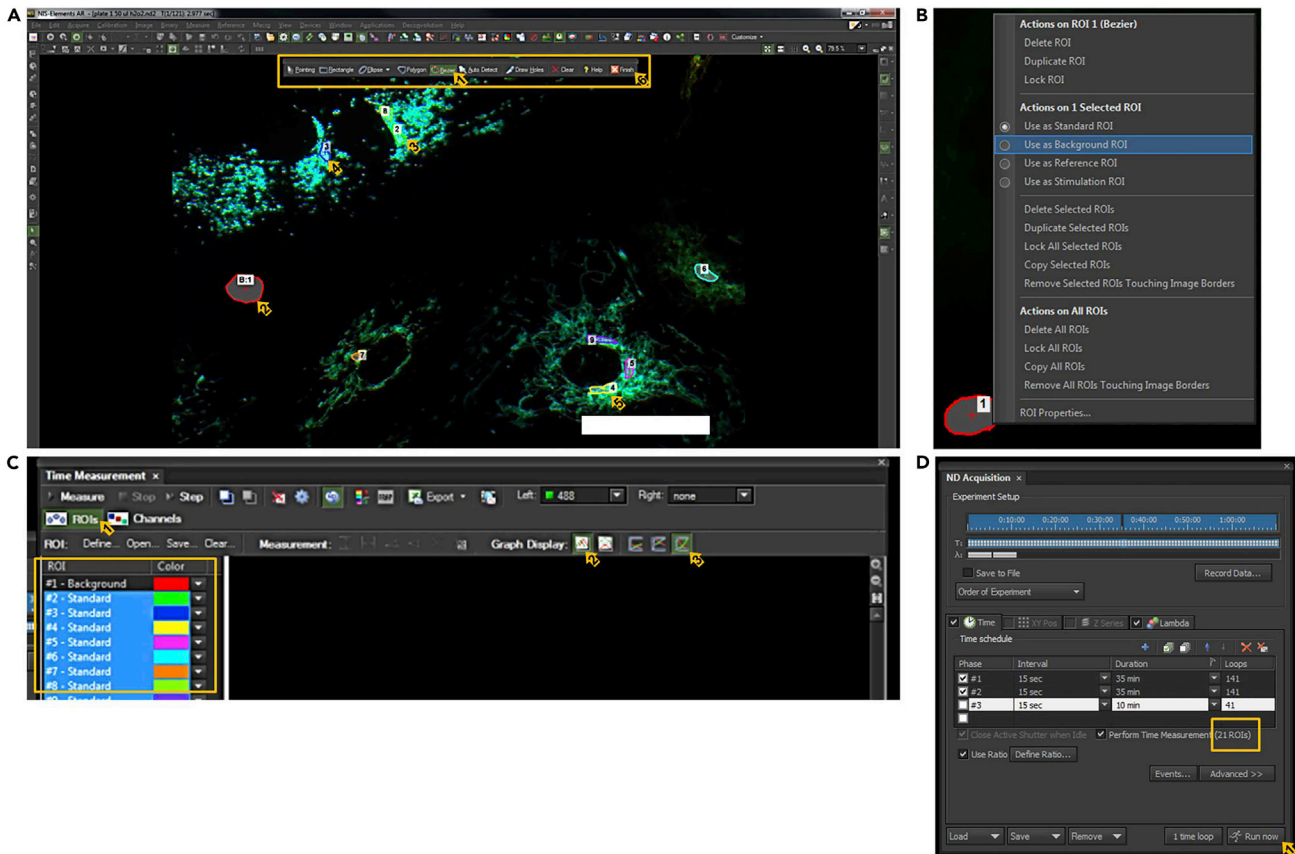


Figure 4. Defining the regions of interest (ROIs)

Screenshots of steps 7g (A) (B), 7h and 7i (C) (D) in NIS elements. Scale bar represents 50 μm .

- e. At the ND Acquisition window, click on the subfield named as “Lambda” (Figure 2D, 1). Confirm that only FITC and Mito-roGFP channels are selected, as indicated in Figure 2D, 2. Check the “close active shutter during filter change” box, as indicated in Figure 2D, 3. At the OC Panel window, check that the right filters are selected in the filter block for both 488 and 405 nm channels (Figures 3A and 3B). At the main window, click on “measure” and then on “Time measurement” (Figure 3C, 1-2). A new window will open; keep aside until noted otherwise.
- f. Bring a control sample to the microscope, replace the media by 500 μL HBSS from tube 1, and put 2-3 cells in focus. Check FITC and mito-roGFP channels and adjust exposure and gain, if necessary. We suggest a gain of 1.0–3.5, and exposure range of 100–300 ms for 488nm filter (FITC) and of 500–900 ms for 405 filter (mito-roGFP). Note that the visualized fluorescence intensity should be similar in both channels to result in a baseline fluorescence ratio close to 1.
- g. At the main window, click on “measure” and then on “Simple ROI (Region Of Interest) Editor” (Figure 3D, 1-2). A small toolbox will popup (as shown by the yellow box in Figure 4A). Select the “Bezier” tool to draw each ROI (Figure 4A, 1). To draw your background ROI, select a region with no cells (Figure 4A, 2). After drawing, use the right click button function of your mousepad on top of the ROI and select “use as background ROI”, as shown in Figure 4B. Next, use the Bezier tool to draw each test ROI (Figure 4A, 3-5). We recommend drawing around 20 test ROIs, always in the regions near the nuclei, given that EC extremities move considerably in response to DTT and H_2O_2 , which may result in unusable ROIs. When done, click the “finish” button in the toolbox (Figure 4A, 6).

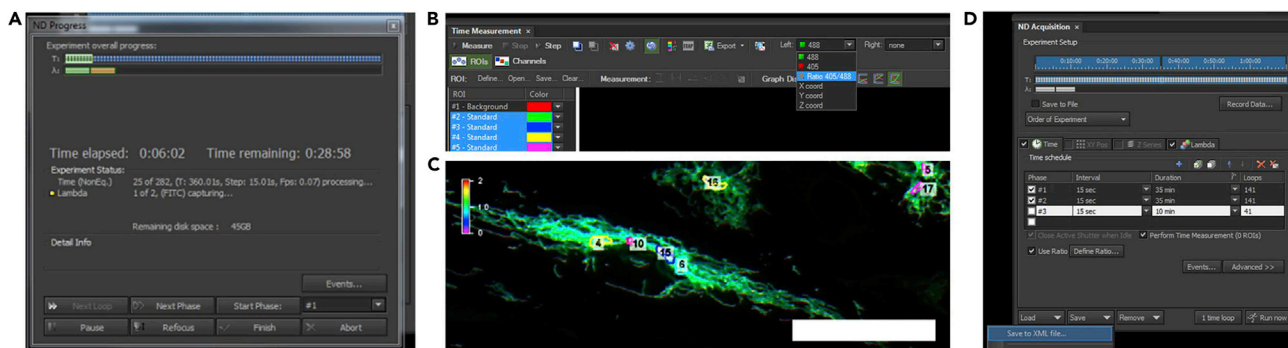


Figure 5. Evaluating the ratio and color of ROIs

Screenshots of steps 7a (A) (B) and 7b (B) (C) (D) in NIS elements. Scale bar represents 50 μm .

- h. At the time measurement window, click the button "ROIs" (Figure 4C, 1). The selected ROIs will appear at the left side, as shown by the yellow square in Figure 4C. All ROIs selected should be highlighted in blue (Figure 4C, yellow square). At this point, the ND Acquisition window should show the number of ROIs selected for measurement (Figure 4D, yellow square). This number should equal the total number of ROIs selected, including background ROI.
- i. Organize all open windows so they are clearly seen without overlapping. At the time measurement window, select: "Graph display" as "No split" and "<min – max>", as indicated by arrows 2 and 3 in Figure 4C. At the ND Acquisition window, click on the "Run now" button (Figure 4D, arrow 1). A new window will pop up (Figure 5A) and ratiometric measurements will start at the time measurement window. At the time measurement window, select: "Left" as "ratio 405/488" (Figure 5 B).
- j. Check if the ratio of all ROIs is, on average, close to 1 (in addition, cells at the main window should present with a green color close to that shown in the reference color bar (around the value 1) of 405/488 ratio channel, as seen in Figure 5C). If yes, write down the exposure and gain used for FITC and Mito-roGFP and proceed to the next step. If not, abort recording, adjust exposure and gain of FITC and Mito-roGFP channels, and repeat step 6, i-j. You may wish to save the experimental setup as a template, for that, please follow steps as shown in Figure 5D.

Pause point: record cells for a minimum of three minutes before proceeding to step 7.

Optional: You may add treatments to the cells prior to step 7. Please see the third table under [materials and equipment](#) for treatment suggestions based on available literature.

7. Prepare to pipette DTT solution.
 - a. Add 400 μL of DTT stock solution to the tube named as "2". After 3 min of baseline recording, use the filter change interval (15 s) to transfer all volume (500 μL) from the tube to the sample. This has to be done by slowly spreading the DTT into the plate but without touching the plate. Note that even the slightest move of the plate may displace your ROIs. The ratiometric values in the time measurement window should immediately go down and cells will appear in purple color at the main window. If cells do not show a purple or darker blue color or move away, the DTT concentration will need to be optimized.

Pause point: Watch the cells for the next 15 minutes, annotating any ROIs that have been displaced due to cell movement. These ROIs will acquire unreliable results and should be excluded from analysis (please refer to item 1 under the heading "limitations" for further information). Simultaneously, prepare for the next step.

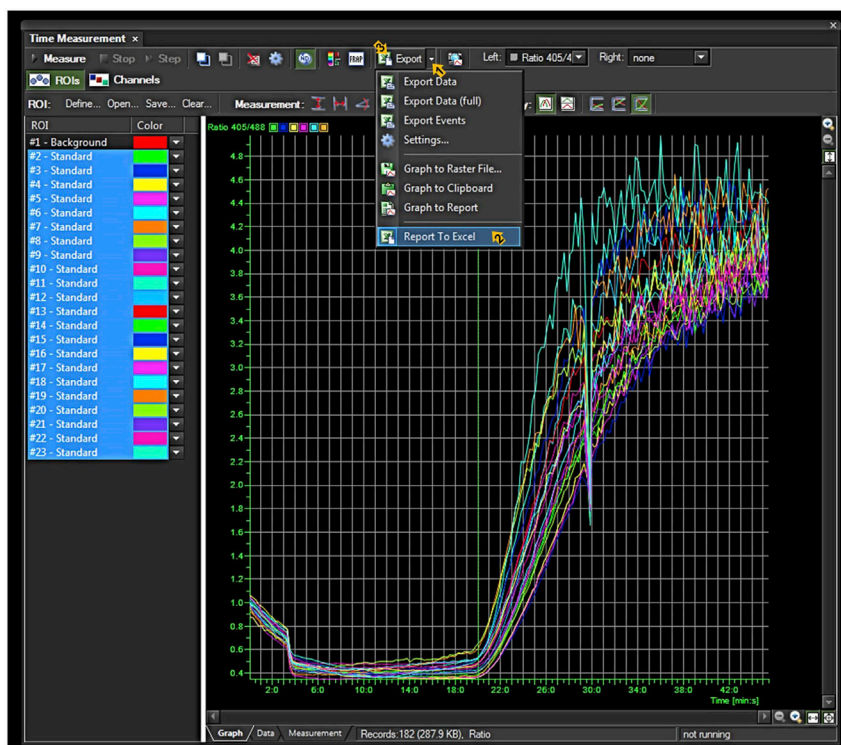


Figure 6. Exporting the experimental report

Screenshot for step 10 in NIS elements.

8. Prepare to pipette H_2O_2 solution.
 - a. Add 47.5 μL of 3% H_2O_2 to the tube named as "3". Fifteen minutes after adding DTT, transfer all volume (100 μL) from the tube to the sample, without touching the plate.

Pause point: Usually, the ratiometric values as well as the cell color will slowly change over the course of the next 10 minutes. Continue recording until the cells consistently present with red color. This time may vary for test samples based on the different pre-treatments applied as part of your experiments (please refer to item 2 under the heading "limitations" for further information).

- b. After seeing predominance of the red color throughout the entire cell (please refer to [Figure 7](#) for example of acceptable visualization of maximum oxidation) for at least 5 frames, you may stop recording.
9. Save and export all data in an ND2 format for later review, *properly identifying your sample*. In addition, export an experimental report to excel, as shown in [Figure 6](#), arrows 1-3. Save the spreadsheet, properly identifying your sample.
10. Subsequently, close the main window used for acquisition and proceed to the next sample; focus the cells under the microscope and start again from step 6, sub step g of this protocol. Alternatively, we have provided a simple procedure, easy to follow protocol, available in the provided Mendeleev dataset. If there are no additional samples, proceed to quantification and statistical analysis.

EXPECTED OUTCOMES

Please refer to [Figure 7](#) for an example of the expected outcomes of this protocol. In addition, examples of acceptable and unacceptable images are shown in [Figure 7G](#). Note that color may differ in between samples, especially when working with very different samples.

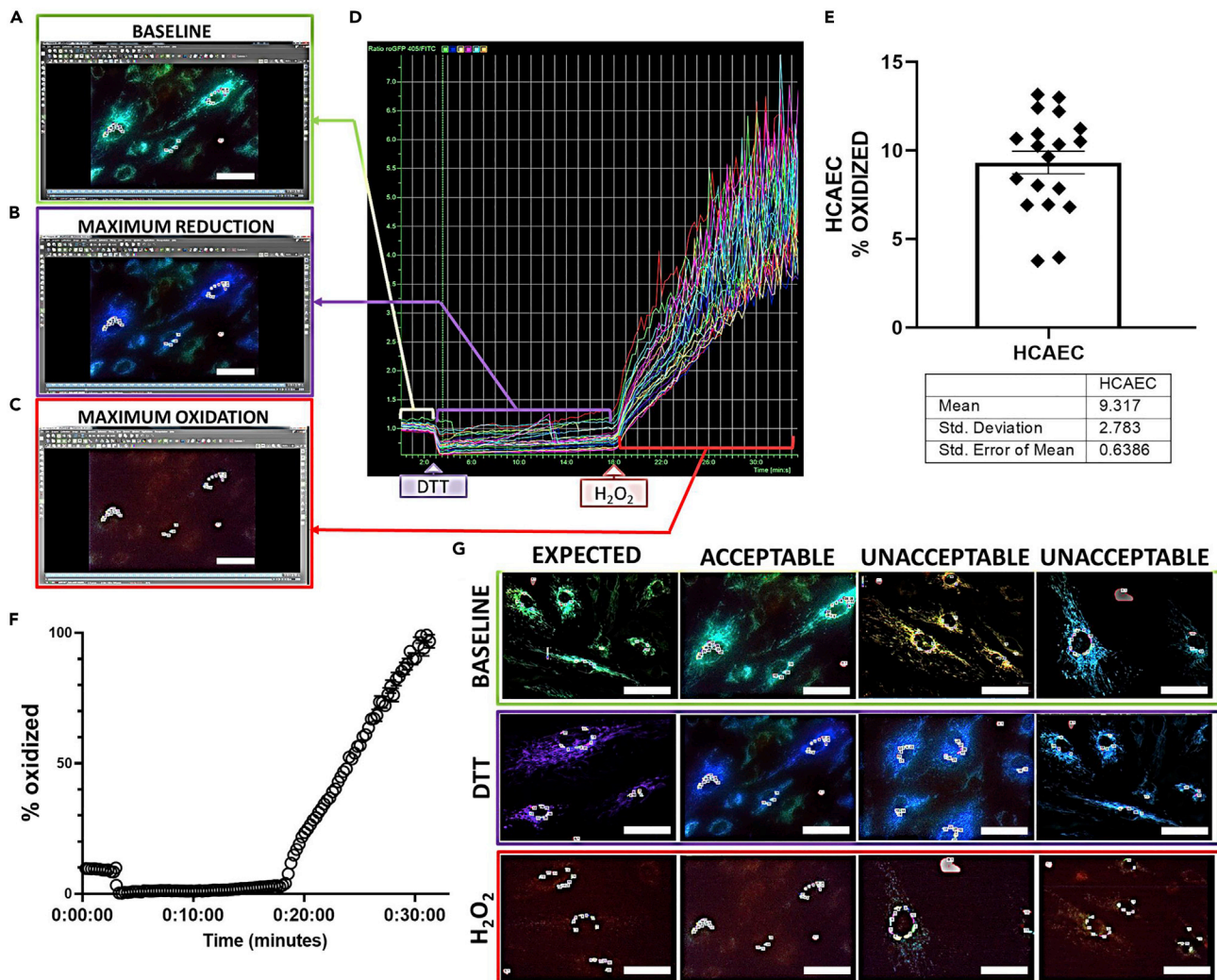


Figure 7. Ratiometric analysis of mitochondrial redox state using mito-roGFP

Representative images show the expected outcomes of this protocol. The expected baseline ratiometric color and respective readings are shown in (A). The addition of DTT should be followed by a decrease in ratiometric reading and a color change from green to a purple or close to purple color, as shown in (B). After adding H_2O_2 , the cells should gradually change to a red color and ratiometric readings should increase, as shown in (C). The overall ratiometric profile expected is shown in (D). The final results after analysis are shown in (E). Alternatively, the ratiometric profile can also be shown as a result, as demonstrated in (F). Reference images of the expected, acceptable, and unacceptable color changes at the ratio channel are shown in (G). Data are represented as mean \pm SEM. Scale bar represents 50 μ m.

QUANTIFICATION AND STATISTICAL ANALYSIS

After clicking on the export button in step 10, a spreadsheet will open, as shown in Figure 8. Note that the first column of your spreadsheet is the information about the order of frames, the second column is the time of each measurement, and the third column refers to your background ROI. For the analysis, you will need to open both the excel and the ND2 file and check your lab notebook notes. If you wish to analyze the data using your personal laptop, you may download NIS Elements viewer, available on: <https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer>. Below, we describe the steps for analysis. An additional example is available in the supplemental Mendeley dataset.

11. Open the .nd2 file and its corresponding excel file. Check your notes in the lab notebook.

No.	Time (min:s.ms)	#1 (Ratio roGFP 405/FITC) (B)	#2 (Ratio roGFP 405/FITC)	#3 (Ratio roGFP 405/FITC)	#4 (Ratio roGFP 405/FITC)	#5 (Ratio roGFP 405/FITC)	#6 (Ratio roGFP 405/FITC)	#7 (Ratio roGFP 405/FITC)	#8 (Ratio roGFP 405/FITC)	#9 (Ratio roGFP 405/FITC)	#10 (Ratio roGFP 405/FITC)
1	00:00.1	5.004	0.953	0.942	0.981	0.992	0.959	0.967	0.999	0.962	0.967
2	00:18.0	4.882	0.943	0.937	0.983	0.992	0.959	0.966	0.989	0.967	0.967
3	00:33.0	4.941	0.95	0.932	0.988	0.977	0.952	0.953	0.987	0.964	0.964
4	00:48.0	4.768	0.943	0.935	0.98	0.979	0.951	0.966	0.991	0.984	0.984
5	01:03.0	4.789	0.924	0.93	0.966	0.988	0.965	0.954	0.991	0.974	0.974
6	01:18.0	4.962	0.931	0.912	0.965	0.988	0.946	0.941	0.989	0.96	0.96
7	01:33.0	4.576	0.941	0.921	0.967	0.977	0.947	0.941	0.975	0.955	0.955
8	01:47.9	4.18	0.933	0.915	0.959	0.973	0.936	0.927	0.961	0.945	0.945
9	02:03.0	4.811	0.921	0.911	0.957	0.979	0.932	0.931	0.961	0.954	0.954
10	02:18.0	5.068	0.925	0.907	0.958	0.964	0.927	0.937	0.956	0.957	0.957
11	02:33.0	4.502	0.908	0.895	0.952	0.952	0.931	0.919	0.954	0.949	0.949
12	02:48.0	5.021	0.917	0.897	0.939	0.955	0.921	0.91	0.943	0.945	0.945
13	03:03.0	5.871	0.465	0.471	0.514	0.513	0.486	0.495	0.51	0.5	0.5
14	03:18.0	5.659	0.47	0.469	0.51	0.512	0.482	0.496	0.502	0.516	0.516
15	03:33.0	5.389	0.473	0.471	0.517	0.519	0.491	0.498	0.517	0.537	0.537
16	03:48.0	5.909	0.473	0.474	0.513	0.524	0.493	0.499	0.519	0.567	0.567
17	04:03.0	5.188	0.491	0.479	0.526	0.538	0.503	0.503	0.525	0.595	0.595
18	04:18.0	5.011	0.495	0.486	0.52	0.537	0.504	0.507	0.536	0.607	0.607
19	04:32.9	5.333	0.495	0.492	0.534	0.545	0.513	0.51	0.544	0.624	0.624
20	04:48.0	5.376	0.501	0.498	0.533	0.558	0.51	0.518	0.554	0.609	0.609
21	05:03.0	4.813	0.516	0.497	0.548	0.557	0.52	0.519	0.565	0.63	0.63
22	05:18.0	5.444	0.519	0.495	0.541	0.562	0.521	0.527	0.567	0.625	0.625
23	05:33.0	4.681	0.53	0.503	0.556	0.578	0.528	0.534	0.568	0.64	0.64
24	05:48.0	5.039	0.52	0.504	0.56	0.576	0.538	0.539	0.584	0.638	0.638
25	06:03.0	5.281	0.53	0.5	0.557	0.578	0.543	0.54	0.585	0.638	0.638
26	06:18.0	5.233	0.536	0.516	0.571	0.58	0.561	0.547	0.59	0.649	0.649

Figure 8. Understanding the output dataset

Screenshot of exported excel file from NIS elements after data acquisition, as described under Quantification and Statistical Analysis section.

12. Identify the different intervals of your acquisition.
 - a. Look for the frame number that corresponds to the time you applied the DTT; e.g., if DTT was added at 3 min, then frame 13 would be already a result of DTT addition, and frames 1 to 12 would be your baseline readings. As a confirmation when reviewing the .nd2 file, you would find that the color of the cells changed from frame 12 to 13 (Figure 9A). In the excel file, mark the frames 1 through 12 as baseline readings. Identify frame 13 as the first frame of DTT.
 - b. Next, look for the frame number that corresponds to the time you applied the H₂O₂; e.g., if H₂O₂ was added at around 18 min and 16 s, then frame 75 would be already a result of H₂O₂ addition, and frames 13 through 74 would correspond to DTT readings. As a confirmation when reviewing the .nd2 file, you will find that the color of the cells changed from frame 74 to 75 (Figure 9B). In the excel file, mark the frames 13 through 74 as DTT readings.
 - c. Identify frame 75 as the first frame of H₂O₂; then look for the frame where cells are entirely red but still visible (frame 94 in our example, Figure 9C). In the excel file, mark the frames 75 through 94 as H₂O₂ readings.
 - d. Next, for each individual ROI in the excel file (with exception of moved ROIs):
 - i. Calculate the average value from baseline readings
 - ii. Calculate the minimum value from DTT readings
 - iii. Calculate the maximum value from H₂O₂ readings
 - iv. Use the following formula to calculate the percent oxidation (Waypa et al., 2010):
 - e. Plot results as a graph. You may consider individual values or the average of all ROIs for each plate/sample. Apply the appropriate statistical tests for your experimental design.

LIMITATIONS

One limitation of this protocol is that HCAECs tend to move upon addition of a reducing (DTT) or oxidizing (H₂O₂) agent. For this reason, displaced ROIs will produce unreliable results and must

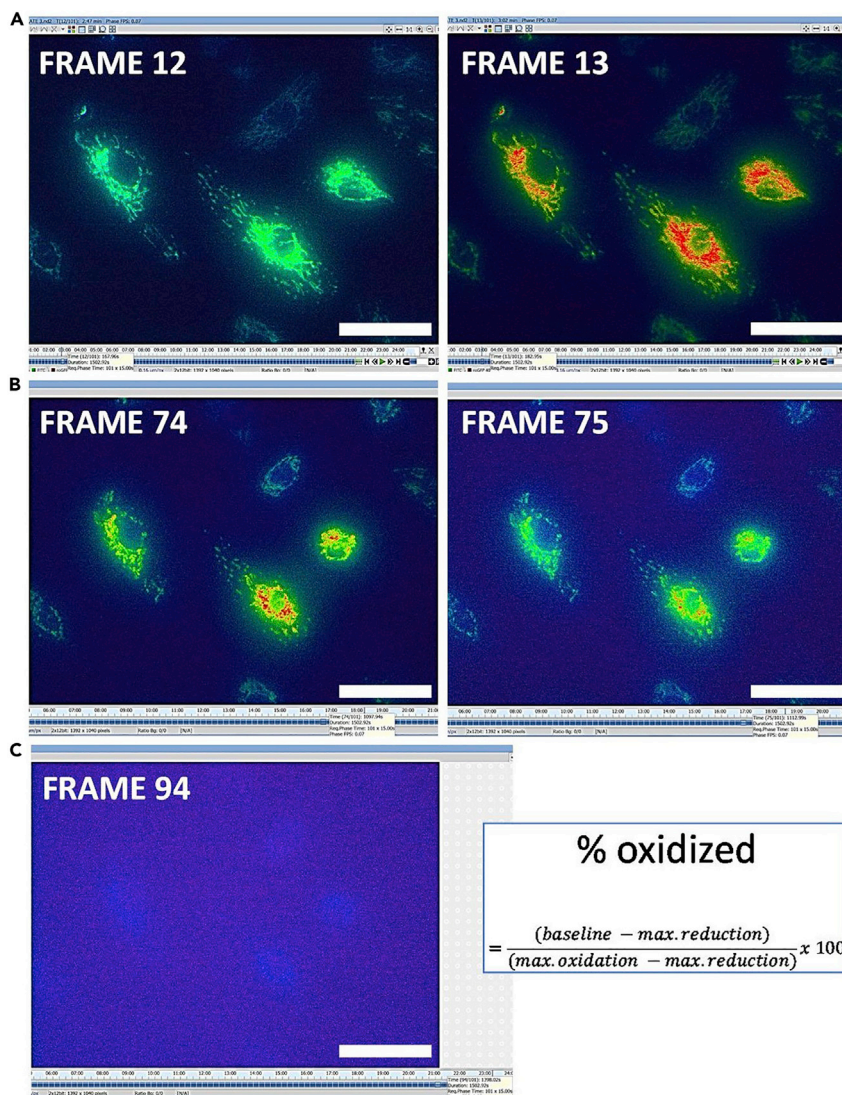


Figure 9. Example of frame selection for identification of the different acquisition intervals

Addition of DTT will result in a color change at the ratio channel, from green (frame 12) to red (frame 13), as shown in (A). This color change can be used for correct selection of the frame corresponding to maximal reduction (Frame 13). After addition of H_2O_2 , the color will gradually change, as seen in frames 74 (before addition of H_2O_2) and 75 (after addition of H_2O_2) (B). However, it can take several minutes to reach full oxidation, which can be identified by the predominance of blue color (frame 94), as shown in (C). In this case, frame 94 corresponds to the maximum oxidation. After correct frame selection, the corresponding values can be used to calculate the % oxidized, as shown in (C). Note that ratio is shown as 488/405 nm. Scale bar represents 50 μ m.

be excluded from analysis. In addition, endothelial cells are resistant to oxidation by nature, which means they may take an extended time to achieve full oxidation following addition of H_2O_2 . This is especially true when using antioxidants as a test treatment. Furthermore, although this protocol works in both highly reduced and highly oxidized samples, the concentration of DTT, H_2O_2 or TBH has to be optimized for both conditions. Finally, we note that it may not be possible to compare highly reduced versus highly oxidized samples as part of one single experiment as they will most likely require very different concentrations of DTT/ H_2O_2 .

TROUBLESHOOTING

Problem 1

Cells are not fluorescent (not expressing roGFP) after transduction. This problem may arise at step 6f.

Potential solution

HCAEC usually express a good level of roGFP 24 h after transduction. Depending on the cell type, e.g., murine EC, a longer incubation time (up to 72 h) may be required after the transduction procedure. In this case, change the media every day and examine the cells under the microscope with FITC filter to check for fluorescence signal. Alternatively, the adenovirus load may need to be increased to achieve the expected fluorescence, however the same concentration of adenovirus should be loaded in both control and test samples to account for changes in baseline ROS induced by the adenovirus transduction. Another solution is to repeat the amplification process or use a new stock vial or single aliquot as mito-roGFP may have been degraded by repeated freeze and thaw cycles. In case of unsuccessful optimization of this protocol in other cell types, we note that there are alternative options with lentivirus and retrovirus-associated mito-roGFP transduction that can access oxidized state of different mitochondrial systems, such as peroxisome and glutathione, some examples are summarized in a Table provided on the Mendeley Data.

Problem 2

ECs are not predominantly exhibiting green color in the 405/488 ratio channel, or the ratio is not close to 1 when starting the acquisition of the standard/control sample. This problem may arise at step 6f.

Potential solution

Adjust the exposure and gain of the 488 and 405 nm channels to a similar level of fluorescence between both channels (please note that the same settings must be used for all samples).

Problem 3

Cells do not change color to purple nor red with the addition of DTT or H₂O₂, respectively. This problem may arise at steps 7a and 8a.

Potential solution

Increase the concentration of DTT or H₂O₂ until you visualize adequate color change without losing the ROIs. As an alternative, H₂O₂ may be replaced by tBH. Although we found inconsistent results using tBH, prior research has shown successful results in other cell types (please refer to the second table under the [materials and equipment](#) section). Please refer to the notebook notes available on Mendeley dataset for an example of H₂O₂ concentration troubleshooting. Note that more oxidized samples will require a higher concentration of DTT (to fully reduce the sensor) and, consequently, H₂O₂ (to fully oxidize the sensor), and more reduced samples may require a lower concentration of DTT and, consequently, of H₂O₂.

Problem 4

Cells shrink and wash away when adding DTT or H₂O₂. This problem may arise at steps 7 and 8.

Potential solution

Reduce the concentration of DTT or H₂O₂ until you visualize adequate color change without losing the ROIs.

Problem 5

Color of cells at the ratio channel suddenly changes without addition of chemicals. This problem may arise at any step during image acquisition at day three.

Potential solution

This may happen due to an error during filter change. Annotate the frame number and exclude that frame from analysis. Observe the cells to see if filters will correctly change in the next frame. If error continues, the recording will have to be restarted. In addition, the interval between filter change may have to be increased depending on the capacity of your imaging system.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. M. Ruhul Abid (ruhul_abid@brown.edu).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

Original data for ND files, spreadsheets in the paper have been deposited to Mendeley Data: <https://doi.org/10.17632/n5s62h3zcc.1>

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, R.B.T. and M.R.A. Validation and formal analysis, R.B.T.; investigation, R.B.T. and C.K.; resources, supervision, project administration, and funding acquisition, M.R.A.; writing – original draft, R.B.T, C.K., and M.S.; writing – review & editing, R.B.T, C.K., M.S., and M.R.A.; visualization, R.B.T. and C.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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