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Detecting Reactive Oxygen Species

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Abstract

Reactive oxygen species are chemically active, oxygen-derived molecules capable of oxidizing other molecules. Because of their reactive nature, there are many deleterious effects associated with unchecked ROS production, including structural damage to DNA and other biological molecules. However, ROS can also be mediators of physiological signaling. There is accumulating evidence that ROS play significant roles in everything from activation of transcription factors to the mediation of inflammatory toxicity that kills foreign pathogens and defend the body.

In this video we will delve into the associations between ROS, metabolism and disease. After establishing their significance, we will discuss the principles and a protocol of a commonly used methodology for measuring ROS levels in cells: the use of non-fluorescent probes that become fluorescent upon oxidation. Lastly, we will review some current applications of this technique in cell biology research.

Transcript

Reactive oxygen species produced in cells have been implicated in tissue homeostasis, cellular aging, and disease states like cancer. As their name implies, these molecules arise from oxygen, which naturally exists as a stable, dioxygen molecule since all its electrons are paired. The addition of one unpaired electron renders it unstable, and leads to formation of the superoxide anion—a form of reactive oxygen species or ROS. Other than the superoxide anion, there are several types of reactive species with unpaired electrons, whose levels the cell aims to tightly control.

In this video, we'll learn how reactive oxygen species are related to cell metabolism and disease, explore the principles behind an assay for its detection using a fluorescent probe, and we'll go over a generalized protocol for this assay. Lastly, we'll investigate how scientists are implementing this method in experiments today.

First, let's discuss how reactive oxygen species are produced, and consider their influence in cell metabolism and disease.

A significant source of cellular reactive oxygen species is the mitochondria. Normally, during cell metabolism electrons are transported through a chain of protein complexes, culminating in the reduction of molecular oxygen to water and simultaneous generation of ATP. Despite the extraordinary regulation of this process, electrons do leak out, resulting in the formation of superoxide anion.

The presence of superoxide anion quickly gives rise to other forms of reactive oxygen species, such as hydrogen peroxide and hydroxyl radical. These radicals, which all possess a highly reactive unpaired electron, can oxidatively damage membranes, DNA, and proteins. To counteract, the cell maintains its own antioxidant stockpile of enzymes like superoxide dismutase, or molecules like vitamin C, that reduce free radicals. Any imbalance in this defense system can result in a potentially fatal positive feedback loop, resulting in a condition of excessive reactive oxygen species known as oxidative stress.

Reactive oxygen species have been implicated in initiation and progression of cancer. Another harmful effect of these molecules is the induction of cellular aging, also known as senescence. The "Free Radical Theory of Aging" proposes that reactive oxygen species produced in cells during normal metabolism evoke cellular senescence and death.

Until now, we discussed the negative aspects of these highly reactive molecules, but they have positive roles in cellular physiology as well. During immune responses when phagocytes engulf pathogens, cells mount a "respiratory burst" during which excessive amounts of reactive oxygen species are generated to oxidatively degrade pathogens. In addition, they are necessary intermediates and regulators of a variety of cell signaling pathways, and can even signal the death of cells that have turned cancerous.

To quantify these influential cellular oxidants, scientists exploit molecules that upon oxidation turn fluorescent. A commonly used probe to detect the reactive oxygen species is H_2DCFDA or dichloro-dihydro-fluorescein diacetate, a non-fluorescent analogue of fluorescein. When added to cells, its cell permeant nature allows it to passively diffuse in.

Then, intracellular esterases catalyze a hydrolysis reaction, which results in cleaving of acetate groups. This makes the compound more polar, so that it is retained within the cell. Upon oxidation, which involves removal of hydrogen atoms by a wide range of reactive oxygen species, the non-fluorescent H2DCFDA is converted to the highly fluorescent dichloro-fluorescein, or DCF. This can be read and quantified by a plate reader, flow cytometer, or fluorescence microscopy.

Now that you know how this assay works, let's see how it's performed in a laboratory setting.

Start by transferring cells grown in culture medium to phosphate buffered saline, followed by centrifugation to wash them. Remove supernatant, and add the fluorescent probe H_2DCFDA solution. Incubate the dye-loaded cells in the dark to prevent photobleaching. After incubation, wash the cells to remove unloaded dye and transfer cells to a plate. At this point, experimental oxidative stress inducers can be added.

When ready for analysis, cells can be inserted into the plate reader. The excitation and emission wavelengths are set for fluorescein. After plates are read, values can be analyzed. Results reveal the relative amount of reactive oxygen species between samples at particular time points.

Now that we've examined the actual protocol, let's look how it's being applied in experiments today.

Researchers often use this method to investigate the mechanics of phagocytosis. This group of scientists wanted to study the ability of zebrafish to mount an immune response at different stages of development. As mentioned earlier, phagocytosis results in the generation of high reactive oxygen species, or “a respiratory burst,” that is used to kill pathogens. Since the enzyme NADPH oxidase is a significant ROS producer in phagocytic cells, these scientists induced the burst response by treating zebrafish with a NADPH inducer. The results demonstrated that amongst zebrafish embryos whose “burst” response had been provoked, those at 72 hours post-fertilization showed higher reactive oxygen species development than those at 48 hours post-fertilization.

Mitochondrial dysfunction due to increased reactive oxygen species is a pathological feature of many diseases. Therefore, researchers can identify mitochondrial dysfunction by measuring the level of oxidative stress. Here, scientists loaded H2DCFDA onto neurons, and then mounted the samples onto a fluorescence microscope. On addition of an oxidative stressor, like hydrogen peroxide, cell bodies displayed a sudden increase in fluorescence, which could be an indication of mitochondrial dysfunction.

Astrocytes have been suggested to protect central nervous system neurons from oxidative stress. Because of this significance, these researchers aimed to develop an assay to detect oxidative stress in astrocytes in the presence of an external inducer. They did this by incubating astrocytes with hydrogen peroxide and the fluorescent probe for reactive oxygen species detection. Subsequent fluorescence generated was analyzed using a flow cytometer. Astrocytes activated for oxidative stress were observed to fall within a region of increased fluorescence intensity, seen shifted to the right.

You’ve just watched JoVE’s video on detecting reactive oxygen species or ROS. To sum up, in this video we discussed the link between reactive oxygen species, cell metabolism, and disease. We then examined the principle and procedure of an assay for reactive oxygen species detection. Finally, we explored how researchers are applying this method to their investigations. The analysis of the still enigmatic roles of reactive oxygen species is of great interest to cell biologists, and reliable measurement with fluorescent probes is proving to be invaluable. As always, thanks for watching!