MICROVOLUMETRIC DETECTION OF REACTIVE OXYGEN SPECIES IN LIVING CELLS

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Abstract. Detecting reactive oxygen species (ROS) in living cells is a difficult task because of their short lifetime, presence of cellular antioxidants, risk of cell death and unwanted transformations of the detector substance. Fluorescence-based techniques are currently most widely used, with high sensitivity and easy processing of samples but the classical instruments need a high amount of samples and consumables. We optimized and validated a microvolumetric fluorescence-based method for ROS detection in living cells, using 2',7'-dichlorodihydrofluorescein acetate (H_2DCFDA) and a microvolumetric fluorospectrometer. In our experimental conditions we determined that a measurement volume of 3 μL containing between 125 and 500 cells was sufficient for a reliable measurement of oxidized 2',7'-dichlorofluorescein fluorescence. The method was applied on H_2O_2 and catalase treated MEF k41 cells. The catalase preloaded cells presented a lower fluorescent signal under H_2O_2 stress. Our results show that this microvolumetric method is suitable for oxidative stress specific detection even when compounds able to discriminate the various molecular categories of ROS are added. The measurements could be performed with high accuracy, demonstrating that the microvolumetric method provides a sensitive, fast and cost-effective means for ROS detection in living cells.

Key words: reactive oxygen species, 2',7'-dichlorodihydrofluorescein acetate, microvolumetric fluorospectrometer.

INTRODUCTION

Most adverse effects exerted on our organism by physical, chemical or biological environmental agents are mediated by an excessive production of reactive oxygen species (ROS) at cellular level. At the same time, proper functioning of a variety of intracellular signaling pathways depends on a controlled

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ROS production. Fast, sensitive and specific detection of reactive oxygen species is essential for the characterization of molecular mechanisms of toxicity and the relationship between ROS and disease.

Several methods have been developed for global or specific evaluation of different reactive species that arise in the cell: electron paramagnetic resonance (EPR) [11], chemical reaction with reduced compounds followed by fluorescent or luminescent evaluation [3, 5, 9]. However, detection of ROS in living cells is challenging because of their very short lifetime and the abundance of antioxidants. Additionally, it is required to speed the analysis in order to minimize cell death and detector's loss or subsequent transformations.

The fluorescence-based methods provide sensitive and specific detection of reactive species and also a convenient laboratory processing of the samples. Most fluorophores used for this purpose are fluorescein, rhodamine or ethidium derivatives (reviewed in [12]). 2',7'-dichlorodihydrofluorescein acetate (H_2DCFDA) is a fluorescein derivative which reacts with most cellular ROS. The compound is non-fluorescent in native, reduced state and becomes fluorescent after removal of acetate groups by the cellular esterases and subsequent oxidation. The fluorescent signal is proportional to the number of molecules of oxidated deacetylated H_2DCFDA (DCF). Thus, the fluorescein-based method represents an easy to use, fast and reliable means for the general evaluation of ROS production in the cell. Additional experimental procedures involving the use of specific antioxidants or enzymes may confer specificity to these protocols.

The equipments used for fluorescence measurement vary from classical, cuvette-based fluorospectrometers to plate readers, fluorescence or confocal microscopes, or flow-cytometers. Each of these has both advantages and disadvantages in terms of sensitivity, specificity, speed and costs.

Our aim was to optimize and validate a microvolumetric method for fluorescence-based detection of reactive oxygen species in living cells exposed to oxidative stress. This method is based on 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) labeling of cultured cells and fluorescence measurement using a microvolumetric fluorimeter. The microvolumetric fluorospectrometers are largely used for the quantification of nucleic acid [2, 4] and proteins [7], but to our knowledge there is no description of use in ROS detection in living cells. The main concern about using small volumes of cell suspensions for fluorescence measurements in a microvolumetric spectrofluorimeter is related to the potential artifacts generated by light scattering in non-homogeneous media. In our study we overcome these limitations by a thorough optimization of cellular and fluorophore concentration.

MATERIALS AND METHODS

CELL CULTURE

The MEF k41 cells (SV40 immortalized murine embryo fibroblasts) were cultured in high glucose Dulbecco's Modified Essential Medium (DMEM-PAA, Austria) supplemented with 10% fetal serum (Biochrom AG, Germany), and 2% L-Glutamine in a humidified incubator with 5% CO_2 atmosphere, at 37°C. The cells were seeded in 12-wells culture plates, at 0.75 x 105 cells/well and let to adhere for 24 h, when the catalase or H_2O_2 treatment were applied. H_2DCFDA labeled cells were detached with a 0,025% Trypsin-EDTA solution (Sigma-Aldrich, EU), counted with a Scepter Handheld Automated Cell Counter (Millipore, Billerica, USA) and analyzed.

H_2O_2 TREATMENT

H_2O_2 was used as a reference oxidant agent, in concentrations of 0, 50, 100, 200, 400 μM. For experimental setup and validation steps, negative control samples were prepared by incubating the cells with catalase (Fluka, EU) in concentration of 1000 units/ml, for 24 h prior to H_2DCFDA labeling and H_2O_2 treatment. Catalase is a H_2O_2 specific scavenger. Another 500 units catalase/ml were added to the cells during their incubation with H_2O_2. Both catalase negative and positive cells were labeled as described below and incubated with H_2O_2 for 5 minutes, in Phosphate Salt solution (pH 7.2, Sigma-Aldrich, EU) (PBS), at room temperature. All solutions were prepared in PBS, with or without 500 units/ml bovine catalase (Fluka) or an equal amount of sterile PBS.

LABELING

Cell labeling was performed for 30 minutes, at 37 °C, in unsupplemented CO_2 independent DMEM (Gibco® – Life Technologies, USA), using H_2DCFDA (Molecular Probes® – Life Technologies, USA). The labeling protocol was optimized by testing several concentrations of H_2DCFDA: 10, 25, 50, 75 and 100 μM. For all the subsequent treatments the 75 μM H_2DCFDA concentration was used. Labeling was performed in the dark and the re-suspended cells were transferred to amber tubes.

FLUORESCENCE MEASUREMENT

Fluorescence measurement was done using a NanoDrop™ 3300 spectrofluorimeter and ND-3300 v.2.7 software (both from Thermo Fischer

Scientific, Wilmington, USA) after automatic selection of sources by using the included Fluorescence Profiler [13]. The blue LED (λ_{ex} = 475 nm) was chosen for excitation and the 523 nm as the emission reading wavelength. The measurement volume recommended by the manufacturer is 1– 5µL. For cellular suspensions we determined that 3 µL is the lowest volume that gave reproducible results. The trypsination protocol was optimized to prevent cell clumps or debris formation, by keeping the cells for 3 minutes at room temperature and microscopic evaluation. Cellular suspensions were mixed by gentle pipetting before each measurement. PBS solution without cells was used for blank measurement. An unlabeled suspension of MEF k41 cells was measured in order to exclude false positive signals derived from cell autofluorescence. Serial dilutions of labeled cells were tested in order to evaluate the sensitivity of fluorescence measurement.

RESULTS

OPTIMIZATION OF CELLULAR LABELING AND FLUORESCENCE MEASUREMENT

The choice of the best excitation source has been done using the fluorescence profiler application, incorporated in ND-3300 software. This application compares the excitation and emission spectra for all three possible sources, giving the emission values for every variant. The selection procedure showed that the excitation source which gives the highest emission signal from the oxidized DCF is the blue LED and the highest emission intensity was recorded at 523 nm (Fig.1A).

As shown in Fig. 1A, the UV source gave no signal and the intensity of the emission generated by the white LED was much lower than the blue one.

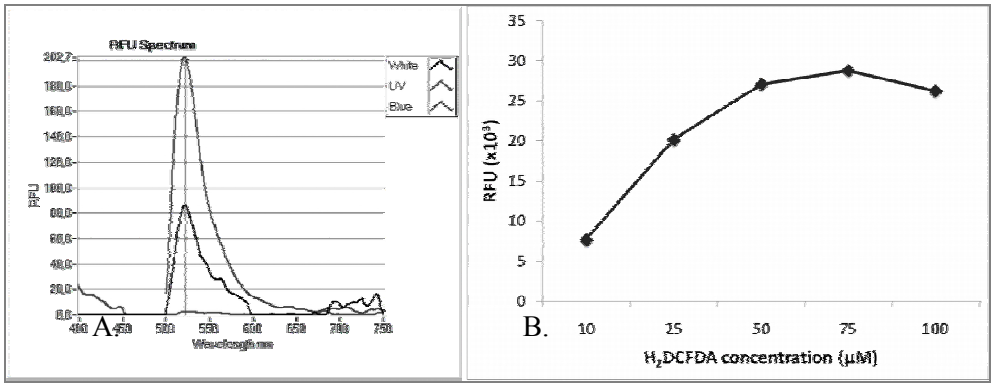

Fig. 1. Optimisation of H_2DCFDA labeling; A. excitation source selection – the highest emission is obtained for the blue LED; B. optimisation of DCFDA concentration.

H_2DCFDA concentration was optimized to give the maximum performance while limiting the unwanted phenomena like: auto-oxidation, quenching, extracellular hydrolysis. The MEF k41 cells were incubated for the same period of time with H_2DCFDA in concentrations of 10, 25, 50, 75 and 100 μM. The same cellular suspension was divided into 5 different samples and two separate tests were performed in parallel. In both cases, the highest fluorescence intensity was obtained for a concentration of 75 μM H_2DCFDA (Fig. 1B). This concentration has been used for further labeling and measurements.

As a negative control, unlabeled cells were measured in the same conditions in order to eliminate a potential false positive signal generated by cellular autofluorescence. No signal was detected above background at the 523 nm wavelength, corresponding to the maximum emission of oxidized dichlorofluorescein (DCF). Extracellular hydrolysis and oxidation of the fluorophore was excluded by measuring the fluorescence of the labeling solution in the absence of cells. The intensity of the fluorescence was also below the background level (less than 30 RFU).

The usual detection methods employed in reactive species investigation need at least several thousands of cells for the signal to rise above the detection threshold. We determined the lowest cell number that can be correctly evaluated by this microvolumetric method and the cell concentration range allowing an approximately linear increase of the fluorescent signal with the cell number. For this purpose, serial dilutions of a cellular suspension were prepared and measured. As we show in Fig. 2, DCF fluorescence reaches the background threshold for as little as 50 cells/3 μL measurement volume. On the contrary, a reliable fluorescence detection may be performed with 125 – 500 cells / 3 μL measurement volume.

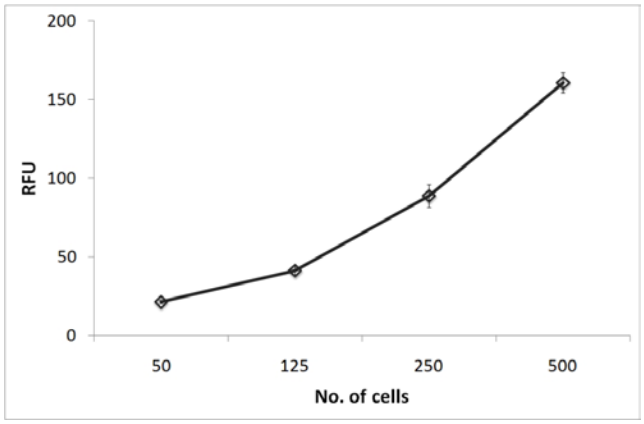

Fig. 2. Evaluation of the sensitivity of the NanoDrop™ 3300 based detection of the oxidized DCF fluorescence as a function of cell concentration in the 3 μL measurement volume.

In all cases, the results represent the media for three readings of the same sample and two different experiments.

VALIDATION OF THE MICROVOLUMETRIC METHOD USING H_2O_2 AS REFERENCE OXIDANT

In basal conditions the living cells produce low amounts of reactive species as a result of metabolic and signaling processes. Interaction with an oxidant stressor induces an increased accumulation of reactive species, which overcome cellular antioxidant capacity and generate an increased fluorescence signal from DCF oxidation.

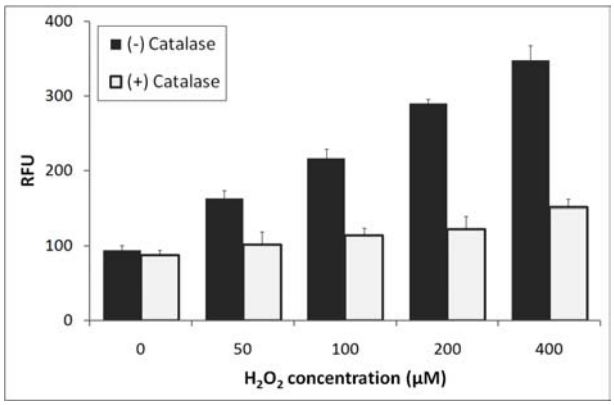

Fig. 3. DCF fluorescence induced by H_2O_2 incubation in catalase treated and untreated MEF k41 cells.

To validate our method we used H_2O_2 as a reference oxidant, applied in different concentrations on H_2DCFDA labeled MEF k41 cells. Fluorescence signal increased proportionally with the concentration of H_2O_2. The very weak increase of fluorescence signal obtained for catalase preloaded cells incubated with even high concentrations of H_2O_2 confirms that the fluorescence was generated by H_2O_2 and it was not an artifact.

Moreover, in order to diminish possible artifacts generated by different intervals of incubation and cell processing until the fluorescent measurement, the samples were labeled and incubated sequentially with H_2O_2 and the measurements were performed immediately after cell preparation for fluorescence evaluation. Additionally, we checked the DCF fluorescence of the control sample at the end of measurement process (data not shown). Less than 10% increase of the signal was registered at this time.

The basal level of reactive species is different in different cell types, a small variation of ROS level may arise for the same kind of cells due to cell cycle phase or culturing conditions. In order to estimate the variations due to cell-to-cell variability, we measured the basal ROS level for cells grown in the same conditions. To evaluate how this characteristic is reflected by NanoDrop™ measurement, we seeded the MEF k41 cells in 8 wells of a 12-wells plate, labeled

them with H_2DCFDA in the same conditions and analyzed them as previously described. For all the 8 wells we obtained a comparable basal fluorescent signal (Fig. 4).

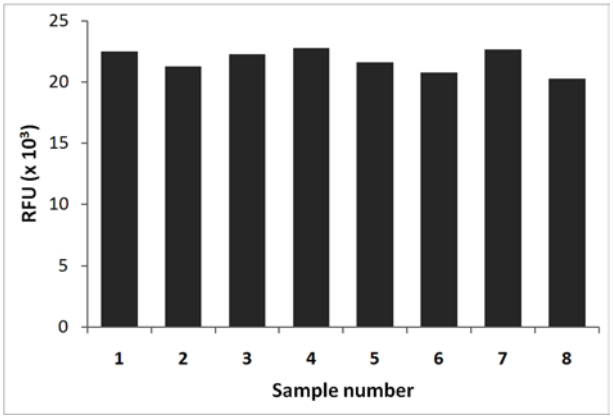

Fig. 4. Basal DCF fluorescence obtained for eight different catalase untreated samples of MEF k41 cells.

The time needed for fluorescent measurement of one sample was about 30 seconds. As shown in Fig. 4, within the same experimental category, the fluorescence variations were small and comfort us that the manipulation / cell processing procedures were controlled.

DISCUSSION

Our study is addressed to the need for a sensitive, robust and cost efficient detection of reactive species in small samples by testing and validating a microvolumetric fluorescence-based method. Our data show that the microvolumetric fluorimeter NanoDrop™ 3300 can be efficiently used for the detection of reactive species in living cells labeled with H_2DCFDA in samples containing at least 125 cells suspended in a 3 µL measurement volume. This also allows significant cost and time reductions compared to a regular cuvette-based fluorimeter as no other consumables are needed and a measurement can be performed as fast as 30 seconds. Additionally, less than 1000 cells are enough for a good evaluation, saving precious samples for other tests.

Moreover, a short time measurement when using cellular suspensions reduces problems related to cell death, dye leaking out of the cells, pH or temperature change or non-intended transformations of such a sensitive fluorophore like H_2DCFDA. On the other hand, measurement of whole cells in suspension in a volume as low as 3 µL may be influenced by light scattering, especially for less homogeneous solutions when cell clumps or debris are present, when imprecise

pipetting and small air bubbles are introduced when pipetting the sample onto the measurement pedestal. This is why it is important to ensure a good separation of cells and to limit cell death by a gentle treatment during labeling and subsequent manipulations. Because the measurement volume is very small (3 μL) and the detector is in close contact with the sample, our method diminishes the signal loss as a result of absorption processes in large volumes of buffer or due plastic irregularities as it is the case with classical cuvette-based detection methods.

When ROS evaluations are performed by fluorescence measurements using H_2DCFDA several limitations due to this fluorophore must not be neglected [5]. H_2DCFDA and its reactive derivative, $DCFH_2$, do not react with the superoxide radical, but this radical may increase the DCFDA fluorescence indirectly, through the action of a catalyst like iron [12]. This is why presence of iron should be considered when analyzing DCFDA data [10]. The evaluation of results should take into account that the changes in fluorescence may sometimes reflect the changes in antioxidants and not in ROS level [12].

Proper use of negative and positive controls ensures the reliability of results. In our experiments we eliminated possible artifacts generated by cellular autofluorescence or dye degradation and auto-oxidation, by measuring both the fluorescence of non-labelled cells and of a dye solution without cells. Transformations induced by prolonged incubation and manipulation were excluded by final re-evaluation of the first sample and by the reduced measurement time.

H_2DCFDA does not specifically detect H_2O_2 and can be oxidized also by other reactive species like peroxinitrite or hydroxyl radical [8, 6]. In our experimental set-up, we used H_2O_2 to induce oxidative stress in living cells. H_2O_2 enters freely in cells where it oxidizes the $DCFH_2$ to DCF which subsequently emits fluorescence. The specificity of this reaction was confirmed by the similar fluorescence levels for controls and catalase pre-loaded cells exposed to H_2O_2. In our experiment catalase, a specific H_2O_2 scavenger was used to demonstrate that the fluorescent signal was due to a ROS accumulation under the H_2O_2 treatment and not to a non-specific oxidative condition. There are studies showing non-ROS induced modifications of $DCFH_2$ in UVA irradiated cells, probably induced by light exposure [1]. Working in the dark and limiting the light exposure of our samples prevented the light-induced $DCFH_2$ oxidation.

In conclusion our method allows sensitive, cost and time-effective detection of reactive oxygen species at the same time limiting the unwanted alterations induced by experimental conditions. When compared with classical cuvette-based fluorescence measurement, microvolumetric detection insures significant cost reduction because of reduced reagent volumes and little number of consumables needed. There is an efficient use of this method for potentially limited samples, less than 1000 cells are enough for three independent measurements, even more, different tests may be done from the same limited sample when various biological evaluations must be correlated. Moreover, due to no interfering factors present

between sample and detection system (e.g, the plastic of the cuvette wall), a high reproducibility is obtained. In association with specific antioxidant treatments, this microvolumetric method can be used for specific detection of various reactive species.

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