



Using BioTek's Synergy™ HT Reader to Measure Reactive Oxygen Species (ROS) Generation in Stimulated Cells

Detecting Cell Growth Stimulation

Paul Held Ph. D¹, and Kheng Newick²

¹Senior Scientist, Applications Dept., BioTek Instruments, Inc.

²Graduate Student, Cell and Molecular Biology Program, University of Vermont

The investigation of biological agents that can stimulate or inhibit cell growth is of great interest to cellular biologists. One such measure is the formation of Reactive Oxygen Species (ROS) as a result of cell stimulation. While there are numerous methodologies to assess ROS species, the use of fluorescent probes has become the preferred method. Here we describe the measurement of DCF fluorescence using a Synergy™ HT to detect and analyze the production of ROS as a result of cell growth stimulation.

Introduction

Reactive Oxygen Species (ROS) is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen. Reactive oxygen species were originally thought to be only released by phagocytic cells as part of their role in host defense. Recent work has demonstrated that ROS have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades [1].

While the specific role that ROS plays is poorly understood, their physical nature makes them ideal candidates for being second messengers with cells. These molecules are small in size and diffuse only a short distance before they dissipate. In addition, there are several controlled mechanisms by which they are generated that work in conjunction with multiple means of their degradation.

While several enzymes are recognized as being able to produce ROS moieties, NADPH oxidase is the most significant [1]. NADPH oxidase activity is controlled by a complex regulatory system that involves the G-protein Rac [2]. In resting cells a membrane embedded heterodimer of two polypeptides (p22-*phox* and gp91-*phox*), which also contains two heme groups as well as a FAD group, enables the transfer of electrons from cytosolic NADPH across the membrane to molecular oxygen without NADPH oxidase activity [1]. It is believed that the charge compensation occurs when gp91-*phox* polypeptide also acts as a proton pump. Upon stimulation, a number of polypeptides (p47-*phox*, p67-*phox* and p40-*phox*) translocates to the inner face of the plasma membrane to form a fully active enzyme complex that contains NADPH oxidase activity (Figure 1).

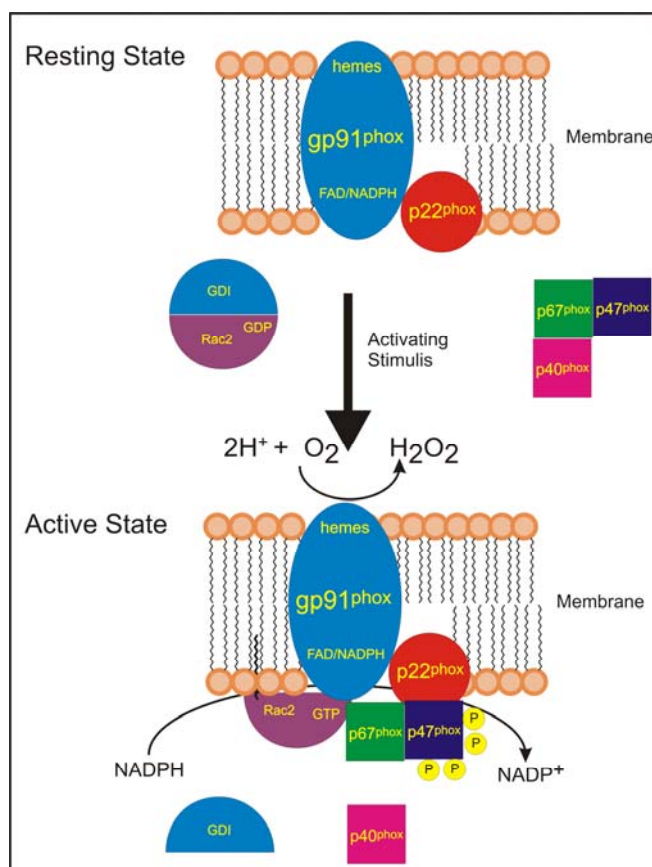


Figure 1. Schematic illustration of the activation of NADPH oxidase.

Part of the active complex is the G-protein Rac, which upon stimulation dissociates from GDI, binds GTP and associates with the membrane [2]. The assembled complex then catalyzes the formation of hydrogen peroxide (H_2O_2) from oxygen and hydrogen ions (Figure 1).

DCF reactions

In order to observe the formation of reactive oxygen species a fluorescent detector is employed. The acetate ester form of 2',7' dichlorodihydrofluorescein diacetate ($H_2DCFDA-AM$) is a membrane permeant molecule that passes through the cell membrane. Once inside the cell, cellular esterases act on the molecule to form the non-fluorescent moiety H_2DCFDA , which is ionic in nature and therefore trapped inside the cell. Oxidation of H_2DCFDA by ROS converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence signal over time (Figure 2).

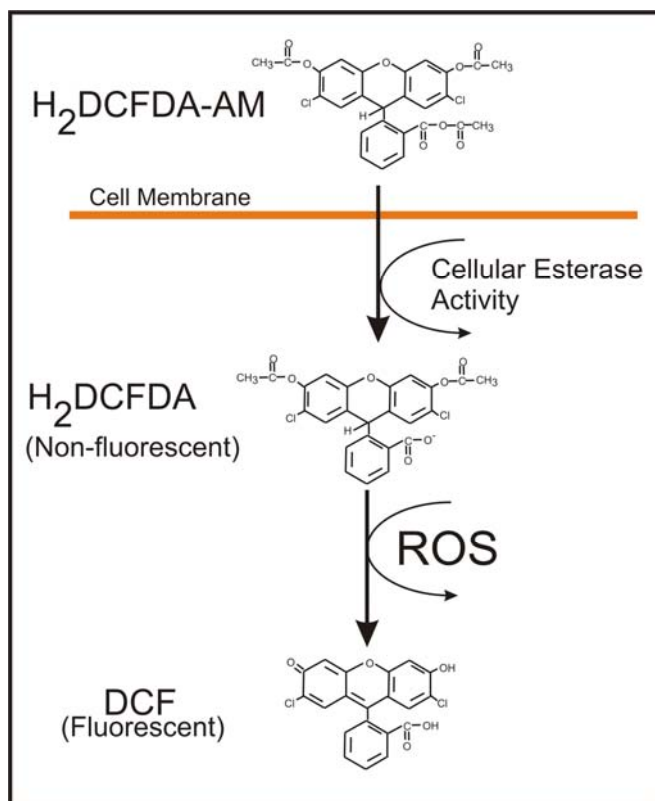


Figure 2. Formation of fluorescent Compound DCF by ROS.

Materials and Methods

Primary mesothelioma cells were cultivated in DMEM (10% FCS). Cell lines were trypsinized, counted and resuspended in fresh media at a density of 30,000-cells/mL. Using a MicroFlo peristaltic pump dispenser (BioTek Instruments), cells were seeded into Corning 24-well plates (catalogue number 3337) at $37^{\circ}C$. The following day, the media was replaced with serum free DMEM and placed back in the incubator overnight.

Cells were loaded with dye by replacing media with fresh phenol red free DMEM containing $5 \mu M$ $H_2DCFDA-AM$ for 30 minutes at $37^{\circ}C$, in a 5% CO_2 environment. After loading, unincorporated dye was removed by washing two times with fresh media. After washing, media containing experimental conditions was added and the fluorescence of the wells measured kinetically.

Fluorescence measurements were made using a Synergy HT Microplate Reader (BioTek Instruments) set to $37^{\circ}C$. Measurements were made using a 485/20 excitation and a 528/20 emission filter pair and a PMT sensitivity setting of 55. Readings were made from the bottom every 30 seconds for a total of 45 minutes.

Results

Figure 3 demonstrates the ability of the Synergy HT to detect cell stimulation using DCF fluorescence under the defined experimental conditions. Phorbol 12-myristate 13-acetate (PMA) and serum are known to stimulate cell growth in quiescent cells. Both of these agents induce the formation of DCF fluorescence over time, while unstimulated cells do not show an increase. Glutathione oxidase, which generates ROS without cellular stimulation is used as a positive control. As demonstrated in Figure 3, agents that are known to stimulate cellular proliferation also induce the formation of ROS, which in turn leads to the production of DCF fluorescence. The increase in fluorescence is not limited by the availability of substrate. Wells with glutathione oxidase, which generates the ROS, H_2O_2 , as a byproduct of the oxidation of glutathione, demonstrate a continuous increase in fluorescence throughout the experiment. This indicates that DCF substrate is not the limiting agent in the assay.

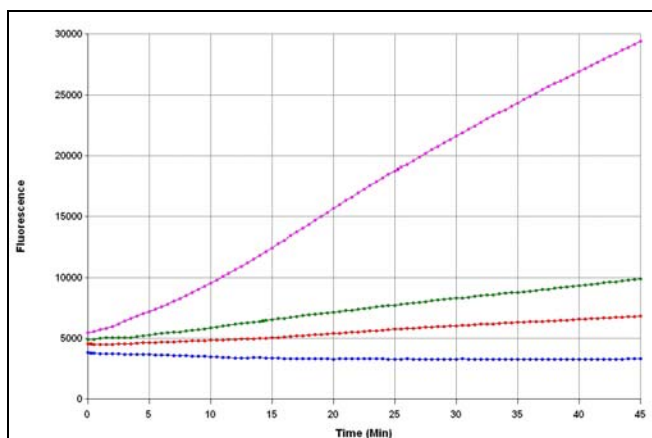


Figure 3. DCF Fluorescence in Stimulated Cells Measured over Time. Cells were treated with 1 mU/mL of Glutathione oxidase (magenta); $10 \mu M$ PMA (green); 0.25% FBS serum (red); or untreated (blue)

Figure 4 demonstrates end point fluorescence 30 minutes after the initiation of treatment. Both PMA and serum demonstrate a marked increase in fluorescence when compared to the unstimulated control. When the background fluorescence, depicted by the no DCF control is subtracted, the increase by 0.25% serum represents a 3-fold increase, while the $1 \mu M$ PMA stimulation was nearly 5-fold.

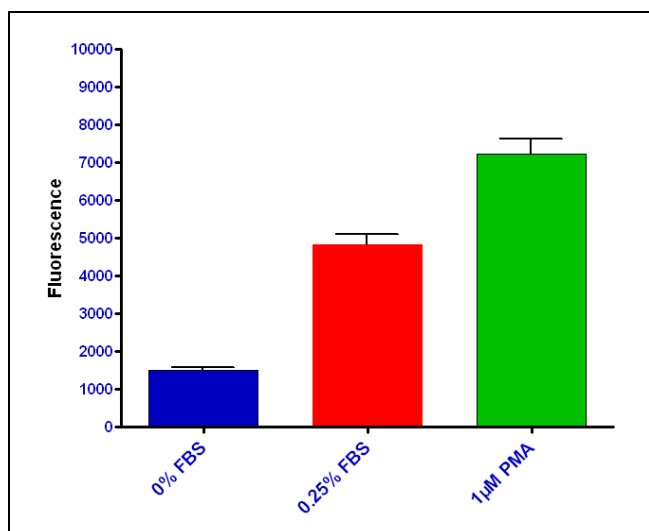


Figure 4. PMA & serum induction of H₂O₂ as measured by endpoint fluorescence after 30 min.

Discussion

These data demonstrate that the Synergy HT can be used to detect the formation of intracellular ROS as a result of the stimulation of primary mesothelioma cells. Increases in DCF fluorescence measured by the Synergy HT follow expected cellular stimulation patterns with differences in signal from background being evident almost immediately.

There are a number of different compounds that can be used to assess ROS production. However, many of these compounds are not necessarily suitable for measurements of live cells *in vivo*. In order to be useful, the reaction components must cross intact cell membranes. For example, homovanillic acid reacts with H₂O₂ in the presence of horse radish peroxidase to form an intensely fluorescent compound [6], but the reaction mixture cannot pass through the membrane. Under similar conditions, Amplex Red can also be used to detect ROS. These reaction mixtures would only be appropriate for detecting secreted ROS. The compound DCF does pass through intact cell membranes and interacts with ROS species such as H₂O₂ to form a fluorescent moiety, but is not necessarily specific for this molecule. DCF is actually more reactive with radical ions such as hydroxyl radical (HO•) or peroxy radical (ROO•) than hydrogen peroxide (H₂O₂). The development of H₂O₂ specific probes such as Peroxyl Green 1 will allow for specific analysis of the formation of H₂O₂. [5]

It is currently impossible to convert DCF fluorescence to actual intracellular concentrations of ROS. DCF interacts with several different ROS with differing efficiencies. However relative changes brought about by experimental conditions within an experiment can be compared and “fold differences” can be reported.

The Synergy family of readers provides an excellent platform for making ROS determinations in stimulated cells. The readers provide highly sensitive fluorescence detection through the use of dedicated optics in conjunction with deep blocking bandpass filters and a photomultiplier tube (PMT). In

these experiments tight temperature control is maintained at 37°C, but the reader is capable of temperatures up to 50°C. Gen5 Data reduction software (BioTek Instruments) was used to control reader function, as well as collect and plot the data. This software package allows for most commonly used data reduction transformations, curve fitting and data analysis.

References

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Acknowledgments

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