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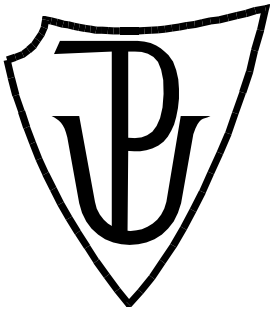

Charakterizace tvorby reaktivních forem kyslíku v lidských buňkách

DIPLOMOVÁ PRÁCE

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PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Biochemistry

Characterization of reactive oxygen species (ROS) generation in human cells

DIPLOMA THESIS

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Supervisor:	**M.Sc. Ankush Prasad, PhD**
Year:	2020

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Abstrakt

Buněčná kultura U937 je pro-monocytární buněčná linie lidského histiocytového lymfomu. Tyto monocyty se mohou diferencovat na dendritické buňky nebo makrofágy. Diferenciace závisí na vlastnostech použitých látek/induktorů, například bylo zjištěno, že buňky U937 aktivované v přítomnosti forbol 12-myristátu 13-acetátu (PMA) se v morfologii buněk změnily; buňky se shromažďovaly, adherovaly a vytvářely pseudopodii. O makrofágech je také známo, že během fagocytózy generují reaktivní formy kyslíku. V této studii zkoumání tvorby intracelulárních ROS v buňkách U937 bylo provedeno za použití fluorescenčních sond, konkrétně Dihydroethidium (DHE) a 3'-p-(hydroxyfenyl) fluoresceinu (HPF) pro detekci superoxidového anionového radikálu ($O_2^{\bullet -}$) a hydroxylového radikálu ($HO^{\bullet}$). Signál ukazující tvorbu ROS pochází hlavně z buněk podstupujících procesy diferenciace a/nebo apoptózy. K ověření výsledků byla použita elektronová paramagnetická rezonance (EPR). Zvýšené hladiny reaktivních forem kyslíku detekovaných v diferencované U937 buněčné kultuře ukazují podporu mnoha aspektů vývoje a progrese nádoru. Tato studie si klade za cíl zvýšit porozumění generování ROS a jejich charakterizaci v buňkách U937.

Klíčová slova	Buňky U937; reaktivní formy kyslíku; oxidační stres; monocyty; apoptóza; konfokální laserová skenovací mikroskopie; EPR spektroskopie
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Abstract

The cell culture U937 is a pro-monocytic, human histiocytic lymphoma cell line. These monocytes can differentiate into either dendritic cells or macrophages. The differentiation depends on the characteristic properties of the substances/inducers applied, for instance, U937 cells activated in the presence of phorbol 12-myristate 13-acetate (PMA) were found to be changed in the cell morphology with cells gathering, adhering generously and creating pseudopodia. Macrophages are also known to generate reactive oxygen species (ROS) during phagocytosis. In the current research, the study on the generation of intracellular ROS in U937 cells was done using fluorescent probes, namely Dihydroethidium (DHE) and 3′-p-(hydroxyphenyl) fluorescein (HPF) for the detection of superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$), respectively. The signal showing the formation of ROS mainly goes from cells undergoing differentiation and/or apoptosis. To validate the results, electron paramagnetic resonance (EPR) spectroscopy was used. Elevated rates of ROS detected in U937 differentiated cell culture indicates the promotion of many aspects of tumor development and progression. This current thesis aims to increase our understanding of ROS generation and its characterization in U937 cells.

Keywords	U937 cells; reactive oxygen species; oxidative stress; monocytes; apoptosis; confocal laser scanning microscopy; EPR spectroscopy
Number of pages	49
Number of appendices	04
Language	English

Contents

Aims of Diploma thesis

- To learn the procedures for the cultivation/storage of human cell lines.
- To understand growth/differentiation pattern and effects of solvents of U937 cells.
- Cell differentiation and its relation to reactive oxygen species generation.
- To learn different ROS detection methods and get familiar with the confocal laser scanning microscopy and EPR spectroscopy.

1 Introduction

Monocytes are circulatory precursor cells which can differentiate into dendritic cells or macrophages by migrating from the blood to tissues and are known to be professional antigen-presenting cells (Italiani and Boraschi, 2014). Monocytes and macrophages provide key important functions in the immune system. These are phagocytosis, antigen presentation and production of cytokines (Germic *et al.,* 2019). The U937 cells are pro-monocytic cell line isolated from the human histiocytic lymphoma and serve as a model line which is extensively used in biomedical research and clinical studies. Cell differentiation in monocytes depends on the characteristics of the tissue microenvironment which it experiences; U937 cells mature and differentiate reacting to a range of stimuli, creating the morphology and characteristics of mature macrophages (Zamani *et al.,* 2013). Several cell lines have been studied, however, U937 cells remain the preferred choice due to ease in handling, they can be stored for years and restored without any evident effects on cell viability and monocyte-macrophage characteristics among other unique features (Chanput, Mes and Wichers, 2014). The cells can also be easily maintained in a culture medium, with a replication time between 20-48 h (Marino-Merlo *et al.,* 2016). The U937 cells in suspension show a circular shape with meager cytoplasm, big beam-shaped nucleus and small microvilli.

The differentiation can be stimulated by a range of substances including but not limited to phorbol 12-myristate 13-acetate (PMA), retinoic acid, Zn^{++}, 12-O-tetradecanoylphorbol-13-acetate (TCA) and low concentration of glutamine (Mendoza-Coronel and Castanon-Arreola, 2016, Chun *et al.,* 2001, Zamani *et al.,* 2013). The U937 cells activated in the presence of PMA are accompanied by growth delay and induction of a differentiation pathways, in which the cells experience several morphological and functional changes such as adhering, forming an irregular shape and creating pseudopodia on their surface (Karina Chimal-Ramirez *et al.,* 2016). Subsequently, this process is accompanied by the formation of ROS. In the current study, we provide evidence on the intracellular ROS generation specifically, superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) in U937 cells. The signal showing the formation of these radical species mainly goes from cells going through differentiation and/or apoptosis process during the PMA treatment. Reactive oxygen species (ROS) have been known to be associated with several human diseases and ailments when it is excessively produced, or if the balance between the scavenging activities mediated by

antioxidants is disturbed, adversely affect the living organisms (Halliwell, 2007; Pospíšil, Prasad and Rác, 2019). The ROS disproportionately generated in the cell or organisms are known to oxidize important biological components including lipids, proteins, and nucleic acid. The oxidative damage of these biological components is in recent years an extensive area of research concerning its participation in a wide range of diseases including cancer, diabetes, high blood pressure, ageing and lifestyle-related diseases, such as arteriosclerosis. Thus, more specific and sensitive detection methods of ROS are required for evaluation and monitoring of oxidative stress which can be utilized in disease detection and diagnosis.

In the current study, the peculiar morphology of mature macrophages under the effect of differentiation inducer has been investigated. In addition to this, the spatial localization of ROS ($O_2^{\bullet -}$ and $HO^{\bullet}$) formed was done using a confocal laser scanning microscope. The results were validated using electron paramagnetic resonance (EPR) spectroscopy. Elevated levels of ROS were detected in U937 differentiated cell culture which can indicate the promotion of tumor development and its progression. This study helps to overlook the ROS formation and its detection in human cell lines.

2 Current state of the topic

2.1 Reactive oxygen species

The term ROS is used to define a number of reactive molecules and free radicals derived from molecular oxygen (O_2) (Fig. 1). These molecules are well known to be produced as byproducts during the mitochondrial electron transport of aerobic respiration by enzymes oxidoreductases and metal-catalyzed oxidation. Reactive oxygen species like $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), $HO^{\bullet}$ and singlet oxygen ($^1O_2$) are the most known examples (Fig. 2). They can either be radicals or non-radicals (Table 1). Free radicals consist of at least one unpaired electron in their outer shell, which makes them short-lived and highly reactive (Phaniendra, Jestadi and Periyasamy, 2015), thus, not all ROS are free radicals (Halliwell and Gutteridge, 2007). Superoxide anion radicals and $HO^{\bullet}$ are examples of radical ROS, while H_2O_2 is an example of non-radical ROS.

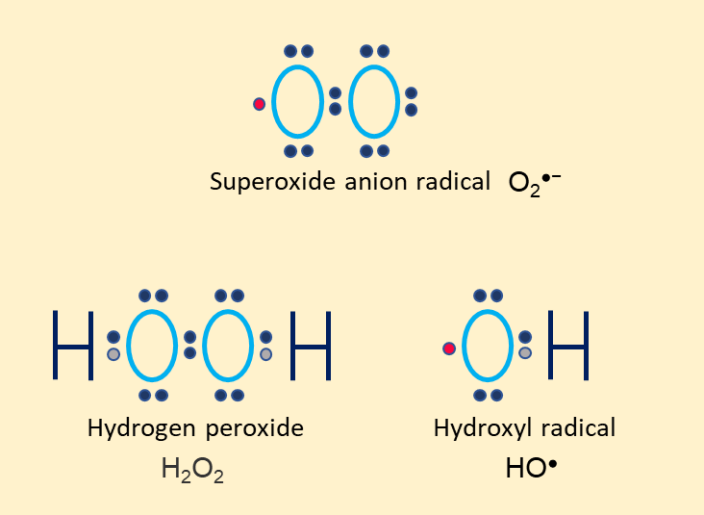


Figure 1: Electron structures of ROS. The structures are provided with its name and the red • designates an unpaired electron/s.

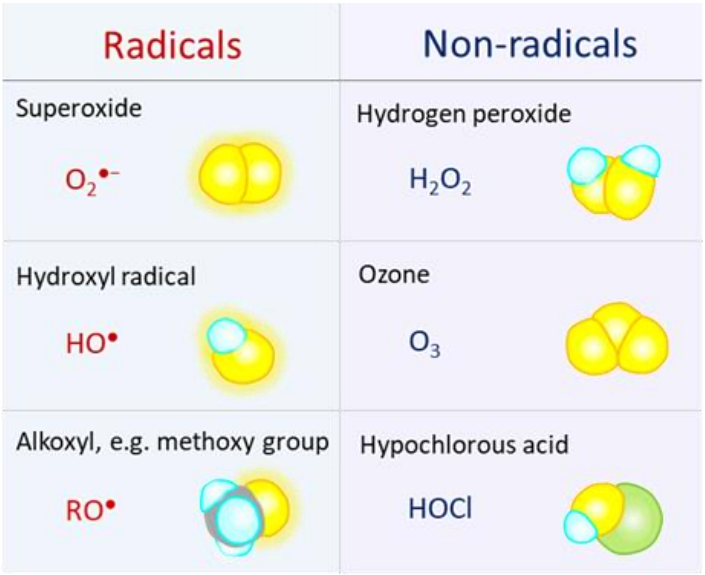


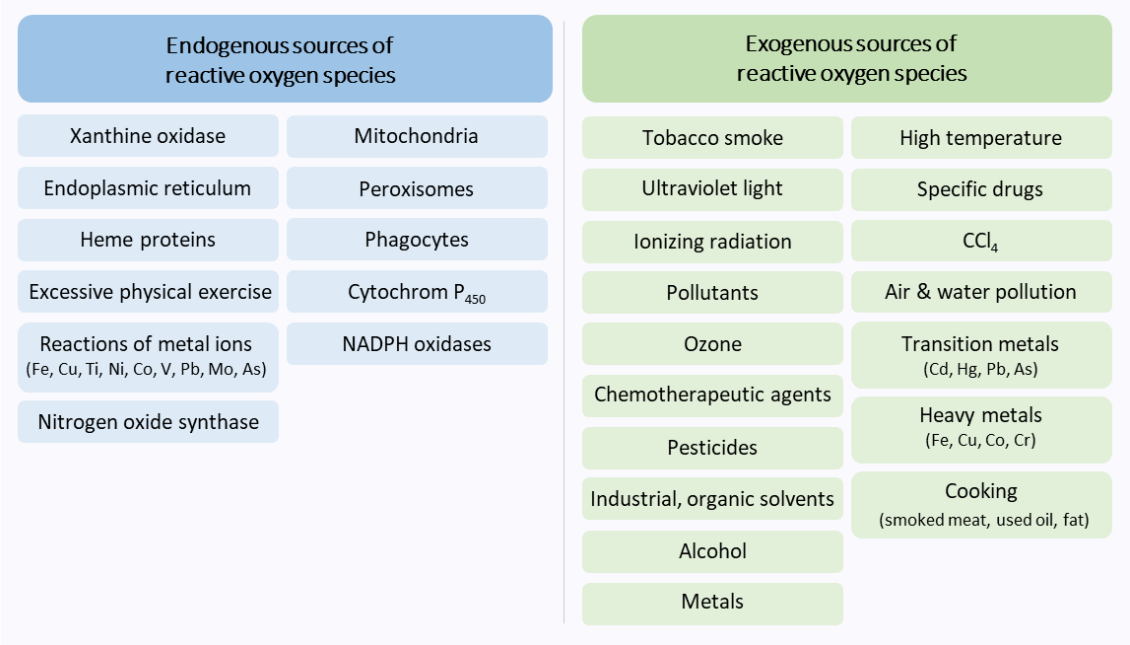
Figure 2: Radical and non-radical ROS.

2.2 Sources of reactive oxygen species

There exist multiple sources of ROS and can be either endogenous or exogenous (Table 1). The endogenous sources include cellular components such as mitochondria, peroxisomes and endoplasmic reticulum where there is a higher consumption of oxygen (Phaniendra *et al.*, 2015). The main contributor of endogenous ROS is the mitochondria in which two major sites complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome *c* reductase) contribute to the production of $O_2^{\bullet-}$ which can be subsequently be converted to H_2O_2 by mitochondrial superoxide dismutase (Mn-SOD) (Murphy, 2009). In the case of peroxisomes, the transfer of an electron from various metabolites to O_2 leads to the generation of H_2O_2. Peroxisomal enzymes (acyl CoA oxidases, D-amino acid oxidase, L-α-hydroxy oxidase, urate oxidase, xanthine oxidase, D-aspartate oxidase) have been presented to produce different ROS (Walker *et al.*, 2018). Besides mitochondria and peroxisomes, the enzymes of endoplasmic reticulum are known to contribute to the formation of ROS (Yoboue, Sitia and Simmen, 2018).

In addition to endogenous sources, ROS are produced in the living cells by various exogenous factors listed in Table 1.

Table 1: ROS generated from endogenous and exogenous sources.



2.3 Significance of reactive oxygen species

When ROS were firstly assimilated into biomedical concepts, it was assumed that they caused toxic effects and were associated mostly with pathologies and it has long been known that ROS can destroy cells (Di Meo *et al.*, 2016). During the last decades, studies have pointed out new roles for ROS in health and disease. Continuous exposure to a high level of ROS may cause non-specific damage to biomolecules (lipids, proteins and nucleic acids) by usually initiating irreversible functional changes and destructions; low to intermediate ROS concentrations exert their effects rather by regulation of cell signaling cascades. Reactive oxygen species are known to bear a key role in human's physiological and pathophysiological processes such as redox regulation of protein phosphorylation, transcription factors and ion channels. In addition, ROS are also required for biosynthetic processes, including the production of thyroid hormone and extracellular matrix crosslinking (Brieger *et al.*, 2012). They also participate in combating against infection and invasion by microbes, acting as a messenger in cell signal transduction and cell cycling, including apoptosis and regulating different intracellular and intercellular signaling cascades. When ROS are in high concentration, overwhelming the defense system by antioxidants, oxidative stress in the cell results and this leads to cellular dysfunction via biomolecule oxidation which includes lipid peroxidation, protein oxidation and DNA damages (Pospíšil, Prasad and Rác 2014, Pospíšil *et al.*, 2019).

Previous studies on the role of ROS in tumor initiation showed that it acts as DNA-damaging agents, increasing the cells mutation rate and thereby promoting oncogenic transformation (Storz, 2005). The ROS-dependent mutations in DNA have been known to include modifications in bases, DNA sequences rearrangements, miscoding of DNA including damage of bases, single-strand DNA breaks and double-strand DNA breaks (Waris and Ahsan, 2006). The larger part of mutations induced by ROS is associated with modification of guanine, leading to transversion from G→T. When it is linked with critical genes such as oncogenes or tumor suppressor genes, initiation/progression can result. Indeed, these species can act at numerous steps in different stages of carcinogenesis. Thus, it is believed that ROS are involved in the initiation and progression of cancer (Liou and Storz, 2010). Most of the DNA-associated damage by ROS is because of $HO^{\bullet}$, which has the capacity to act in a non-specific, destroying manner (Storz, 2005).

2.4 Oxidative stress, antioxidants and defense mechanisms

The generation of ROS in cells profoundly affects several critical cellular functions, and the absence of effective cellular mechanisms of detoxification, which remove these radical species can lead to progression of diseases (Storz, 2005). Thus, organisms have developed methods to combat the process. The degradative action of ROS action is normally balanced by the antioxidative capacity of a cell and any disruption of this delicate equilibrium in favor of a prooxidant state is referred to as oxidative stress. Oxidative stress is thus an imbalance in the ratio between oxidants (ROS) and antioxidants (Rahal *et al.*, 2014). During the carcinogenesis process, the level of ROS in cancer cells increases and the level of antioxidants decreases (Liou and Storz, 2010). Reactive oxygen species in these cells can increase under the effect of intrinsic or extrinsic factors, leading to induction of gene mutations and changes in transcriptional processes as well as changes in signaling pathways and, finally, the occurrence of cancer. Studies have demonstrated that oxidative stress affects several signaling pathways associated with cell proliferation. Among them, the epidermal growth factor receptor signaling pathway (EGFR) can be mentioned, in which proteins such as the nuclear factor erythroid 2-related factor 2 (Nrf2) and Raf are involved. Additionally, the mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), phospholipase C and protein kinase C are affected by oxidative stress. ROS also change the expression of the p53 tumor suppressor gene (Weng *et al.*, 2018). Thus, oxidative

stress caused by alterations in gene expression, cell proliferation, apoptosis, and angiogenesis play a substantial role in tumor initiation and progression (Matsuzawa and Ichijo, 2008; Nguyen, Nioi and Pickett, 2009).

The damaging effect of ROS on biomolecules is prevented by the action of the antioxidant defense systems which includes both non-enzymatic and enzymatic types. The non-enzymatic antioxidant systems include low-molecular-weight metabolites such as ascorbate, tocopherols, carotenoids, glutathione, coenzyme Q10 etc., whereas enzymes such as the superoxide dismutase family of enzymes (Fe-SOD, Ni-SOD etc.) and the peroxidase family of enzymes (cytochrome *c* peroxidase, glutathione peroxidase and catalase, ascorbate peroxidase etc.) are involved in enzymatic scavenging (Das and Roychoudhury, 2014; Aruoma, 1998). Innate antioxidant defenses are not always sufficient to maintain the right ROS balance. The process of ageing includes a contribution from ROS, leading to the proposition that the senescence results partially from oxidative stress. Oxidative stress relates to the general ageing process and cell death, influencing all principal organ systems and ROS have a part in many age-associated diseases, including Parkinson's disease, Alzheimer's disease, and almost all cardiovascular diseases (Liguori *et al.*, 2018). There is a thin balance between appropriate redox states and oxidative stress, based on the relative rates of production and degradation. For specificity in signaling, biological systems identify the quantity and type of ROS, as well as the spatial and temporal distribution (Turkan, 2018). The ROS-related disease can be either because of a shortage of ROS (e.g. autoimmune disorders) or a surplus of ROS (e.g. neurodegenerative and cardiovascular diseases) (Brieger *et al.*, 2012).

2.5 Reactive oxygen species in disease

During the last decades, an increased interest has been seen in the role of ROS in health and disease. From basic research to clinical trials, the biomedical researchers have rapidly advanced toward a better understanding of ROS-metabolizing systems and their contribution to definite conditions. ROS contribute to a wide range of pathologies and many of the diseases which occur, as a result, are lethal (Fig. 3). The overproduction of ROS has been associated with various diseases such as cancer, respiratory, neurodegenerative, and digestive diseases among others (Fig. 3).

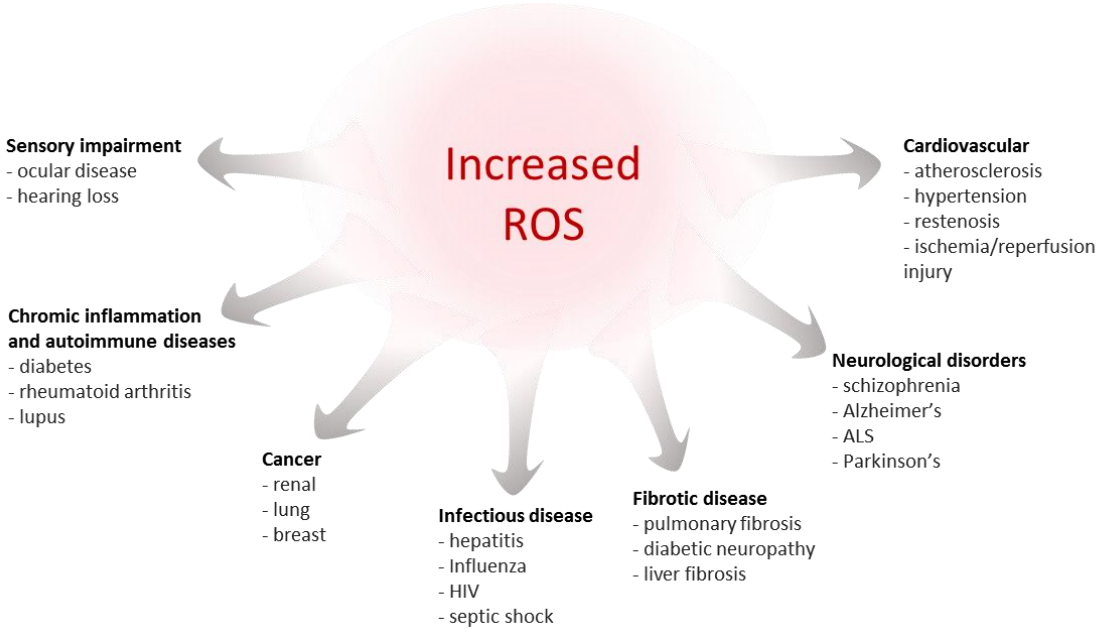


Figure 3: Overproduction of ROS and its involvement to various diseases.

2.6 Human cell line as a model system

U937 cells are pro-monocytic, human myeloid leukaemia cell line isolated from the histiocytic lymphoma of a 37-year-old male (Sundstrom and Nilsson, 1976). U937 cells are used as a model cell line to study the behaviour and differentiation of monocytes. An unlimited number of cells can be produced and they are comparatively uniform. These leukaemia cells have been used as an experimental model to understand the mechanisms of monocyte and differentiation. In addition to U937 cells, THP-1, HL-60, ML-2 among other cells are used in similar studies. The main difference between U937 and THP-1 cells being the origin and maturation stage. U937 cells have a tissue origin and are at a more mature stage, while THP-1 cells arise from a blood leukaemia origin at a less mature stage (Chanput *et al.*, 2014). As U937 cell culture has been used more frequently, we focus here on this cell line. The U937 growth in cell suspension can be described as cells possessing round shape and short microvilli, scarce cytoplasm and a large beam-shaped nucleus (Pagliara *et al.*, 2005). As these monocytes have the potential of differentiating into either macrophages or dendritic cells they are commonly used to study the monocytes behavior and differentiation. The U937 cells differentiation depends on the characteristics of the tissue microenvironment, they mature and differentiate as a reaction to a range of stimuli by adopting the morphology and characteristics of mature macrophages (Italiani and Boraschi, 2014; Kigerl *et al.*, 2009). U937 cells can be influenced or induced to differentiation by a large number of substances: PMA, nicotinamide adenine dinucleotide

phosphate (NADPH), SOD and others (Yamamoto *et al.,* 2009). After the action of these differentiation inducers, U937 cells adhere to a plastic substrate coated or not with matrix components, like bovine serum albumin (BSA), fibrinogen, collagen etc. The changes in environmental conditions and substance concentrations can influence the normal cells differentiation processes. Thus, we used a well-established differentiation model system of human U937 myelomonocytic leukaemia cells that allows investigation of differentiation-dependent alterations, is easy to use, can be stored for several years and can be recovered without any obvious effects on monocyte-macrophage features and cell viability (Richter *et al.,* 2016).

2.7 U937 cells and PMA treatment

Exposure of U937 cells to PMA, which can transform monocytic cells toward the macrophage pathway, is accompanied by a delay in growth and induction of differentiation pathways. During this pathway, the cells undergo several morphological and functional modifications (Song *et al.,* 2015; Karina Chimal-Ramirez *et al.,* 2016). Upon treatment with PMA, the non-adherent U937 cells adjust their cell adhesion molecules to attach to the surface and differentiate. The monocytes treated with PMA are named as "macrophage-like," because the properties of the transformed cell line are not yet completely understood (Daigneault *et al.,* 2010).

Phorbol 12-myristate 13-acetate treated U937 cells express elevated levels of CD11b and protein kinase C (PKC) because it mimics diacylglycerol (DAG) which is PKC activator. PMA initially starts to induce adherence and cell cycle arrest following differentiation by activation of PKC that results in activation of AP1 binding activity and other transcriptional factors including NF-*k*B, AP-1, AP-2, AP-3 etc. that binds to *cis*-acting elements and involved in macrophage differentiation, as a result, enhances the expression of adhesion receptors (b2-integrin family) that has a key role in the interaction among leukocyte and endothelium during the immune reaction (BaierBitterlich *et al.,* 1996). In addition, PMA also activates the calcium and phospholipid-dependent isoforms of PKC and induces cyclic adenosine monophosphate (AMP) metabolism, which leads to maturation into a macrophage-like morphology (Otte *et al.,* 2011) (Fig. 4). The presence of non-viable cells after treatment with PMA can be observed because of APAF-1 (apoptotic protease activating factor 1) activation after activation of PKC and other pro-apoptotic proteins (Bak and Bcl-Xs) (Ways *et al.,* 1994).

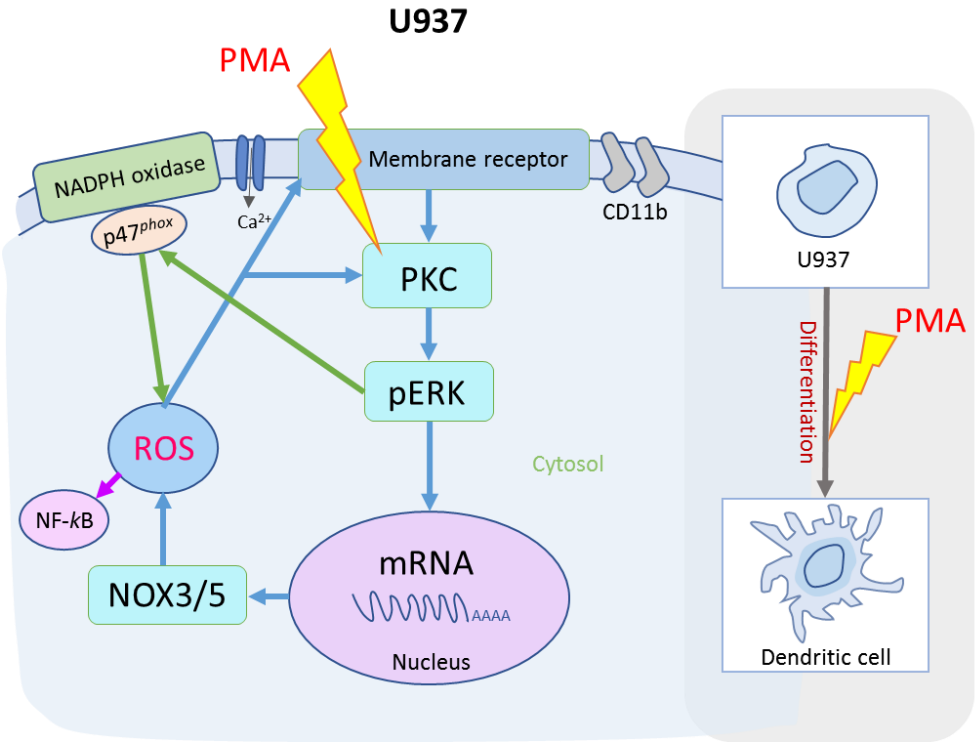


Figure 4: A schematic illustration of PMA contribution to U937 cells differentiation.

2.8 ROS detection in living systems

It is very critical to carry out a reliable measurement of the concentration or relative level of ROS (Zhang, Dai and Yuan, 2018). Several methods are available for ROS detection as outlined in Fig. 5, such as the classical and mostly used spectrophotometry methods where the reaction of ROS with redox substances leads to change in absorbance; employment of fluorescent-dependent methods where oxidant sensitive probes give enhanced fluorescence under oxidative stress; chemiluminescence probes where photon emission is monitored resulting from probes reaction with ROS; and electron spin resonance (ESR/EPR), which provides direct identification of ROS etc. Also, more recently, electrochemical sensors (biosensors) has been designed for monitoring the real-time production of ROS (Prasad *et al.*, 2015; Prasad *et al.*, 2016). Considering the fact that all methods mentioned has its pros and cons during the application, the rational use of more than one technique is crucial for better testing of the ROS in cells/organisms.

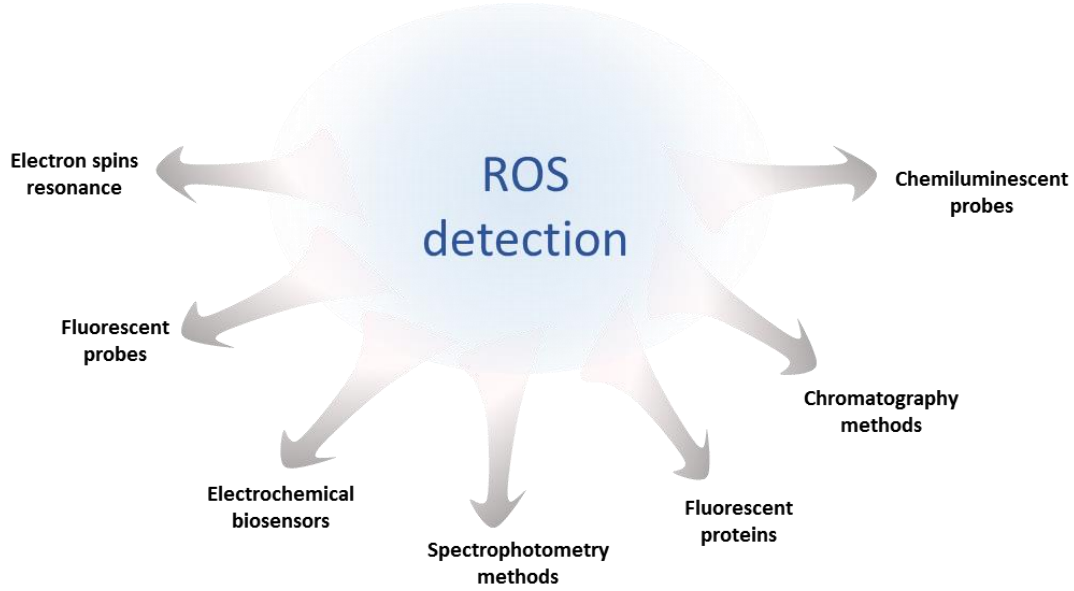


Figure 5: Reactive oxygen species detection methods.

In this study, we primarily focus on confocal laser scanning microscopy and EPR spin-trapping spectroscopy. Confocal laser scanning microscopy is a powerful tool to visualize and localize the ROS in cells (Xu *et al.*, 2013). It is one of the most important advances accomplished during last few decades in the field of fluorescence imaging (Rodrigues *et al.*, 2008). With the development of specific and sensitive fluorescent probes, the confocal laser scanning microscopy has paved its way in free radical research to a large extent. The EPR spin-trapping is another powerful method which has been used in the current study was developed in the early 1970s and allowed the analysis of short-lived species. This methodology has been frequently used during the past decades as one of the most effective tools for ROS related studies (Kohno, 2010). The direct detection of ROS is quite challenging because of their short half-lives (Suzen, Gurer-Orhan and Saso, 2017). For this reason, chemical compounds (spin probes and spin traps) have been used that form stable adducts with ROS.

3 Materials and methods

3.1 U937 cell line and culture condition

U937 cell line which is a human pro-monocytic myeloid leukaemia cell line was maintained in suspension culture (RPMI-1640) supplemented with L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum and antibiotics [1% penicillin/streptomycin]. All products utilized in cell culture and maintenance were purchased from Biosera, Nuaille, France. The cells were cultured in culture flasks (Techno Plastic Products AG, Zollstrasse 7, Switzerland) with incubation at 37° in 5% CO_2 in a humidified atmosphere (Fig. 6).

3.2 Cell density, growth and viability

The cell growth pattern in bulk over several days was measured using Bio-Rad TC20 Automated Cell Counter (Bio-Rad Laboratories, Hercules, California). The TC20 cell counter provides a total cell count and monitors cell viability via trypan blue exclusion. The trypan blue dye (Sigma-Aldrich, GmbH, Germany) exclusion test is utilized to determine the number of viable cells present in a cell suspension. It is based on the property that lives cells which possess intact cell membranes to exclude dyes such as trypan blue, eosin and propidium iodide, whereas it penetrates the dead cells. For the estimation, equal parts of 0.4% trypan blue dye are added to the cell suspension, mixed and measured after 2 min of incubation. The cell viability was measured for the following reasons: 1). to understand the growth curve and 2). to monitor the effect due to the presence of different solvents/chemicals used for solubilisation and measurements (Fig. 6).

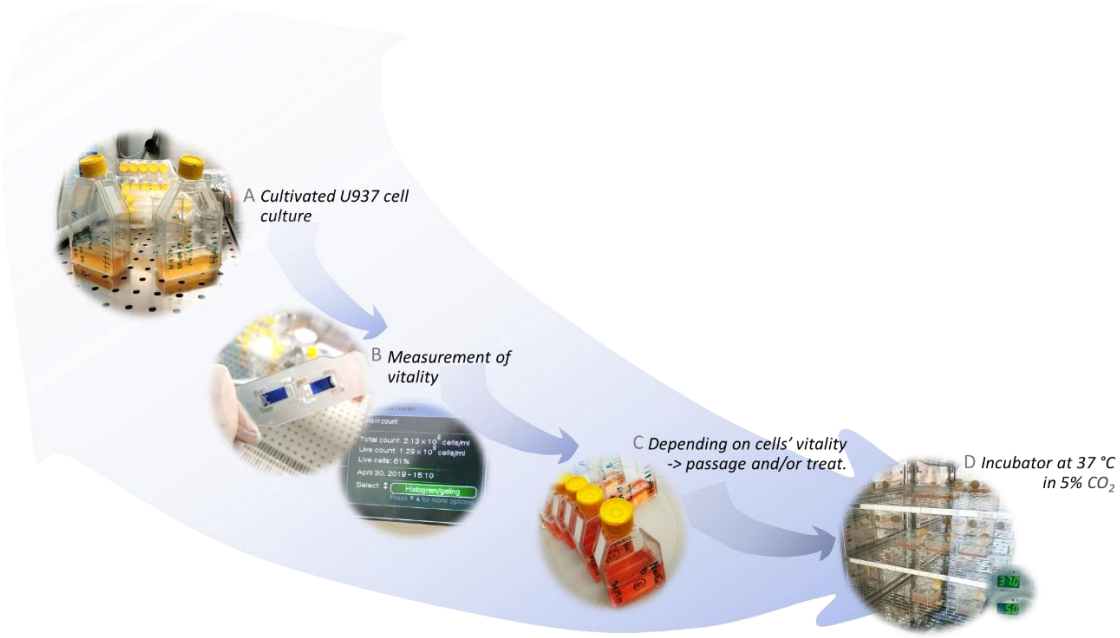


Figure 6: (from left to right) (A) Culture flasks with U937 cells maintained in suspension culture in RPMI-1640 medium supplemented with L-glutamine, fetal bovine serum and antibiotics; (B), Measurement of cell viability using Bio-Rad TC20 automated cell counter; (C), Passage or cell treatment based on viability and (D), cell incubation at 37° in 5% CO_2 in a humidified atmosphere.

3.3 Growth medium and chemicals

- RPMI-1640 medium also known as RPMI medium was developed by Moore *et al.*, at Roswell Park Memorial Institute. It was used in combination with fetal bovine serum and antibiotics as a growth medium for U937 cells.
- Dimethyl sulfoxide which is an organosulfur compound was used as a solvent for PMA (Sigma Aldrich, GmbH Germany). Its final concentration in cell suspension was always maintained below 1% to minimize its toxic effects described in the later sections.

3.4 Fluorescent probes

- Dihydroethidium (DHE) (Sigma Aldrich, GmbH, Germany), also known as hydroethidium is a cell-permeant fluorescent probe utilized for detection of intracellular ROS. It is a known $O_2^{\bullet-}$ indicator, which exhibits blue fluorescence in the cytosol until oxidized to 2-hydroxyethidium. In addition to its interaction with $O_2^{\bullet-}$, it can also intercalate with the cell's DNA, staining its nucleus. It

provides fluorescence with excitation/emission maxima ~500/590 nm as presented in Fig. 7.

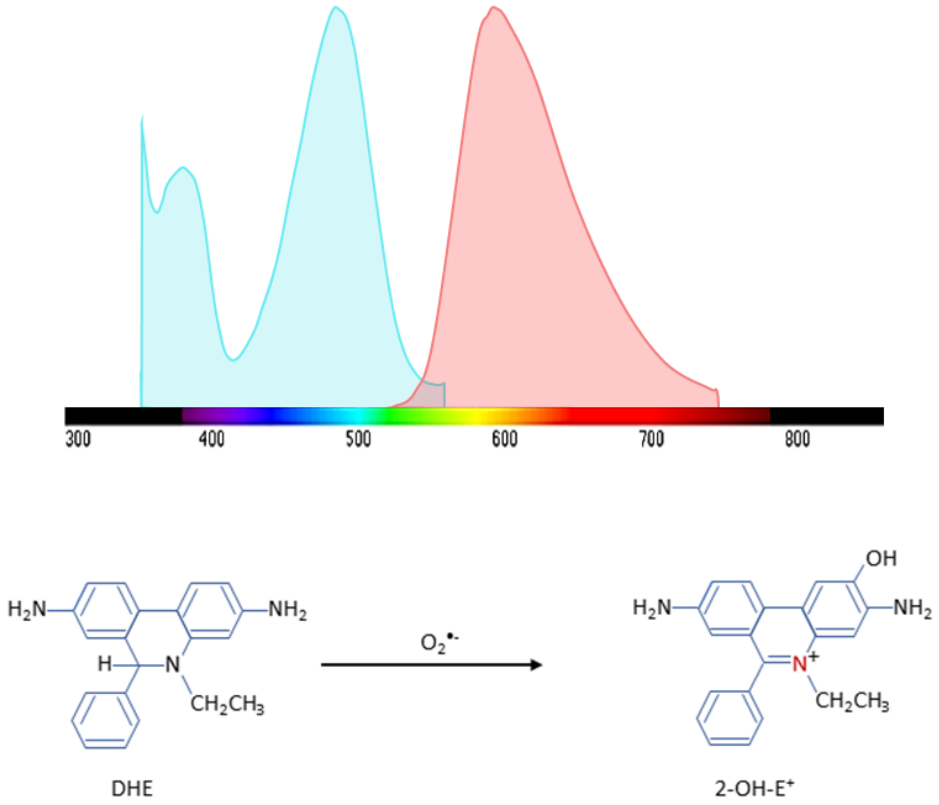


Figure 7: Dihydroethidium oxidation by $O_2^{\bullet -}$ forming 2-hydroxyethidium (2-OH-E^+) providing fluorescence with excitation/emission maxima ~500/590 nm [adopted from Prasad *et al.*, Frontiers in Plant Science 10:1660 (2020)].

- 3′-p-(hydroxyphenyl) fluorescein (HPF) (ThermoFisher Scientific, Paisley, UK) is a fluorescent probe with great specificity and stability for $HO^{\bullet}$. HPF is known to show limited non-selective reactivity and is resistant to autoxidation by induced light. HPF is nonfluorescent until it reacts with $HO^{\bullet}$ and exhibits bright green fluorescence upon oxidation (HPFox) (excitation/emission maxima ~490/515 nm) (Fig. 8).

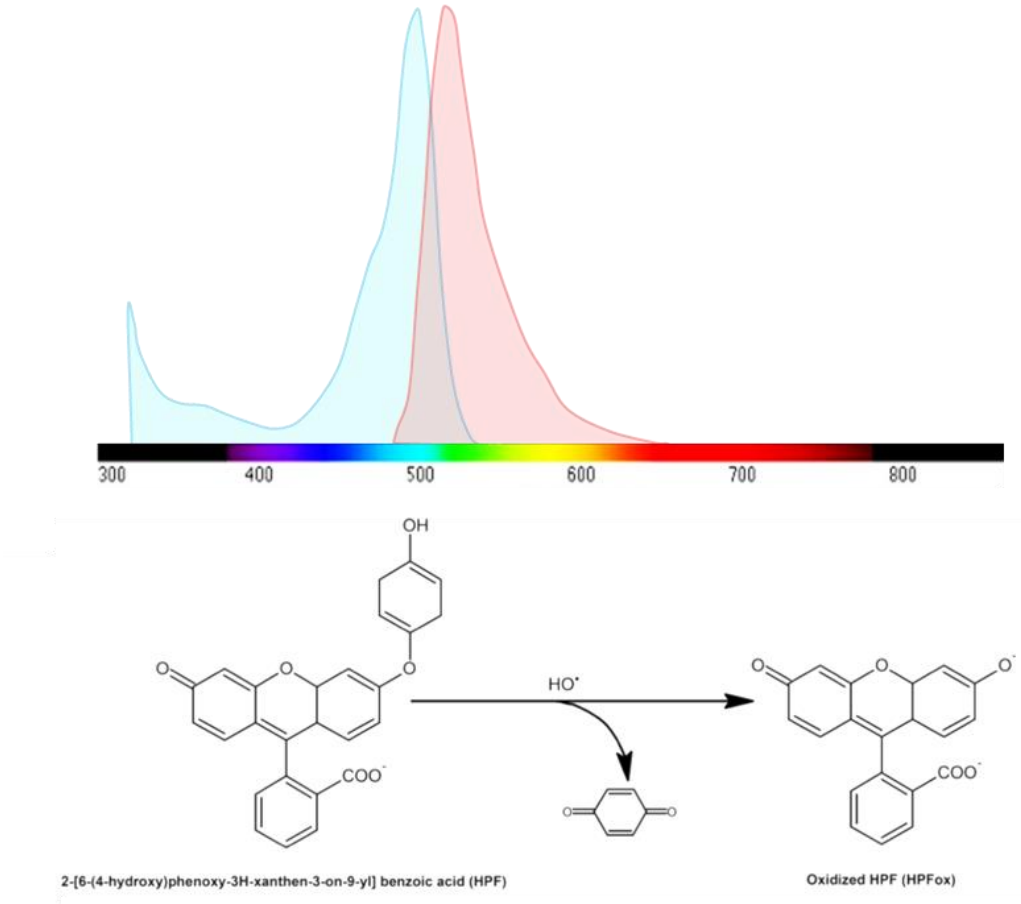


Figure 8: 3′-p-(hydroxyphenyl) fluorescein oxidation by $HO^{\bullet}$ providing fluorescence with excitation/emission maxima ~490/515 nm. [adopted from Kumar *et al.*, Data in Brief 21, 2246-2252 (2018)].

3.5 Confocal laser scanning microscopy

The morphology of U937 cells was visualized using diode laser excitation (405 nm) by transmitted light detection module and differential interference contrast filters. The cells were visualized after transferring the cells on a glass slide from the culture flask and duration between the two steps were kept minimum in order to minimize any exogenous stress on the cells. The cells were visualized using Fluorview 1000 unit attached to IX80 microscope (Olympus Czech Group, Prague, Czech Republic) (Fig. 9). The $O_2^{\bullet -}$ indicator DHE and $HO^{\bullet}$ indicator HPF were added to cell culture in the presence and absence of 100nM PMA. The duration of incubation with fluorescent probes in the absence or presence of inducer was in the range of 0-48 h as described in the result section. The excitation of DHE and HPF was performed using an argon laser (488 nm) and the emission was detected by a 505–605 nm filter.

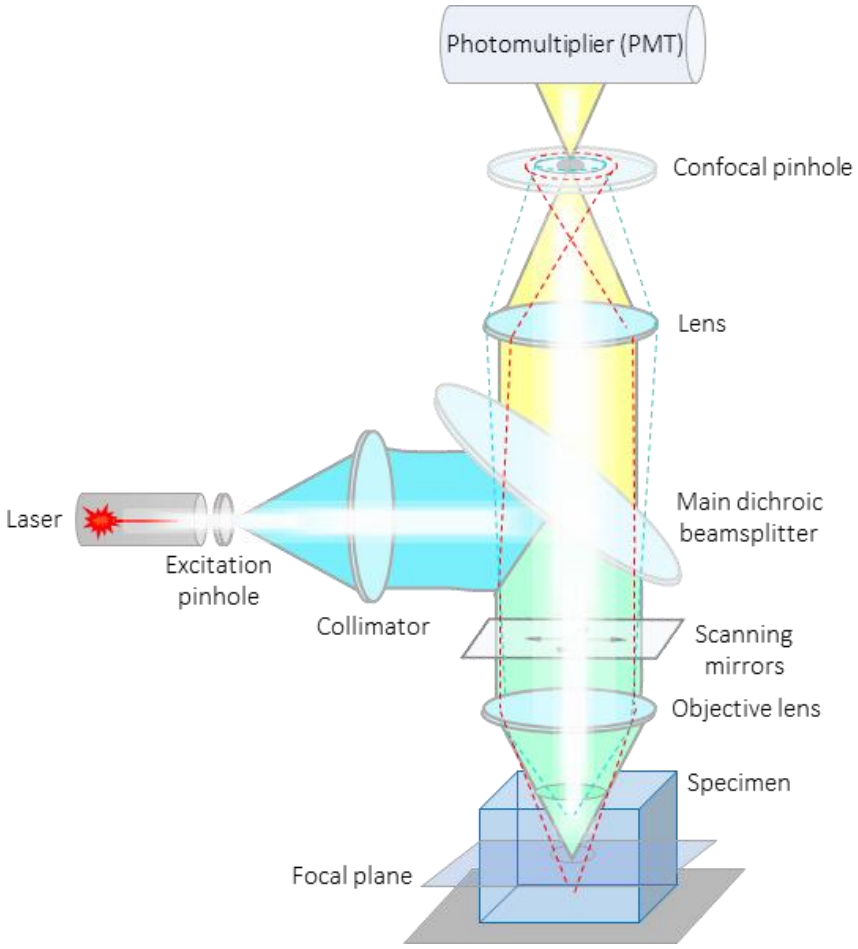


Figure 9: Schematics on working principle of confocal laser scanning microscope.

3.6 EPR spin-trapping spectroscopy

Electron paramagnetic resonance spin-trapping spectroscopy allows the direct detection of ROS. It bears the ability to detect short-lived species with high sensitivity and specificity. In the current study, detection of $HO^{\bullet}$ was performed using MiniScope MS400 (Magnettech GmbH, Berlin, Germany). Hydroxyl radical was detected using 4-pyridyl-1-oxide-N-tert-butylnitrone (POBN)/ethanol spin-trapping system in a glass capillary tube (Blaubrand intraMARK, Brand, Germany). The duration of cell incubation with spin trap (POBN/ethanol) in the absence or presence of inducer (PMA, 200nM) was for the duration of 30 min in an ultrasonic bath (37°C, 30 min). EPR spectra were recorded under the following parameters: 10mW, microwave power; 1G modulation amplitude; 100 KHz, modulation frequency; 100 G, sweep width; 1.62 G s^{-1}, scan rate.

4 Results

4.1 Growth curve of U937 cells

In order to determine the growth and behavior of cell culture, the cell division and growth curve was monitored (Fig. 10). The growth characteristics, cell viability and proliferation rates of the cell line was followed from day 1 till day 11. A typical growth curve for cultured cells shows a sigmoid pattern of cell proliferation. Since any changes in cellular growth patterns can indicate towards a substantial problem within the cell line and if undetected, can lead to error in experimental results. Thus, we monitored the growth curve to assure the concerns. Based on the growth curve, we standardized the protocol for cell passage and/or treatment with inducer. This helped us to assure the maximum cell count and minimize fluctuations during the experiments.

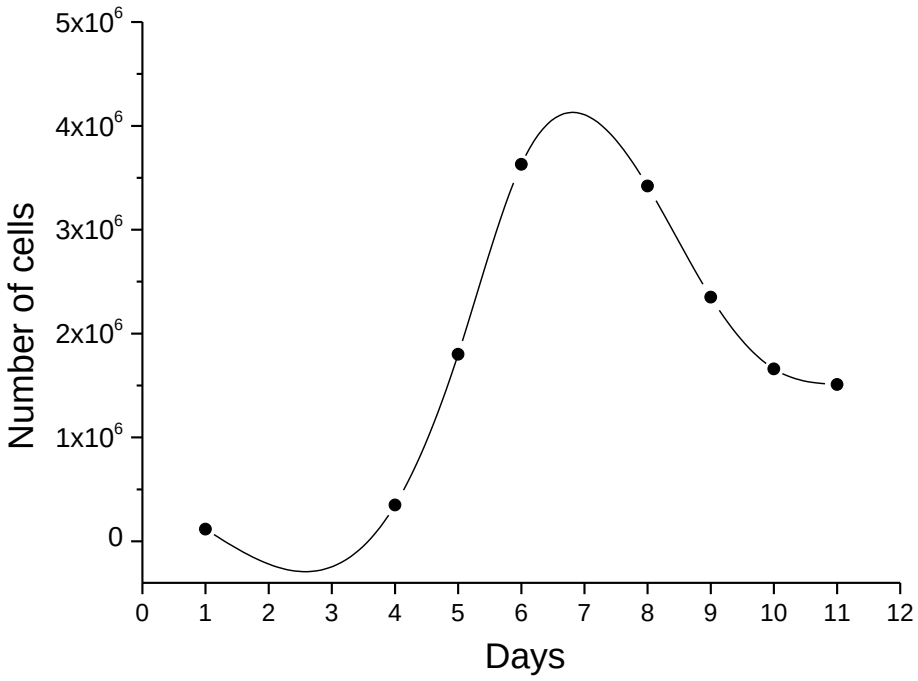


Figure 10: Growth curve of U937 cell following inoculation at density of 1.17×10^5cells/ml. The cell count was done using a counting slide (dual chamber for cell counter, Bio-Rad) on an automated cell counter.

4.2 Effect of solvents/chemicals on U937 cells

In order to avoid any interference and error during the measurement, it is pre-requisite to check the interference of solvents and/or chemicals utilized. Thus, in the current study, the effect of DMSO alone, which is used in following experiments to solubilize PMA and DHE on U937 cells was tested by monitoring the cell morphology and viability (Fig. 11).

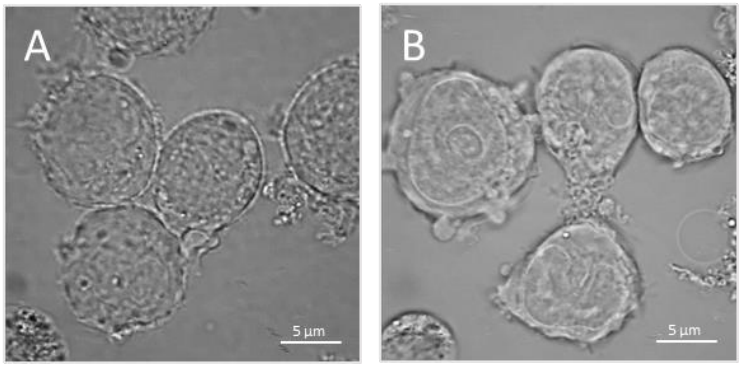


Figure 11: Images of U937 cells in the absence and presence of DMSO.

The PMA dissolved in DMSO showed cell differentiation and a reduction in cell viability by about 10% which is similar as in the case of DMSO alone (Figs. 12 and 13). The cells with DHE dissolved in DMSO showed a decrease in the proportion of living cells by about 20% while HPF alone by about 5% (Fig. 13). Application of the chemicals/solvent separately or in combinations maintained the cell viability equal or above 80% and thus their impact can be considered within the acceptable limits.

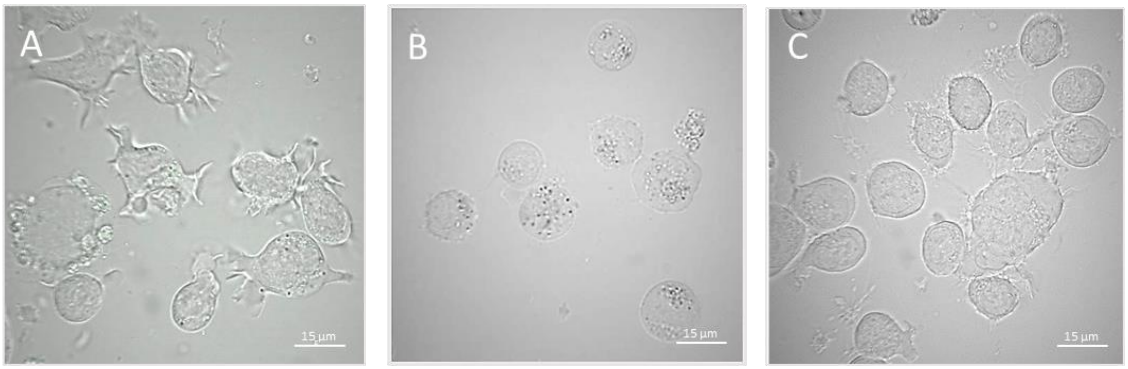


Figure 12: U937 cells under the effect of different chemicals: (A) PMA; (B) DHE and (C) HPF.

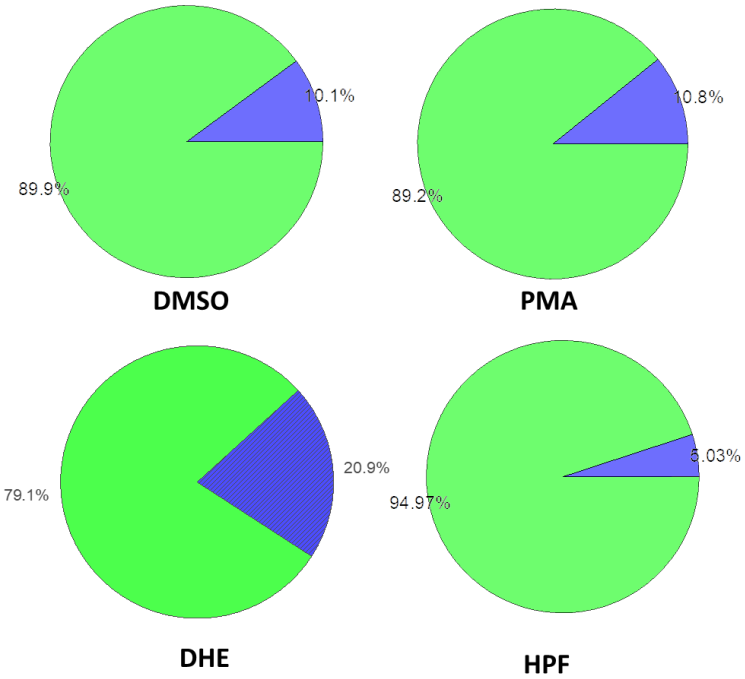


Figure 13: Effect of DMSO, PMA (100 nM); DHE (50 µM) and HPF (1µM) on viability of U937 cells. The viability was measured following 24 h of incubation following which trypan blue (0.4%) was mixed in the ration 1:1. The ratio of alive:dead cell was measured using a counting slide (dual chamber for cell counter, Bio-Rad) on an automated cell counter.

4.3 U937 cell differentiation using phorbol 12-myristate 13-acetate

The visual changes in relation to differentiation and apoptosis of U937 cells after treatment with PMA to U937 cells were evaluated. As shown, PMA addition did not show any immediate change and cells were round and translucent (Fig. 14A). U937 pro-monocytes differentiate into mature monocytes or into macrophages upon PMA-treatment which happens in the range to several hours till days. Typical exposure to PMA is for 48 h. After treatment with PMA for 48 h, the cell morphology was changed, with cells gathering and adhering generously and creating pseudopodia (Fig. 14B).

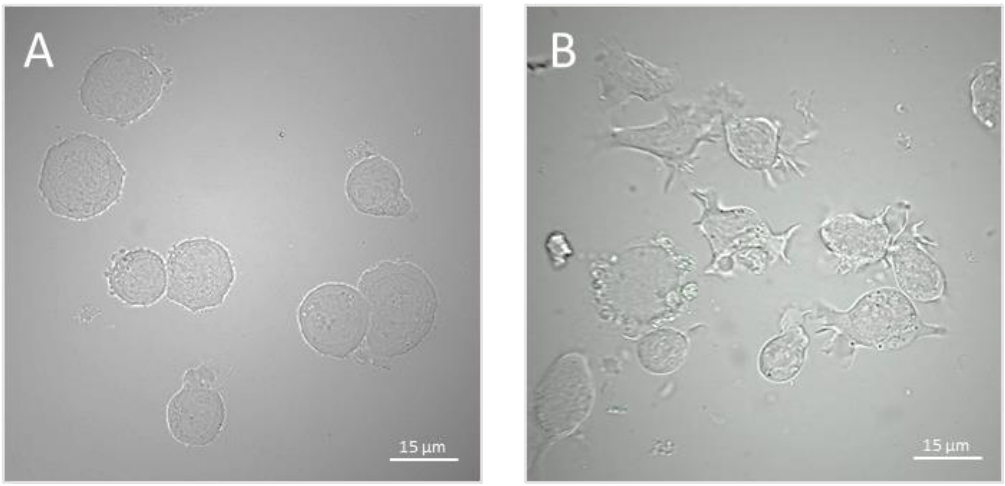


Figure 14: Morphology of U937 cells in the presence of PMA on U937 cells: (A), 100 nM PMA, 0 h (B), 100 nM PMA, 48 h.

4.4 Imaging of superoxide anion radical using confocal microscopy

The confirmation of the intracellular generation of ROS in U937 cells was done using fluorescent probe DHE for the detection of $O_2^{\bullet-}$ after 48 h of PMA treatment. It can be observed that following PMA treatment (100nM) for 48h in the presence of DHE showed an increase in DHEox fluorescence compared to control (Fig. 15). These results indicate that differentiation and $O_2^{\bullet-}$ formation are associated phenomenon. DHE fluorescent probe has been in criticism for its non-specificity (Patsoukis, Papapostolou and Georgiou, 2005). Nevertheless, many studies have reported the use of DHE in detecting $O_2^{\bullet-}$. The interference from other oxidative radicals prevented them from measuring $O_2^{\bullet-}$ accurately. Moreover, it has also been identified to intercalate with DNA (Fig. 16) which makes it complex to use and thus it is always recommended to validate with other techniques. Thus, considering this fact, we decided to follow the formation of $HO^{\bullet}$. It is well-known that dismutation of $O_2^{\bullet-}$ can lead to the formation of H_2O_2 and thus it can act as a precursor for the generation of $HO^{\bullet}$. The results were validated using EPR spin-trapping spectroscopy.

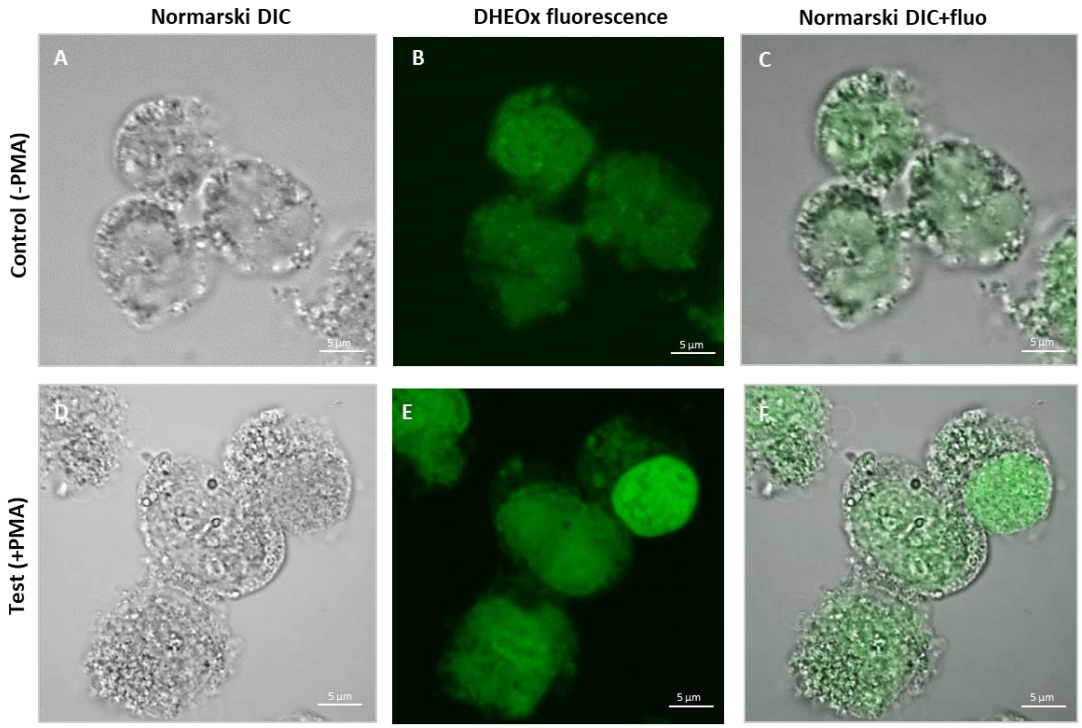


Figure 15: ROS ($O_2^{\bullet-}$) detection with DHE fluorescent probe in U937 cells. DHE: 50µm; PMA: 100nM (48 h); cell suspension: RPMI medium. From left to right are Nomarski DIC channel, DHEox fluorescence and combination of Nomarski DIC channel + DHEox fluorescence.

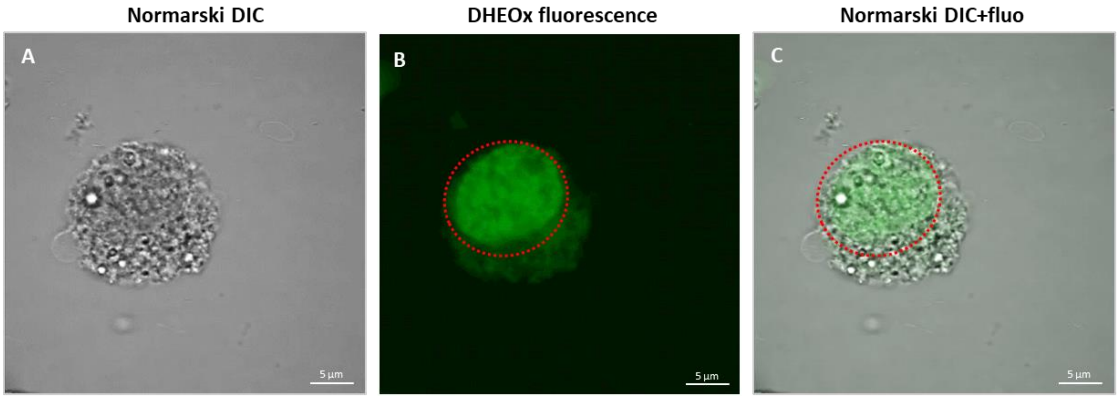


Figure 16: ROS ($O_2^{\bullet -}$) detection with DHE fluorescent probe in U937 cells. Conditions: DHE: 50µm; PMA: 100nM (48 h); cell suspension: RPMI medium. From left to right are Nomarski DIC channel, DHEox fluorescence and combination of Nomarski DIC channel + DHEox fluorescence.

4.5 Imaging of hydroxyl radical using confocal microscopy

The confirmation of ROS generation in U937 cells was done using fluorescent probe HPF to specifically detect $HO^{\bullet}$. PMA treated U937 cells exhibited higher HPFox fluorescence signal compared to non-PMA treated indicating generation of $HO^{\bullet}$ during differentiation. The signal showing the formation of ROS such as $HO^{\bullet}$ mainly goes from cells undergoing apoptosis processes. The intracellular ROS generation in U937 cells after 48 h is shown in Figure 17 and Supplementary data 1. The mature differentiated U937 cells can be seen to acquire all the peculiar features of macrophage-like cells including irregular shape, a large number of pseudopodia on their surface etc. These results clearly indicate that differentiation/apoptosis and $HO^{\bullet}$ are associated phenomenon.

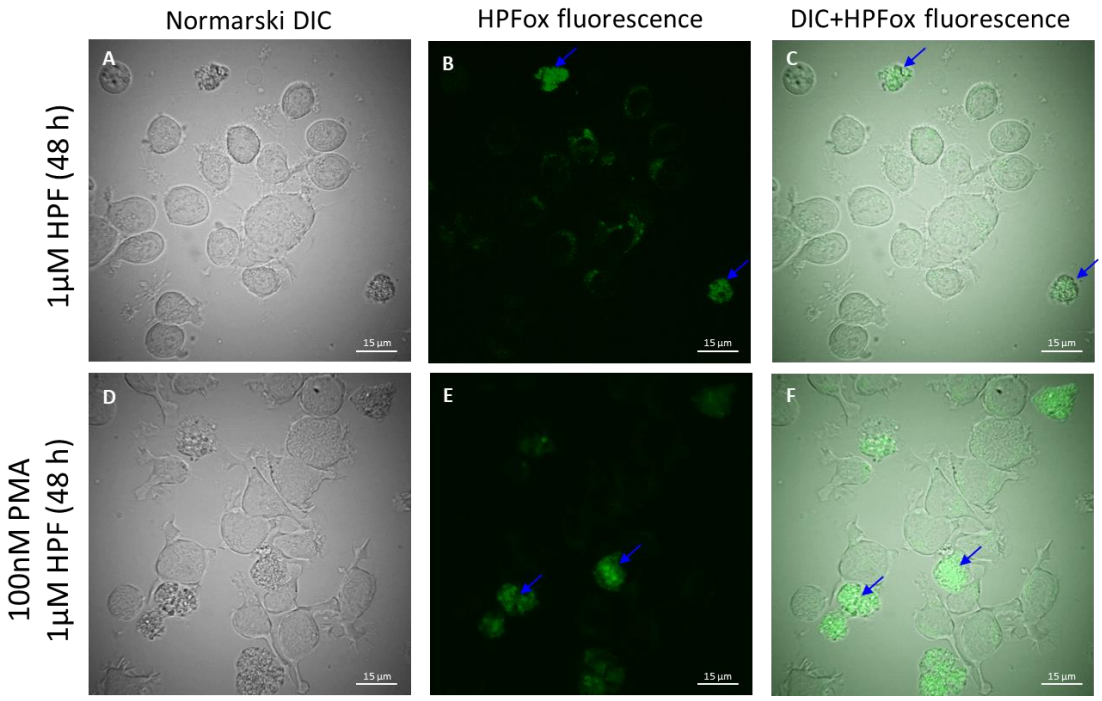


Figure 17: Hydroxyl radical imaging using HPF in U937 cells by confocal laser scanning microscopy after 48 h treatment with PMA. HPF: 1 µM in the absence (upper panel) and presence (lower panel) of PMA (100nM, 48 h). From left to right are Nomarski DIC channel, HPFox fluorescence and combination of Nomarski DIC channel + HPFox fluorescence. Blue arrows indicate damaged/apoptotic cells.

4.6 Hydroxyl radical detection by EPR spin-trapping spectroscopy

The generation of $HO^{\bullet}$ in U937 cells was measured by EPR spectroscopy using spin trap system (POBN/ethanol). In the absence of PMA, negligible intensity of EPR signal was observed in U937 (Fig. 18A, trace a and 18B), whereas treatment with PMA (200 nM, 30 min) to pre-differentiated cells resulted in the formation of the α-hydroxyethyl radical adduct of POBN [POBN-CH(CH_3)OH adduct] EPR signals (Fig. 18A, trace b and 18B). Based on the observation, it can be concluded that $HO^{\bullet}$ is formed as a result of cell differentiation in U937 cells.

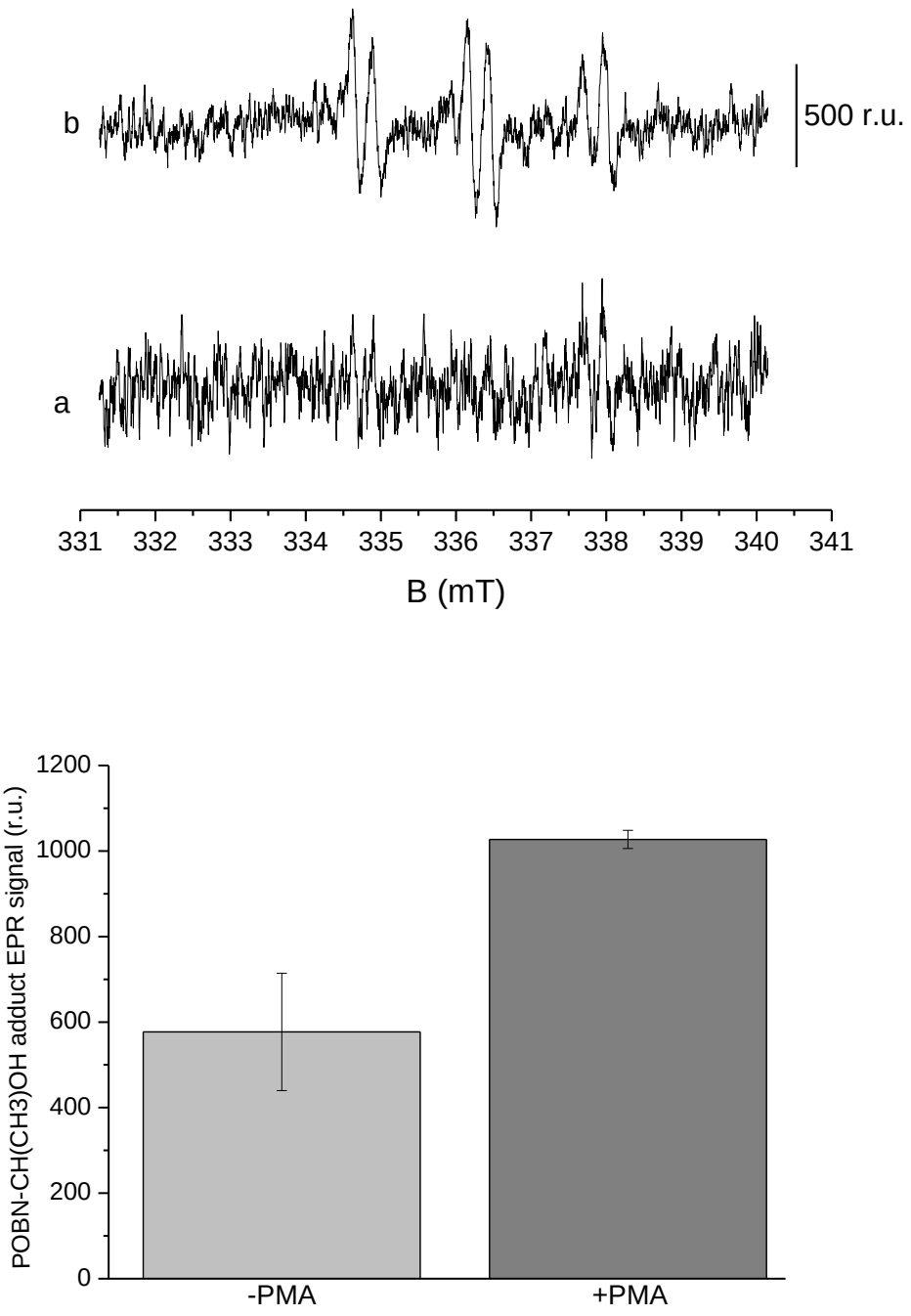


Figure 18: Detection of $HO^{\bullet}$ by using EPR spin-trapping spectroscopy. PMA induced POBN (4-pyridyl-1-oxide-N-tert-butylnitrone)-OH adduct EPR spectra: differentiated U937 cell with 50 mM POBN in the absence (18A, trace a) and presence of 200 nM PMA (18A, trace b). POBN-OH adduct EPR spectra were after 30min of sonication performed in the presence of 200 nM PMA. The lower panels show the standard deviation of EPR signal intensity (where, n=2).

5 Discussion

The current project was aimed to understand the growth and differentiation pattern of U937 cells under the influence of a differentiation inducer. Following the basic standardization, the relation of cell differentiation and ROS generation was followed. The generation of ROS was confirmed by using different detection techniques including confocal laser scanning microscopy and EPR spin-trapping spectroscopy. The U937 culture cells in the presence of DMSO showed no/minimum effect on U937 cells morphology. Control experiments with fluorescent probe DHE showed the effect on cellular integrity and HPF showed minimum effect which was also validated by cell viability measurements. In the current study, the signal showing the formation of $O_2^{\bullet-}$ and $HO^{\bullet}$ radicals mainly goes from cells undergoing differentiation and/or apoptosis during the PMA treatment. The results presented in this project was validated using EPR spin-trapping spectroscopy.

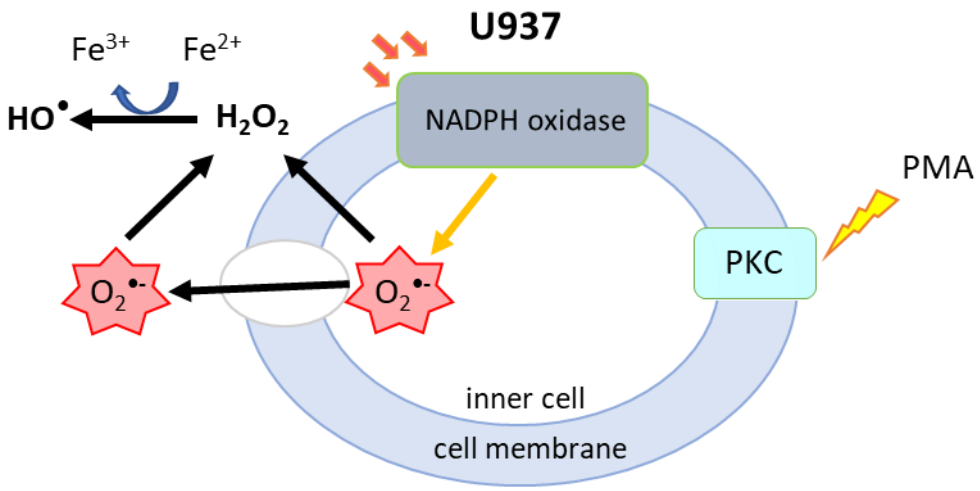


Figure 19: Schematic on ROS generation in U937 cells.

The highly sensitive fluorescent probe, DHE, was used to detect weak intracellular signals of $O_2^{\bullet -}$. Phorbol 12-myristate 13-acetate treated U937 cells showed higher DHEox fluorescence signal compared to non-PMA treated cells indicating the generation of $O_2^{\bullet -}$during differentiation process. Though many studies have described the application of DHE in detecting $O_2^{\bullet -}$ in cells or systems, the interference from other oxidative radicals prevented them from quantifying $O_2^{\bullet -}$ accurately (Patsoukis *et al.*, 2005; Chen, Rogers and Kavdia, 2013). Moreover, it has also been identified to intercalate with DNA, which makes it even more complicated to use. Thus, it is always suggested to validate with other techniques. Taking into consideration this fact, we decided to study the formation of $HO^{\bullet}$ in U937 cells.

U937 cells treated for 48 h with PMA showed higher HPFox fluorescence signal compared to control (Fig. 17). These results indicate that differentiation/apoptosis and $HO^{\bullet}$ formation are associated phenomenon. The EPR spectroscopy is often used to measure the formation of stable paramagnetic spin adducts by the reaction of $HO^{\bullet}$ with spin trap compounds. The usage of these compounds can also be valuable in that they can allow for simultaneous detection and quantification of ROS through the formation of different spin adducts, each with its distinctive EPR spectrum (Abdel-Rahman *et al.*, 2016). In our study, we used POBN/ethanol spin trap system to measure the formation of $HO^{\bullet}$ in U937 cells. A negligible intensity of EPR signal was observed in U937 cells in the absence of PMA, while incubation with PMA (200 nM) to pre-differentiated cells for 30 min resulted in the formation of the α-hydroxyethyl radical adduct of POBN [POBN-$CH(CH_3)OH$ adduct] EPR signals.

6 Conclusion

In conclusion, although further studies are needed to better understand the details of ROS generation in human cells, it nevertheless appears clear that differentiation of U937 cells to monocytes is accompanied by ROS generation. Cytotoxicity caused by PMA is mediated by the formation of ROS and subsequent activation of the ROS-dependent caspase. In this study, we characterized the response of U937 cells to a differentiating inducer (PMA) by means of *in-vivo* ROS imaging and characterization with confocal laser scanning microscopy with the utilization of DHE and HPF fluorescent probes and EPR spin-trapping spectroscopy, respectively. Despite the enormous efforts taken by several researchers in developing and establishing the detection methods of ROS, precise and specific measurement in cells and tissues remains still a challenge because of their short lifespan and very low level in cells. Knowing the mechanism and quantitative estimation of ROS formation is crucial as it is a valuable oxidative stress biomarker that can reflect the physiological status of an organism. Therefore, improvements and development of new specific methodologies are always necessary and demandable.

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Article citation:

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9 List of Appendices

9.1 List of abbreviations.

9.2 Supplementary data.

9.3 Article under submission (co-author) (Journal: Frontiers in Physiology) - part of the results presented in the thesis are included in the manuscript.

9.4 Published article (co-author) (Journal: Frontiers in Plant Science).

Appendix 1:

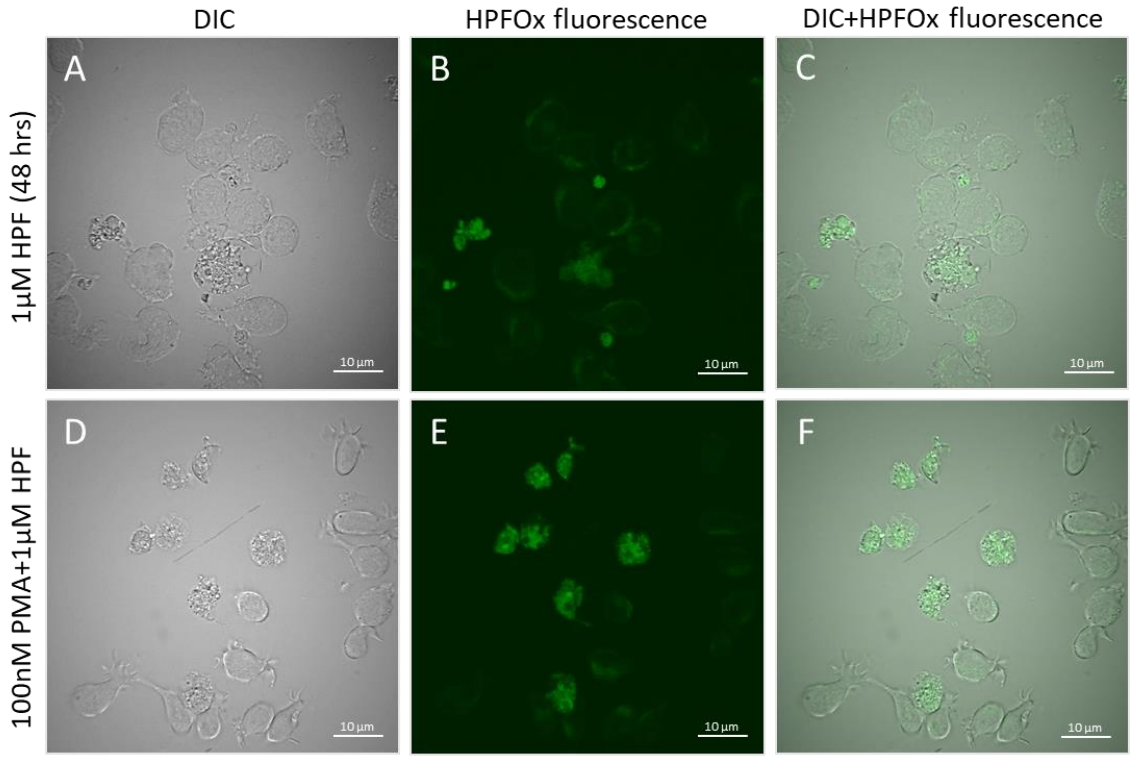
List of abbreviations

$^1O_2$	singlet oxygen
2-OH-E+	2-hydroxyethidium
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP1	activator protein 1, transcription factor
APAF-1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
b2-integrin	integrin beta 2
BSA	bovine serum albumin
CD11B	cluster of differentiation molecule 11B
DHE	dihydroethidium
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DUOX2	dual oxidase 2
EGFR	epidermal growth factor receptor signaling pathway
EPR	electron paramagnetic resonance
H_2O_2	hydrogen peroxide
HIF-1α	hypoxia-inducible factor 1-alpha
$HO^{\bullet}$	hydroxyl radical
HPF	3′-p-(hydroxyphenyl) fluorescein
MAPKs	mitogen activated protein kinases
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NF-kB	nuclear factor kappa B
Nrf2	nuclear transcription factor 2
$O_2^{\bullet-}$	superoxide anion radical
O_3	ozone
PI3K	phosphatidyl inositol 3-kinase
PKC	protein kinase C

PMA	phorbol 12-myristate 13-acetate
POBN	4-pyridyl-1-oxide-N-tert-butylnitrone
ROS	reactive oxygen species
RPMI-1640 medium	Roswell Park Memorial Institute 1640 medium
SOD	superoxide dismutase

Appendix 2:

Supplementary data 1



Supplementary data 1: ROS ($HO^{\bullet}$) detection with HPF fluorescent probe in U937 cells by confocal laser scanning microscopy after 48 h treatment with PMA. Conditions: HPF: 1 mM; absence (upper panel) and presence (lower panel) of PMA: 100nM (48 h PMA); cell suspension: RPMI1640 medium. From left to right are Nomarski DIC channel, HPFox fluorescence and combination of Nomarski DIC channel + HPFox fluorescence.

Appendix 3:

Article under submission (co-author) - The author contributed in data curation.

Reactive oxygen species imaging in U937 cells

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Appendix 4:

Published article (co-author) - the author contributed in data curation and participated in the drafting of the first version of the manuscript.

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ORIGINAL RESEARCH
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Reactive Oxygen Species as a Response to Wounding: *In Vivo* Imaging in *Arabidopsis thaliana*

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Mechanical injury or wounding in plants can be attributed to abiotic or/and biotic causes. Subsequent defense responses are either local, i.e. within or in the close vicinity of affected tissue, or systemic, i.e. at distant plant organs. Stress stimuli activate a plethora of early and late reactions, from electric signals induced within seconds upon injury, oxidative burst within minutes, and slightly slower changes in hormone levels or expression of defense-related genes, to later cell wall reinforcement by polysaccharides deposition, or accumulation of proteinase inhibitors and hydrolytic enzymes. In the current study, we focused on the production of reactive oxygen species (ROS) in wounded Arabidopsis leaves. Based on fluorescence imaging, we provide experimental evidence that ROS [superoxide anion radical ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$)] are produced following wounding. As a consequence, oxidation of biomolecules is induced, predominantly of polyunsaturated fatty acid, which leads to the formation of reactive intermediate products and electronically excited species.

Keywords: Arabidopsis, confocal microscopy, fluorescent probes, mechanical injury, wounding

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INTRODUCTION

In biological systems, the metabolism is affected by non-physiological conditions which lead to stress reactions (Foyer et al., 1994; Cramer et al., 2011). The stress conditions in plants are categorized as biotic or abiotic; the former include herbivory, viral, bacterial, and fungal infections and damage by pests while the later include extreme environmental factors such as temperature, UV radiation, light, water availability, pH, salinity, toxic chemicals, burning, and mechanical injury among others (Garces et al., 2001; Johansson Jankanpaa et al., 2013; Kasai et al., 2019). The stressors can act independently or in various combinations (Savatin et al., 2014).

In plants, active i.e. biochemical defense responses have been well known to occur under the condition of wounding. Activation of local response to repair the damages occurs *via* stress-responsive gene, oxidative burst linked with cell wall reinforcement, deposition of callose, suberin, synthesis of various phenolics, defensive proteins, lectins, accumulation of phytoalexins etc. (Reymond et al., 2000; Savatin et al., 2014; Rehrig et al., 2014). The defense responses are known

Abbreviations: ROS, reactive oxygen species; CCD, charge-coupled device; $O_2^{\bullet-}$, superoxide anion radical; LOOH, lipid hydroperoxide; $^1O_2$, singlet oxygen.