CHAPTER THIRTEEN

Methods to Monitor ROS Production by Fluorescence Microscopy and Fluorometry

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Contents

Abstract

Mitochondria are considered one of the main sources of reactive oxygen species (ROS). The overgeneration of ROS can evoke an intracellular state of oxidative stress, leading to permanent cell damage. Thus, the intracellular accumulation of ROS may not only disrupt the functions of specific tissues and organs but also lead to the premature death of the entire organism. Less severe increases in ROS levels may lead to the nonlethal

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oxidation of fundamental cellular components, such as proteins, phospholipids, and DNA, hence exerting a mutagenic effect that promotes oncogenesis and tumor progression. Here, we describe the use of chemical probes for the rapid detection of ROS in intact and permeabilized adherent cells by fluorescence microscopy and fluorometry. Moreover, after discussing the limitations described in the literature for the fluorescent probes presented herein, we recommend methods to assess the production of specific ROS in various fields of investigation, including the study of oncometabolism.

1. INTRODUCTION

To date, seven distinct sites of reactive oxygen species (ROS) production have been identified in mammalian mitochondria. The two sites that have been studied most extensively are complexes I and III of the mitochondrial respiratory chain (Brand, 2010; St-Pierre, Buckingham, Roebuck, & Brand, 2002). It has also been suggested that the age-related deterioration of complex II can lead to superoxide production (Ishii, Miyazawa, Hartman, & Ishii, 2011). The involvement of complex II in ROS production was also discussed in the context of diabetes (Nishikawa et al., 2000), and it was suggested that mutations in complex II might also result in superoxide overproduction (Ishii et al., 2005). Additionally, the role of mitochondrial complex II in ROS production during hypoxia has been discussed by Paddenberg et al., (2003) and Yankovskaya et al., (2003). Other documented sources of ROS production in the mitochondria include monoamine oxidase and dihydroorotate dehydrogenase (Cadenas & Davies, 2000; Lambertucci et al., 2008). Furthermore, the flavoproteins acyl-CoA dehydrogenase and glycerol phosphate dehydrogenase can generate ROS in tissues during the oxidation of lipid-derived substrates (Lambertucci et al., 2008; St-Pierre et al., 2002). Both pyruvate and α-ketoglutarate dehydrogenase contain flavoenzyme dihydrolipoyl dehydrogenase subunits and can also participate in ROS production (Starkov et al., 2004; Tahara, Barros, Oliveira, Netto, & Kowaltowski, 2007). However, Brown and Borutaite (2011) present a number of examples supporting the hypothesis that mitochondria cannot be considered as the primary source of ROS. It is important to note that mitochondria are not the only sites of intracellular ROS production. Microsomes (e.g., cytochrome P450 and diamine oxidase), peroxisomes (e.g., enzymes involved in fatty acid oxidation), and some enzymes in the plasma membrane (e.g., NADPH oxidase and lipooxygenase) have been identified as ROS generators as well.

Apart from the aforementioned harmful effects, ROS also can have a beneficial role in cell physiology. Under physiological conditions, ROS can act as mediators and regulators of metabolism. ROS affect the transmission of signals to and throughout the cell, and they can be considered second messengers during growth, differentiation, and cell death. ROS can activate proteins involved in cell division (mitogen-activated proteins) and participate in the body's immune responses, as well influence the expression of many genes or regulate the synthesis of prostanoids (Bartosz, 2009; Dröge, 2002).

Many different methodological approaches have been described for monitoring extra- and intracellular ROS production. For comprehensive reviews and original papers concerning ROS measurement using the fluorescent probes described later, as well as equipment and many other methods, refer the following references: Zielonka and Kalyanaraman (2010), Dikalov and Harrison (2014), Grisham (2013), Halliwell and Whiteman (2004), Kalyanaraman et al. (2012), and Winterbourn (2014).

2. MEASUREMENT OF REACTIVE OXYGEN SPECIES PRODUCTION IN INTACT ADHERENT CELLS USING FLUORESCENT PROBES

2.1. Concept

One of the fastest, easiest, most user-friendly, and accessible methods for monitoring ROS production is based on the detection of ROS-sensitive fluorescent probes using a fluorescence microplate reader. There are several analytical, fluorescence approaches used to detect and characterize different ROS.

Later, we present the application of dihydroethidium (DHE), MitoSOX Red, and 5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate (CM-H_2DCFDA) to measure cytosolic superoxide, mitochondrial superoxide, and hydrogen peroxide production, respectively, in intact adherent cells. These probes are all oxidized to form intermediate probe-derived radicals that are successively oxidized to generate the corresponding fluorescent products (Winterbourn, 2014). Their reactivity toward different oxygen species is briefly described, as well as the strengths and limitations of their use (for more detailed analysis, refer to the following: Dikalov & Harrison, 2014; Wardman, 2007; Winterbourn, 2014). Despite criticism and the limitations described in the literature, the presented methods can be useful for basic and quick screening experiments to provide an overview of general ROS production.

DHE is the reduced form of the widely used DNA dye ethidium bromide. Due to its desirable quality of passively diffusing into cells, along with its high reactivity, DHE has been commonly used to detect cytosolic superoxide (Gomes, Fernandes, & Lima, 2005; Wardman, 2007; Zhao et al., 2003). Upon its reaction with the superoxide anion, DHE forms a red fluorescent product, 2-hydroxyethidium (Kalyanaraman et al., 2012; Zielonka, Vasquez-Vivar, & Kalyanaraman, 2008), with maximum excitation and emission peaks at 500 and 580 nm, respectively (Table 13.1). DHE itself displays blue fluorescence in the cell cytoplasm; however, the oxidized form 2-hydroxyethidium intercalates into DNA and exhibits red fluorescence (Bucana, Saiki, & Nayar, 1986). DHE is known to be the most specific fluorescent probe for superoxide detection (Wardman, 2007); however, apart from superoxide-mediated oxidation to 2-hydroxyethidium, DHE can also undergo unspecific oxidation by $ONOO^-$ or $^{\bullet}OH$ into ethidium (Gomes et al., 2005; Kalyanaraman et al., 2012; Patsoukis, Papapostolou, & Georgiou, 2005; Wardman, 2007; Zielonka, Hardy, & Kalyanaraman, 2009). Moreover, DHE can be oxidized by cytochrome *c* (Gomes et al., 2005; Zielonka, Srinivasan, et al., 2008). Because the fluorescence spectra of ethidium and 2-dihydroxyethidium differ only slightly, fluorometric measurements are not sufficient to distinguish between these two products, and this should be taken into account when interpreting the results. To verify the superoxide specificity of the signal, other analytical methods should be applied, such as HPLC and mass spectrometry (Zielonka, Vasquez-Vivar, et al., 2008).

MitoSOX Red is a dye used to measure superoxide production in the mitochondrial matrix. Because MitoSOX Red is a cationic derivative of DHE, its reaction with superoxide anions is very similar to that of DHE (Kalyanaraman et al., 2012). However, it reacts with superoxide faster than DHE and is rapidly targeted to the mitochondria (due to the positive charge of the cationic triphenylphosphonium substituent), where it is oxidized by superoxide to form 2-hydroxymitoethidium, which excites and emits at 510 and 580 nm, respectively (Robinson, Janes, & Beckman, 2008; Zielonka, Vasquez-Vivar, et al., 2008). Similar to DHE, MitoSOX Red can also undergo unspecific reactions with other oxidants to form mito-ethidium, which overlaps the fluorescence peak of 2-hydroxymitoethidium (Kalyanaraman et al., 2012; Zielonka & Kalyanaraman, 2010). Interestingly, it has been shown that the superoxide-specific product of MitoSOX Red oxidation has a specific excitation peak at ~400 nm (Robinson et al., 2006). Moreover, the user should be aware of the cyto- and mitotoxicity

Table 13.1 The list of fluorescent probes used to detect ROS with the use of microplate reader

Fluorescent probe	ROS detected	Excitation/emission wavelengths (nm)	Fluorescent product
Dihydroethidium (DHE)	Cytosolic superoxide but also $ONOO^-$ or $^{\bullet}OH$	535/635	2-Hydroxyethidium but also ethidium
MitoSOX Red	Mitochondrial superoxide but also other oxidants	510/595 alternatively: 400/595	2-Hydroxymitoethidium but also mito-ethidium
5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate (CM-H_2DCFDA)	Hydrogen peroxide but also hydroxyl radical, carbonate radical, and nitrogen dioxide	495/520	Dichlorofluorescein (DCF)

Excitation/emission wavelengths and fluorescent products are presented.

of these probes (Gomes et al., 2005; Kalyanaraman et al., 2012; Zielonka & Kalyanaraman, 2010), the potential modification of mitochondrial morphology, and the redistribution of fluorescence to the nuclei at higher probe concentrations. For more complex and detailed analyses, additional, more supportive methods such as fluorescence spectroscopy are also recommended (Nazarewicz, Bikineyeva, & Dikalov, 2013).

CM-H_2DCFDA is often called a "hydrogen peroxide-detecting probe for the measurement of hydrogen peroxide in intact cells"; however, the range of ROS detected by this probe is much broader. CM-H_2DCFDA freely permeates the plasma membrane and is hydrolyzed in the cytosol to form the DCFH carboxylate anion (Gomes et al., 2005; Kalyanaraman et al., 2012). Oxidation results in the formation of fluorescent DCF, which is maximally excited at 495 nm and emits at 520 nm (Kalyanaraman et al., 2012). The oxidation of H_2DCF to DCF is a two-step process: first, the DCF radical is formed, and then it is further oxidized to DCF in a reaction with molecular oxygen (Wardman, 2007). The first step of H_2DCF oxidation can be mediated by different radical species: hydroxyl radical, carbonate radical, and nitrogen dioxide, as well as by thiyl radicals resulting from thiol oxidation (Halliwell & Whiteman, 2004; Kalyanaraman et al., 2012; Winterbourn, 2014). H_2O_2 does not react with H_2DCF directly but requires the presence of peroxidases or other enzymes containing transition metals. Moreover, an alteration of the signal can be caused by antioxidant enzymes or superoxide that competes with the probe for the ROS (Winterbourn, 2014). Moreover, the superoxide radical is formed in the second step of probe oxidation and can then be dismutated to hydrogen peroxide and cause self-amplification of the signal (Grisham, 2013).

Despite the limitations described earlier, H_2DCFDA, DHE, and MitoSox Red are still the convenient probes for measuring general aspects of intracellular oxidative stress and redox status, provided that the constraints imposed by probe chemistry are taken into account when interpreting the results. Furthermore, to avoid data misinterpretation due to variations in cell numbers, normalization of the data based on protein amount is required, and a sulforhodamine B (SRB)-based method is also presented.

2.2. Experimental setup

The complete measurement buffer consists of the following: phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}, pH 7.4.

Directly before the measurement, 5 m*M* glucose is added.

Stock solutions

- DHE (on the day of the experiment, a working solution is prepared from 10 m*M* DMSO stock (1 mg in 317 μl of DMSO), diluted in DMEM)—protect from light
- MitoSOX Red (on the day of the experiment, a working solution is prepared from 5 m*M* DMSO stock (50 μg in 13 μl of DMSO), diluted in measurement buffer)—protect from light
- CM-H_2DCFDA (on the day of the experiment, a working solution is prepared from 5 m*M* DMSO stock (50 μg in 20 μl of DMSO), diluted in measurement buffer)—protect from light
- Glucose, 1 *M* stock

2.3. Procedure

Preparation of cells

Cells cultured in standard Dulbecco's modified Eagle medium (DMEM) are seeded into multiwell plates (approximately 25,000–30,000 cells per well). The medium should be changed the day before the experiment. Cells should reach 80–90% of confluence on the day of the experiment. The user should adjust the number of seeded cells and the culture duration to suit the specific cell type used.

2.3.1 Measurements

2.3.1.1 Measurement of cytosolic ROS production with the use of DHE

Cytosolic superoxide can be measured using the DHE fluorescent probe. Cells grown in multiwell plates should be incubated for 20 min (needed to allow the probe to enter the cell and start the reaction) at 37 °C in 0.5 ml of DMEM containing 0.5 μ*M* DHE. After the incubation, cells should be washed twice with PBS. The fluorescence can be measured in the measurement buffer using a microplate reader set to 535 nm excitation (Ex bandwidth: 25 nm) and 635 nm emission (Em bandwidth: 35 nm) wavelengths. This is a single-read measurement that does not define the kinetics of the reaction. Figure 13.1A shows the results obtained with this method.

2.3.1.2 Measurement of mitochondrial ROS production with the use of MitoSOX Red

Mitochondrial superoxide production can be measured using the MitoSOX Red fluorescent probe. Cells grown in multiwell plates should be washed twice with PBS to remove the medium and subsequently incubated for 10 min (needed to allow the probe to enter the cell and start the reaction

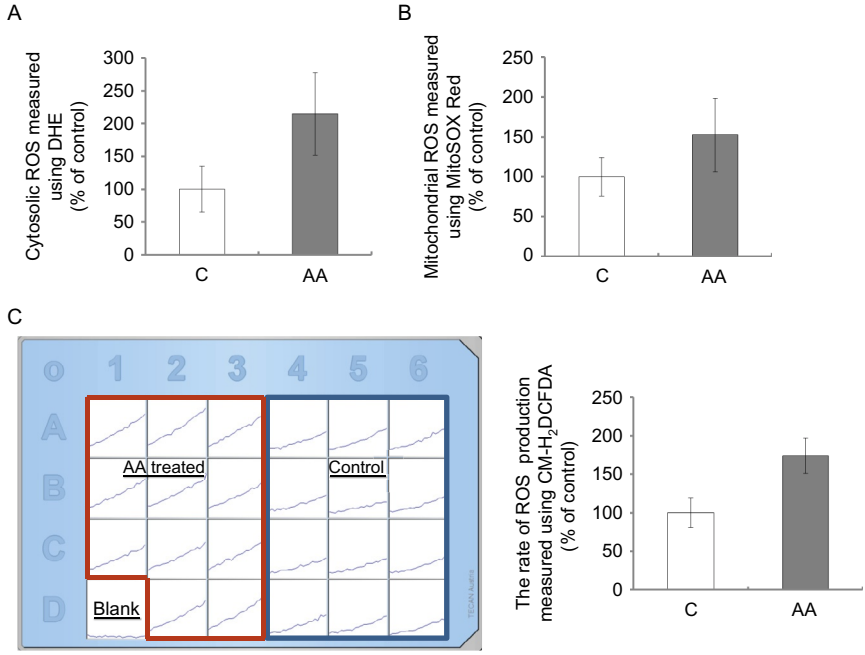

Figure 13.1 *Effect of antimycin A on ROS production in intact human fibroblasts measured with the use of a microplate reader*. C—control fibroblasts, AA—fibroblasts treated with antimycin A; (A) cytosolic ROS production measured using DHE; (B) mitochondrial ROS production measured using MitoSOX Red; (C) ROS generation measured using CM-H_2DCFDA. After the measurements, it is necessary to determine the cellular protein content via the sulforhodamine B (SRB) assay, as described by Vichai and Kirtikara (2006), which enables the standardization of ROS production as a function of cell number (protein level). A defect in the mitochondrial respiratory chain results in higher cytosolic and mitochondrial superoxide production. The rate of H_2O_2 production is also increased.

within the mitochondria) at 37 °C in 0.5 ml of measurement buffer containing 5 μ*M* MitoSOX Red. After the incubation, the cells should be washed twice with PBS. The fluorescence can be monitored in the measurement buffer with a microplate reader set to 510 nm excitation (Ex bandwidth: 10 nm) and 595 nm emission (Em bandwidth: 35 nm) wavelengths. Alternatively, if the signal is high enough, the fluorescence can also be measured using 400 nm excitation to measure only the superoxide-specific product of MitoSOX Red oxidation. This is a single-read measurement that does not define the kinetics of the reaction. Avoid exposing the MitoSOX Red stock solution and dye-loaded cells to light before measuring. Figure 13.1B shows the results obtained with this method.

2.3.1.3 Measurement of ROS production with the use of CM-H_2DCFDA

The rate of H_2O_2 production can be measured using the CM-H_2DCFDA fluorescent probe. Cells grown in multiwell plates should be washed twice with PBS to remove the medium. Next, add 0.5 ml of measurement buffer containing 2 μ*M* CM-H_2DCFDA. Immediately after addition, start measuring the fluorescence in kinetic mode at 485 nm excitation (Ex bandwidth: 20 nm) and 520 nm emission (Em bandwidth: 10 nm) wavelengths. Figure 13.1C shows the results obtained with this method.

3. QUANTIFICATION OF PROTEIN LEVEL WITH THE USE OF THE SRB ASSAY

The protein concentration in each well should be determined after the measurements described earlier to standardize ROS level/production as a function of the protein level (i.e., cell number) in each well. This can be performed using the SRB assay, as described by Vichai and Kirtikara (2006).

3.1. Experimental setup

Stock solutions

- Methanol
- Acetic acid
- Tris (10 m*M*), pH 10
- SRB

3.2. Procedure

The following protocol has been optimized for 24-well plates. Directly after the measurement of ROS described in Section 2, fix the cells by gently adding a cold solution of 1% (v/v) acetic acid in methanol and incubating for at least 1 h at −20 °C. Next, dry the plate (e.g., in an incubator). Add 0.5% (w/v) SRB solution in 1% acetic acid to each well and incubate for 1 h at 37 °C (gentle shaking is recommended). Aspirate the SRB solution and gently wash the cells with 1% (v/v) acetic acid. Repeat this step a few times until the remaining SRB solution is removed. Dry the plate (e.g., in an incubator). To the dried wells, add 1 ml of 10 m*M* Tris to dissolve the dye and gently shake for 15 min. Afterward, transfer 200 μl of the solution from each well into a 96-well plate and measure the absorbance at 540 nm using the microplate reader.

4. MEASUREMENT OF MITOCHONDRIAL ROS PRODUCTION IN PERMEABILIZED CELLS WITH THE AMPLEX RED FLUORESCENT PROBE

4.1. Concept

Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) is a nonfluorescent derivative of dihydroresorufin that is used to detect ROS in various experimental systems. This probe is oxidized to resorufin (excitation/emission maxima = 563/587 nm) in a peroxidase-catalyzed reaction with H_2O_2 (Zhou, Diwu, Panchuk-Voloshina, & Haugland, 1997). The probe is characterized by high sensitivity (down to 50 n*M* H_2O_2) and product stability. Amplex red is cell impermeable and is thus mostly applied to measure ROS production in isolated organelles (usually isolated mitochondria because these are considered an important intracellular source of ROS) or to detect extracellular H_2O_2 release (Dikalov, Griendling, & Harrison, 2007). Amplex red can also be used to monitor mitochondrial ROS production *in situ* using permeabilized cells, which allows the investigation of mitochondrial function in a more physiological environment than in the case of isolated organelles because it preserves the interactions of mitochondria with other organelles and avoids the mitochondrial fragmentation that occurs during isolation (Kuznetsov et al., 2008). Additionally, the number of cells used for the assay is much smaller than that needed when isolating mitochondria. Apart from ensuring the access of the probe to the mitochondria, cell permeabilization allows the manipulation of mitochondrial respiratory chain function through the application of appropriate substrates and inhibitors. Additionally, permeabilization leads to the dilution of cytosolic enzymes in the measurement medium, which diminishes the influence of cytosolic ROS-removing enzymes, such as cytosolic superoxide dismutase 1 (SOD1) and catalase, on the amount of ROS detected by the probe. Therefore, the ROS levels measured do not reflect actual intracellular ROS levels. However, this method is very convenient for investigating the relationship between the functional state of the mitochondria and the amount of ROS released by these organelles.

Later, we present the application of an Amplex red–horseradish peroxidase system to compare mitochondrial ROS production in C_2C_{12} myoblasts and myotubes. It should be mentioned that nonmitochondrial ROS sources (such as peroxisomes and plasma membrane NADPH oxidases) can also contribute somewhat to the results. However, the influence of

nonmitochondrial ROS is minimized due to the strong dilution of their substrates on cell permeabilization. The addition of particular substrates to the measurement medium (in this case, substrates fuelling mitochondrial respiration) makes it possible to control which source of ROS will contribute the most to the ROS production being measured.

Because Amplex red oxidation is also observed in the presence of superoxide (Malinska, Kudin, Debska-Vielhaber, Vielhaber, & Kunz, 2009), measurements are performed in the presence of exogenous superoxide dismutase to ensure the conversion of mitochondrially released superoxide into H_2O_2 and avoid the bias caused by potential differences in the sensitivity of the probe to these two ROS species. Another source of error, particularly when working with permeabilized cells, could arise from the oxidation of Amplex red in the presence of HRP and NAD(P)H. Such oxidation can also be prevented using superoxide dismutase (Votyakova & Reynolds, 2004). This phenomenon has to be accounted for while working with permeabilized cells because it could interfere with the results.

4.2. Experimental setup

The complete measurement buffer consists of the following: 120 m*M* KCl, 5 m*M* KH_2PO_4, 5 m*M* EGTA, 10 m*M* HEPES, pH 7.4.

Directly before the measurement, add 5 m*M* $MgCl_2$.

Stock solutions

- Amplex red (on the day of the experiment, a working solution is prepared from 20 m*M* DMSO stock diluted 100 times in the measurement medium)
- Horseradish peroxidase (4000 U/ml in measurement medium)
- Superoxide dismutase (7000 U/ml in measurement medium)
- Catalase
- Digitonin (10 mg/ml in water; to dissolve, heating may be necessary)
- H_2O_2 (10 μ*M*, freshly diluted from 30% solution)

Respiratory substrates and inhibitors: 1 *M* potassium succinate and 1 mg/ml antimycin A.

4.3. Procedure

Preparing the cells

C_2C_{12} myoblasts and myotubes are detached by trypsinization and spun down, and the cell pellet is suspended in PBS (pellet from one 10 cm culture plate in 100 μl of PBS).

4.3.1 Measurement

The measurement is performed in the presence of 1 μ*M* Amplex red, 20 U/ml horseradish peroxidase, and 35 U/ml superoxide dismutase. Amplex red oxidation is monitored with a fluorometer at $\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm. The reaction is initiated by adding the cell suspension (ca. 0.3 mg/ml protein), and the cells are then permeabilized by the addition of 40 μg/ml digitonin. An increase in Amplex red oxidation is observed due to the dilution of cytosolic SOD1, which makes more of the produced H_2O_2 available to react with the probe. Next, the appropriate respiratory substrates and inhibitors are applied to facilitate measurement of the changes in mitochondrial ROS production induced by changes in the redox state of particular respiratory chain reaction centers. Calibration is performed by adding known amounts of H_2O_2 to the cells to account for endogenous catalase activity within the cells. This enables the calculation of the actual amount of ROS released from the mitochondria. The sensitivity of this method to H_2O_2 is verified by catalase addition.

Figure 13.2 shows a comparison of H_2O_2 release from the mitochondria of C_2C_{12} myoblasts and myotubes using succinate as the respiratory substrate

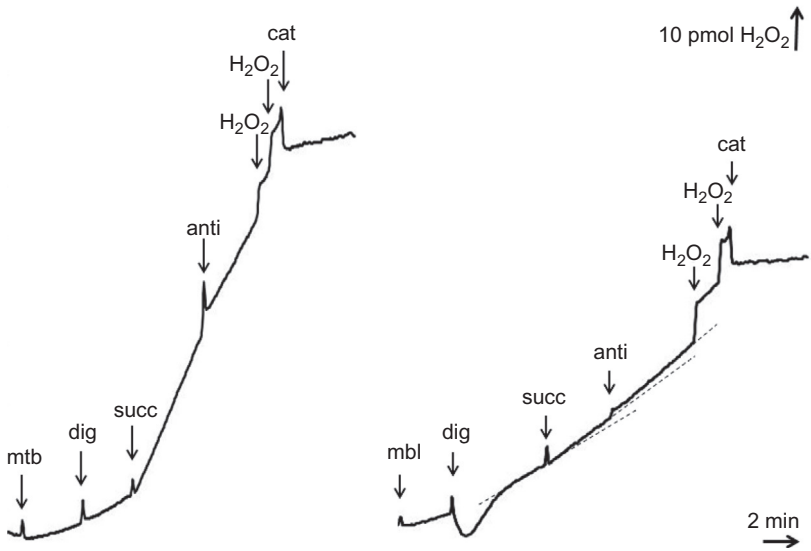

Figure 13.2 *Mitochondrial ROS generation in digitonin-permeabilized C_2C_{12} myotubes (left panel) and myoblasts (right panel) measured with the Amplex red–horseradish peroxidase system*. mbl—myoblasts, 0.3 mg/ml protein, mtb—myotubes, 0.3 mg/ml protein, dig—digitonin, 10 μg/ml, succ—succ, 10 m*M*, anti—antimycin A, 1 μg/ml, H_2O_2—10 pmol of hydrogen peroxide, cat—catalase, 8 kU/ml.

and in the presence of antimycin A, an inhibitor of respiratory complex III. In highly polarized mitochondria, oxidation using succinate as a predominant substrate is known to lead to reverse electron transport from complex II to complex I, resulting in strong reduction of complex I and enhanced superoxide production by this complex (Ksenzenko, Konstantinov, Khomutov, Tikhonov, & Ruuge, 1983). Antimycin A blocks electron transfer at complex III, leading to depolarization of the mitochondria (thereby abolishing reverse electron transport) and to stimulation of superoxide production by respiratory complex III (Votyakova & Reynolds, 2001). The experiment shows generally higher mitochondrial ROS production in C_2C_{12} myotubes in comparison to myoblasts and also shows the higher susceptibility of myotube mitochondria to the occurrence of reverse electron transport.

5. MEASUREMENT OF ROS BY FLUORESCENCE MICROSCOPY

5.1. Concept

Despite the many advantages of the plate reader format in measuring ROS (as described in Section 2), some experimental conditions require a more precise readout, even at the cost of lower throughput. Fluorescence microscopy would then be more appropriate to use in these cases. Microscopy is especially useful in cases with a nonhomogenous sample, in which only some cells needs to be analyzed (i.e., differentiating cells, a sample undergoing gene expression manipulation, a cell cycle phase-specific assay, etc.), or when a nonhomogeneous subcellular compartment needs to be analyzed. Several fluorescence microscopy techniques have been successfully used to investigate ROS, ranging from wide field to spinning disk confocal microscopy. The following procedure can be adapted to measure ROS in adherent cells using most common fluorescence microscopy techniques.

5.2. Experimental setup and procedure

Modified Krebs Ringer Buffer (KRB): 135 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 0.4 m*M* KH_2PO_4, 1 m*M* $MgSO_4$, 20 m*M* HEPES, pH 7.4.

Directly before the measurement, 5.5 m*M* glucose should be added.

5.2.1 Cell preparation

Plate cells on cover slips or glass-bottom petri dishes and let the cells grow in complete medium for at least 24 h at a density that allows the cells to reach a

Table 13.2 The list of most common fluorescent reporters compatible with CM-H_2DCFDA, DHE, and MitoSOX RED and dedicated filter sets for laser scanning (LSCM) or wide-field microscopy

		LSCM		Wide-field microscopy	
Dye	**Compatible reporter**	**Excitation**	**Emission**	**Excitation**	**Emission**
CM-H_2DCFDA	RFP, DsRed, mCherry	488—525/20, 543—LLP560		490/20—525/ 20,545/25607/70	
DHE/MitoSOX Red	EGFP, MitoTracker GREEN	514—LP560, 488—525/20		500/20—LP560, 490/20—525/20	
DHE/MitoSOX Red	MitoTracker DEEP RED	514—LP560, 633—LP650		490/20—580/65, 630/20—LP650	

maximum of 80% confluence on the day of the experiment. Proper seeding conditions are extremely dependent on cell type and have to be optimized by each experimenter; nevertheless, a general requirement is that cells should be seeded as uniformly as possible, avoiding the formation of cell aggregates. If the experimental condition requires DNA transfection, a certain degree of toxicity due to the transfection method has to be considered. In some experiments, transfection with fluorescent reporters may be recommended. A list of fluorescent reporters compatible with the discussed probes is presented in Table 13.2.

5.2.2 Counterstaining

To more precisely analyze MitoSOX Red data, mitochondrial counterstaining can be performed. The best counterstaining can be achieved through the use of targeted fluorescent proteins; however, if cells are refractory to transfection, MitoTracker staining is suggested. A large number of fluorescent probes are available for staining mitochondria. Due to the particular properties of the MitoSOX Red spectrum, we suggest MitoTracker Deep Red as the best option for staining mitochondria without interfering with the MitoSOX Red signal. However, if MitoTracker Deep Red cannot be acquired, MitoTracker GREEN should be used instead.

5.2.3 Procedure and signal recording

On the day of the experiment, prepare a loading solution composed of CM-H_2DCFDA, DHE, or MitoSOX Red, depending on the ROS to be measured (see Table 13.1 for working concentrations), 0.2% (v/v) pluronic acid and (in the case of mitochondrial counterstaining) 100 μM

MitoTracker Deep Red/GREEN in modified KRB; the solution can be stored at 4 °C for a few hours. If the stimulation or inhibition of ROS production is part of the experimental design, a 10 × stock of any required compounds (e.g., Antimycin A, Rotenone, or CCCP) should be prepared in modified KRB solution. Wash the cells twice to remove dead cells and cell debris, add 1 ml of the loading solution (prewarmed at room temperature) to the cover slip, and incubate for 10 min at 37 °C. After loading, the cover slip should be mounted in a metal cage or other appropriate support depending on the microscope model and covered with 900 μl of modified KRB. Mount the sample on an inverted microscope equipped with a thermostatic chamber and a magnification ranging from 20 × to 63 ×. CM-H_2DCFDA and DHE will be loaded in several compartments, but the signal will appear mostly cytosolic due to the resolution limit of fluorescence microscopy. As such, a high-resolution setup is not necessary, and a lower magnification is in fact preferred to allow the analysis of a higher number of cells. Excitation sources and emission filters for the ROS probes are suggested in Table 13.3.

ROS-sensitive dyes are irreversibly oxidized by ROS and trapped in the cells. The kinetics of this accumulation directly represents the ROS production rate. Based on this, a time series should be acquired using a delay of at least 15–30 s to avoid photoactivation not related to ROS production. The basal rate could be estimated by acquiring unstimulated cells for 5–10 min. Chemical stimulation can then be performed by adding 100 μl of the 10 × stock solution (e.g., Antimycin A or Rotenone), allowing the substance to diffuse through the chamber and continuing to record for the desired time.

5.2.4 Data analysis

Image analysis can be performed offline using a variety of commercial (e.g., Metamorph, NIS, and Volocity) and open-source (e.g., ImageJ, Bioimage

Table 13.3 The list of commonly used dyes for ROS production measurement with suggested filter sets for confocal (LSCM) and wide-field microscopy as well as their working concentrations

	LSCM		Wide-field microscopy		
Dye	**Excitation**	**Emission**	**Excitation**	**Emission**	**Working concentration (μ*M*)**
CM-H_2DCFDA	488	LP505	490/20	LP510	5
DHE	514	LP530	490/20	LP510	5
MitoSOX Red	514	LP530	490/20	LP510	0.2

XD, and Cell Profiler) bioimaging software. The first step that needs to be performed is background subtraction. Next, a region of interest (ROI) has to be drawn within each cell. If counterstaining is available, the counterstained image can be binarized and used as a mask to filter the original image (see Figure 13.3). Once ROIs have been drawn, the average intensity within each ROI can be measured for each time point and exported to a

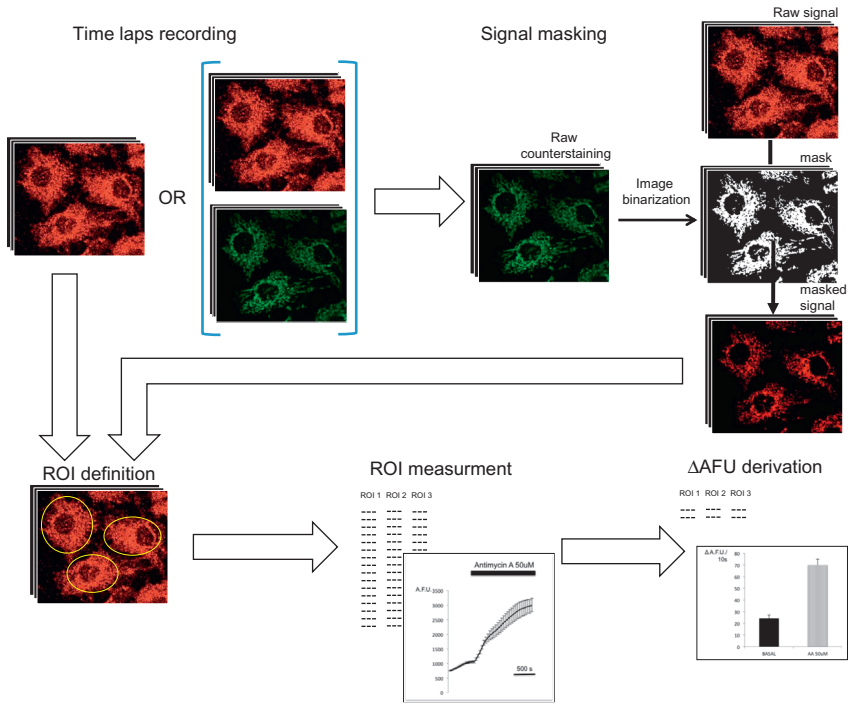

Figure 13.3 *Overview of ROS imaging and analysis workflow*. A time-lapse series is acquired with the preferred dye alone or in combination with counterstaining for the specific subcellular regions (e.g., mitochondria) or with other conditions (e.g., co-transfection with an oligonucleotide or cDNA). For counterstaining, the raw counterstaining images should be converted into a mask by image binarization. Such a mask is used to filter the raw signal images for the ROS probe, thereby allowing only the overlapping regions to be considered in the analysis. Regions of interest (ROIs) should then be defined in the raw or filtered images and the average fluorescence intensity calculated for each ROI at each time point; such data will be collected in a spreadsheet for further analysis. The delta value is then determined by calculating the difference between the intensity at each time point and the intensity of the preceding time point. Finally, the average delta intensity, expressed in arbitrary fluorescence units (ΔAFU), of each ROI has to be calculated for each experimental condition.

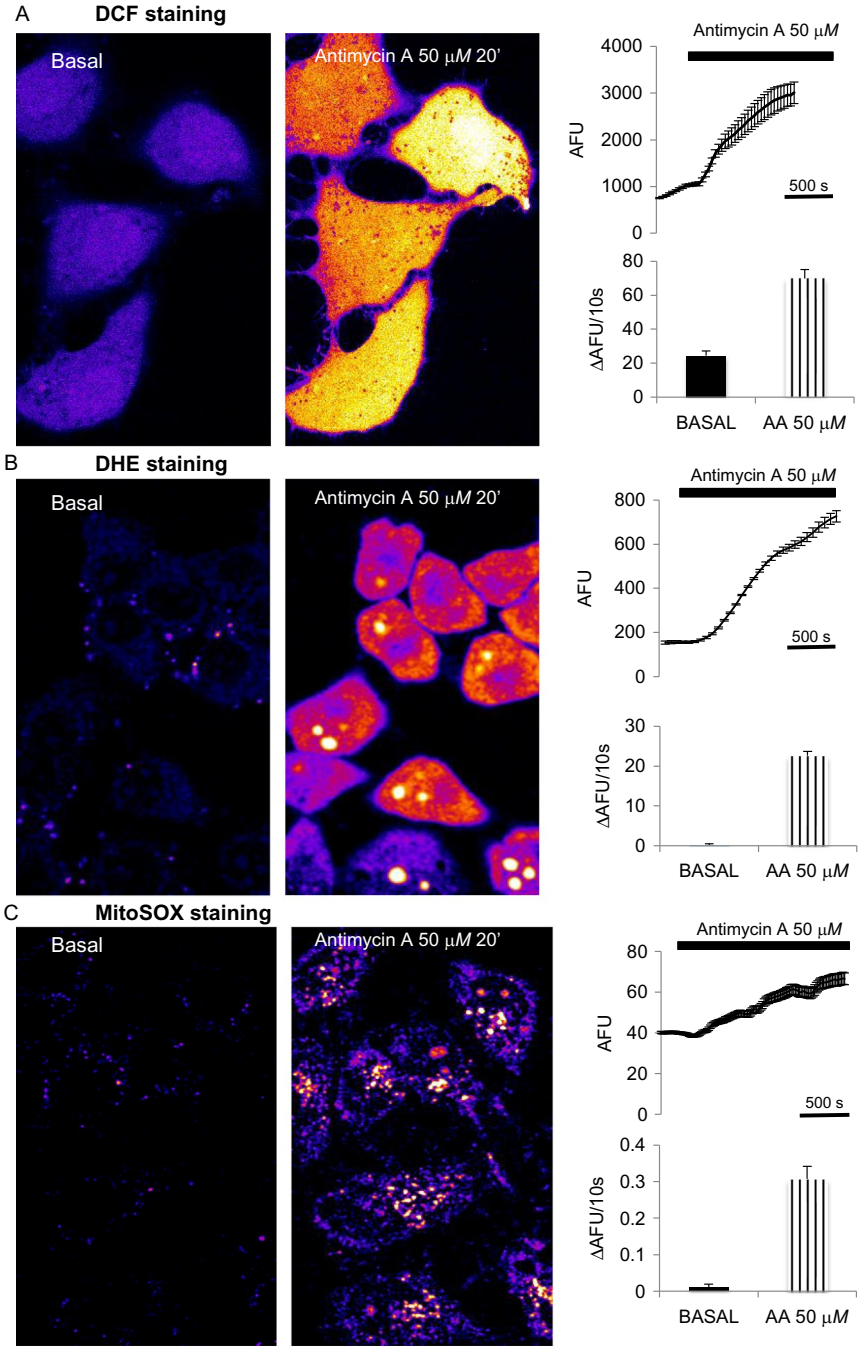

Figure 13.4 *Image-based measurement of ROS production after stimulation*. Representative measurements of cytosolic ROS production by CM-H_2DCFDA (A), cytosolic ROS by DHE (B), and mitochondrial ROS by MitoSOX Red (C) before and after stimulation with 50 μ*M* antimycin A (AA) in HeLa cells by laser scanning confocal microscopy. For each group, representative false-color images are displayed, along with graphs of the kinetics and ROS production rates.

spreadsheet. With the use of software such as Microsoft Excel and Libreoffice Calc, the ΔF can be calculated for each cell to represent the rate of ROS production. An average ΔF has to be calculated for each phase of the experiment if different stimulants or treatments were used. Such a value (expressed as ΔAFU) could be collected for different replicates and compared between different conditions (as an example, see Figure 13.4).

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