

## Video Article

# Visualization of Vascular Ca<sup>2+</sup> Signaling Triggered by Paracrine Derived ROS

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## Abstract

Oxidative stress has been implicated in a number of pathologic conditions including ischemia/reperfusion damage and sepsis. The concept of oxidative stress refers to the aberrant formation of ROS (reactive oxygen species), which include O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and hydroxyl radicals. Reactive oxygen species influences a multitude of cellular processes including signal transduction, cell proliferation and cell death<sup>1-6</sup>. ROS have the potential to damage vascular and organ cells directly, and can initiate secondary chemical reactions and genetic alterations that ultimately result in an amplification of the initial ROS-mediated tissue damage. A key component of the amplification cascade that exacerbates irreversible tissue damage is the recruitment and activation of circulating inflammatory cells. During inflammation, inflammatory cells produce cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and IL-1 that activate endothelial cells (EC) and epithelial cells and further augment the inflammatory response<sup>7</sup>. Vascular endothelial dysfunction is an established feature of acute inflammation. Macrophages contribute to endothelial dysfunction during inflammation by mechanisms that remain unclear. Activation of macrophages results in the extracellular release of O<sub>2</sub><sup>-</sup> and various pro-inflammatory cytokines, which triggers pathologic signaling in adjacent cells<sup>8</sup>. NADPH oxidases are the major and primary source of ROS in most of the cell types. Recently, it is shown by us and others<sup>9,10</sup> that ROS produced by NADPH oxidases induce the mitochondrial ROS production during many pathophysiological conditions. Hence measuring the mitochondrial ROS production is equally important in addition to measuring cytosolic ROS. Macrophages produce ROS by the flavoprotein enzyme NADPH oxidase which plays a primary role in inflammation. Once activated, phagocytic NADPH oxidase produces copious amounts of O<sub>2</sub><sup>-</sup> that are important in the host defense mechanism<sup>11,12</sup>. Although paracrine-derived O<sub>2</sub><sup>-</sup> plays an important role in the pathogenesis of vascular diseases, visualization of paracrine ROS-induced intracellular signaling including Ca<sup>2+</sup> mobilization is still hypothesis. We have developed a model in which activated macrophages are used as a source of O<sub>2</sub><sup>-</sup> to transduce a signal to adjacent endothelial cells. Using this model we demonstrate that macrophage-derived O<sub>2</sub><sup>-</sup> lead to calcium signaling in adjacent endothelial cells.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3511/>

## Protocol

Reactive oxygen species can be measured in live cells using oxidation sensitive dyes (1 &amp; 2) or using plasmid sensors (3 &amp; 4) by confocal microscopy.

### 1. Visualization of cytosolic ROS in J774 cells

1. Grow J774.1 mouse monocyte-derived macrophages (10<sup>6</sup> cells/ml) on glass bottom 35-mm dishes (Harvard Apparatus) for 48 h.
2. Challenge cells with TLR agonists (2  $\mu$ g/ml, Lipo-teichoic acid-TLR2 agonist; 10 $\mu$ g/ml, Poly (I:C)-TLR3 agonist; 1  $\mu$ g/ml LPS-TLR4 agonist) for 6 h at 37°C.

Note: Macrophages treated under similar conditions were used for co-culture model Ca<sup>2+</sup> mobilization.

3. Add the oxidation-sensitive dye H<sub>2</sub>DCF-DA (Invitrogen) to give a final concentration of 10  $\mu$ M in ECM (Extracellular medium: 121 mM NaCl, 5 mM NaHCO<sub>3</sub>, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose and 2.0% dextran, pH 7.4; all obtained from Sigma) containing 2%BSA to dishes and incubate cells for 20 min before visualization under confocal microscopy.
4. After dye loading, visualize cells placed on a temperature controlled stage of an appropriate imaging system. We use Carl Zeiss LSM 510 Meta confocal imaging system coupled with Argon ion laser source at an excitation of 488 nm for DCF fluorescence using 40x oil objective<sup>13</sup> (Fig. 1A and D).
5. Analyze images using ImageJ software (1.44), freely available software from the National Institutes of Health (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009).

### 2. Visualization of mROS in J774 cells

1. Grow J774.1 mouse monocyte-derived macrophages (10<sup>6</sup> cells/ml) on glass bottom 35-mm dishes (Harvard Apparatus) for 48 h.

2. Challenge the cells with TLR ligands to induce mROS production (2 µg/ml, Lipo-teichoic acid-TLR2; 10µg/ml, Poly (I:C)-TLR3; 1 µg/ml LPS-TLR4) for 6 h at 37°C.
3. Add mitochondrial superoxide indicator MitoSOX Red (Invitrogen) to give a final concentration of 5 µM in ECM containing 2%BSA to dishes and incubate cells for 30 min at 37°C to allow loading of MitoSOX Red.
4. After dye loading, visualize cells on a temperature controlled stage of a Carl Zeiss LSM 510 Meta confocal imaging system couple with Argon ion laser source at an excitation of 561 nm for MitoSOX Red fluorescence using 40x oil objective (Fig 1B and E).
5. For control experiments, after dye loading add PBS or DMSO (negative control) or 2µM Antimycin A (as mitochondrial superoxide generator-positive control) to cells prior to image the sample as described in 2.4 (Fig 1C).
6. Analyze the images using ImageJ software (1.44).

### 3. Visualization of cytosolic ROS in stable HyPer-cyto MPMVECs

1. We use pHyPer-cyto (Evrogen) a mammalian expression vector encoding a fluorescent sensor HyPer that localizes in the cytoplasm and specifically senses submicromolar concentration of H<sub>2</sub>O<sub>2</sub><sup>14</sup>.
2. Transfect Murine Pulmonary Microvascular Endothelial cells (MPMVECs) with pHyPer-cyto vector using either electroporation or transfection reagents. We use 10 x 10<sup>6</sup> cells and transfect with 10µg of pHyPer-cyto via electroporation using BioRad Gene pulser electroporator system (250V; 500 µF). Culture MPMVECs in DMEM medium supplemented with 10% FBS, nonessential amino acids, endothelial growth supplement and antibiotics.

Note: Following transfection, plate cells at low density to facilitate colony growth.

3. Replace medium with complete culture medium supplemented with 500 µg/ml G418, 48 hours after transfection. Screen for clones that have highest percentage of HyPer positive cells and passage HyPer positive clones to increase the number of HyPer positive cells (Fig. 2).

Note: We use serial dilution of cells for clonal selection of single cells and screen for stable cells highly expressing HyPer.

4. Grow stable HyPer-cyto MPMVECs on 0.2% gelatin coated coverslips (80% confluence). Next day challenge cells with TLR ligands (2 µg/ml, Lipoteichoic acid-TLR2; 10µg/ml, Poly (I:C)-TLR3; 1 µg/ml LPS-TLR4) for 6 h at 37°C. Untreated cells serve as control.
5. After treatment, mount the coverslips onto Attofluor cell chamber (Invitrogen). Add 1ml of pre warmed Hanks balanced salt solution (HBSS) to the chamber and place the chamber on a temperature controlled stage of Carl Zeiss LSM 510 Meta confocal imaging system. Visualize and image fluorescence change at an excitation of 488 nm using 40x oil objective (Fig. 3A and D).
6. Analyze images using ImageJ software (1.44) (see section 6).

### 4. Visualization of mitochondrial ROS in stable HyPer-dMito MPMVECs

1. We use pHyPer-dMito (Evrogen) a mammalian expression vector encoding mitochondria-targeted HyPer that localizes in the mitochondria and specifically senses submicromolar concentration of H<sub>2</sub>O<sub>2</sub>.
2. Transfect MPMVECs with pHyPer-dMito and generate stable HyPer-dMito MPMVECs following steps described in section 3.2-3.3.
3. Grow stable HyPer-dMito MPMVECs on 0.2% gelatin coated coverslips (80% confluence).
4. Next day challenge cells with TLR ligands (2 µg/ml, Lipoteichoic acid-TLR2; 10µg/ml, Poly (I:C)-TLR3; 1 µg/ml LPS-TLR4) for 6 h at 37°C. Untreated cells serve as negative control. Add 2µM Antimycin A (mitochondrial superoxide inducer-positive control) prior to visualizing sample.
5. After treatment, mount the coverslips onto Attofluor cell chamber (Invitrogen). Add 1ml of pre-warmed Hanks balanced salt solution (HBSS) to the chamber and place the chamber on a temperature controlled stage of Carl Zeiss LSM 510 Meta confocal imaging system. Visualize and image fluorescence change at an excitation of 488 nm using 40x oil objective (Fig. 3B, C and E).
6. Analyze images using ImageJ software (1.44) (see section 6).

### 5. Co-culture model for visualization of paracrine-derived ROS triggered Ca<sup>2+</sup> signaling in endothelial cells

For the visualization and measurement of [Ca<sup>2+</sup>]<sub>i</sub> changes we use a Ca<sup>2+</sup> sensitive fluorescent dye fluo-4 AM (Invitrogen).

1. Grow MPMVECs on 0.2% gelatin coated 25mm glass coverslips (80% confluence).
2. Incubate the cells grown on coverslips at room temperature for 30 min in ECM containing 2%BSA and 0.003% pluronic acid with 5 µM fluo-4 AM (Invitrogen) final concentration to allow loading of fluo-4 AM. After loading, wash cells with experimental imaging solution (ECM containing 0.25% BSA) containing sulfinpyrazone to minimize dye loss.
3. After loading fluo-4, mount coverslips onto Attofluor cell chamber (Invitrogen). Add 800µl of pre warmed experimental imaging solution to the chamber and place it on a temperature controlled stage of Carl Zeiss LSM 510 Meta confocal imaging system.
4. In a separate tube label the nonactivated or activated macrophages isolated from either wild-type or gp91<sup>phox-/-</sup> mice (treated with LPS for 6 hr) with cell tracker red CMTPIX (500ng/ml) (Invitrogen) in ECM containing 2%BSA and 0.003% pluronic acid. Wash and resuspend cells with 200µl of experimental imaging solution.
5. Add labeled macrophages on to fluo-4 AM loaded MPMVECs in the chamber assembly.
6. Record the green and red fluorescence every 3s for 10-20 min using Carl Zeiss LSM510 Meta confocal imaging system with a Argon ion laser source with excitation at 488 and 561 nm for fluo-4 AM and cell tracker red fluorescence respectively ( Fig.4 A-C).

Note: Alternatively, J774.1 cell line could be used to elicit MPMVECs Ca<sup>2+</sup> mobilization.

7. Analyze fluo-4 fluorescence intensity changes using ImageJ software (1.44) (see section 6).

### 6. Data analysis using ImageJ software

Transfer the images obtained from the confocal system as a .ism format file for data analysis. ZEN 2009 and ZEN 2010 software provided with LSM 510 Meta confocal microscope or LSM 710 multiphoton confocal microscope respectively saves files in .ism format. We recommend using this native .ism files for image analysis as original quality is retained. This file format can be directly opened in image analysis program Image J. The following steps provide an example quantitative analysis of single image. No additional plugins are required for this data analysis.

1. Open .ism or .TIFF file in Image J program using FILE>OPEN
2. Click the tab IMAGE>COLOR>SPLIT CHANNELS and split the green and DIC channels. Use the image from the green channel for subsequent analysis.
3. Double click and select the 'Elliptical brush selection" tab and set the pixel value to 20.
4. For Hyper-cyto images, click on a single cell to define the circular area of measurement. Then press "M" key to measure the intensity of the selected area. The values are retained in a new results window.
5. Next by holding "Shift" key click on a different cell to select another area, followed by pressing "M" key. The intensity is recorded in the result window. Repeat the steps 4-5 on 50 different cells.
6. For Hyper-d-mito images, set the pixel value to 10 in the Elliptical brush selection. Region of interest (ROI) has to be manually traced including the mitochondria of particular cells.
7. Using freehand, trace the region of around the mitochondria and then press "M". Repeat the steps 6-7 on 50 different cells.
8. Copy the mean values from the results window and paste in a Excel sheet or any software to plot the results. We use either SigmaPlot or Graphpad PRISM to plot the data as graph.

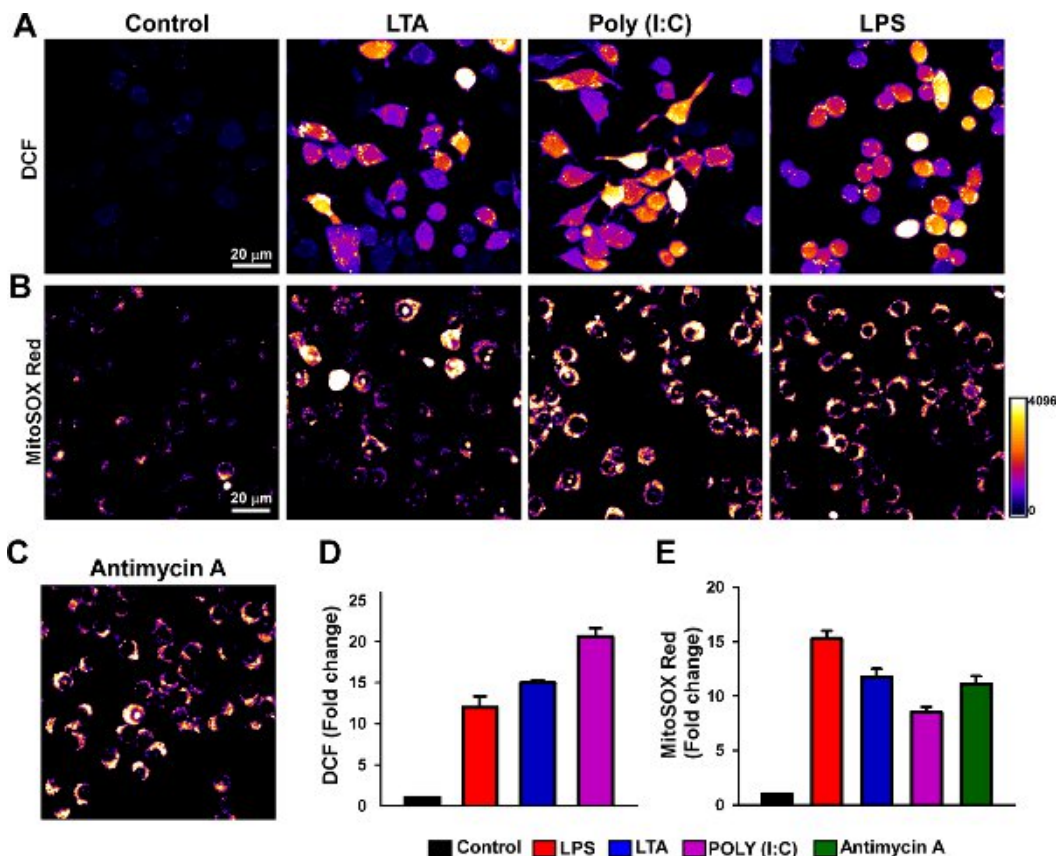
## 7. Representative Results

Figure 1 shows cytosolic and mitochondrial ROS production upon challenge with TLR ligands. Representative confocal images show increase of DCF (cytosolic) and MitoSOX Red (mitochondria) fluorescence after 6 hr of stimulation. Fluorescence changes were normalized and expressed as fold change.

Figure 2 shows the generation of stable HyPer-cyto and HyPer-dMito endothelial cells. Mouse endothelial cells were transfected with pHyPer-cyto or pHyPer-dMito plasmid constructs via electroporation. Cells stably expressing plasmids were selected with G418 for two weeks. After selection, diluted cells were seeded in 96 well plates. Individual colonies were isolated and imaged for homogeneous expression.

Figure 3 shows the measurement of endothelial cytosolic and mitochondrial H<sub>2</sub>O<sub>2</sub> levels. Representative confocal images show increase of HyPer-cyto (cytosolic) and HyPer-dMito (mitochondria) fluorescence after 6 hr of stimulation. Fluorescence intensity changes were normalized and expressed as fold change.

Figure 4 shows the assessment of macrophage-derived ROS induced endothelial cytosolic Ca<sup>2+</sup> mobilization. LPS-stimulated macrophages were added onto pulmonary microvascular endothelial cells (PMVEC) that had been previously loaded with the intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) indicator dye Fluo-4. Application of wild type LPS-activated macrophages evoked an [Ca<sup>2+</sup>]<sub>i</sub> rise in PMVECs that was attenuated by knockout of functional NADPH oxidase (gp91<sup>phox-/-</sup>).



**Figure 1. Visualization of macrophage generated cellular ROS and mitochondrial superoxide. J774.1 cells were challenged with TLR2, 3 and 4 ligands.** (2 µg/ml, Lipoteichoic acid-TLR2; 10µg/ml, Poly (I:C)-TLR3; 1 µg/ml LPS-TLR4) for 6 h at 37°C. After treatment cells were loaded with (A) cellular ROS indicator (H<sub>2</sub>DCF-DA; green fluorescence) or (B) mitochondrial superoxide indicator (MitoSOX Red; red fluorescence). (C) Antimycin A was used as a positive control for mitochondrial ROS production. Fluorescence intensity changes were assessed using confocal microscopy. (D) and (E) Quantitation of mean fluorescence change.

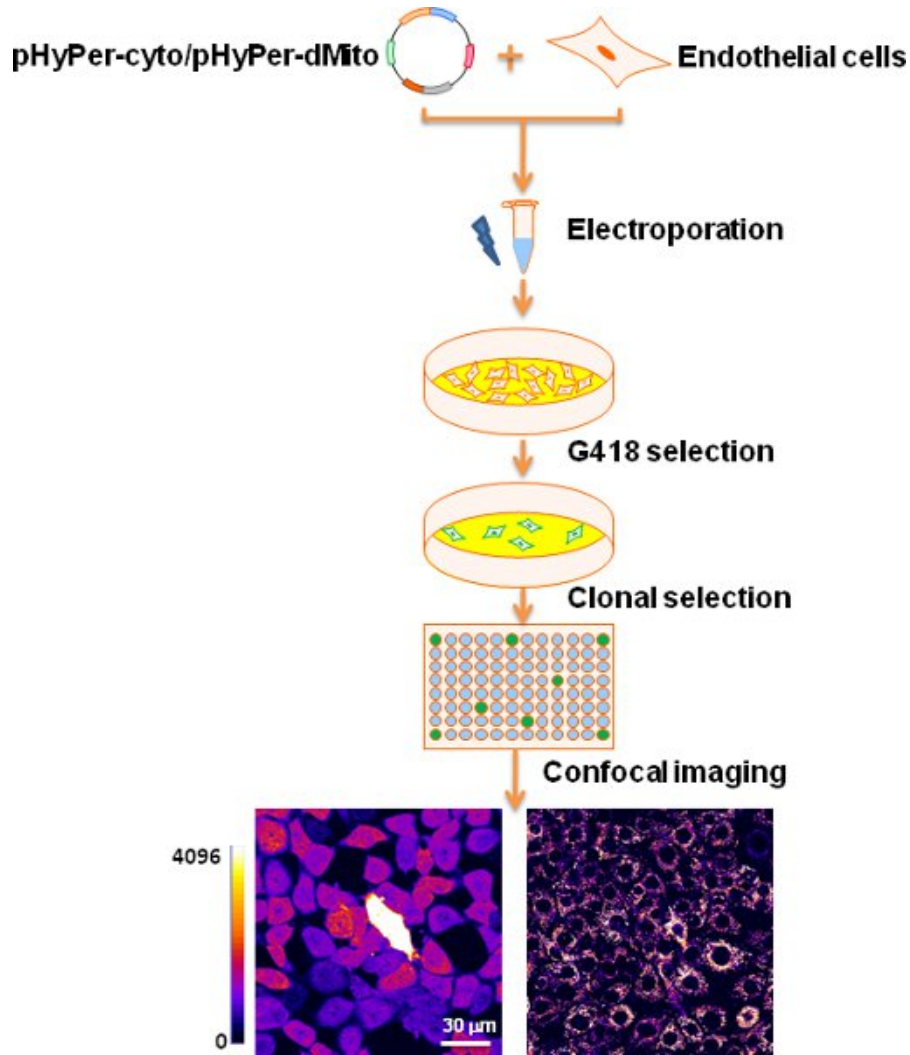
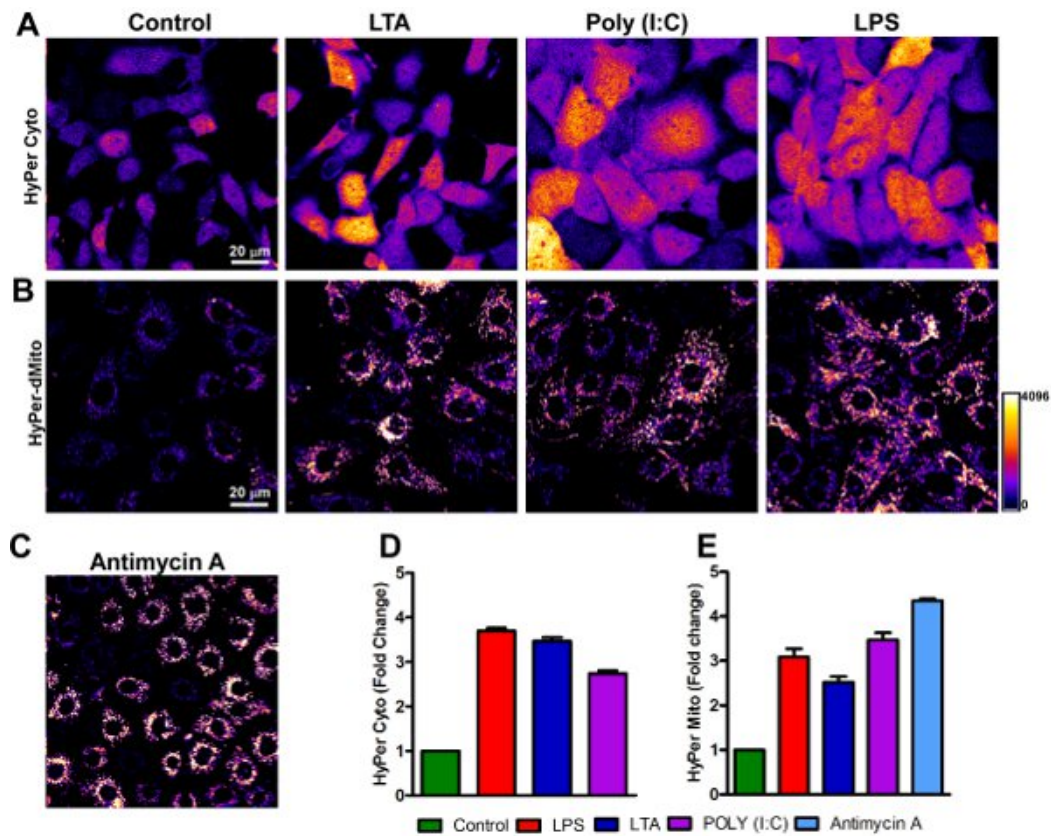


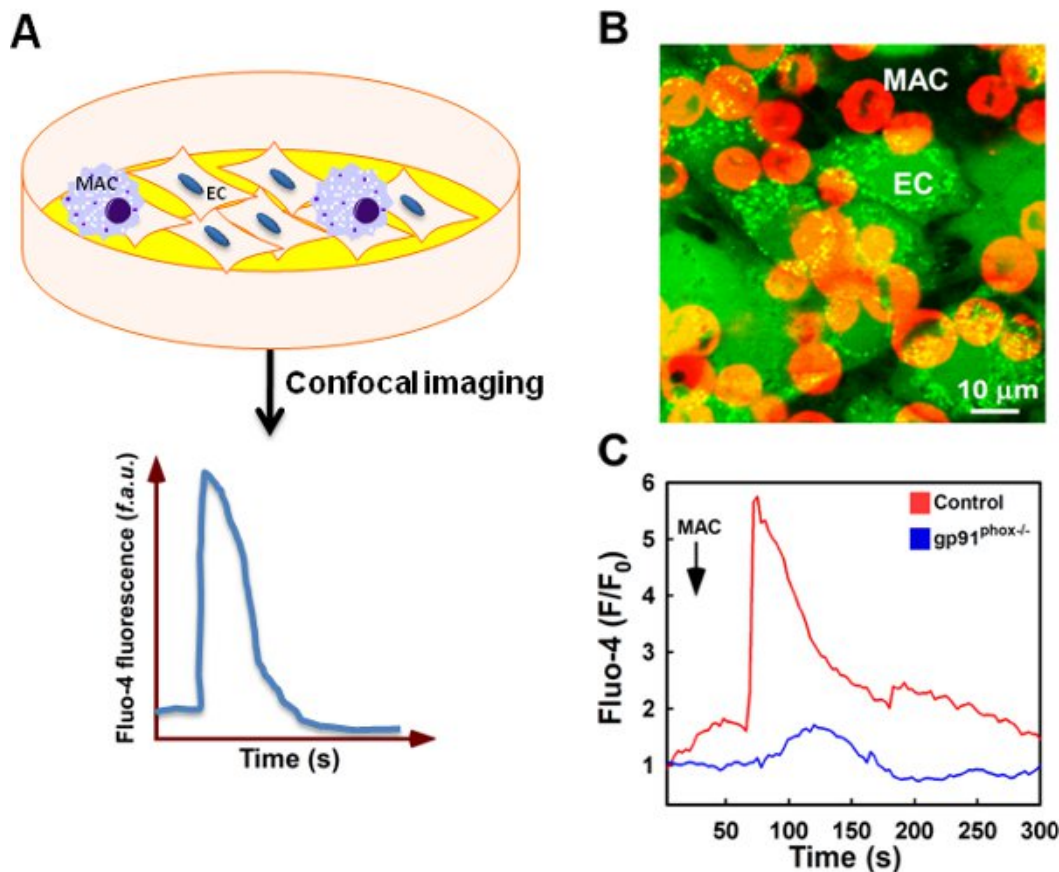
Figure 2. Schematic representation of generating stable expression of endothelial cell cytosolic and mitochondrial ROS indicators.





**Figure 3. Visualization of endothelial cytosolic and mitochondrial H<sub>2</sub>O<sub>2</sub> levels.** MPMVECs stably expressing (A) HyPer-cyto or (B) HyPer-dMito were challenged with TLR2, 3 and 4 ligands (2 µg/ml, Lipoteichoic acid-TLR2; 10µg/ml, Poly (I:C)-TLR3; 1 µg/ml LPS-TLR4) for 6 h at 37°C. (C) Antimycin A was used as a positive control for mitochondrial ROS production. After treatment, fluorescence intensity changes were assessed using confocal microscopy. (D) and (E) Quantitation of mean fluorescence change.

## Endothelial cells/macrophage co-culture model study



**Figure 4. Macrophage-derived ROS elicit impaired endothelial cell Ca<sup>2+</sup> mobilization.** (A) Schematic representation of endothelial cells/macrophage co-culture model study. (B) Freshly isolated murine macrophages from both WT and gp91<sup>phox</sup> null mice (The Jackson Laboratory) were activated by 1  $\mu$ g/ml LPS for 6 h. Macrophages were labeled with cell-tracker Red (red) and added onto Fluo-4 loaded PMVECs (green) to assess paracrine O<sub>2</sub><sup>-</sup> signaling. (C) LPS-activated macrophages isolated from WT mice triggered large and rapid Ca<sup>2+</sup> mobilization in PMVECs compared to macrophages from gp91<sup>phox</sup> null mice. Validation of macrophage activation has been demonstrated previously.

### Discussion

The method described here allows rapid and quantitative measurement of reactive oxygen species in living cells either using oxidation sensitive dyes or plasmid sensors. Agonists of TLRs (Toll-like receptors) are compounds that stimulate the cells through the TLRs present on the cell surface and trigger the downstream signaling pathways<sup>15</sup>. In our protocol, we used three different TLR agonists viz., Lipo-teichoic acid-TLR2 agonist; Poly (I:C)-TLR3 agonist; LPS-TLR4 agonist which are reported to induce ROS as a model system to demonstrate the ROS measurement. Measurement of ROS using oxidants sensitive dye is rapid and can be used to measure variety of ROS species. The use of plasmid based sensor HyPer selectively detects H<sub>2</sub>O<sub>2</sub> levels. H<sub>2</sub>O<sub>2</sub> is more stable oxidant and also the use of HyPer cyto and HyPer-dMito in a stable cell setting adds the advantage over photo-oxidation of ROS sensitive fluorescent dyes. HyPer does not cause artifactual ROS generation and can be used for detection of fast changes of H<sub>2</sub>O<sub>2</sub> concentration in different cell compartments under various physiological and pathological conditions. Cells stably expressing the HyPer sensors are used for clonal selection of population that offers more reliable and accurate measurements of H<sub>2</sub>O<sub>2</sub> levels. Importantly, this method also describes the detection and quantitative measurement of Ca<sup>2+</sup> mobilization in live cells triggered by paracrine derived ROS from the neighboring cells such as innate immune cells. This experimental procedure combined with confocal microscopy may be very useful to reveal important spatio-temporal changes in cell signal transduction events especially in instances where two different cell types are involved. Though, the representative experiments detailed in this paper are just one example of the type of interaction studied, this technique can be easily adapted to study cellular interactions between multiple live cell types.

### Disclosures

No conflicts of interest declared.

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