**Title: A plate reader-based measurement of the cellular ROS production using Dihydroethidium and MitoSOX**

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**Abstract**

Intracellular reactive oxygen species (ROS) acts as an important signalling transductor in cells, regulating almost every expects of cell biology. Measurements of ROS production, thus, offer links oxidative stress and cell pathophysiology. Here, we describe a simple screening assay in intact adherent cells by fluorescence microplate readers, using Dihydroethidium (DHE) and MitoSOX to measure cytosolic superoxide and mitochondrial superoxide production, respectively. This assay enables a quick and reliable assessment of ROS generation in a well-controlled environment.

**Keywords**

Reactive oxygen species, dihydroethidium, MitoSOX, mitochondria, plate reader

**Introduction**

Mitochondria are the main sources of reactive oxygen species (ROS). Intracellular ROS plays important role in cell signalling, regulating cell growth/death, differentiation and metabolism under pathophysiological conditions. Aberrant ROS production also causes oxidative stress, damaging proteins and nucleic acids (such as DNA). Thus, measurement of the intracellular accumulation of ROS is therefore essential for providing links between mitochondrial dysfunction and oxidative stress in neurodegeneration diseases.

Here, we describe a simple method using Dihydroethidium (DHE) and MitoSOX for measuring cytosolic superoxide and mitochondrial superoxide production, respectively, in intact adherent cells by fluorescence microplate readers. This method is useful for screening experiments and can provide an overview of general ROS production.

DHE is an intracellular superoxide indicator which is oxidised by superoxide to forms a red fluorescent product, 2-hydroxyethidium. MitoSOX is a cationic derivative of DHE targeted to

the mitochondria and thus used to measure superoxide production in the mitochondrial matrix, which reacts with superoxide anions in a similar way with DHE. Despite some limitations [1], DHE and MitoSox are still convenient probes for measuring intracellular oxidative stress and redox status.

**Materials**

1. A fluorescent microplate reader with filter sets capable of measuring fluorescence at Excitation (ex) = 480-520 nm, Emission (em) = 570-600 nm.
2. Black, clear-bottom, tissue culture-treated 96-well plates.
3. Recording media: DMEM no phenol red with HEPES is used for cancer cell linens and fibroblasts; DMEM/F12 no phenol red with HEPES is used for neuron-progenitor cells (NPC) and neurons (**Note 1** and **2**). Adjust nutrients, such as glucose, sodium pyruvate and L-glutamine, according to your culture conditions/experimental settings.
4. Dihydroethidium (DHE): make a 5 mM stock solution in DMSO and aliquot to avoid freeze thaw cycles. Prepare a working solution of 5 µM DHE in recording media by diluting the stock solution (**Note 3**). Protect from light.
5. MitoSOX: make a 5 mM stock solution in DMSO and aliquot to avoid freeze thaw cycles. Prepare a working solution of 5 µM MitoSOX in recording media by diluting the stock solution (**Note 4**). Protect from light.
6. Antimycin A (AA): AA is a mitochondrial complex III inhibitor, which causes excessive ROS production when applied. Make a 10 mM stock solution in ethanol and prepare a 150 µM working solution by diluting 15 µl of the stock solution into 1 ml recording media.
7. N-acetyl Cysteine (NAC): NAC is an antioxidant that raises intracellular glutathione levels by providing cysteine, thereby upregulating the rate-limiting step in the synthesis of the glutathione tripeptide. Freshly make a 300 mM NAC working solution by dissolve 10 mg NAC in 200 µl recording media. Discard after experiments.

**Methods**

1. Two days before the experiments, cells are seeded into the clear-bottom 96-well plates (approximately 10,000 cells per well for cancer cell lines and 30,000-40,000 cells per well for fibroblasts and NPC; **Note 5**) and cultured in the media suited for users' cell lines. Ensure that cells reach around 80-90% of confluence on the day of the experiment (**Note 6**).

2. Designate at least two wells as positive (AA) and two wells as negative (NAC) controls (**Note 7**).
3. Carefully wash the positive and negative control wells with PBS twice and add 150 µl recording media.
4. Add 10 µl NAC to negative control wells and incubate for 30 min at 37°C.
5. After that, add 10 µl AA to positive control wells and incubate for an additional hour at 37°C.
6. Carefully wash all the wells with PBS twice and add 100 µl of 5 µM either DHE or MitoSOX to each well.
7. Set a plate reader to ex = 480-520 nm/em = 570-600 nm (**Note 8**). and measure the fluorescence at intervals of 2-5 min for 30-40 min (**Note 9** and **10**).
8. Plot the fluorescence intensity against time points of each well (**Fig. 1**). The slope of the linear range was used for analysis to determine and represents the rate of ROS production (**Note 11** and **12**) [2].

**Notes**

1. Other media without phenol red can also be used if it is more appropriate for specific cell types.
2. HEPES is not necessary If 5% $CO_2$ can be delivered by a plate reader.
3. Working concentration of DHE for measuring ROS production ranges from 0.5-5 µM [1–5]. Therefore, conducting a titration for DHE working concentrations to optimise your results is encouraged. The cyto- and mitotoxicity and the redistribution of fluorescence to the nuclei at higher concentrations of these probes should be considered.
4. Working concentration of MitoSOX ranges from 2-10 µM [1–4]. Therefore, conducting a titration to optimise your results is encouraged.
5. The initial cell numbers and the duration can and should be adjusted for specific cell types. As cell size and volume vary in cell types, performing a seeding titration is recommended.
6. If the confluence varies among wells with different cell types/treatments, normalisation of the ROS production should be considered. For example, DAPI/ Hoechst 33342 staining or BCA assay to obtain the relative cell numbers/amount of protein in each well.

7. Blank wells (without cells) can also be designated to remove the background change in fluorescence intensity caused by spontaneous oxidation of the probe to its fluorescent metabolite [6].
8. DHE itself (reduced form) displays blue fluorescence with ex/em = ~370/420. Monitoring the blue fluorescence change of DHE simultaneously can be used as an internal control.
9. The interval depends on the measurement speed of a plate reader and the number of wells.
10. Studies suggest that DHE is stable within 30 min [4] and MitoSOX tends to accumulate in the nucleus after approximately 40 min[3].
11. If blank wells are designated, remove average fluorescence background from the measurements at each time point.
12. DHE and mitoSOX can also undergo unspecific oxidation into ethidium, which is hard to distinguish from 2-dihydroxyethidium because of fluorescence spectra overlapping. This should be taken into account when interpreting the results [1].

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**Figure legends:**

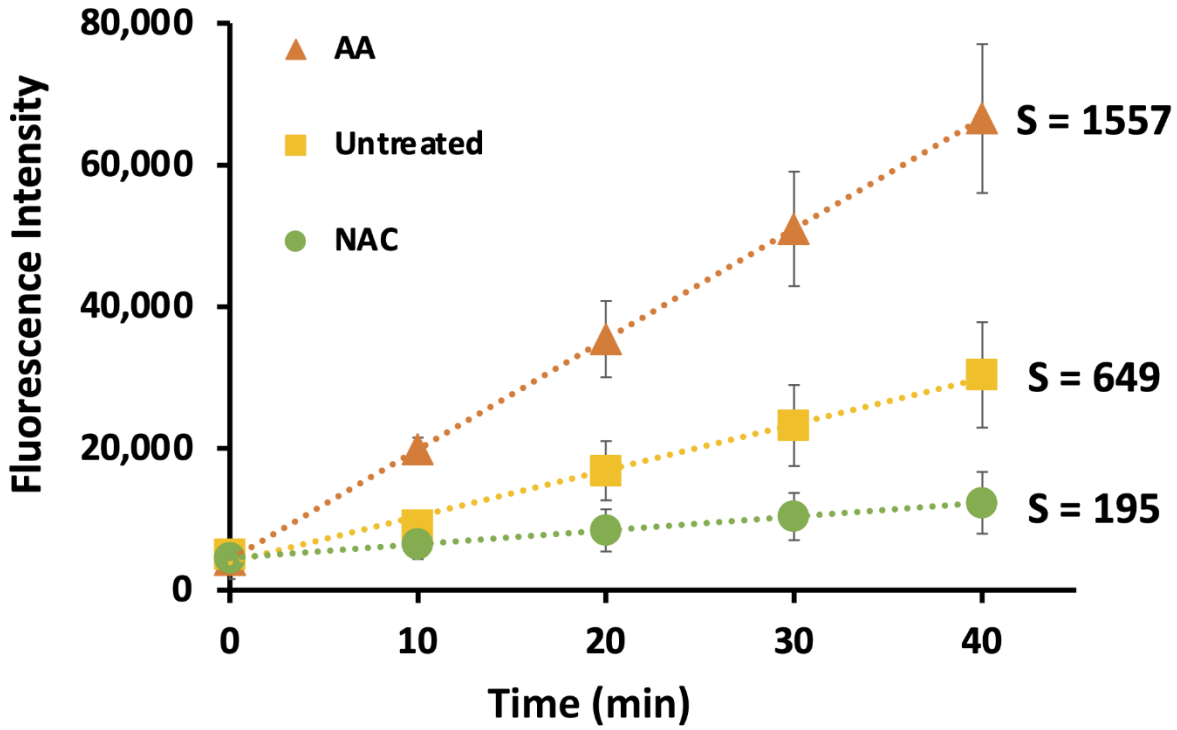


**Figure 1**. A scheme describing how kinetics of ROS production is measured in intact adherent cells, as the rate of increase in red fluorescence intensity over 40 min incubation with DHE/MitoSOX.