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Detecting reactive oxygen species in free-living symbiotic dinoflagellates exposed to nanoparticles

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Synthetic coral HDR

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2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a popular fluorescent probe for the detection of oxidative stress in cells. Since the probe can be prone to auto-oxidation, carboxy-2',7'-dichlorodihydrofluorescein-diacetate (carboxy-H₂DCF-DA) which is more stable and easily penetrates cell membranes is often reported in the literature instead. Upon crossing the cell membrane, esterases hydrolyse DCFH-DA to DCFH, which remains trapped within cells. The oxidation of DCFH yields DCF, a fluorescent compound which can be measured using excitation/emission wavelengths of 485-495/520-530 nm.

Due to several cases of interference when used in cellular systems, DCFH-DA is a general marker of the cellular oxidative stress rather than a specific indicator H₂O₂ formation or other ROS.

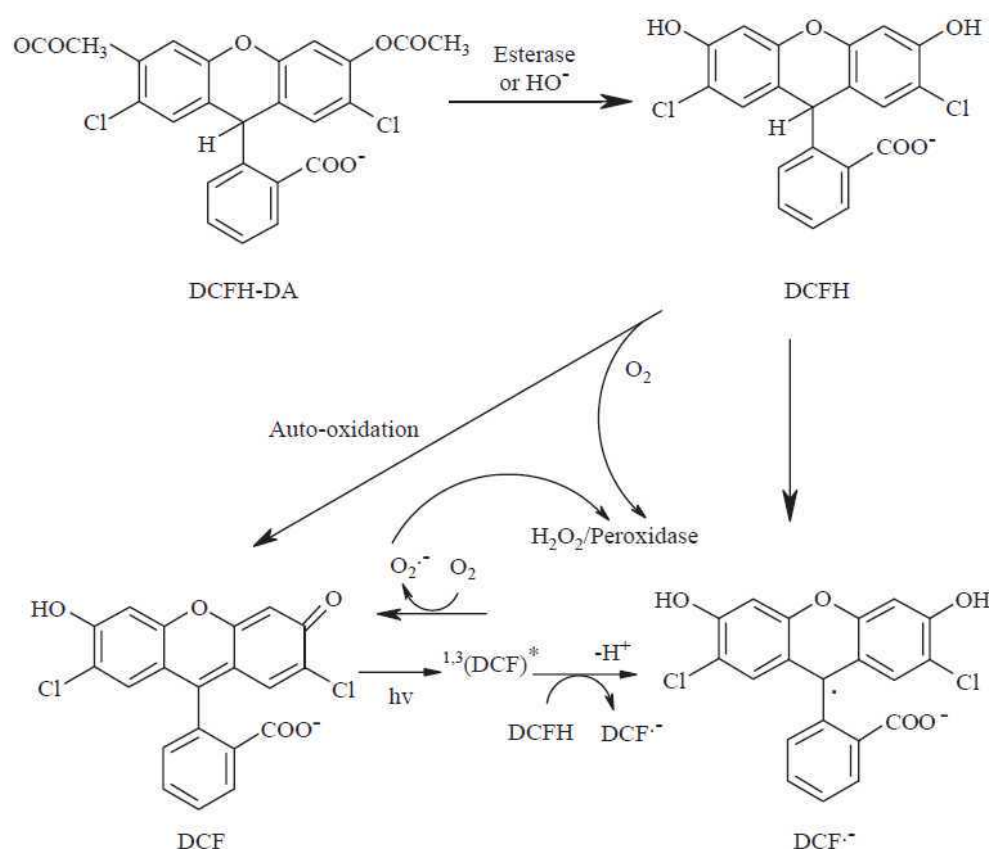


Image credit: Gomez et al. 2005 (J. Biochem. Biophys. Methods, 65, 45–80)

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DCFH-DA, free-living, symbiotic dinoflagellate, nanoparticle, antioxidant, ROS, peroxide

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All cell culture work should be conducted in the biological cabinet with standard personal protection equipment (lab coat, gloves, goggles).

CHEMICALS:

- 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, CAS Number 4091-99-0, MW 487.29, Product Number D6883)
- Hydrogen peroxide (H₂O₂, 30%, CAS Number 7722-84-1, MW 34.01, Product Number 216763)
- BD DifcoTM Marine Broth 2216 (dehydrated culture media, REF 279110)
- Methanol (XXXX)
- MilliQ water
- Nanoparticle suspension
- Cell suspension (here *Breviolum minutum*, wild type, marine dinoflagellate algae)

EQUIPMENT:

- 15 mL conical tubes
- 50 mL conical tubes
- 1.5 mL or 2 mL Eppendorf tubes
- Multichannel pipettor (at least 8 positions) with 300µL volume/pipette
- 1 mL pipettor and tips
- 20-200 µL pipettor and tips
- 10-100 µL pipettor and tips
- 0.5 to 2.5 µL pipettor and tips
- 96 well dosing plate, round bottom, sterile
- 96 well plate, black, sterile (note: clear 96-well plate can also be used but a black plate will prevent bleed-through between wells)
- Troughs (reagent container)
- Aluminium foil
- Microplate reader

- cytochrome c has been reported to catalyze the oxidation of DCFH, therefore using

DCFH-DA to probe oxidative stress during cell apoptosis could result in higher fluorescence without any change in cellular peroxide levels should due to the rise in cytosolic cytochrome c levels. (from Lawrence et al. J Biolog. Chem. 2003, 278, 32, 29410-29419)

- DCFH oxidation also occurs by action of H_2O_2 in presence of Fe(II), with $HO\cdot$ responsible for the oxidation.
- DCF can suffer photo-reduction in presence of visible light or by action of UVA.
- DCFH has been used as an indicator of oxidative bursts in macrophages and neutrophils.

Due to several cases of interference when used in cellular systems, DCFH-DA is a general marker of the cellular oxidative stress rather than a specific indicator H_2O_2 formation or other ROS.

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Chemicals

- 1 - 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, CAS Number 4091-99-0, MW 487.29, Product Number D6883)
 - Hydrogen peroxide (H_2O_2 , 30%, Sigma-Aldrich, CAS Number 7722-84-1, MW 34.01, Product Number 216763)
 - BD Difco™ Marine Broth 2216 (dehydrated culture media, Fisher Scientific, REF 279110)
 - Methanol (Fisher Chemical, A454-1, 0.2 micron filtered)
 - MilliQ water
 - Nanoparticle suspension
 - Cell suspension (here *Breviolum minutum*, wild type, marine dinoflagellate algae)

Equipment

- 2 - 15 mL conical tubes
 - 50 mL conical tubes

- 1.5 mL or 2 mL Eppendorf tubes
- Multichannel pipettor (at least 8 positions) with 300 µL volume/pipette
- 1 mL pipettor and tips
- 20-200 µL pipettor and tips
- 10-100 µL pipettor and tips
- 0.5 to 2.5 µL pipettor and tips
- 96 well dosing plate, round bottom, sterile
- 96 well plate, black, sterile (note: clear 96-well plate can also be used but a black plate will prevent bleed-through between wells)
- Troughs (reagent container)
- Aluminium foil
- Microplate reader

Free-living symbiotic dinoflagellate complete culture medium:

- 3 Dissolve 37.4 g of dehydrated BD Difco™ Marine Broth 2216 in 1 L of MilliQ water. Autoclave final solution.
Composition of BD Difco™ Marine Broth 2216: <https://www.fishersci.com/shop/products/bd-difco-dehydrated-culture-media-marine-broth-2216/DF0791174>

H2O2 working solution:

- 4 Dilution 1: In a 2 mL Eppendorf tube, add 113 µL of stock solution of H₂O₂ and 886 µL sterile^{1m} marine broth to achieve a final concentration of 1M H₂O₂.
Dilution 2: in a 2mL Eppendorf tube, add 30 µL of dilution 1 and 270 µL of sterile marine broth to achieve a final concentration 0.1M H₂O₂.

DCFH-DA solutions:

- 5 Stock solution: Dissolve 24.4 mg of DCFH-DA in 50mL methanol to make 1mM DCFH-DA^{2m} concentrated stock solution.
This solution can be stored at -20°C for up to 4 months.
- 6 Working solution: in a 15 mL conical tube, add 100 µL of stock solution and 9.9 mL of sterile marine broth.

DCFH-DA loading and plating cells

1h

- 7 In a 15 mL conical tube, add 6 mL of cell suspension (at 1x10⁶ cells/mL) and 60 µL of DCFH-DA working solution. Wrap the tube in aluminium foil for 60 min dark incubation at room temperature. This is for half a 96-well plate at 150 µL per well (row B-G, columns 1-6)^{1h}
- 8 After 60 min dark incubation, centrifuge the solution at 2000 rpm for 3 min at room temperature. Remove to supernatant and add 6 mL of fresh sterile marine broth. Plate cells in row B through G, columns 1 through 6 with 150 µL per well. Rows A and H (column 1 through 6) should be filled with 150 µL of sterile marine broth as blanks. Wrap plate in aluminium foil to^{5m}

prevent photoactivation or bleaching of DCFH, if you are not ready to dose the cells yet. Best practice would be to prepare the dosing plate during the DCFH-DA loading period so that you are ready to dose the cells as soon as the DCFH-DA loading is completed.

Nanoparticle working solution (CeO₂)

- 9 The nanoparticles tested here are composed of CeO₂ with a poly(acrylic acid) coating and Dil^{2m} fluorescent labeling.
When synthesized, the colloid solution concentration was 1.3M.
To obtain the working solution:
- dilution 1: in an Eppendorf tube, add 1 µL of colloid solution and 999 µL of marine broth;
 - dilution 2: in an Eppendorf tube, add 1 µL of dilution 1 and 999 µL of marine broth;
 - dilution 3: in an Eppendorf tube, add 10 µL of dilution 2 and 990 µL of marine broth (final concentration 3.6 µM)

Nanoparticle Dosing Plate prep

- 10 Prepare a nanoparticle working solution from your colloid solution (nanoparticles in suspension) to a final concentration of 1.3M. [Note: this protocol was developed for testing CeO₂ nanoparticles and their potential for scavenging ROS in *Breviolum minutum* (symbiotic dinoflagellate often associated with *Aiptasia* anemones and *Acropora* corals)] 20m

10.1 Preparation of the dosing plate:

- in a round-bottom 96 well plate transfer 250 µL of complete sterile marine broth to column 1 to 6 in row B;
- H₂O₂ replicates:
 - add 225 µL of sterile marine broth to columns 1 to 3 in row C;
 - add 225 µL of sterile marine broth to columns 1 to 3 in row D;
 - add 225 µL of sterile marine broth to columns 1 to 3 in row E;
 - add 225 µL of sterile marine broth to columns 1 to 3 in row F;
 - add 297 µL of sterile marine broth to columns 1 to 3 in row G;
 - add 3 µL of H₂O₂ working solution (dilution 2) to columns 1 to 3 in row G;
- serial dilution: transfer 25 µL of wells G1-G3 to F1-F3 (using multichannel pipettor), repeat process from F1-F3 to E1-E3, from E1-E3 to D1-D3, from D1-D3 to C1-C3;
- CeO₂ replicates:
 - add 150 µL of sterile marine broth to columns 4 to 6 in row C;
 - add 125 µL of sterile marine broth to columns 4 to 6 in row D;
 - add 125 µL of sterile marine broth to columns 4 to 6 in row E;
 - add 125 µL of sterile marine broth to columns 4 to 6 in row F;
 - add 250 µL of CeO₂ (3.6 µM in sterile marine broth) to columns 1 to 3 in row G;
- serial dilution: transfer 125 µL of wells G4-G6 to F4-F6 (using multichannel pipettor), repeat process from F4-F6 to E4-E6, from E4-E6 to D4-D6, and 100 µL from D4-D6 to C4-C6;

Procedure

1d 4h 53m

11

3m

Centrifuge the 96-well plate with plated cells: 2000 rpm for 3 min at room temperature.

- 11.1 Remove supernatant (from columns 1 to 6, rows B to G) carefully using a fine^{5m} tip plastic dropper pipette or standard pipettor;
[Be careful not to aspirate cells because the dinoflagellates do not attach]
- 11.2 Using a multichannel pipettor, transfer 150 μ L from the dosing plate to the^{5m} 96-well plate, well for well (from columns 1 to 6, rows B to G). Wrap the plate in aluminium foil until you analyze it in the plate reader. The analysis should be done directly after. The reaction starts as soon as you transfer the reagents from dosing plate to the plate with cells so the longer you wait the more of the reaction you miss.
- 11.3 Measure fluorescence of DCF using excitation/emission wavelengths of^{3h} 485/530nm using a microplate reader at desired time points (every minute for up to 3H for kinetics or 1H for initial reaction);
- 11.4 Report peroxide production as the fluorescence intensity variation of treated cells relative to untreated cells;