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Detection and Quantification of Programmed Cell Death in *Chlamydomonas reinhardtii*: The Example of S-Nitrosoglutathione

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Abstract

Chlamydomonas (*Chlamydomonas reinhardtii*) is a unicellular model alga that has been shown to undergo programmed cell death (PCD) that can be triggered in response to different stresses. We have recently shown that *Chlamydomonas* is particularly well suited to the study and quantification of PCD. We have shown for the first time that S-nitrosoglutathione (GSNO), a nitric oxide (NO) donor, is able to induce PCD and can be used as a study system in *Chlamydomonas*. In this article, we provide a simple and robust protocol for quantifying GSNO-induced PCD, which can be adapted to any other treatment. We explain how to detect NO production in the cell following GSNO treatment. We show how PCD can be identified simply by analyzing the degradation profile of genomic DNA. We also provide an easy and reproducible cell death quantification protocol, which makes it possible to follow the course of PCD over time and highlight very fine differences in the number of affected cells between different samples.

Key features

- Use of S-nitrosoglutathione (GSNO) as a means to study programmed cell death (PCD) in *Chlamydomonas.*
- Discrimination of PCD vs. necrosis.
- In vivo determination of NO production in the cell.
- A simple, robust protocol for PCD quantification.

Keywords: *Chlamydomonas reinhardtii*, Programmed cell death, S-nitrosoglutathione, Nitric oxide, DNA fragmentation, Evans blue staining

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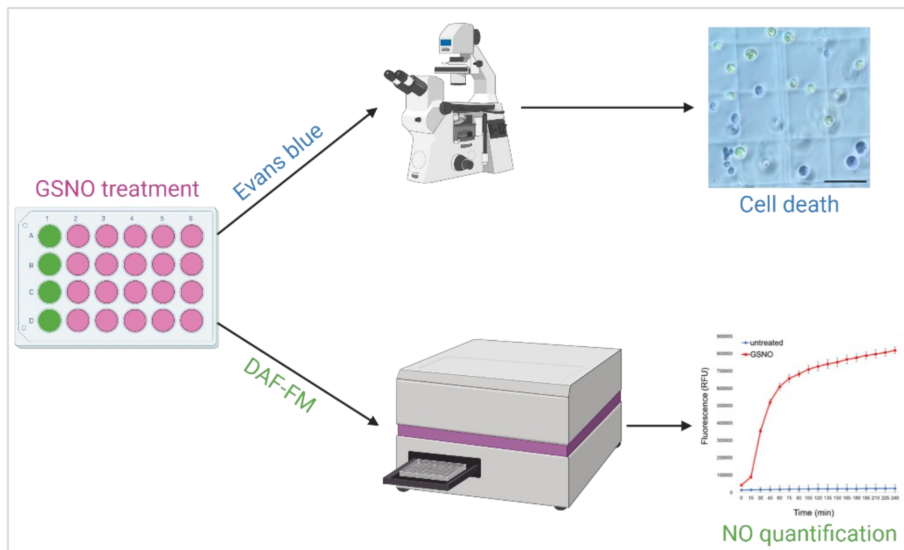
Graphical overview



Background

Programmed cell death (PCD) is a crucial process identified in animals in 1972 [1], which plays a role in a wide range of biological processes. Understanding targeted disappearance of cells whose presence is no longer desired is straightforward in multicellular organisms. However, the concept of PCD is more difficult to apply to a unicellular organism, since the death of the cell corresponds to the death of the organism. In recent years, however, it has been shown that PCD does exist in unicellular organisms such as *Chlamydomonas*, where it was found to be an altruistic mechanism that allows the survival of the population [2,3]. Nitric oxide (NO) has been shown in several studies to be an important molecule for PCD in *Chlamydomonas* [4,5]; this is why we used the main source of NO in the cell, S-nitrosoglutathione (GSNO), as a system for studying PCD in *Chlamydomonas* [3]. Several specific criteria can be used to discriminate programmed cell death from necrosis; one of the simplest is to analyze the DNA degradation profile during death [6]. If PCD occurs, DNA migrates as multiples of 180 bp in gel electrophoresis, resulting in a DNA ladder; in the case of necrosis, continuous DNA degradation represented by a smear will be observed [7]. We describe how to analyze the DNA degradation profile in *Chlamydomonas*, as well as a simple and robust method for calculating the percentage of dead cells in a population. In the case of GSNO-induced PCD, it is important to be able to quantify NO in the cell after treatment; here, we explain how to implement a method for doing so in vivo, using a fluorescent probe. Our protocol outlines the steps to detect and quantify PCD in *Chlamydomonas* using Evans blue staining, whether you are using GSNO as an inducer or any other treatment.

Materials and reagents

Biological material

1. We used *Chlamydomonas reinhardtii* D66 (CC-4425) [8] and CLiP library (CC-4533) [9] strains, but any strain could be used

Reagents

Note: Unless specified otherwise, the reagent can be stored at room temperature.

1. TAP (tris-acetate-phosphate) solution (Life Technologies, catalog number: T8050)
2. S-nitrosoglutathione (GSNO) produced in our laboratory but also available for purchase (Sigma, catalog number: N4148), stored at -20 °C
3. Evans blue powder (Sigma, catalog number: E2129)
4. Phenol:chloroform:isoamyl (25:24:1) (Sigma, catalog number: 77617), stored at 4 °C
5. Ethanol 100% (Sigma, catalog number: 32205-M)
6. Trizma® base (Sigma, catalog number: 93350)
7. Hydrochloric acid (HCl) (Sigma, catalog number: 258148)
8. Sodium chloride (NaCl) (Sigma, catalog number: S7653)
9. Ethylenediaminetetraacetic acid tetrasodium (EDTA) (Sigma, catalog number: E6511)
10. Sodium dodecyl sulfate (SDS) 20% (Sigma, catalog number: 05030)
11. Sodium acetate (Sigma, catalog number: S2889)
12. RNase A, 10 mg/mL (Thermo Scientific, catalog number: EN0531), stored at -20 °C
13. Agarose (Sigma, catalog number: A9539)
14. Tris borate EDTA (TBE) buffer (Sigma, catalog number: T4415)
15. Ethidium bromide solution (Sigma, catalog number: 46067)
16. GeneRuler 100 bp Plus DNA ladder (Thermo Scientific, catalog number: SM0321), store at 4 °C
17. DAF-FM diacetate (4-amino-5-methylamino-2’,7’-difluorescein diacetate) (Invitrogen, catalog number: D-23844), store at -20 °C, protected from light

Solutions

1. 50 mM GSNO solution (see Recipes)
2. DNA extraction buffer (see Recipes)
3. Sodium acetate 3.3 M (see Recipes)
4. TBE 0.5× solution (see Recipes)
5. Evans blue solution (see Recipes)

Recipes

1. 50 mM GSNO solution

a. Weigh 20 mg of GSNO and dissolve it in 800 µL of TAP medium [10] in a 1.5 mL tube.

b. Verify the concentration of the 100-fold diluted solution using the molar extinction coefficient of GSNO (922 $M^{-1}\cdot cm^{-1}$ at 335 nm) [11].

Note: It is better to prepare fresh GSNO for each experiment to avoid its oxidation.

2. DNA extraction buffer (for 500 mL)

Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.5)	200 mM	100 mL
NaCl (1 M)	250 mM	125 mL
EDTA (0.5 M, pH 8)	25 mM	25 mL
SDS (20%)	0.5%	12.5 mL
MilliQ water	n/a	262.5 mL
Total	n/a	500 mL

Note: It is recommended to autoclave extraction buffer (add SDS after autoclaving) before proceeding to DNA extraction.

3. Sodium acetate 3.3 M (for 100 mL)

Reagent	Final concentration	Amount
Sodium acetate	3.3 M	27 g
MilliQ water	n/a	100 mL

Total	n/a	100 mL

a. Dissolve using a magnetic stirrer and stir bar.
b. Filter sterilize (0.22 µm).

4. **TBE 0.5× solution**

Reagent	Final concentration	Amount
TBE (10×)	0.5×	100 mL
MilliQ water	n/a	1.9 L
Total	n/a	2 L

5. **Evans blue solution**

Reagent	Final concentration	Amount
Evans blue powder	0.5 %	0.5 g
TAP	n/a	100 mL
Total	n/a	100 mL

Filter sterilize (0.22 µm).

Laboratory supplies

1. Spectrophotometer semi-micro cuvette (Biosigma, catalog number: BSA002)
2. 24-well plates (Evergreen Labware, Medical Caplugs, catalog number: 222-8044-01F)
3. Surgical tape (Micropore™, catalog number: 1530-0)
4. Counting chamber Neubauer (Blaubrand, catalog number: BR717805)

Equipment

1. Spectrophotometer (Implen Nanophotometer)
2. Microplate reader (ClarioStar Plus, BMG LABTECH)
3. Orbitron Rotary shaker (Infors)
4. Olympus BX43 microscope
5. Thermomixer (Eppendorf, catalog number: 5382000015)
6. Vortex (Dutsher Vortex Genie 2, catalog number: 079008)
7. Electrophoresis tanks (Embitec® runOne™, model: EP-2000)
8. Camera (Q-IMAGING Micropublisher 3.3 RTV)
9. NanoDrop One (Thermo Scientific)
10. GelDoc Go Imaging System (Bio-Rad)

Software and datasets

1. Image Lab v6.1 for the GelDoc Go Imaging System (Bio-Rad)
2. Smart control data analysis for ClarioStar Plus (MARS, BMG LabTech)

Procedure

A. Cell culture

1. Cultivate *Chlamydomonas* cells in TAP liquid media at 25 °C, under continuous light (50 µmol

photons/m²/s) with shaking at 120 rpm until you obtain a concentration of 4×10^6 to 6×10^6 cell/mL.

2. Transfer 1 mL of culture to as many wells as required in a 24-well plate.
3. Add 20 µL of a 50 mM fresh solution of GSNO per well.
4. Close the 24-well plate with Micropore surgical tape and place it at 25 °C under continuous light (50 µmol photons/m²/s) with shaking at 120 rpm.

B. NO detection after GSNO treatment

1. Add the DAF-FM diacetate to your sample at a final concentration of 5 µM (e.g., 1 µL of a 5 mM solution in a final volume of 1 mL) and place the cells under low light (e.g., 10 µmol photons/m²/s) at 25 °C under agitation (120 rpm) for 30 min.
2. Centrifuge cells at 2,300× *g* for 3 min, remove the supernatant, and replace it with the same volume of TAP media. Repeat twice.
3. After a 15 min incubation period, add GSNO to your sample at a final concentration of 1 mM (e.g., 20 µL of a 50 mM solution in a final volume of 1 mL).
4. NO can be detected as soon as 30 min after GSNO treatment in a plate reader, using wavelengths corresponding to fluorescein (excitation 483 ± 14 nm and emission at 530 ± 30 nm) (Figure 1).

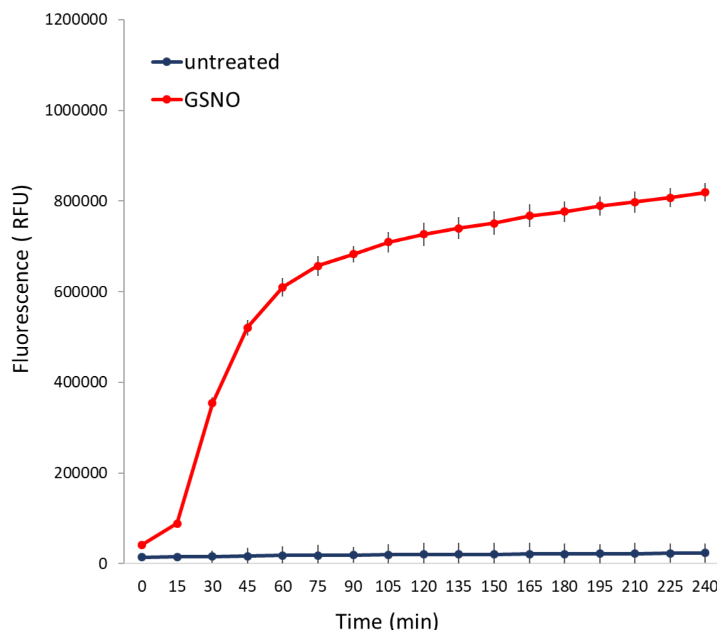


Figure 1. Example of nitric oxide (NO) quantification after S-nitrosoglutathione (GSNO) treatment. DAF-FM fluorescence was measured every 15 min for 4 h in a sample treated with GSNO (1 mM) and in the untreated control. Values represent the average of four biological replicates; error bars indicate ± SEM.

C. DNA degradation profile analysis

1. Eight hours after the beginning of the GSNO treatment, transfer the contents of two wells (2 mL) of the 24-well plate into a 2 mL Eppendorf tube.
2. Harvest the cells by centrifuging the tubes at 2,300× *g* for 5 min.
3. Remove the supernatant and dissolve the pellet in 600 µL of extraction buffer by placing it for 10 min at 37 °C under 1,400 rpm agitation in a thermomixer.
4. Centrifuge at 17,000× *g* for 3 min to pellet the debris.
5. Transfer 400 µL of the supernatant into a new 1.5 mL Eppendorf tube.

6. Add 500 µL of phenol:chloroform:isoamyl solution.
7. Mix the solution by vortexing for at least 30 s.
8. Centrifuge at 16,000× *g* for 5 min.
9. Carefully collect 300 µL of the upper phase and transfer it to a new 1.5 mL Eppendorf tube.
10. Add 750 µL of 100% ethanol and 45 µL of sodium acetate 3.3 M, mix gently turning the tube over a few times, and leave in ice for 30 min.
11. Centrifuge at 17,000× *g* for 30 min at 4 °C to pellet the DNA.
12. Remove all supernatant and rinse the pellet with 500 µL of ethanol 70%. Centrifuge at 17,000× *g* for 5 min at 4 °C.
13. Remove all supernatant and dry the pellet by placing the tube upside down on absorbent paper for 10 min. *Note: No residues of ethanol should remain at this stage. If needed, the tubes can be dried for a longer time.*
14. Add 100 µL of water and dissolve the pellet by shaking at 1,400 rpm for 5 min at 50 °C in a thermomixer. *Note: The pellet can be hard to dissolve. If needed, use a pipette tip and increase agitation time.*
15. DNA concentration (typically 200–500 ng/µL) and A_{260nm}/A_{280nm} ratio (expressing protein contamination, typically between 1.8 and 2) are estimated spectrophotometrically using a NanoDrop.
16. Digest 10 µg of DNA with 1 µL of RNase A for 15 min at 37 °C.
17. Load 10 µg of your DNA samples and 4.5 µL of GeneRuler 100 bp Plus DNA ladder on a 2% agarose gel.
18. Check the DNA degradation profile after 20 min of migration at 100V and estimate the size of the DNA fragments, using GelDoc Go and Image Lab (Figure 2).

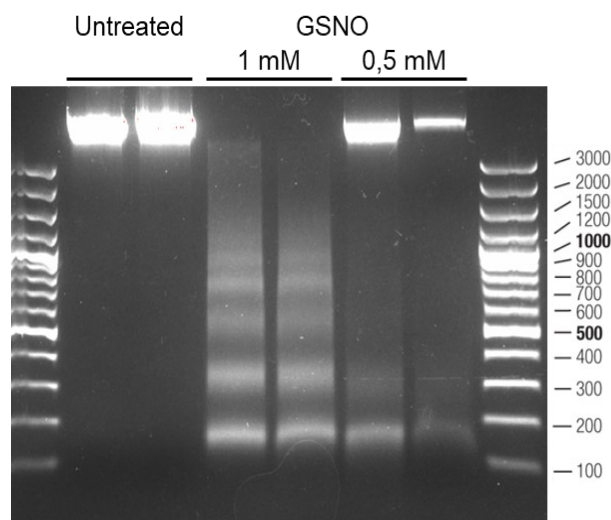


Figure 2. Analysis of DNA degradation profile in untreated cells compared with cells treated with S-nitrosoglutathione (GSNO) 0.5 mM and 1 mM, 8 h after treatment. For each condition, two independent samples are shown. The size of the marker bands is expressed in base pairs.

D. Death quantification procedure

1. At different times after GSNO treatment (we recommend 4, 8, and 24 h), take 20 µL of culture from each well and add 8 µL of Evans blue 0.5%.
2. Observe the cells under a microscope (at 200× or 400× magnification) using a slide with sufficient space to avoid crushing the cells (e.g., Counting chamber Neubauer) and take a representative photo of each sample.
3. Determine the percentage of dead cells over a population of at least 100 cells by counting the number of blue (dead cells) and living cells (green cells) (Figure 3A). The percentage of dead cells is equal to the number of dead cells divided by the total number of cells, multiplied by 100 (Figure 3B).

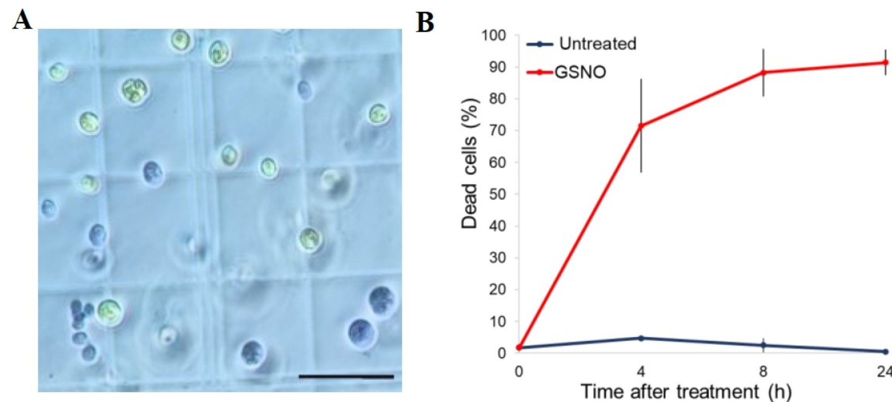


Figure 3. Cell death quantification using Evans blue staining. A. Living cells remain green, while dead cells appear blue after treatment with Evans blue. Scale bar represents 50 µm. B. Percentage of dead cells at different times after S-nitrosoglutathione (GSNO) treatment compared with control. Values represent the average of six biological replicates; error bars indicate ± SEM. Adapted from Lambert et al. [12].

Validation of protocol

This protocol or parts of it has been used and validated in the following research article:

- Lambert et al. [12]. Type II metacaspase mediates light-dependent programmed cell death in *Chlamydomonas reinhardtii*. *Plant Physiology* (Figures 1, 2–6).

In this article, the percentage of death was assessed in different *Chlamydomonas* populations, using between four and six biological replicates and a Student's *t*-test to highlight significant differences. In this way, we were able to reveal quite fine statistical differences between the different samples tested (e.g., Figure 1A).

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Competing interest

The authors declare no conflicts of interest.

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