Chapter 2
Pitfalls of Reactive Oxygen Species (ROS) Measurements by Fluorescent Probes and Mitochondrial Superoxide Determination Using MitoSOX

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Abstract Intracellular and mitochondrial superoxide formation is detected using phenanthrene-based dyes such as hydroethidine, mitochondria-targeted hydroethidine, or MitoSOX. HE and MitoSOX are redox probes, which undergo two-electron oxidation forming ethidium (E^+) and Mito-ethidium (Mito-E^+). The two-electron oxidation products derived from these probes exhibit the characteristic fluorescence that aids in fluorescence microscopy or flow cytometry or related techniques that are used to detect and determine superoxide (sometimes referred to as ROS, mitochondrial ROS, or mROS). This chapter briefly addresses the pitfalls of fluorescence-based techniques for detecting the intracellular superoxide.

Keywords Reactive oxygen species measurement · Pitfalls of ROS detection and determination · Fluorescent probes for ROS · Mitochondrial superoxide determination · MitoSOX ROS determination pitfall · Hydroethidine mitochondrial ROS

Phenanthrene-based dyes (e.g., hydroethidine, mitochondria-targeted hydroethidine, or MitoSOX) have been used to detect intracellular and mitochondrial superoxide formation [1, 2]. Both HE and MitoSOX are redox probes and undergo two-electron oxidation forming ethidium (E^+) and Mito-ethidium (Mito-E^+). The two-electron oxidation products derived from these probes exhibit characteristic fluorescence, and therefore, fluorescence microscopy or flow cytometry or related techniques have been used to detect and quantitate superoxide (sometimes referred to as ROS, mitochondrial ROS, or mROS). Most recently, MitoSOX-derived fluorescence was used to detect mitochondrial ROS formed in activated T cells [3]. This short commentary addresses the pitfalls of fluorescence-based techniques for

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detecting intracellular superoxide. Readers are referred to the previous reviews on this topic [4–8].

Many years ago, we showed that superoxide reacts with hydroethidine and other analogs including hydropropidine (HPr) and MitoSOX, forming a characteristic hydroxylated product (e.g., 2-hydroxyhydroethidium or 2-OH-E^+ and Mito-2-OH-E^+), but not the corresponding ethidium (E^+ or Mito-E^+) [9, 10]. The fluorescence parameters of 2-OH-E^+ and Mito-2-OH-E^+ are significantly different, and therefore, monitoring the red fluorescence of HE or MitoSOX in cells will not measure intracellular superoxide formation, and the increase in fluorescence intensity is merely indicative of increased oxidation of HE and MitoSOX. Although the exact mechanism of oxidation is not determined, it is conceivable that redox metal ions (iron, for example) and/or peroxidatic mechanism is responsible for the oxidation of HE to E^+ and MitoSOX to Mito-E^+. Therefore, the use of MitoSOX to measure mitochondrial superoxide formation, using the fluorescence technique, is incorrect and flawed.

Evidence for one-electron oxidant formation in extracellular and intracellular settings was obtained by determining dimeric product formation (e.g., E^+-E^+, Mito-E^+-Mito-E^+) using HPLC and LC-MS techniques [11]. The dimeric products are not fluorescent.

Another caveat is the uptake of redox dyes into cells. The intracellular uptake varies depending upon the experimental conditions (changes in oxidative profile, membrane potential, apoptosis). Measuring the intracellular concentration of the fluorescent probes is critical for interpreting the results. At the same rate of intracellular oxidant generation, an increase or decrease in probe uptake will alter the amount of product formation.

Improper use of these probes during extraction or incubation procedure can induce hydrolysis, like in other assays, and give rise to confounding results [12]. We have used isotopically labeled oxidants (e.g., O-18-labeled superoxide) and unequivocally shown that the oxygen atom in 2-OH-E^+ is incorporated from molecular oxygen and not from water (unpublished results).

In conclusion, irrespective of the probes (HE, Mito-SOX or HPr) used, it is important to obtain a global profile of probe uptake and oxidation products in order to fully assess the extracellular, intracellular, and mitochondrial oxidant formation.

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