Chapter 18

Live-Cell Assessment of Reactive Oxygen Species Levels Using Dihydroethidine

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Abstract

Reactive oxygen species (ROS) play an important role in cellular (patho)physiology. Empirical evidence suggests that mitochondria are an important source of ROS, especially under pathological conditions. Here, we describe a method for ROS measurement using dihydroethidium (HEt) and live-cell microscopy.

Key words MitoSOX Red®, Mitochondrial membrane potential, Fluorescence imaging

Abbreviations

FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
HEt	dihydroethidium
HT	HEPES-Tris
mito-HEt	mito-dihydroethidium
ROS	reactive oxygen species
TPP	triphenylphosphonium
$\Delta\psi$	mitochondrial membrane potential

1 Introduction

Reactive oxygen species (ROS) can damage cellular biomolecules, but are also increasingly recognized as signaling molecules [1–4]. Among other sources, mitochondria are considered to be important contributors to ROS generation during healthy and pathological conditions [5]. The primary ROS superoxide ($O_2^{\cdot -}$) is produced by membrane-bound parts of the respiratory chain and a number of soluble mitochondrial proteins. Although $O_2^{\cdot -}$ cannot traverse membranes and has only limited reactivity with biological targets, it might function as a redox signal in mitochondria

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[6–8]. Since mitochondrial signaling and cellular function are intricately linked, it is important to measure mitochondrial $O_2^{\cdot-}$ in the proper context—that is, the living cell. However, it has proven difficult to develop fluorescent probes or sensors that are specific for $O_2^{\cdot-}$ and sensitive enough to compete with superoxide dismutase (SOD), which converts $O_2^{\cdot-}$ to hydrogen peroxide at an extremely high rate [9]. Quantifying the oxidation of dihydroethidium (HEt) is a widely applied strategy to detect $O_2^{\cdot-}$ in living cells. HEt is membrane-permeable and reacts with $O_2^{\cdot-}$ to form the specific fluorescent product 2-hydroxyethidium (2-OH-Et^+) (Fig. 1a). In addition, HEt can act as a hydride acceptor, leading to oxidative formation of the nonspecific fluorescent product ethidium (Et^+) [10–12]. Whereas the fluorescence excitation peaks of 2-OH-Et^+ (Fig. 1b; upper blue curve) and Et^+ (lower blue curve) overlap at around 500 nm, 2-OH-Et^+ has one additional peak at 396 nm which has been used for more specific detection of 2-OH-Et^+ [9]. Still, the latter approach should be considered semiquantitative and not fully $O_2^{\cdot-}$-specific given the unknown reactivity of $O_2^{\cdot-}$ with SOD, the possible oxidation of HEt by cytochrome *c* and because HEt can catalyze $O_2^{\cdot-}$ dismutation [9, 13]. Given its lipophilic nature, HEt oxidation can in principle occur everywhere in the cell. Both 2-OH-Et^+ and Et^+ are positively charged and therefore accumulate: (1) in the nucleus where they intercalate with nucleic acids, and (2) in the mitochondrial matrix due to the inside-negative membrane potential ($\Delta\psi$) of this organelle. In an attempt to utilize $\Delta\psi$, a mitochondria-targeted variant of HEt was developed (MitoSOX Red®, aka Mito-HEt). The latter consists of HEt extended with a cationic triphenylphosphonium (TPP) side group [9]. Below we describe the critical loading procedure for different intact cells and methods to measure HEt-oxidizing ROS using live cell microscopy.

2 Materials

2.1 General

1. Cells cultured on a glass coverslip (ø24 mm, Thermo Scientific, Etten-Leur, The Netherlands) placed in a 35-mm CellStar tissue culture dish (Sigma-Aldrich) or disposable incubation chamber (Willco Wells BV) (*see* **Note 1**).
2. A microscope system with the following setup or similar; a monochromator (Polychrome IV, TILL Photonics) allowing for excitation with 405 and/or 490 nm light, a 525DRLP dichroic mirror (Omega Optical Inc.) and 565ALP emission filter (Omega Optical Inc) and an image capturing device (e.g., a CoolSNAP HQ monochrome CCD-camera (Roper Scientific)). The microscope should be equipped with an environmental control system to sustain cell viability.

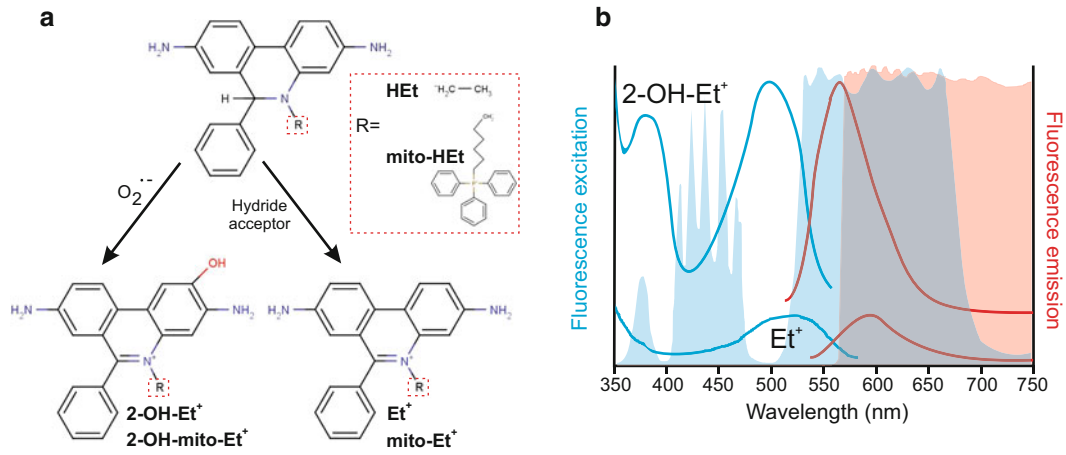

Fig. 1 Chemical structures and excitation spectra of HEt and mito-Het. (**a**) HEt and mito-HEt specifically react with $O_2^{\cdot -}$ to form the reaction product 2-OH-(mito-)Et^+ and (mito-)Et^+ as a nonspecific by-product. The inset shows the HEt- and mito-HEt-specific groups. (Figure was adapted from [17]). (**b**) Fluorescence excitation (blue) and emission (red) spectra of 2-OH-Et^+ (top traces) and Et^+ (lower traces) superimposed with the excitation and emission filters used in our microscope setup. (Figure was adapted from [18]. This figure was reproduced (with permission) from Ref. 19)

3. 5× HEPES buffer: 662.2 mM NaCl, 50 mM HEPES, 21.06 mM KCl, 6.1 mM $MgCl_2$ (*see* **Note 2**).
4. 1× HEPES-Tris buffer: 10 mM HEPES, 132 mM NaCl, 4.2 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM D-glucose. Adjust the pH to 7.4 using Tris-base (*see* **Note 3**).

2.2 Dihydroethidium (HEt)

1. HEt stock solution (31.7 mM in DMSO): Dissolve 1 mg of HEt powder (Invitrogen) in 100 µl DMSO. Prepare 10 µl aliquots in brown Eppendorf tubes, overlay with N_2 gas and store at −20 °C (*see* **Note 4**).
2. HEt working solution (5 mM in DMSO): Thaw an aliquot of HEt stock solution and add 53.4 µl of DMSO to yield the 5 mM HEt working solution.

2.3 Mito-Dihydroethidium (Mito-HEt)

Mito-HEt working solution (500 µM in DMSO): Dissolve 50 µg of MitoSOX Red® powder (Invitrogen) in 132 µl DMSO. Prepare 4 µl aliquots in brown Eppendorf tubes, overlay with N_2 gas and store at −20 °C.

3 Methods

3.1 Microscopy Imaging of Dihydroethidium (HEt) Oxidation

1. Seed the cells at such densities that the cells are 70–80% confluent at the time of imaging. This allows for subtraction of the background signal (*see* **Note 5**).

2. Transfer the cells to an incubator close to the microscope system at least 1 h prior to imaging (*see* **Note 1**).
3. Aliquot 2 μl of HEt working solution in Eppendorf tubes.
4. Take 1 ml of medium from the culture dish, add it to the aliquoted HEt and vortex for 5–10 s to prepare a final concentration of 10 μM (*see* **Note 6**).
5. Replace the rest of the medium from the dish by the medium–HEt solution.
6. Incubate the cells in a 37 °C, 5% CO_2 incubator for exactly 10 min (*see* **Note 7**).
7. Wash the coverslip three times with 1 ml PBS.
8. Replace the PBS by prewarmed HT buffer and mount the coverslip in a Leiden chamber on the microscope [14]. When using oil-based objectives be careful to remove all excess buffer from the bottom of the coverslip with a tissue to avoid optical artifacts.
9. Start loading of a next coverslip as soon as loading of the current one is complete.
10. Preferably, take images at both 405 and 490 nm excitation. Set the exposure time to 100 ms.
11. Record at least 10 different images, each containing ~15 cells (*see* **Notes 8** and **9**).

3.2 Microscopy Imaging of Mito-Dihydroethidium (Mito-HEt) Oxidation

1. Seed the cells at such densities that the cells are 70–80% confluent at the time of imaging. This allows for subtraction of the background signal.
2. Transfer the cells to an incubator close to the microscope system at least 1 h prior to imaging.
3. Thaw the 4 μl mito-HEt contents of a brown Eppendorf tube.
4. Take 1 ml of medium from the culture dish, add it to mito-HEt and vortex for 5–10 s to prepare a final concentration of 2 μM (*see* **Note 10**).
5. Replace the rest of the medium from the dish by the medium–mito-HEt solution.
6. Incubate the cells in a 37 °C, 5% CO_2 incubator for exactly 10 min (*see* **Note 7**).
7. Wash the coverslip three times with 1 ml phosphate buffered saline (PBS).
8. Replace the PBS by prewarmed HT buffer and mount the coverslip in a Leiden chamber on the microscope [14]. When using oil-based objectives be careful to remove all excess buffer from the bottom of the coverslip with a tissue to avoid focusing problems.

9. Start loading of a next coverslip as soon as loading of the current one is complete.
10. Preferably, take images at both 405 and 490 nm excitation. Set the exposure time to 500 ms.
11. Record an image sequence of one field of view, acquiring one image every 5 or 10 s for a total of at least 2 min (*see* **Note 11**).

3.3 Image Analysis

1. Open the raw images in an image analysis program such as Metamorph® (Molecular Devices Corporation, Palo Alto, CA, USA), Image Pro Plus (Media Cybernetics) or the open-source software FIJI (http://fiji.cs/).
2. Draw circular regions of interest (ROIs) in: (1) a mitochondria-dense area, (2) surrounding the nucleus, and (3) just outside each individual cell to correct for background intensity (*see* **Note 9**).
3. Export the average ROI gray value to a spreadsheet program such as Excel (Microsoft) and calculate the background-subtracted values of the mitochondria and the nucleus of each cell.

4 Notes

1. Due to the positive charge of mito-HEt and the reaction products of HEt oxidation, the fluorescence intensity measured in the mitochondria is $\Delta\psi$-dependent (Fig. 2). Since the mitochondrial membrane potential is very sensitive to environmental changes (e.g., temperature, pH), we advise to culture the cells in separate dishes and to allow for cells to recover in an incubator close to the microscope system at least 1 h prior to imaging. In addition, we have noticed that HEt can react with residues on a number of glass-bottom culture dishes, leading to fluorescent "spots" in the background. Therefore, always check background levels for disturbances.
2. We store a large volume of 5× HEPES buffer at 4 °C, which is stable for at least 6 months.
3. We advise to make the 1×HT buffer supplemented with the required substrates at the day of imaging. We routinely use 5.5 mM D-glucose for human skin fibroblasts and myoblasts/fibers, whereas HEK293 cells are imaged in HT buffer containing 25 mM D-glucose. In addition, one can consider supplementing pyruvate and/or glutamine. If necessary, the HT buffer can be stored for ~1 week at 4 °C.
4. Always keep HEt solutions protected from light and air.

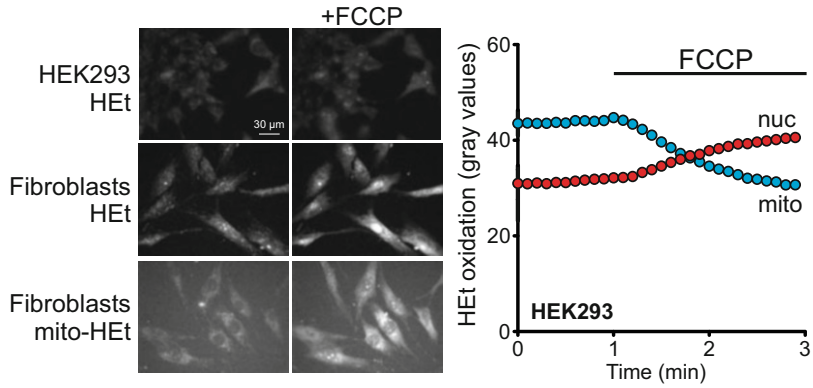

Fig. 2 Localization of HEt oxidation products is dependent on the membrane potential. Addition of the mitochondrial protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 0.5 µM) acutely induces translocation of fluorescent HEt and mito-HEt oxidation products from the mitochondria to the nucleus in HEK293 cells and human skin fibroblasts. (This figure was reproduced (with permission) from Ref. 19)

5. Proper background correction close to the cells is of particular importance when the fluorescent signal is close to the background intensity. Such a background correction is challenging when confluency exceeds 80%. Therefore, adjust the seeding condition according to the number of days the cells are cultured.
6. We prefer to load the cells with HEt in the collected cell culture medium to detect ROS levels in the exact cell culture conditions. However, we have noticed lower fluorescence intensities in medium-loaded cells as compared to cells loaded with HEt in the HT buffer. This is possibly caused by binding of the HEt to proteins present in the medium (i.e., serum), but might also be due to increased fluorescence levels in HT medium (Fig. 3a).
7. The HEt incubation time should be determined experimentally for each cell line separately. To be able to semiquantitively measure HEt oxidation, the increase in fluorescence intensity from oxidation products should be linear. To that end, we mount unloaded cells onto the microscope system in HT buffer, start imaging every 10 s and add the required HEt in HT buffer in 1:1 ratio. We then calculate the maximum time of incubation in which the increase is still linear. An incubation time of 10 min is most often used for HEK293 and human skin fibroblasts (Fig. 3b). However, in some cell types such as primary mouse myotubes it appears to be safer to use shorter incubation times because the signal increase deviates from linearity within 10 min (Fig. 3c).
8. When attempting to image HEt oxidation using 405 nm excitation, we advise to use the 490 nm light to locate cells and find

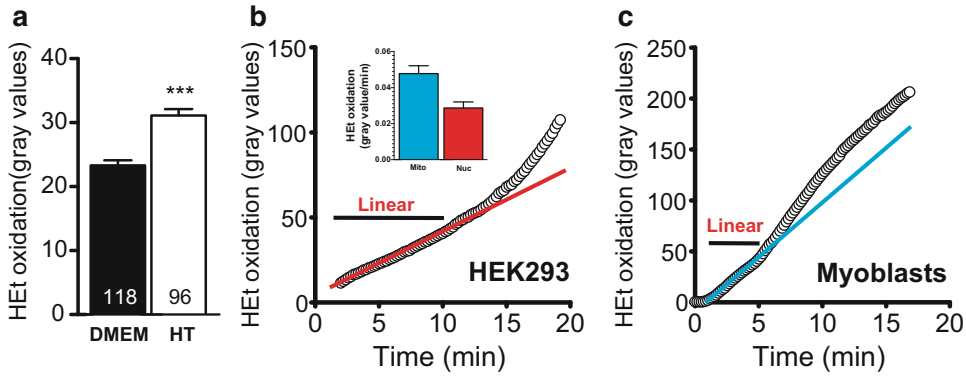

Fig. 3 HEt loading procedure in intact cells. (**a**) HEK293 cells were incubated with HEt for exactly 10 min in the collected cell culture medium (DMEM) or washed and incubated for exactly 10 min in HT buffer. HEt oxidation was measured at 490 nm. The data presented represent the mean ± SE of two different experiments. Statistical significance was assessed using a Mann–Whitney test. Numerals within bars indicate the number of analyzed cells. (**b**) Representative trace of nuclear HEt oxidation fluorescence intensity at 490 nm excitation light in HEK293 cells. Up to approximately 10 min the increase remains linear. Note that the mitochondrial increase occurs faster in these cells than in the nucleus. (**c**) Representative trace of mitochondrial HEt oxidation fluorescence intensity at 490 nm in myoblasts. The increase is linear during 4.5 min. (This figure was reproduced (with permission) from Ref. 19)

a good focus, because in our experimental setup fluorescence intensity is higher at 490 nm than at 405 nm excitation (Fig. 4a). Moreover, we routinely only measure fluorescence from 490 nm excitation since, in some cell types, the 405 nm signal is too low for reliable quantification. However, relative changes in emission fluorescence observed at 405 and 490 nm excitation are similar, justifying the use of 490 nm as a measure of HEt oxidation [15].

9. It is sufficient to have only a portion of the cells in the field of view, since only part of the cell is analyzed.
10. We advise to use a mito-HEt concentration that is as low as possible, but still revealing detectable levels of fluorescence. In theory, the positive charge accumulating in the mitochondrial matrix might interfere with mitochondrial bioenergetics and therefore report incorrect HEt oxidation values. In addition, it was reported that nuclear fluorescence occurred at concentrations as low as 2 μM and therefore to use a concentration of mito-HEt between 0.1 and 2.5 μM [9].
11. Removing excess HEt after incubation effectively removes all nonoxidized HEt and therefore the signal remains stable for at least 10 min of continuous imaging [16]. The TPP moiety of Mito-HEt induces accumulation of nonoxidized Mito-HEt in the mitochondrial matrix that is insensitive to washing. Consequently, the fluorescence signal continues to increase after washing and therefore we advise to measure the slope of the increase after loading and washing (Fig. 4b).

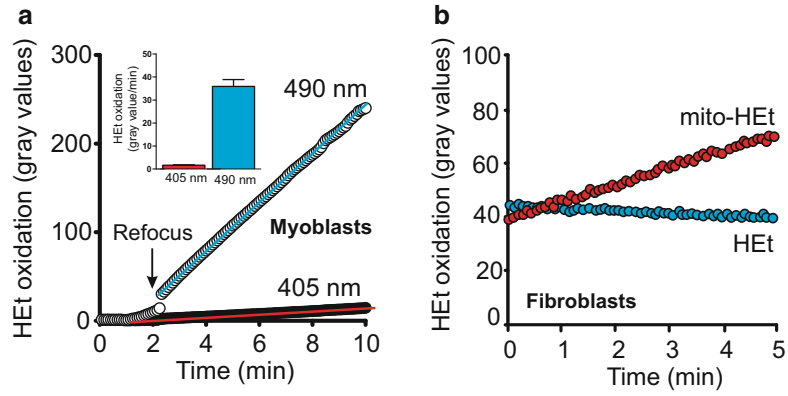

Fig. 4 Fluorescence intensity from 405 and 490 nm excitation and measurement of HEt versus mito-HE oxidation. (**a**) Simultaneous measurement of HEt oxidation using 405 and 490 nm excitation light in myoblasts. (**b**) Human skin fibroblasts were incubated with 10 μM HEt or 1 μM mito-HEt, washed, and imaged every 5 s using 490 nm excitation light. Staining with HEt yields end-point signals, whereas mito-HEt signals continue to increase in time. (This figure was reproduced (with permission) from Ref. 19)

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