Detection of Reactive Oxygen Species in Preimplantation Bovine Embryos

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Principle

5-(and-6)-carboxy-2',7'-dihydrofluorescein diacetate (H_2DFFDA) is converted from a nonfluorescent form to a fluorescent derivative when acetate groups are removed by intracellular esterases and oxidation occurs within the cell (**Figure 1**). Oxidation of the probe in embryos can be detected by monitoring the increase in fluorescence using an epifluorescent microscope with FITC filters.
(Source: *ROS Detection Reagents - Invitrogen*)

Materials

Culture Plates: Make 48 µl microdrops of modified KSOM or other culture medium and cover drops with mineral oil. Allow medium to equilibrate for at least 4 hours in incubator (typically 5% O_2, 5% CO_2, 38.5°C) before transferring embryos. **Note:** avoid serum as it contains esterases that can cleave the probe.

Stock solution 1 (carboxy-H_2DFFDA): Dissolve carboxy-H_2DFFDA (Invitrogen™, catalog number C13293) to a final concentration of 1 x 10^{-5} *M* in KSOM or other culture medium. The solution must be prepared immediately before use. Always use a freshly-prepared solution. ***Note:*** *We weigh 1.0 mg per 80 µl DMSO for a stock solution of 2.51 x 10^{-2} M carboxy-H_2DFFDA. This stock can be aliquoted into 5 µl portions in 0.6 ml or smaller microcentrifuge tubes and stored at -20°C in the dark. Immediately before use, 195 µl KSOM or other culture medium is added to the 5 µl DMSO-carboxy-H_2DFFDA stock. 112 µl of this dilution is then added to 6888 µl culture medium for a final concentration of 1 x 10^{-5} M carboxy-H_2DFFDA. The final dilution can be scaled to obtain the volume needed for the experiments.*

PBS-PVP: 1mg/ml polyvinyl pyrollidone in 10 mM PO_4 buffer, pH 7.4 containing 0.9% (w/v) NaCl. ***Note****: Prepare 1 L 100 mM sodium phosphate monobasic ($NaH_2PO_4.H_2O$) and 100 mM sodium phosphate dibasic (Na_2HPO_4) solutions. Mix 100 ml monobasic with about 300 ml dibasic solution until pH 7.4. Dilute 100 ml of this buffer with ~ 500 ml ddH_2O, add 9 g NaCl, add water to bring the final volume to ~900 mL adjust pH for 7.4, and bring volume to 1000 mL.*

72-well MiniTrays (Nunc™, catalog number 136528) –for examining embryos under the microscope.

Procedures

Invitrogen has prepared a booklet entitled Reactive Oxygen Species Detection Reagents for working with H_2DFFDA and other probes for reactive oxygen species

- Positive control: maintain embryos at 38.5°C and 5% or 20% (v/v) O_2; incubated with 2% hydrogen peroxide (v/v) before ROS detection;
- Negative control: maintain embryos at 38.5°C and 5% O_2 environment and exclude the fluorescent probe.
- Expose embryos to treatments. ***Note****: For heat shock, we have cultured for 3 hours at 41°C and 20% O_2 before adding probe and incubating another hour. For chemical treatments, all of the solutions including stocks and dilutions are made up from the medium containing the final concentration of 1 x 10^{-5} M carboxy-H_2DFFDA.*
- Incubate embryos in their respective treatment for 1 hour with the probe.
- Wash embryos four times in PBS-PVP to remove the excess dye by transferring embryos from drop to drop.
- Organize embryos in PBS-PVP drops in the 72-well MiniTrays. ***Note****: It is also possible to place drops on the cover of Intergrid plates or in microscope slides. Do not cover embryos with cover slips. Try to have few embryos per drop (5-10) to facilitate embryo searching while working in the microscope. Also, watch the drops constantly to avoid drying.*
- Take embryos immediately to the epifluorescence microscope room for examination of fluorescence using a FITC filter.

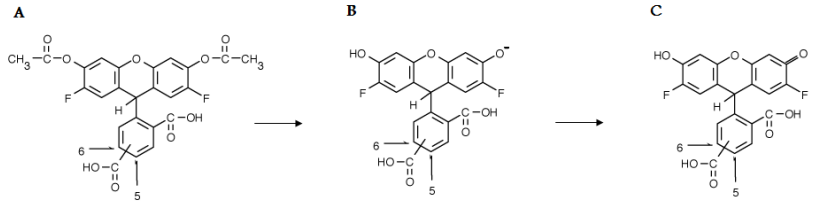

Figure 1: Fluorescence generation by carboxy-H_2DFFDA deacetylation and oxidation. Panel A: nonfluorescent carboxy-H_2DFFDA. Panel B: nonfluorescent deacetylated carboxy-H_2DFF. Panel C: the fluorescent, deacetylated and oxidized product, carboxy-DFF. Modified from *Reactive Oxygen Species Detection Reagents*

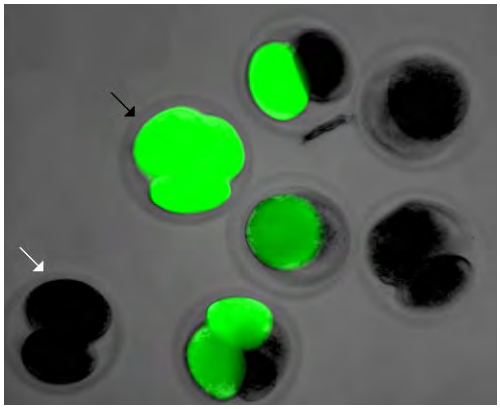

Figure 2: Epifluorescent image of 2-4 cell bovine embryos submitted to mild oxidative stress (20% O_2 environment) and incubated with 1.3 mM carboxy-H_2DFFDA. Green fluorescence indicates embryos positive for ROS production. Black arrow: ROS production detected; White arrow: no ROS production detected. Note that embryos were also photographed under differential interference contrast.

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