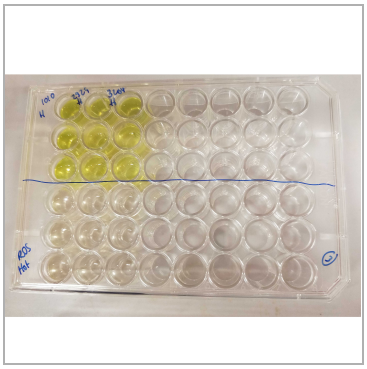

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Protocol status: Working
We use this protocol and it's working

Created: Dec 01, 2020

Quantifying Reactive Oxygen Species in diatoms

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ABSTRACT

This protocol us designed to assess the relative concentration of reactive oxygen species in diatoms using a fluorescent dye.

This protocol is based on methods from:

Knauert, S., and Knauer, K. (2008). The role of reactive oxygen species in copper toxicity to two freshwater green algae *J. Phycol.* 44**,** 311-319.

Szivák, I., Behra, R., and Sigg, L. (2009). Metal-induced reactive oxygen species production in *Chlamydomonas reinhardtii* (Chlorophyceae) *J. Phycol.* 45**,** 427-435.

This method was used in:

Argyle, P. A., Walworth, N. G., Hinners, J., Collins, S., Levine, N. M., & Doblin, M. A. (2021). Multivariate trait analysis reveals diatom plasticity constrained to a reduced set of biological axes. *ISME Communications*, *1*(1), 59.

Argyle, P. A., Hinners, J., Walworth, N. G., Collins, S., Levine, N. M., & Doblin, M. A. (2021). A high-throughput assay for quantifying phenotypic traits of microalgae. *Frontiers in microbiology*, *12*, 706235.

IMAGE ATTRIBUTION

Phoebe Argyle

Last Modified: Feb 25, 2023

PROTOCOL integer ID: 45069

Keywords: Reactive oxygen species, phytoplankton, diatoms, ROS

GUIDELINES

This protocol has been developed using *Thalassiosira* spp. diatoms and may be applied to other taxa, however the staining concentration may need adjusting according to the permeability of cells to the dye.

As the incubation is performed in the dark, the effects of differential light treatments on cultures may not be captured.

MATERIALS

H2DCFDA (H2-DCF, DCF) **Thermo Fisher Catalog #D399**

DMSO **P212121 Catalog #GB-D-360**

Equipment

Breathe-Easy® sealing membrane	NAME
Plate seal	TYPE
Breathe-Easy®	BRAND
Z380059-1PAK	SKU
https://www.sigmaaldrich.com	LINK

Equipment

Infinite® M1000 Pro	NAME
Microplate reader	TYPE
Tecan	BRAND
n/a	SKU
https://lifesciences.tecan.com/microplate-readers	LINK
Note M1000 Pro no longer in production, newer models such as the Spark are available and will serve the same purpose.	SPECIFICATIONS

Equipment	
48 well Clear TC-treated Multiple Well Plates	NAME
Tissue culture plate	TYPE
Costar	BRAND
3548	SKU
https://ecatalog.corning.com/	LINK

BEFORE START INSTRUCTIONS

Ensure your cultures are at the correct growth phase for measurement. We developed the protocol with cells growing in exponential growth but depending on the requirements of the researcher this may not be applicable.

Preparation of stain stock solution

1h

1 Add 12.5mg of H2DCFDA (H2-DCF, DCF) **Thermo Fisher Catalog #D399** to 5 mL DMSO **P212121 Catalog #GB-D-360** to create a [M] 2.5 mg/mL stock solution.

When not in use store at -20 °C in the dark.

Initiation of assay

30m

2 Remove algae cultures from growth conditions/incubator.

Transfer 2 x 500 µL aliquots of microalgae culture into separate wells of a 48-well tissue culture plate.

Equipment	
48 well Clear TC-treated Multiple Well Plates	NAME
Tissue culture plate	TYPE
Costar	BRAND
3548	SKU
https://ecatalog.corning.com/	LINK

One well will act as a blank, the other as the treatment. Do this for all cultures being assayed.

3 Add 2 µL of 2',7'dichlorodihydrofluorescein (H_2DCF) stock solution from step 1 to the treatment wells as quickly as possible.

4 Seal the plate with a Breathe-Easy sealing membrane to prevent evaporation during incubation.

Equipment	
Breathe-Easy® sealing membrane	NAME
Plate seal	TYPE
Breathe-Easy®	BRAND
Z380059-1PAK	SKU
https://www.sigmaaldrich.com	LINK

5 Gently shake plate by hand to ensure even mixing of the stain within the culture.

Incubation

30m

6 Wrap the whole plate in tin foil and incubate in the experimental conditions (return to culturing incubator) for 02:00:00 2h

Note

As H2DCFDA (H2-DCF, DCF) **Thermo Fisher Catalog #D399** is a fluorescent dye and thus light-sensitive, any differences in light between culturing conditions may not be accurately reflected in this assay. E.g. a 'high light' vs. 'low light' treatment.

Analysis

7 Read the fluorescence of all wells (treatments and blanks) on a plate reader (e.g. Tecan Infinite® M1000 Pro) at excitation/emission of 488nm excitation 525nm emission.

Equipment

Infinite® M1000 Pro	NAME
Microplate reader	TYPE
Tecan	BRAND
n/a	SKU
https://lifesciences.tecan.com/microplate-readers	LINK
Note M1000 Pro no longer in production, newer models such as the Spark are available and will serve the same purpose.	SPECIFICATIONS

The complete list of settings that we used for our experiments were as follows:
Multiple reads per well 4x4 (circle, filled), border 1000 µm
Excitation wavelength: 488 nm
Emission wavelength: 525 nm
Excitation bandwith: 5 nm

Emission bandwith 5 nm
Gain: 100
Number of flashes: 50
Flash frequency: 400 Hz
Integration time: 20 µs
Lag time: 0 µs
Settle time: 10 ms

Gain settings will depend on the specific plate reader being used and on the density of the culture. If the culture is very dense the fluorescence will read as "OVER" in which case reduce the gain settings and read again. However, it is very important to maintain the same gain setting across an experiment in order to cross-compare different cultures.

ROS per well calculation

30m

8 Calculate raw fluorescence (Relative fluorescence units RFU) for ROS with the following calculation:
Fluorescence of stained well - Fluorescence of the blank well

ROS per cell calculation

1d

9 Estimate the number of cells in each aliquot using flow cytometry (cite other protocol). Divide the value calculated in step 6 by the number of cells in the aliquot to obtain a ROS per cell measure (in RFU).

Note

If comparing between diatom taxa of different sizes, size correction may be applicable. In this instance forward scatter measures from the flow cytometer may be used as a proxy for cell size. A size correction can be done by dividing the ROS per cell measure from step 7 by the median forward scatter or the estimated cell size (when using size beads).