Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60682R1
Full Title:	Detection of Total Reactive Oxygen Species in Adherent Cells by 2',7'-Dichlorodihydrofluorescein Diacetate Staining
Section/Category:	JoVE Biochemistry
Keywords:	Oxidative stress; 2',7'-Dichlorodihydrofluorescein diacetate; reactive oxygen species; adherent cell; fluorescence microscope; fluorescence plate reader
Corresponding Author:	Xiang Xue University of New Mexico Albuquerque, New Mexico UNITED STATES
Corresponding Author's Institution:	University of New Mexico
Corresponding Author E-Mail:	xxue@salud.unm.edu
Order of Authors:	Hyeoncheol Kim
	Xiang Xue
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Albuquerque, NM

TITLE:

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

AUTHORS AND AFFILIATIONS:

Hyeoncheol Kim[1], Xiang Xue[1]

[1]Department of Biochemistry and Molecular Biology, University of New Mexico, Albuquerque, NM, USA

Corresponding Author:

Xiang Xue (xxue@salud.unm.edu)

Email Address of Co-Author:

Hyeoncheol Kim (jun582@unm.edu)

KEYWORDS:

oxidative stress, 2',7'-dichlorodihydrofluorescein diacetate, reactive oxygen species, adherent cell, fluorescence microscope, fluorescence plate reader

SUMMARY:

Here, we present a protocol to detect total cellular reactive oxygen species (ROS) using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This method can visualize cellular ROS localization in adherent cells with a fluorescence microscope and quantify ROS intensity with a fluorescence plate reader. This protocol is simple, efficient and cost-effective.

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[2-4]. 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 used to detect cellular ROS for studying various conditions[6-8]. 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

1.1. Seed 2 x 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.

1.2. 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

2.1. Dissolve 4.85 mg of DCFH-DA in 1 mL of dimethyl sulfoxide (DMSO) to make a 10 mM stock solution.

2.2. Dilute the stock solution with pre-warmed DMEM into 10 µM working solution right before adding it to the wells.

2.3. Vortex the working solution for 10 s.

3. DCFH-DA staining

3.1. Remove the drug containing medium and wash once with DMEM.

3.2. Add 500 µL of the DCFH-DA working solution into each well and incubate at 37 °C for 30 min.

3.3. Remove the DCFH-DA working solution. Wash once with DMEM and 2x with 1x phosphate-buffered saline (PBS).

3.4. Add 500 µL of 1x PBS to each well.

4. Imaging acquisition and intensity measurement

4.1. Take representative fluorescent images for each well using the green fluorescent protein (GFP) channel on a fluorescence microscope.

4.2. After taking images, remove PBS and add 200 µL of radioimmunoprecipitation assay (RIPA) buffer to each well.

4.3. Incubate on ice for 5 min, then collect cell lysate into 1.5 mL tubes.

4.4. Centrifuge at 21,130 x *g* for 10 min at 4 °C.

4.5. 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.

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

4.7. 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.

FIGURE LEGEND:

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. * = $p < 0.05$; *** = $p < 0.001$.

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 1x PBS or 1x 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.

ACKNOWLEDGMENTS:
This work was supported in part by the National Institutes of Health (K01DK114390), a Research Scholar Grant from the American Cancer Society (RSG-18-050-01-NEC), a Research Pilot Project Grant from University of New Mexico Environmental Health Signature Program and Superfund (P42 ES025589), a Shared Resources Pilot Project Award and a Research Program Support Pilot Project Award from UNM comprehensive cancer center (P30CA118100), and a new investigator award from the Dedicated Health Research Funds at the University of New Mexico School of Medicine.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Birben, E. et al. Oxidative stress and antioxidant defense. *World Allergy Organization Journal*. **5** (1), 9-19 (2012).

2. Kim, G.H. et al. The Role of Oxidative Stress in Neurodegenerative Diseases. *Experimental Neurobiology*. **24** (4), 325-40 (2015).
3. Sullivan, L.B., Chandel, N.S. Mitochondrial reactive oxygen species and cancer. *Cancer & Metabolism*. **2**, 17 (2014).
4. Formentini, L. et al. Mitochondrial ROS Production Protects the Intestine from Inflammation through Functional M2 Macrophage Polarization. *Cell Reports*. **19** (6), 1202-1213 (2017).
5. Rakotoarisoa, M. et al. Curcumin- and Fish Oil-Loaded Spongosome and Cubosome Nanoparticles with Neuroprotective Potential against H_2O_2-Induced Oxidative Stress in Differentiated Human SH-SY5Y Cells. *ACS Omega*. **4** (2), 3061-3073 (2019).
6. Mateen, S. et al. Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. *PLoS One*. **11** (4), (2016).
7. Kim, H. et al. The interaction of Hemin and Sestrin2 modulates oxidative stress and colon tumor growth. *Toxicology and Applied Pharmacology*. **374**, 77-85 (2019).
8. Wang, S.H. et al. Sotetsuflavone inhibits proliferation and induces apoptosis of A549 cells through ROS-mediated mitochondrial-dependent pathway. *BMC Complementary and Alternative Medicine*. **18**, 235 (2018).
9. Kruger, N.J. The Bradford Method For Protein Quantitation. In *The Protein Protocols Handbook*. Edited by Walker, J.M., 17-24, Humana Press. Totowa, NJ (2009).
10. Tetz, L.M. et al. Troubleshooting the dichlorofluorescein assay to avoid artifacts in measurement of toxicant-stimulated cellular production of reactive oxidant species. *Journal of Pharmacological and Toxicological Methods*. **67** (2), 56-60 (2013).
11. Rong, L. et al. Hydrogen peroxide detection with high specificity in living cells and inflamed tissues. *Regenerative Biomaterials*. **3** (4), 217-22 (2016).
12. Liu, L.Z. et al. Quantitative detection of hydroxyl radical generated in quartz powder/phosphate buffer solution system by fluorescence spectrophotometry. *Guang Pu Xue Yu Guang Pu Fen Xi*. **34** (7), 1886-9 (2014).

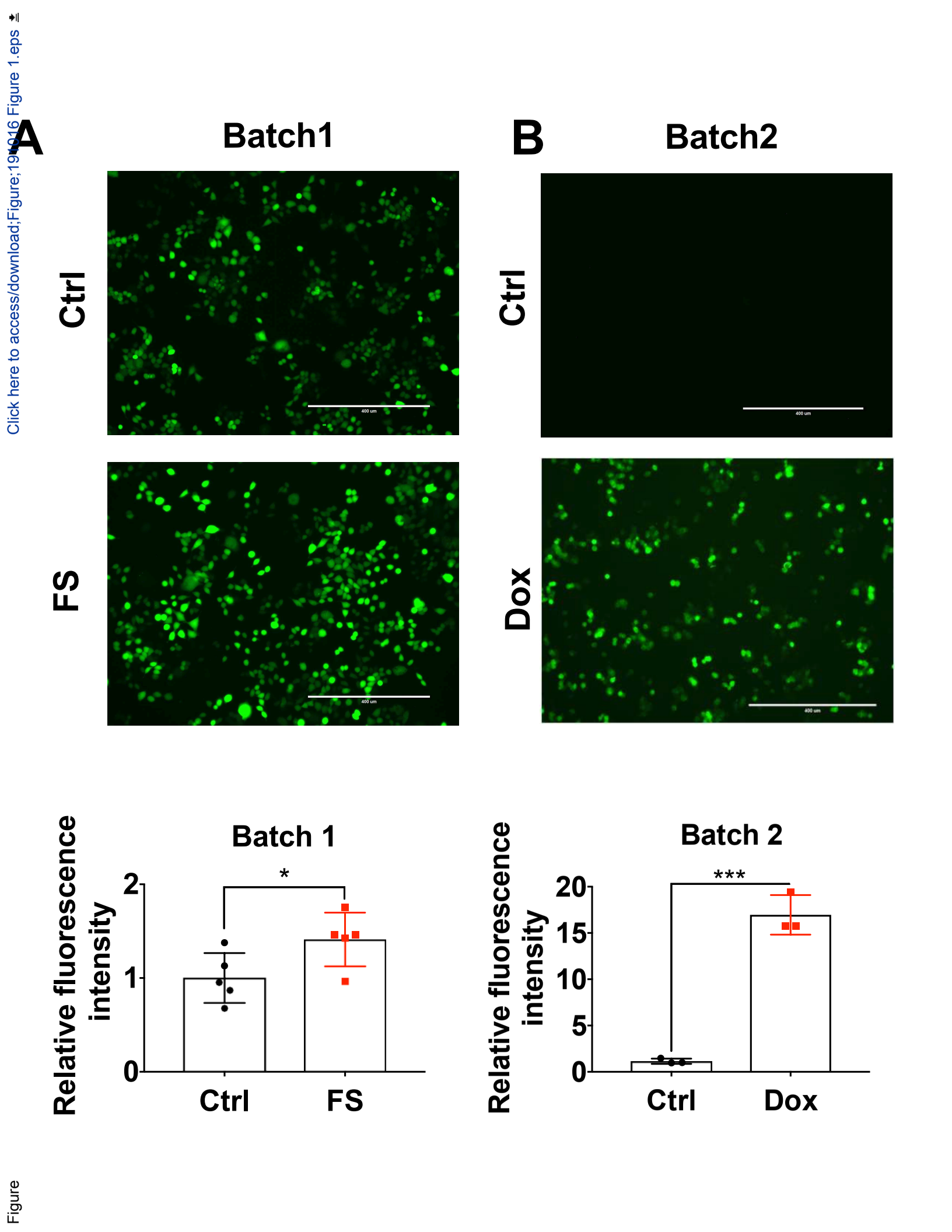

Table of Materials

Click here to access/download;Table of Materials;60682_Materials.xlsx

Name of Reagent/Material	Company	Catalog Number
2',7'-Dichlorofluorescein diacetate	Cayman Chemical, Ann Arbor, MI	20656
Doxorubicin hydrochloride	TCI America, Portland, OR	D4193-25MG
Dulbecco's Modified Eagle Medium	Corning, Corning, NY	45000-304
Ferrous Sulfate Heptahydrate	VWR, Radnor, PA	97061-542
Invitrogen EVOS FL Auto Imaging System	Thermo Fisher Scientific Waltham, MA	AMAFD1000
Protein assay Bradford solution	Bio-Rad, Hercules, CA	5000001
SpectraMax M2 Microplate Reader	Molecular Devices, Radnor, PA	89429-532

Comments

or any other fluorescence microscope

or any other fluorescence microplate reader

Reply to editorial and reviewers' comments:

Ms. No.: JoVE60682
Title: "Detection of total reactive oxygen species by 2',7'-Dichlorodihydrofluorescein diacetate staining"

We are grateful for the comments provided by the Editors and the Reviewers. Below is a point-by-point discussion to address the concerns and suggestions by the Editors and the reviewers; our responses are highlighted in blue font.

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Re: Thank you very much for this kind reminder.

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Re: We have thoroughly proofread the manuscript.

2. Keywords: Please provide at least 6 keywords or phrases.

Re: We have added three more keywords: adherent cells; fluorescence microscope; fluorescence plate reader.

3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"

Re: We have added a summary section before abstract:

Here, we present a protocol to detect total cellular reactive oxygen species (ROS) using 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). This method can visualize cellular ROS localization with a fluorescence microscope in adherent cells and quantify ROS intensity with a fluorescence plate reader. This protocol is simple, efficient and cost-effective.

4. Introduction: Please revise to include all of the following:
a) A clear statement of the purpose of this method
b) The rationale behind the development and/or use of this method
c) The advantages over alternative methods with references to relevant studies
d) The context of the method in the wider body of published literature
e) Information to help readers decide whether the method described is appropriate for them.

Re: We appreciate the editorial comments.

a) The purpose of this method is to determine total cellular ROS in adherent cells using 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining.

b) The rationale is oxidation of DCFH-DA to 2'-7'dichlorofluorescein (DCF) has been used extensively for total ROS detection including hydroxyl radicals (•OH) and nitrogen dioxide (•NO2).
c) Compared with detection of fluorescence with flow cytometry and other alternative methods, the advantages of this method using a fluorescence microscope and a plate reader include clear visible fluorescent images, easy to perform, efficient and cost-effective.
d) This method has been widely used to detect cellular ROS for studying various conditions.
e) This protocol is used for detecting total ROS for adherent cells, using this method to detect ROS in suspension cells may need some modifications.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Cayman Chemical, Invitrogen™ EVOS™, Thermo Fisher Scientific, SpectraMax, Molecular Devices, BioRad, etc.

Re: We have changed commercial language and added company name and catalog number to the table of materials.

6. 1.2: Please provide specific values (e.g., drug used and the incubation time) to be used here. We cannot film a generalized protocol; we need specific settings of a specific experiment.

Re: We have provided specific values in the protocol, "Replace the culture medium with or without 100μM ferrous sulfate containing medium and incubate for 24 hours."

7. 2.1: Please list an approximate volume of stock solution to prepare.

Re: We have changed the sentence to: "Dissolve 4.85 mg DCFH-DA in 1mL DMSO to make a 10mM stock solution."

8. 4.4: Please list all centrifugation speeds in terms of centrifugal g-force instead of rpm: e.g., 100 x g.

Re: We have changed "14,000 rpm" to "21,130 × g".

9. Figure 1: Please define the scale bars and error bars in the figure legend.

Re: We have defined the scale bar in the figure legend: 400 μM.

10. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Re: We have changed it according to the suggestion.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I.,

LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl] imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

Re: We have ensured that the format of references are the same as indicated above.

12. Table of Materials: Please revise to include information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

Re: We have added all relevant supplies in the Table of Materials as a .xls file and sorted the materials alphabetically by material name.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Following the dynamic changes in the levels of ROS is very important in many studies, and this manuscript describes a step-by-step protocol for measuring cellular ROS using DCFH-DA.

Major Concerns:
1. Lack of negative control: should include NAC-treated cells as a negative control;

Re: We appreciate this great comment from the reviewer #1. Actually we have published our results with NAC-treated cells in “Toxicol Appl Pharmacol, 2019. 374: p. 77-85.”, so we didn’t include NAC in this protocol to make it straight-forward. We have added this point in our discussion though.

2. Lack of flow cytometry measurement of DCF fluorescence;

Re: The purpose of this manuscript is to describe a cost-effective and easy method to measure cellular ROS. We consider that flow cytometry method would be equivalent or more complicated than a fluorescence plate reader method.

3. Lack of description of the equipment used in Fig 1B.

Re: We have indicated that representative fluorescent images were taken by a fluorescence microscope and intensity quantification were performed on a fluorescence microplate reader.

Minor Concerns:
The resolution of Fig 1A is too low.

Re: We have provided the figure as a vector image file to ensure high resolution throughout production: (.eps) and ensured that the image is 300 dpi according to instructions from the editor.

Reviewer #2:
Manuscript Summary:
Authors demonstrated 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.

Major Concerns:
It is an employed method worldwide. In fact, a similar article in JoVE (Danli Wu and Patricia Yotnda, J Vis Exp. 2011; (57): 3357) also demonstrated how to use this kind of probe. The only difference is that they used 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (also a generic ROS-sensitive molecular probe) instead of 2',7'-Dichlorodihydrofluorescein diacetate used in the present manuscript. Then, I consider that there is no need of a second paper in the same Journal about this method.

Re: We appreciate the comments from Reviewer #2. There are some critical differences between our protocol and the above-mentioned protocol:
1. They used human leukemia cell line, which is a suspension cell line.
2. They treated cells first with DCFH-DA and then incubate drug with H2O2 only for 1h.
3. They used cell culture media with 2% serum.

For #1, we used adherent cell lines.

For #2, We also tried to treat cells with DCFH-DA first and then ferrous sulfate or hemin. Unfortunately, after several hours of drug treatment (1-4h), DCFH-DA's green fluorescence was decreased significantly. Considering many experiments need to treat the cells for more than 1 hour, we believe our protocol condition would be better fitting widely.

For #3, they used serum at a lower concertation than ours. One advantage of our protocol is that we don't need to prepare special cell culture medium.

The representative result reported (with hemin treatment) is already published in Kim, H., et al., The interaction of Hemin and Sestrin2 modulates oxidative stress and colon tumor growth. Toxicol Appl Pharmacol, 2019. 374: p. 77-85.

Re: We appreciate the comments from reviewer #2. Now we have deleted the results with hemin treatment and include results with Doxorubicin treatment.

Minor Concerns:
DCFH-DA solution preparation. a) About dilution of the stock solution: "pre-warmed DMEM" is free of serum or not?

Re: "pre-warmed DMEM" contains 10% serum.

DCFH-DA staining. a) Wash with DMEM: free of serum?. b) After removal of the DCFH-DA working solution and washings: authors should indicate that PBS is added in each well. Imaging taking and intensity measurement. c) Centrifugation must be expressed in g, not rpm.

Re: We appreciate the comments.
a) Wash with DMEM: 10% serum was added to the medium.
b) We have added it according to the reviewer's suggestion.
c) We have changed the centrifugation speed according to the reviewer's suggestion.

In the discussion: a) Authors said "But, in our case of using HCT116 and RKO, diluting DCFH-DA stock solution with PBS and fetal bovine serum free DMEM generated high background signal even in non-treated cell". So, they used DMEM with fetal bovine serum? That is it? But

the incubation is during 30 min, how this short period without serum could disturb cells and affect the measurement? b) Discussion about detection of superoxide is out of the scope of this protocol. c) Discussion about variations of the protocol would be more interesting.

a) Yes, we used DMEM with 10% serum. We are still not sure why the short period incubation with 1x PBS disturbed the cell, but the protocol presented here gave us robust and reproducible results.
b) Superoxide is processed by superoxide dismutases to generate H2O2. H2O2 is known for generating hydroxyl radical which is also captured by DCFH-DA. So, we believe our discussion for superoxide is related to this protocol.

c) This is a great point. We have included discussion about controlling variations. As shown in Figure 1, two different batches of experiments could result in different representative images even in untreated control groups. To control the experimental variations, we suggested to treat cells with diluted DCFH-DA working solution as we presented above instead of adding stock solution directly onto the cells. Also, images should be taken at fields with similar cell densities and at the same exposure time. Finally, performing studies for all the comparison groups at the same time would be very important.