

Jan 22, 2025

Measuring ROS production by neutrophils using DCFH-DA probe and flow Cytometry

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvj9j3blk5/v1>

Valeria Duarte de Almeida¹

¹Faculty of Health Sciences



Valeria Duarte de Almeida

Faculty of Health Sciences, Universidade do Estado do Rio Gr...

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.n2bvj9j3blk5/v1>

Protocol Citation: Valeria Duarte de Almeida 2025. Measuring ROS production by neutrophils using DCFH-DA probe and flow Cytometry. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.n2bvj9j3blk5/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working.



Created: January 22, 2025

Last Modified: January 22, 2025

Protocol Integer ID: 118815

Keywords: ROS, Neutrophils, ros production by neutrophil, intracellular ros level, cytometry neutrophil, neutrophil, stimulated neutrophil, specificity of ros detection, flow cytometry, ros detection, measuring ros production, increased fluorescence intensity, indicating higher ros production, ros production, fluorescence intensity, higher ros production, fluorescence, significant reduction in fluorescence, ro, using dcfh

Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to [protocols.io](https://www.protocols.io) is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with [protocols.io](https://www.protocols.io), can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Abstract

Neutrophils are isolated, stained with the DCFH-DA probe, and incubated with or without stimuli (e.g., PMA or fMLP) to induce ROS production. After incubation, fluorescence is measured by flow cytometry to assess intracellular ROS levels. Stimulated neutrophils should exhibit increased fluorescence intensity compared to unstimulated controls, indicating higher ROS production. The presence of inhibitors like DPI should result in a significant reduction in fluorescence, confirming the specificity of ROS detection.

Guidelines

To ensure the accuracy, reproducibility, and safety of the experiment, the following guidelines should be followed:

- Use anticoagulants like EDTA or heparin to prevent clotting.
- Process blood samples promptly (within 2–4 hours) to ensure neutrophil viability.
- Ensure >95% cell viability using trypan blue or propidium iodide staining before proceeding with the assay.
- Handle cells gently to avoid activation or damage.
- Always include appropriate controls (negative control, positive control with PMA, and inhibitor control with DPI) to validate the experiment.
- Use biological replicates (at least 3) to ensure statistical significance.
- Maintain consistent cell concentrations across samples (e.g., 1×10^6 cells/mL).
- Use freshly prepared reagents to prevent degradation and variability.
- Prepare DCFH-DA solutions under low light conditions to prevent premature oxidation.
- Aliquot reagents to avoid repeated freeze-thaw cycles.
- Set appropriate forward and side scatter parameters to properly gate neutrophil populations.
- Conduct experiments under identical conditions, such as temperature, incubation times, and reagent batches.
- Use standardized protocols for sample preparation and data analysis.
- Work in a sterile environment to prevent bacterial contamination, which may alter results.
- Regularly clean flow cytometry fluidics to avoid cross-contamination.

Materials

- Neutrophils suspension (isolated from human peripheral blood or animal models)
- DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) probe
- Phosphate-buffered saline (PBS), pH 7.4
- RPMI 1640 medium (with 10% fetal bovine serum, optional)
- Stimuli for positive control: Phorbol 12-myristate 13-acetate (PMA)
- Inhibitors (if needed): Diphenyleneiodonium (DPI, NADPH oxidase inhibitor)
- Flow cytometer with excitation/emission filters for FITC (488/530 nm)
- FACS tubes or 96-well plates
- Refrigerated centrifuge and 37°C water bath

Safety warnings

- ❗ **Biological Hazards:** Handle blood-derived neutrophils with care to avoid potential infections. Disinfect work surfaces before and after the experiment.
- **Chemical Hazards:** PMA and DPI are potentially hazardous; handle them inside a fume hood and dispose of them properly.
- **Laser Safety:** Avoid direct exposure to the laser beams of the flow cytometer to prevent eye damage.

Ethics statement

Human Samples:

- Obtain informed consent from donors in compliance with institutional and national ethical regulations.
- Ensure anonymity and confidentiality of donor data.
- Dispose of biohazardous waste following institutional protocols.

Animal Studies:

- Conduct experiments according to ethical guidelines outlined by relevant regulatory bodies (e.g., ARRIVE, IACUC).
- Use the minimum number of animals required to achieve statistical power.

If approval was obtained, please include the name of the IACUC or equivalent ethics committee and any relevant permit numbers.

Before start

Before starting the experiment, ensure the following conditions are met:

1. **Personal Protective Equipment (PPE):** Wear gloves, lab coat, and safety goggles when handling blood samples and reagents.
2. **Biosafety:** Follow biosafety level 2 (BSL-2) guidelines if working with human blood or primary cells.
3. **Instrument Calibration:** Verify that the flow cytometer is properly calibrated and compensated to avoid false-positive signals.
4. **Reagent Preparation:** Prepare fresh DCFH-DA working solutions to prevent auto-oxidation and ensure accurate results.
5. **Sample Collection Ethics:** Ensure all human samples are collected with informed consent and under the approval of an Institutional Ethics Committee.



Neutrophil Isolation and Preparation

3h 30m

- 1 Isolate neutrophils from whole blood using density gradient centrifugation (e.g., Ficoll-Paque).
- 2 Wash cells twice with PBS to remove any residual isolation medium.
- 3 Count the cells and adjust the concentration to approximately **1×10^6 cells/mL** in RPMI 1640 or PBS.

3h



30m



Staining with DCFH-DA

45m

- 4 Prepare the DCFH-DA working solution at a final concentration of **$5\text{--}10 \mu\text{M}$ in PBS**.
- 5 Add the DCFH-DA to the neutrophil suspension and incubate for **20–30 minutes at 37°C in the dark**, allowing the probe to penetrate the cells and be hydrolyzed to non-fluorescent DCFH.
- 6 After incubation, wash cells with PBS to remove excess probe.

5m

30m



10m



ROS Stimulation and Measurement

1h 20m

- 7 **Divide cells into different experimental groups**, such as:
 - Unstimulated control (no treatment)
 - Positive control (treated with 100 nM PMA)
 - Experimental groups (treated with stimuli of interest, e.g., fMLP)
 - Inhibitor groups (pretreated with DPI before stimulation)
- 8 Incubate cells with the desired stimulus for **30–60 minutes at 37°C**.
- 9 After incubation, immediately place cells on ice to stop the reaction

1h





- 10 Analyze fluorescence using a flow cytometer, with excitation at **488 nm** and emission at **530 nm** (FITC channel).

20m



Flow Cytometry Analysis

20m

- 11 Set up the flow cytometer using unstained cells (gate) to establish background fluorescence.

2m

- 12 Acquire at least **10,000 events** per sample.

- 13 Analyze data in terms of:
- **Mean fluorescence intensity (MFI)**, which reflects the level of ROS production.
 - Percentage of positive cells producing ROS.

18m



- 14 Compare results across conditions to determine changes in ROS levels.



Data Analysis and Interpretation

- 15
- Increased fluorescence indicates higher ROS production.
 - Compare MFI values between treated and control samples.
 - Use statistical tests (e.g., ANOVA or t-test) to assess significance.



Protocol references

Ito, Y., Lipschitz, D.A. (2002). Assay of Intracellular Hydrogen Peroxide Generation in Activated Cytometry Individual Neutrophils by Flow. In: Armstrong, D. (eds) Oxidants and Antioxidants. Methods in Molecular BiologyTM, vol 196. Humana Press. <https://doi.org/10.1385/1-59259-274-0:111>

Eruslanov, E., Kusmartsev, S. (2010). Identification of ROS Using Oxidized DCFDA and Flow-Cytometry. In: Armstrong, D. (eds) Advanced Protocols in Oxidative Stress II. Methods in Molecular Biology, vol 594. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60761-411-1_4

Muñoz-Sánchez G, Godínez-Méndez LA, Fafutis-Morris M, Delgado-Rizo V. Effect of Antioxidant Supplementation on NET Formation Induced by LPS In Vitro; the Roles of Vitamins E and C, Glutathione, and N-acetyl Cysteine. Int J Mol Sci. 2023 Aug 24;24(17):13162. doi: 10.3390/ijms241713162. PMID: 37685966; PMCID: PMC10487622.