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Optimization of ROS Measurement in PANC-1 Cells

Merve Gündoğdu[1], Didem Tecimel[1], Ömer Faruk Bayrak[2], Didem Seven[2]

[1] Department of Bioengineering and Genetics, Yeditepe University Faculty of Medicine, İstanbul, Türkiye; [2] Department of Medical Genetics, Yeditepe University School of Medicine, İstanbul, Türkiye

Abstract

Objective: Accurate measurement of reactive oxygen species (ROS) is essential for understanding oxidative stress-related cellular responses. Among the available detection methods, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is widely used due to its sensitivity and ease of application. However, signal variability due to differences in incubation time can impact data reliability. This study aimed to optimize the incubation time of H_2DCFDA for accurate detection of intracellular ROS levels in PANC-1 cells using flow cytometry.

Materials and Methods: PANC-1 cells were cultured under optimized conditions and incubated with 10 µM H_2DCFDA from 5 minutes to 90 minutes. Following incubation, cells were detached, washed, and analyzed using flow cytometry (FITC channel). Three independent biological replicates were performed for each time point.

Results: A time-dependent increase in intracellular ROS fluorescence was observed. At 5 minutes, 19.79% of cells were ROS-positive, which increased from 30.65% to 65.70% until 25 minutes, respectively. After that point, ROS-signal was saturated and measured approximately 90-95%, like positive control. The strongest fluorescence signal, was detected at 30 minutes, indicating a peak in probe oxidation and ROS detection efficiency.

Conclusion: This study demonstrates that H_2DCFDA provides reliable time-sensitive ROS detection in PANC-1 cells, with 30-minute incubation offering optimal signal intensity without additional chemical induction. However, variations in ROS dynamics among different cell types underscore the need for cell-specific optimization of assay conditions.

Keywords: H2DCFDA, ROS assay, flow cytometry, oxidative stress, PANC-1

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Correspondence Didem Seven **E-mail** didem.seven@yeditepe.edu.tr

INTRODUCTION

Oxidative stress is a condition that arises when the production of reactive oxygen species (ROS) during cellular metabolism exceeds the antioxidant defense capacity (1). ROS comprise a variety of reactive molecules and free radicals, including superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) (2). Under physiological conditions, low levels of ROS play regulatory roles in intracellular signaling pathways; however, excessive ROS can lead to oxidative damage of lipids, proteins, and DNA contributing to inflammation, tissue injury, and organ dysfunction (3). Furthermore, oxidative stress plays a key role in the pathogenesis of various chronic diseases, including cancer progression, atherosclerosis, neurodegenerative disorders, and diabetes (1). Recent studies have shown that although a certain amount of well-regulated ROS production contributes to immune homeostasis, uncontrolled elevation of ROS levels may cause cellular damage. As a result, the biology of ROS and oxidative stress has become an intensive area of investigation across multiple disciplines such as cancer research (2).

One One of the most used techniques for detecting intracellular ROS levels is the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA, non-fluorescent, is a cell-permeable compound that is hydrolyzed by intracellular esterases and, upon oxidation, in the presence of ROS, is converted into the highly fluorescent product DCF. The H_2DCFDA method is advantageous due to its relative ease of use, high sensitivity, and low cost (3). When combined with flow cytometry, fluorescence intensity can be quantified at a single-cell level, enabling precise identification of cells undergoing elevated oxidative stress within heterogeneous populations (2).

However, the H_2DCFDA-based ROS detection method has some important drawbacks. The H_2DCFDA probe does not respond equally to all types of ROS; for instance, it does not react directly with superoxide anions ($O_2^{\bullet -}$) but can be oxidized indirectly in the presence of catalytic metal ions such as iron particularly Fe (II). As a result, fluorescence signals may not always reflect actual ROS levels under certain experimental conditions. Furthermore, H_2DCFDA can be oxidized by other reactive species, including nitric oxide (NO) and peroxynitrite, and its fluorescence signal may also be influenced by changes in intracellular antioxidant levels, such as glutathione (4). To ensure accurate ROS measurement, factors such as light sensitivity of the fluorophore, esterase variability among cells, and mechanical stress during cell handling must be carefully controlled (5). Taken together, while H_2DCFDA serves as a valuable and dynamic indicator of oxidative activity, careful consideration of its limitations is essential for accurate interpretation of data.

The biological effects of ROS depend not only on their concentration but also on the duration of exposure. While transient increased ROS levels may serve as physiological signals, prolonged and widespread ROS accumulation is often associated with pathological outcomes. Besides, the kinetics of ROS production, persistence, and clearance are critical for the accurate interpretation of experimental results. Inadequate temporal optimization may lead to misinterpretation, for instance, mistaking a transient ROS spike for sustained oxidative stress. Hence, precise control and optimization of temporal parameters in ROS measurements are essential for elucidating underlying mechanisms and identifying effective therapeutic targets (6). In this regard, the duration of incubation is a key variable when using fluorescent probes such as H_2DCFDA for ROS detection. Short incubation periods may yield insufficient signal intensity, whereas excessively long exposures can result in signal saturation, photobleaching, or loss of cellular viability, all of which compromise data reliability (7). Moreover, metabolic activity, esterase expression, and ROS dynamics vary across cell types, necessitating specific optimization for each experimental system. Researchers can ensure that ROS measurements accurately reflect the biological state of the cells and remain within the linear detection range of the probe by systematically calibrating incubation times (8).

Since oxidative stress plays an important role in cellular physiology as well as in pathophysiology, the accurate, sensitive, and consistent measurement of ROS levels is essential. However, the technical limitations and interpretability of ROS detection protocols can significantly impact the reliability of experimental outcomes. In many studies, H_2DCFDA incubation time is used as a fixed value, even though it's not always tested for the specific cell type or experimental setup. However, this can compromise data accuracy—short incubations may yield weak signals, whereas extended durations can lead to fluorescence saturation, phototoxicity, or cell stress. In this context, we optimized the H_2DCFDA incubation time for PANC-1 cells using flow cytometry, as incubation conditions can strongly influence baseline ROS measurements. As no ROS-inducing agent was used in this study, it is particularly important to tailor the protocol to each cell type to ensure reliable detection of intrinsic ROS levels.

MATERIALS AND METHODS

Cell Culture

PANC-1 pancreatic cancer cells (American Tissue Cell

Culture [ATCC], Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37°C. For passages, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution is used.

Preparation of H2DCFDA

To prepare the stock solution, 0.0097 g of H_2DCFDA powder (MedChemExpress, HY-D0940) was accurately weighed and dissolved in 2 mL of dimethyl sulfoxide (DMSO) with the aid of ultrasonic agitation, yielding a 10 mM concentration. For experimental use, the working solution was freshly prepared by diluting the stock in 1X phosphate-buffered saline (PBS) to achieve a final concentration of 10 µM. This solution was then applied directly to the cells for ROS detection.

Incubation with H_2DCFDA

Intracellular ROS levels were assessed using the fluorescent probe H_2DCFDA, 24 hours after seeding the cells at approximately 70% confluency, with or without treatment. Cells were incubated with 2 mL of a 10 µM H_2DCFDA solution prepared in 1X PBS for 5-10-15-20-25-30-40-50-60 and 90 minutes at 37°C in the dark. As a positive control, cells were also treated with 500 µM H_2O_2 for 30 minutes. After incubation, cells were detached by trypsinization and collected in 5 mL of 1X PBS. The suspension was then centrifuged at 300 g for 3 minutes at room temperature. The resulting cell pellet was gently resuspended in 100 µL of 1X PBS, and samples were immediately analyzed by flow cytometry to measure ROS-associated fluorescence. Three biological replicates were carried out.

Flow Cytometry Acquisition and Analysis Parameters

Flow cytometric analysis was performed using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). H_2DCFDA was excited using a 488 nm argon-ion laser, and emission was detected in the FITC channel (FL1; 530/30 nm bandpass filter). A minimum of 10,000 events were collected per sample. Instrument settings, including forward scatter (FSC), side scatter (SSC), and FITC voltages, were optimized using untreated (unstained) control cells. Data acquisition and analysis were conducted using FlowJo™ software v10 (BD Biosciences, Ashland, OR, USA).

RESULTS

Flow cytometric analysis using H_2DCFDA staining revealed a time-dependent increase in intracellular ROS levels. Histograms obtained from the FITC-A channel demonstrated a progressive rightward shift in fluorescence intensity with increasing incubation time. At 5 minutes, 19.79% of the cells exhibited ROS positivity, which

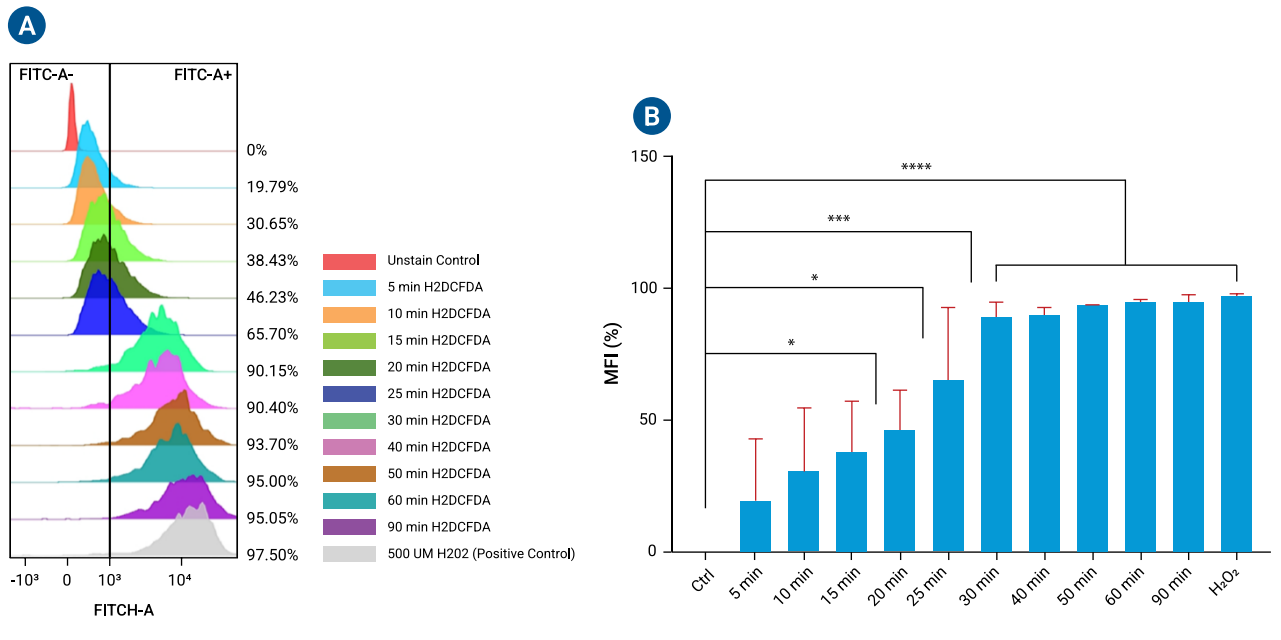

FIGURE 1. (A) Representative FITC-A histograms of PANC-1 cells stained with H_2DCFDA for various incubation durations (5 to 90 minutes), along with negative (unstained) and positive (H_2O_2-treated) controls. (B) Quantitative analysis of ROS levels presented as mean fluorescence intensity (MFI), normalized to the control group. Data are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical comparisons were performed relative to the unstained control group.
*p<0.05, ***p<0.01.

increased to 30.65%, 38.43%, 46.23%, 65.70% and %90.15 at 10, 15, 20, 25 and 30 minutes, respectively. ROS signal levels appear to plateau, indicating that the probe reaches saturation and no additional ROS accumulation is detected beyond 30 minutes, significantly (p>0.05) (Figure 1). All statistical comparisons were performed relative to control samples that were not treated with H_2DCFDA.

DISCUSSION

In this study, intracellular ROS levels were measured in PANC-1 cells using H_2DCFDA-based flow cytometry method to detect the most appropriate incubation time. The findings showed that there was a gradual increase in FITC-A fluorescence signal depending on the incubation time and the ROS positive cell ratio increased significantly especially at the 30th minute (Figure 1). After that point, fluorescence signal reached a saturation point and there was no significant difference for the following time points. These results suggested that H_2DCFDA is a suitable indicator for monitoring intracellular oxidative stress levels in a time-sensitive manner.

This increase in ROS levels indicates that cells are exposed to increasing oxidative stress over time and suggests that H_2DCFDA accurately reflects dynamic ROS changes. The 19.79% positivity detected at 5 minutes shows that H_2DCFDA is sensitive to ROS even in short-term incubations, while the 90.15 % positivity reached at 30 minutes provides quantitative information about the oxidative status of the cells (Figure 1). This linear increase supports the fact that H_2DCFDA is oxidized by increasing ROS over time and becomes fluorescent DCF, which can be measured by flow cytometry, as reported in the literature (9,10). According to our results, ROS levels could be measured even at incubation times as low as 5 minutes, but 30 minutes is generally accepted as the standard time in the existing literature. For instance, Kim and Xue (11) measured ROS levels in colorectal cancer cells by incubating them with a 10 μM H_2DCFDA solution at 37°C for 30 minutes. Also, Bode et al. (3), used H_2DCFDA in measuring ROS by incubating different cells with it for 30 minutes. Our results also demonstrated that a 30-minute incubation time is suitable for PANC-1 cells, as it provided a high ROS signal compared to control cells. Notably, no significant difference was found between the 25- and 30-minute time points, suggesting that saturation occurs over 30 minutes. Moreover, no significant differences were observed among the extended incubation times beyond 30 minutes, further supporting the notion of signal saturation.

On the other hand, the concentration of H_2DCFDA is also a critical factor affecting ROS detection. Wu and Yotnda (12) recommend using a starting dose of 10 μM for the determination of ROS in cancer cells to evaluate the toxicity. In our study, a 10 μM solution was used, which is consistent with the initial recommendations. Soares et al. (13) optimized ROS detection in human blood samples, using a 120 μM solution and an incubation time of 30 minutes. Gonzalez and Salido (14) worked on pancreatic acinar cells to detect ROS levels, with a 10 μM H_2DCFDA solution and incubation time of 40 minutes. These studies demonstrate that incubation time and concentration can significantly affect the results, depending on the cell type.

However, some limitations of using H_2DCFDA should be considered. H_2DCFDA is not equally sensitive to all ROS species; for example, it does not directly respond to $O_2^{\bullet -}$ but can be indirectly oxidized *via* metal ions. This means that in some experimental conditions the measured fluorescence signal may not fully reflect the true ROS concentration. Furthermore, H_2DCFDA also reacts with other reactive species such as NO and peroxynitrite, which may reduce the specificity of the signal (4). In addition, since intracellular antioxidant levels such as glutathione may also affect the signal, standardization of experimental conditions is of great importance. Some studies report that trypsinizing adherent cells and then incubating them with DCFH-DA in suspension can yield higher ROS signals. However, this approach can disrupt cell integrity due to enzymatic disaggregation, which can affect the ROS signal. Incubation with H_2DCFDA directly on adherent cells, as in our protocol, yields results that are closer to those obtained with standard ROS measurement methods in the literature (15,16).

This study demonstrates that H_2DCFDA is a highly sensitive and applicable method for the detection of ROS; however, potential limitations should be considered in the interpretation of the data. In particular, standardization of cell culture duration, incubation time and measurement conditions will increase the comparability and biological significance of the obtained signals. In this respect, the study provides a reliable experimental protocol that can be used in oxidative stress studies and provides a methodological contribution to H_2DCFDA-based analyses in the literature.

CONCLUSION

In conclusion, the need for cell type-specific optimization in the assessment of ROS levels with H_2DCFDA is clear. Among the most important limitations of our study is the lack of a healthy cell line. However, the optimizations for the time and dose used were performed to determine the appropriate conditions for PANC-1 cells. Therefore, it is crucial for each study to experimentally determine the conditions specific to its own cell line to obtain reliable and comparable results.

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Informed Consent: N.A.

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