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Detection of Total Reactive Oxygen Species in Adherent Cells by 2',7'-Dichlorodihydrofluorescein Diacetate Staining

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Abstract

Oxidative stress is an important event under both physiological and pathological conditions. In this study, we demonstrate how to quantify oxidative stress by measuring total reactive oxygen species (ROS) using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining in colorectal cancer cell lines as an example. This protocol describes detailed steps including preparation of DCFH-DA solution, incubation of cells with DCFH-DA solution, and measurement of normalized intensity. DCFH-DA staining is a simple and cost-effective way to detect ROS in cells. It can be used to measure ROS generation after chemical treatment or genetic modifications. Therefore, it is useful for determining cellular oxidative stress upon environment stress, providing clues to mechanistic studies.

Introduction

Three major reactive oxygen species (ROS) produced by cellular metabolism that are of physiological meaning are superoxide anion, hydroxyl radical, and hydrogen peroxide[1]. At low concentrations, they participate in physiological cell processes, but at high concentrations they have adverse effects on cell signaling pathways[1]. Our body has developed antioxidant systems, which are effective against excessive ROS. However, oxidative stress can occur when ROS overwhelm the detoxifying ability of our body, which contributes to many pathological conditions, including inflammation, cancer, and neurodegenerative disease[2, 3, 4]. The purpose of this method is to determine total cellular ROS in adherent cells using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. The rationale is that oxidation of DCFH-DA to 2'-7'dichlorofluorescein (DCF) has been used extensively for total ROS detection including hydroxyl radicals (•OH) and nitrogen dioxide ($•NO_2$). Mechanistically, DCFH-DA is taken up by cells where cellular esterase cleaves off the acetyl groups, resulting in DCFH. Oxidation of DCFH by ROS converts the molecule to DCF, which emits green fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Compared with detection of fluorescence with flow cytometry and other alternative methods[5], advantages of this method using a fluorescence microscope and a plate reader are that it produces clearly visible fluorescent images, and is easy to perform, efficient and cost-effective. This method has been widely

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Disclosures

The authors have nothing to disclose.

used to detect cellular ROS for studying various conditions[6, 7, 8]. This protocol is used for detecting total ROS in adherent cells. Using this method to detect ROS in suspension cells may need some modifications.

Protocol

1. Cell seeding

1. Seed 2 × 10^5 HCT116 colorectal cancer cells per well in a 24-well plate and maintain the cells in Dulbecco's modified Eagle medium (DMEM) overnight at 37 °C.
2. Replace the culture medium with or without 100 μM ferrous sulfate (FS) or 10 μM doxorubicin (DOX) containing medium and incubate for 24 h.

2. Preparation of the DCFH-DA solution

1. Dissolve 4.85 mg of DCFH-DA in 1 mL of dimethyl sulfoxide (DMSO) to make a 10 mM stock solution.
2. Dilute the stock solution with pre-warmed DMEM into 10 μM working solution right before adding it to the wells.
3. Vortex the working solution for 10 s.

3. DCFH-DA staining

1. Remove the drug containing medium and wash once with DMEM.
2. Add 500 μL of the DCFH-DA working solution into each well and incubate at 37 °C for 30 min.
3. Remove the DCFH-DA working solution. Wash once with DMEM and 2× with 1× phosphate-buffered saline (PBS).
4. Add 500 μL of 1× PBS to each well.

4. Imaging acquisition and intensity measurement

1. Take representative fluorescent images for each well using the green fluorescent protein (GFP) channel on a fluorescence microscope.
2. After taking images, remove PBS and add 200 μL of radioimmunoprecipitation assay (RIPA) buffer to each well.
3. Incubate on ice for 5 min, then collect cell lysate into 1.5 mL tubes.
4. Centrifuge at 21,130 × *g* for 10 min at 4 °C.
5. Transfer 100 μL of the supernatant to a black 96 well plate and measure the fluorescence intensity using a fluorescence a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

6. Transfer 1 μL of the supernatant to a clear 96 well plate containing 100 μL of 1× protein assay solution to measure the protein concentration using the Bradford assay[9].

7. Normalize fluorescence intensities with protein concentrations.

Representative Results

HCT116 colorectal cancer cells were treated with 100 μM FS or 10 μM DOX to induce oxidative stress[7]. As shown in Figure 1, green fluorescence was dramatically increased by both FS and DOX as expected. To quantify the relative intensity change, the cells were lysed after taking images and normalized with protein concentrations. The quantified fluorescence intensity was significantly increased by FS or DOX in HCT116 cells.

Discussion

The experimental protocol described here is easily reproducible to measure cellular total ROS. The critical steps include making DCFH-DA solution fresh and avoiding light exposure, minimizing cell status disturbance and extensive PBS washing right before taking images. For the preparation of DCFH-DA working solution, the stock solution should be added into pre-warmed DMEM right before adding into the 24 well plate. The reason is that old solutions that generate high background fluorescence or light exposure will lead to photobleaching. Most studies use 1× PBS or 1× Hanks' balanced salt solution (HBSS) to dilute DCFH-DA and use it as reaction buffer[10]. However, when using HCT116 and RKO, dilution of DCFH-DA stock solution with PBS and fetal bovine serum free DMEM generated high background signal even in untreated cell. This may be due to cell status disturbance. In addition, the DCFH-DA working solution should be added slowly along the well wall. Disturbance of cell status will generate high fluorescence signal compared to the undisturbed nearby area. It is also critical to wash at least twice with PBS before taking images to reduce auto-fluorescence of the phenol containing DMEM. Phenol-free DMEM may be a better choice but we show here that PBS washing was sufficient to minimize auto-fluorescence. As shown in Figure 1, even in untreated control groups two different batches of experiments could result in different representative images. To control experimental variations, we recommend treating cells with diluted DCFH-DA working solution (as in the protocol) instead of adding stock solution directly onto the cells. Also, images should be taken in fields with similar cell densities and the same exposure time. Finally, it is important to perform experiments on all comparison groups at the same time.

Due to the significance of ROS, specific ROS detection, in addition to total ROS detection, has also been developed. For example, cellular production of superoxide can be detected by dihydroethidium, which upon oxidation results in hydroxylation at the 2-position to form 2-hydroxyethidium. As 2-hydroxyethidium intercalates into cellular DNA, red fluorescence with excitation and emission wavelengths of 535 nm and 635 nm, respectively, can be observed. Mitochondrial superoxide can be visualized with the MitoSOX reagent, a cationic derivative of dihydroethidium that enters live cells and specifically targets mitochondria. The oxidation product of Mitosox which generates red fluorescence can intercalates into mitochondrial DNA. Chemoselective fluorescent naphthylimide peroxide probe was

developed for H_2O_2 detection[11]. In addition, detection of hydroxyl radicals using fluorescence spectrophotometry was also reported[12].

In summary, here we described a simple and optimized protocol for detecting cellular total ROS using cost-effective DCFH-DA staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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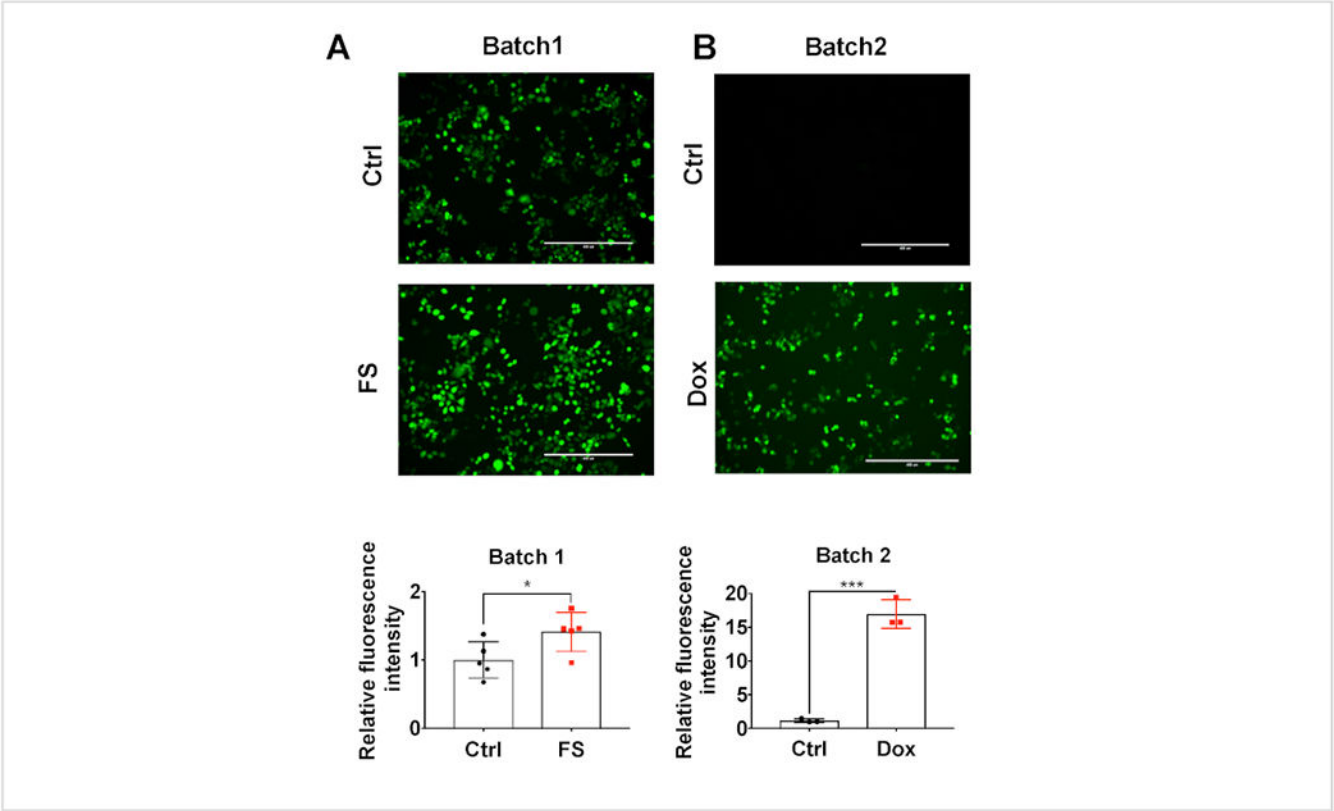

Figure 1: Iron treatment increases ROS in colorectal cancer cells.

Representative fluorescent images taken by a fluorescence microscope and intensity quantification by a fluorescence microplate reader for 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining in HCT116 cells after untreated control (Ctrl), (**A**) 100 μM ferrous sulfate (FS) or (**B**) 10 μM doxorubicin (DOX) treatment for 24 h. Scale bar = 400 μm. * = $p < 0.05$; *** = $p < 0.001$.