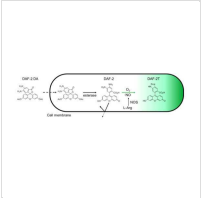

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Detecting nitric oxide in free-living symbiotic dinoflagellates exposed to nanoparticles

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Synthetic coral HDR

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Diaminofuorescein-2 diacetate (DAF-2 DA) is a fluorescent indicator of nitric oxide (NO). The DAF reacts with the nitric anhydride (N_2O_3) which formed by oxidation of NO.
Upon crossing the cell membrane, esterases hydrolyse DAF-2 DA to DAF-2, which remains trapped within cells. The DAF-2 reacts to the oxidation of intracellular NO (or more accurately the N_2O_3) to produce the highly fluorescent triazolofluorescein (DAF-2T) by nitrosation and dehydration (Kojima et al., 1999).

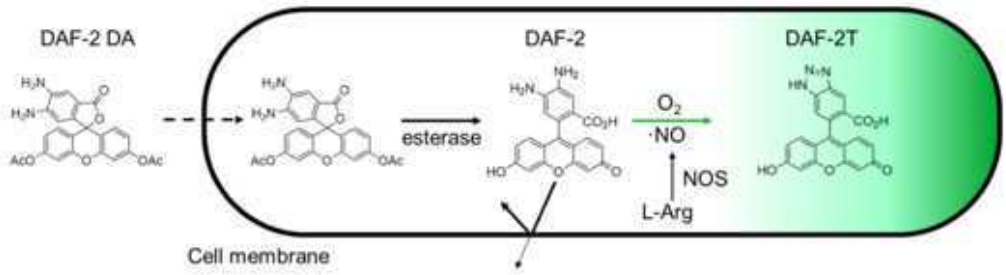

image credit: https://goryochemical.com/en/research-reagent/3717/

Protocol adapted from Bouchard & Yamasaki (2009) and Kojima et al. (1999)

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nitric oxide, RNS, DAF-2 DA, free-living, symbiotic dinoflagellate, antioxidant nanoparticles

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52143

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

CHEMICALS:
- Diaminofluorescein-2 diacetate (DAF-2 DA, $C_{24}H_{18}N_2O_7$, Sigma-Aldrich, CAS Number 205391-02-2, MW 446.4, SKU 251505-1MG-1)
- Sodium nitroprusside dihydrate (SNP, $Na_2[Fe(CN)_5NO] \cdot 2H_2O$, Sigma-Aldrich, CAS Number 13755-38-9, MW 297.95, SKU 71778-25G)
- Sterile Calcium-Magnesium free seawater (CMFSW)
- 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

DAF-2 DA is an irritant (B)

SNP is classed acutely toxic (oral, dermal, inhalation), categories 1,2,3

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DAF-2 DA information:
- Prepare working solutions fresh if possible and protect from light;
- Avoid repeated freeze-thaw cycles and wait until solution has reached room temperature before opening to avoid moisture from entering the vial;
- Best practice would be to prepare and freeze aliquots when the chemical is first opened;
- Addition of bovine serum albumin (BSA), phenol red, calcium ion and vitamins may affect the fluorescence.

Chemicals

1 - Diaminofluorescein-2 diacetate (DAF-2 DA, $C_{24}H_{18}N_2O_7$, Sigma-Aldrich, CAS Number 205391-02-2, MW 446.4, SKU 251505-1MG-1)
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- 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

Calcium-Magnesium free seawater (CMFSW) preparation:

3 Combine
- 23 g/L NaCl
- 0.763 g/L KCl
- 3 g/L NaSO4
- 0.25 g/L NaHCO3

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with DI water and autoclave

Nanoparticle working solution:

4 The nanoparticles tested here are composed of CeO_2 with a poly(acrylic acid) coating and DiI 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 CMFSW;
- dilution 2: in an Eppendorf tube, add 1 µL of dilution 1 and 999 µL of CMFSW;
- dilution 3: in an Eppendorf tube, add 10 µL of dilution 2 and 990 µL of CMFSW (final concentration 3.6 µM)

SNP working solution preparation:

5 Prepared a working solution of 3 mg/mL SNP in DI water (10 mM). Note: SNP is more soluble at >25°C.
Protect from light using aluminium foil

DAF-2 DA loading and plating cells

6 In a 15 mL conical tube, add 6 mL of cell suspension (at $1x10^6$ cells/mL in CMFSW) and 9 µL of DAF-2 DA stock 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)
Note: it is important to use CMFSW because DAF- 2 DA reacts with calcium ions in typical seawater

6.1 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 CMFSW. 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 CMFSW as blanks. Wrap plate in aluminium foil to prevent photoactivation or bleaching of DAF-2T, if you are not ready to dose the cells yet. Best practice would be to prepare the dosing plate during the DAF-2 DA loading period so that you are ready to dose the cells as soon as the DAF-2 DA loading is completed.

Nanoparticle dosing plate prep:

7 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 CeO_2 nanoparticles and their potential for scavenging nitric oxide (NO) in *Breviolum minutum* (symbiotic dinoflagellate often associated with *Aiptasia* anemones and *Acropora* corals)]

7.1 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;
- SNP replicates:

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add 225 µL of sterile CMFSW to columns 1 to 3 in row C;
add 225 µL of sterile CMFSW to columns 1 to 3 in row D;
add 225 µL of sterile CMFSW to columns 1 to 3 in row E;
add 225 µL of sterile CMFSW to columns 1 to 3 in row F;
add 297 µL of sterile CMFSW to columns 1 to 3 in row G;
add 3 µL of SNP 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;

- CeO_2 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 CeO_2 (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;
Avoid direct exposure to light.

Procedure:

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

8.1 Remove supernatant (from columns 1 to 6, rows B to G) carefully using a fine tip plastic dropper pipette or standard pipettor;
[Be careful not to aspirate cells because the dinoflagellates do not attach]

8.2 Using a multichannel pipettor, transfer 150 µL from the dosing plate to the 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.

8.3 Measure fluorescence of DAF-2T using excitation/emission wavelengths of 495/515nm using a microplate reader at desired time points (every minute for 1H);

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