Video Article

Evaluation of Intracellular Location of Reactive Oxygen Species in *Solea Senegalensis* Spermatozoa

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URL: https://www.jove.com/video/55323
DOI: doi:10.3791/55323

Keywords: Developmental Biology, Issue 133, *Solea Senegalensis*, Spermatozoa, Mitochondria, Cryopreservation, Mitochondria Stain, DCFH-DA, DAPI, Confocal Microscopy

Date Published: 3/11/2018

Citation: Valcarce, D.G., Robles, V. Evaluation of Intracellular Location of Reactive Oxygen Species in *Solea Senegalensis* Spermatozoa. *J. Vis. Exp.* (133), e55323, doi:10.3791/55323 (2018).

Abstract

Oxidative stress is one of the important factors in decreasing sperm quality. Developing efficient protocols for detecting reactive oxygen species (ROS) in spermatozoa is of high importance in any species, but these methods are rarely used and even less in teleost. Cryopreservation is a useful technique in aquaculture for different purposes, including gene banking and guaranteed sperm availability throughout the year. Freezing/thawing procedures could cause ROS production and damage the sperm cells. Considering the prospective damage that an excess of ROS production could cause in spermatozoa depending on their localization, here a detailed methodology to detect H_2O_2 and to evaluate its intracellular localization by confocal microscopy is provided. For this purpose, a combination of 3 fluorochromes (2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), a live mitochondria stain and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)) are used to evaluate the co-localization of H_2O_2 with spermatozoa nuclei or mitochondria in *Solea senegalesis* sperm samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/55323/

Introduction

The reactive oxygen species production has been linked with sperm quality recently[1]. Although ROS production in mitochondria can be considered a normal physiological process, oxidative stress by an excess of ROS production is a clear cause of damage in spermatozoa at different levels. In humans, oxidative stress is associated with male infertility, altering motility and the ability to undergo capacitation[2]; in mammals, change of DNA integrity in frozen sperm samples has been also related to synthesis of H_2O_2[3].

Cryopreservation is a common technique for gene banking in aquaculture. This technology is particularly important in species with reproductive problems such as *Solea senegalensis*. This valuable species in the market shows reproductive dysfunction in individuals born in captivity due to a lack of courtship. This fact makes sperm cryopreservation necessary to have sperm availability for artificial fertilization. However, cryopreservation could be a source of oxidative stress that could be detrimental for spermatozoa[4] as studies have reported a beneficial effect of antioxidant supplementation. ROS inhibition through mitochondrial-targeted antioxidant was reportedly beneficial for sperm cryopreservation in yellow catfish[5].

Therefore, the levels of ROS in sperm samples are important to know, particularly after cryopreservation[6,7] because these molecules have been recognized as a drawback for sperm survival and fertility[8]. Moreover, studying the distribution of ROS within the cell could be crucial to infer the level of potential damage. As an example, low levels of ROS in the mitochondria could be assumed normal and compatible with sperm function, but high levels of ROS within the nucleus could be indicators of spermatozoa DNA damage. H_2O_2 is one of the most relevant ROS that could be released from the mitochondria and penetrate the nucleus because it is a small and charge-less molecule[9]. Dichlorofluorescein diacetate (DCFH-DA) can specifically reveal intracellular peroxide emitting green fluorescence. In this article, a detailed protocol for detecting H_2O_2 intracellular localization in *Solea senegalensis* sperm using confocal microscopy is presented.

Protocol

NOTE: Fluorochrome incubation and confocal analysis will take at least 2-3 h for a control and a treated sample. Data processing is not included in this time calculation. Required materials can be found in the **Table of Materials**. This protocol can be applied to fresh or cryopreserved spermatozoa. *Solea senegalensis* is a fish species that spawns in cold water, work always under cold conditions (4-7 °C). See **Figure 1** for a general view of the protocol.

1. Preparatory Work Before the Experiment

1. Prepare a 1 mM mitochondria stain stock solution in analytical grade DMSO.
2. Prepare a 1 µg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stock solution in deionized water.
3. Prepare a 200 mOsm/kg Ringer solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.7)
4. Prepare a 4 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) stock solution in analytical grade methanol.
   NOTE: Keep the stock solutions in aliquots at -20 °C until needed.
5. Set the microcentrifuge at 4-7 °C.

2. Sample Preparation Before Fluorochrome Incubation

NOTE: Gentle handling of spermatozoa is desirable when pipetting and resuspending.

1. If working with cryopreserved cells, prepare a water bath for thawing at 40 °C for *Solea senegalensis* spermatozoa[10]. Immerse the straw samples in the water bath for 7 s.
2. Using scissors to cut, empty the sample into a microfuge tube and centrifuge at 1000 x g, 4-7 °C for 1 min.
3. Remove seminal plasma or seminal plasma with cryoprotectants from the cryopreserved sample by pipetting to discard any component that could interfere with the staining protocol.
4. Resuspend the cells in 50-100 µL of 4 °C 200 mOsm/kg Ringer solution.
5. Determine the sperm concentration with a Neubauer or a similar counting chamber under a stereoscopic microscope.
6. Dilute the cell suspension up to 1-2 x 10^6 cells/mL in a final volume of 0.5 mL in 4 °C Ringer solution.

3. Fluorochrome Incubation

NOTE: Fluorochromes must be handled in low light conditions, especially when in solution.

1. Add 3.125 µL of DCFH-DA stock solution (final concentration in the sample: 25 µM) to the working sample dilution. Incubate at 4-7 °C for 40 min in darkness[10].
2. After 30 min, add 0.5 µL of the DAPI stock solution (final concentration in the sample: 2 µM) and 0.5 µL of the mitochondria stain stock solution (final concentration in the sample: 100 nM) to the sample. Incubate both fluorochromes for 10 min in darkness.

4. Sample Preparation for Confocal Microscopy

1. After the incubation time, centrifuge the cell suspension at 1000 x g, 4-7 °C for 1 min.
2. Discard the supernatant carefully by pipetting.
3. Add a 10-20 µL of 200 mOsm/kg Ringer solution.
4. Place 5 µL of a concentrated cell suspension drop on a slide.
5. Carefully, put a cover slide on the preparation and seal it with polish. Once the polish is dry, the preparation is ready for confocal microscopy.

5. Confocal Setup Prior to the Experiment

NOTE: Depending on the microscope, DCFH-DA could be "burnt". Establish the best conditions with a non-valuable sample first.

1. Turn on the microscope, lasers, camera and the computer running the microscope.
2. Set excitation lasers taking into account the excitation and emission maximums of each fluorochrome:
   1. For DAPI, use an excitation maximum of 358 nm and an emission maximum of 465 nm.
   2. For the mitochondrial stain, use an excitation maximum of 644 nm and an emission maximum of 662 nm.
   3. For DCFH-DA, use an excitation maximum of 504 nm and an emission maximum of 525 nm.
3. Start working with 25X objective to focus on the spermatozoa. Choose the DAPI channel to focus because this fluorochrome reports the highest intensity. Once the cells are focused, use a 63X or 100X objective for a thorough evaluation because *Solea senegalensis* spermatozoa size is around 2 µm.
4. For each channel, optimize the pinhole, the gain and the voltage. In the same way, adjust the digital offset to reduce background. Once all parameters are optimized for the fluorochrome used, save the settings. For a routine experiment for a single *Solea senegalensis* sperm cell we used the following (these parameters may change in each experiment): pinhole of 1 and the gain voltage of 750 V.

6. Acquisition of Images

1. Select the desired quality conditions for the image acquisition. Define acquisition settings like bit depth, image format, light sheet thickness, and choose single sided illumination. For a routine experiment for a single *Solea senegalensis* spermatozoa we used the following (these parameters may change in each experiment): frame size of 1024 px; bits per pixel of 16; scan speed of 2-4).
2. Open and center the scan area to begin and take an image of the field.
3. Using the crop tool, select the region of interest and press live.
4. With the help of the range indicator tool, adjust the digital offset to reduce the background. Do this for each channel and take the image.
5. Check the quality of individual channels for each fluorochrome and their combinations.

7. Creating a 3D Image Video

1. Z-stack acquisition settings.
    1. Choose the Z-stack option in the software.
    2. Select the single cell, group of cells or region of interest and focus it.
    3. Delimit the Z-stack with the 'First Slice' and 'Last Slice' options and establish the z step to interval to 0.2 µm with the help of the fine focus. Choose DAPI channel to focus because this fluorochrome may report the highest intensity and you can easily select the whole spermatozoa head. Choose the optimal acquisition parameters for each channel and run the experiment.
2. Run the Z-stack experiment. Split the channels and observe the localization of each signal within the cells.
3. Z-stack process.
    1. Volume render: Once the experiment is finished, select the options of the Z-stack processing software and choose the volume render option. Select the number of steps and the rotation degrees (360°). Save the file with a video extension.
    2. Stereo anaglyph: Choose stereo anaglyph in the options process window. This tool allows to create a 3D image video with split channels.

Representative Results

Confocal microscopy is an ideal method for intracellular ROS evaluation in teleost sperm. The combination of the three fluorochromes (DAPI, a mitochondria stain and DCFH-DA) presented in this study (**Figure 1**) provides many useful information that can be applied in basic research and can have applications in improving procedures used in industrial aquaculture plants, such as cryopreservation protocols. Different types of analysis may be carried out in order to correlate intracellular ROS presence and other parameters: motility, viability, different patterns between good and bad breeders, activation of motility or seasonal sperm variations among many others. In the present work, two different patterns of intracellular H_2O_2 distribution within the spermatozoa are shown: collocated with mitochondrion or spread in the nucleus (**Figures 2, 3**). Those spermatozoa showing low potential damage produced by ROS showed DCFH-DA labelling only in the mitochondria whereas those suffering DNA damage showed DCFH-DA labelling also in the nuclei. Confocal microscopy software allows to create useful videos and provide easy and fast colocalization graphs.

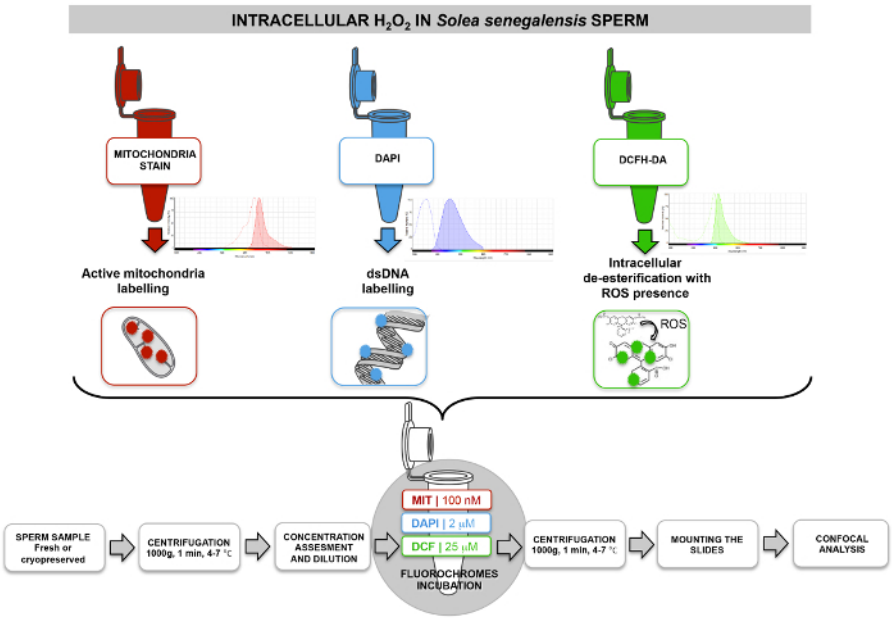

Figure 1. General overview of the protocol. Please click here to view a larger version of this figure.

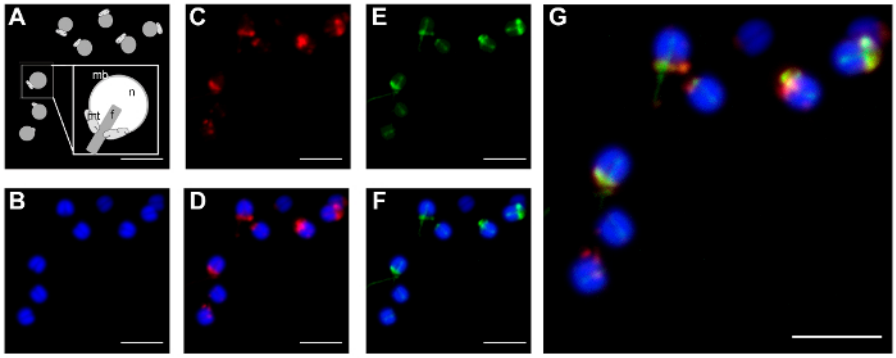

Figure 2. Example of confocal image acquisition. A. Scheme of the sample **B.** DAPI channel (labelling dsDNA). **C.** Mitochondria stain channel (labelling mitochondrion). **D.** Mitochondria stain channel merged with DAPI channel. **E.** DCFA-DH channel (labelling intracellular H_2O_2). **F.** DCFA-DH channel merged with DAPI channel. **G.** Merge of the three channels. Abbreviations: **n**: nuclei, **mt**: mitochondria, **f**: flagellum and **mb**: membrane. Scale bar: 5 µm. Please click here to view a larger version of this figure.

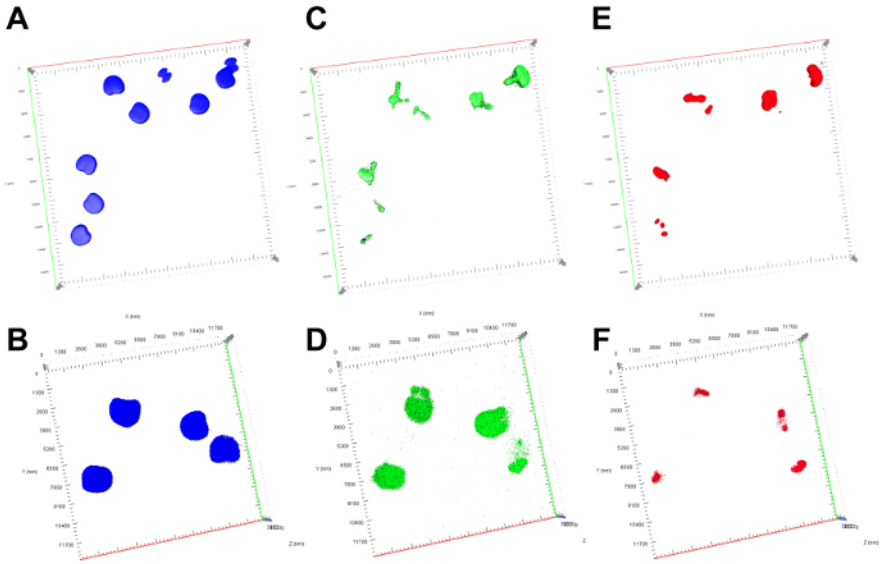

Figure 3. Different patterns of intracellular H_2O_2 in *Solea senegalensis* spermatozoa (Volume render). Resulting channels of spermatozoa showing intracellular ROS in the nuclear fossa and mitochondrion (**A, C, E**). Resulting channels of spermatozoa showing intracellular H_2O_2 also within the nuclei (**B, D, F**). **A** and **B**: DAPI. **C** and **D**: DCFH-DA. **E** and **F**: Mitochondria stain. Please click here to view a larger version of this figure.

Discussion

It is well known that mitochondria are key organelles for sperm motility and function. These organelles are concurrently directly involved in ROS production. Interestingly, controlled levels of ROS are needed for proper sperm function[1]. Positive relationships between fertility and oxidative stress have been shown in mammals[11] but excessive levels affect sperm quality[12]. One crucial factor that could be decisive towards a positive or negative effect is not only ROS levels but also ROS intracellular localization. One of the deleterious effects of ROS is producing DNA damage[9] and therefore the nuclear presence of ROS could be an indicator of potential damage in the nucleus whereas ROS levels could be normal in mitochondria. It is necessary to supplement existing quantitative methods (*e.g.*, flow cytometry) with techniques like confocal microscopy that could provide information about ROS intracellular localization.

Confocal microscopy is an optimal option to visualize the intracellular localization of ROS. The use of specific fluorochromes such as a live mitochondria stain and DAPI allows the visualization of mitochondria and nucleus respectively and the combination of these molecules with DCFH-DA allows the intracellular localization of peroxide.

The critical steps within the protocol are fluorochrome incubation and confocal setup. Both steps should be optimized and carefully performed to obtain reproducible and consistent results. Methodology modifications should be performed at these two levels depending on the seminal plasma used. Therefore, fluorochrome incubation should be adapted. Temperatures and storage conditions significantly differ among sperm samples, and most of the protocols are optimized for mammals.

Here, a protocol for teleost sperm samples, particularly for *Solea senegalensis* spermatozoa, is described (**Figure 1**). Successful labeling of nuclei and mitochondria have been performed using the described protocol and ROS presence has been reveal using DCFH-DA (**Figure 2**). Results indicate that co-localization of H_2O_2 have been found in the nuclei or mitochondria depending on the sperm sample (**Figure 3**). As

previously explained, those samples with a high percentage of spermatozoa displaying high levels of ROS within the nucleus would be prone to DNA damage. This protocol has a unique limitation, the requirement of expensive equipment (confocal microscope), but it could have future utility in selecting sperm samples with low presence of ROS within the nucleus for cryopreservation purposes.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank AQUAGAMETE FA 1205 COST Action. This work was financially supported by AGL201568330-C2-1-R project (MINECO/FEDER). David G. Valcarce was funded by Junta de Castilla y León (EDU1084/2012) and Fondo Social Europeo. Authors acknowledge Dr. Ana Riaza and Stolt Sea Farm S.A., Dr. Paulino de Paz, Dr. Ignacio Martínez Montero and José Ramón Guiérrez. We also thank Paula Fernández Colado for videography.

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