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Real time monitoring and quantification of reactive oxygen species in breast cancer cell line

MCF-7 by 2',7'– dichlorofluorescin diacetate (DCFDA) assay

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Abstract

The detection of reactive oxygen species (ROS) using 2',7'–dichlorofluorescin diacetate (DCFDA) is commonly performed by a single measurement of fluorescence but this fails to capture a profile of ROS generation over time. This study aimed to develop a real-time monitoring method to increase the utility of the assay, to incorporate cytotoxicity screening and to describe the combined effects of DCFDA and the ROS generator, Ter-butyl hydrogen peroxide (TBHP). Breast cancer MCF-7 cells were loaded with DCFDA (0-50μM) for 45min, and then exposed to TBHP (0-50μM). Fluorescence was recorded according to three different schedules: every hour for 6h, or once after 6h or 24h. Viability was assessed in a crystal violet assay and cell morphology was examined by microscopy. TBHP caused a time and dose-dependent increase in ROS and the magnitude of the fluorescent signal was affected by the loading concentration of DCFDA. Reading the fluorescence every hour for 6h did not diminish the emission signal. The most sensitive and reliable combination for this ROS assay was 10μM DCFDA with 25μM TBHP; since higher concentrations of DCFDA compromised cell viability. In conclusion we adapted a single point ROS assay to enable production of a profile of ROS generation over an extended 6h period, and related this to cell viability and morphology.

Introduction

Reactive oxygen species (ROS) are highly reactive molecules with one or more unpaired electrons produced by the reduction of oxygen (Fan & Li, 2014; Gomes, Fernandes, & Lima, 2005; Valko et al., 2007). Common ROS include superoxide radical (O_2^-), hydroperoxyl radical (HO_2^-), hydroxyl radical (HO^-), peroxyl radical (ROO^-), alkoxyl radical (RO^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), nitric oxide (NO) and hypochlorous acid (HOCl) (Fink, 2002; Gomes et al., 2005; Murrant & Reid, 2001).

Exposure to UV light, radiation, chemotherapeutics or infectious agents may induce the production of ROS (Cooke, Evans, Dizdaroglu, & Lunec, 2003; Halliwell, 1991) and ROS are also produced within the body as a by-product of respiratory metabolic processes facilitated by mitochondrial electron transport chains, NADPH oxidase, cytochrome P450 reductase, xanthine oxidase and nitric oxide synthase (Dröge, 2002; Finkel, 2011; Gutteridge & Halliwell, 2010; Halliwell, 1991; Li & Shah, 2004; Valko et al., 2007). ROS also play an essential role as second messengers in different intracellular signalling processes (Li & Shah, 2004). ROS production and elimination is mediated by a wide range of enzymatic and non-enzymatic molecules with strong reductive power (Li & Shah, 2004; Verbon, Post, & Boonstra, 2012). The balance between these two processes (ROS generation and ROS scavenging) is known as cellular "redox state" (Fan & Li, 2014). When there is an alteration of this balance, either by overproduction of ROS or depletion of the antioxidant defence molecules, oxidative stress occurs, which causes the oxidation of lipids, tissue proteins, DNA and other biomolecules, and can lead to aberrant cell signalling, dysfunctional redox control, apoptosis and cell death (Gomes et al., 2005).

Detection of ROS is commonly used to assess the mechanism of action of different drugs and compounds, and is also used as a marker for biological functions and cell cycle progression (Lautraite, Bigot-Lasserre, Bars, & Carmichael, 2003). Some reactive oxygen species have a very short half-life; for instance OH radicals have a $T_{1/2}$ of approximately 10^{-9} seconds; therefore they exert effects close to their site of generation (Forkink, Smeitink, Brock, Willems, & Koopman, 2010; Valko et al., 2007). Other molecules like O_2^- and H_2O_2

have longer half-lives, with approximate values of 10^{-6} and 10^{-5} seconds respectively; these molecules have wider diffusion distances, moving within and between cells to interact with redox-sensitive molecules that in turn regulate cell cycle progression (Giorgio, Trinei, Migliaccio, & Pelicci, 2007). Although the precise mechanism of ROS signalling is poorly understood, it is currently thought that local ROS generation induces reversible post-translational changes at cysteine (Miki & Funato, 2012), selenocysteine (Hawkes & Alkan, 2010), methionine (Hoshi & Heinemann, 2001) and histidine (Lee & Helmann, 2006) protein residues with subsequent modifications to protein conformation, binding sites and surface properties (Woolley, Stanicka, & Cotter, 2013). It is also well known that ROS can modulate the response of many important cell-signalling molecules including mitogen-activated protein kinases, nuclear factor-kB, tumour suppressor protein p53, and other cell cycle check point proteins (Li & Shah, 2004; Menon & Goswami, 2007; Vurusaner, Poli, & Basaga, 2012). Intracellular changes in ROS levels can therefore influence whether cells progress through or withdraw from the cell cycle, or undergo apoptosis (Fan & Li, 2014). The rate at which the cell cycle is promoted or inhibited will ultimately depend on ROS levels, and varies according to the type of cell, extracellular stimuli and the duration of exposure (Fan & Li, 2014).

Different techniques have been used to quantify ROS, including electrochemical quantification, electro spin resonance and fluorescent signalling (Fan & Li, 2014). The fluorescence methodology is based on the use of suitable probes to measure ROS generation. Brandt and Keston (Brandt & Keston, 1965) used 2',7'– dichlorofluorescin diacetate (DCFDA) as a fluorometric probe for the detection of H_2O_2. It was later demonstrated that DCFDA is oxidized by other reactive oxygen species including peroxyl and hydroxyl radicals (Gomes et al., 2005). DCFDA is a non-fluorescent, lipophilic and non-ionic compound capable of diffusing and crossing the cell membrane into the cytoplasm. Once inside the cells, DCFDA is deacetylated by intracellular esterases, producing 2',7'– dichlorofluorescin (DCFH), a non-fluorescent and membrane-impermeable compound that reacts with intracellular ROS (Brandt & Keston, 1965; Rosenkranz et al., 1992; Rota, Chignell, & Mason, 1999; Rota, Fann, & Mason, 1999). Intracellular levels of non-fluorescent

DCFH might vary widely with cell type, initial incubation time, DCFDA concentration, esterase activity and possible subsequent leakage (Chen, Zhong, Xu, Chen, & Wang, 2010).

Experimental conditions in the DCFDA assay often vary in different studies due to the requirements of the experimental design and/or the preference of individual researchers. The lack of uniformity of experimental conditions makes it difficult to compare results from different studies, as it has been shown that the slope of the ROS dose-response curves differ greatly depending on the DCFDA concentration in the cell culture media (Wan, Zhou, & Kennedy, 2003; Wan, Zhou, Ware, & Kennedy, 2005). DCFDA concentrations range from 5-50μM (Chen et al., 2010; Valko et al., 2007) and cells can be incubated with DCFDA from 30min to 1h (Chen et al., 2010) before or after challenging the cells with a ROS analogue or inducer. In the presence of ROS, DCFH is oxidised to fluorescent 2', 7'–Dichlorofluorescin (DCF). The emitted fluorescence is measurable by spectrofluorometry (J. H. Kim et al., 2005) or flow cytometry (Epling et al., 1992), with the magnitude of DCF fluorescence proportional to ROS activity (Halliwell & Whiteman, 2004). In most cases the emitted fluorescence is measured once at a single designated time-point and the result used as a qualitative marker of cellular oxidant stress (Chen et al., 2010). Gong et al. (Gong, Yu, Yang, & Zhang, 2016) examined real-time ROS production in mouse macrophages (RAW264) by incubating the cells with phosphorus and nitrogen doped carbon dots (PN-CDs) for 24h at 37°C. Real time confocal imaging was used to continuously monitor the cells for 50min after lipopolysaccharide (LPS) exposure to induce ROS. There was a significant fluorescence emission which decreased gradually after exposure to LPS. Oparka et al. (Oparka et al., 2016) investigated ROS-dependent M-H$_2$DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate) oxidation in fibroblasts by measuring fluorescence every 1.5 minutes for 30 minutes, but to our knowledge there are no reports of studies that monitor ROS generation over extended periods of time in the same cells using a microplate fluorescence spectrophotometer. The development of a real-time measurement method for the determination of intracellular ROS concentration would be valuable for the elucidation of cell cycle progression and the mechanism of action of different drugs; furthermore, using a

microplate reader will give the advantage of *in situ* measurement, without the need for trypsinization; provide a large amount of data in a short period of time and allow parallel cytotoxicity screening of drugs and other compounds.

Ter-butyl hydrogen peroxide (TBHP) is a short-chain analogue of lipid hydroperoxides and is widely used as pro-oxidant agent *in* vitro because it mimics the toxic effect of peroxidized fatty acids (Kučera et al., 2014; Martín et al., 2001).TBHP is well known for its damaging effects towards DNA, lipids and other macromolecules, and for causing oxidative stress to cells (Alia, Ramos, Mateos, Bravo, & Goya, 2005). Some studies have also suggested that TBHP promotes peroxidation of membrane lipids more efficiently than H_2O_2 (Guidarelli, Cattabeni, & Cantoni, 1997), which makes this hydroperoxide an excellent compound to use when establishing and characterising optimal conditions for an assay to measure ROS. TBHP diffuses through cell membrane easily and generates intracellular alkoxyl and peroxyl radicals, as well as H_2O_2, in a reaction assisted by metal ions (Alia et al., 2005; Guidarelli et al., 1997). These RO species induce several physiological alterations such as lipid peroxidation, oxidative DNA damage, the depletion of intracellular glutathione, and cell death by apoptosis or necrosis (Guidarelli et al., 1997; J.-A. Kim, Kang, Kim, Lee, & Lee, 1998; Sandström, 1991). Kweon et al., (Kweon, Jung, & Sung, 2004) studied the cytotoxic effect of TBHP in Bovine aortic endothelial cells (BAECs). BAECs were exposed to 1mM TBHP for 1-24h, and then assessed for cell viability using the MTT assay, and ROS generation with a DCF fluorometric assay. TBHP-initiated ROS generation preceded cell death and the oxidative stress induced by 1mM TBHP caused significant cell death (>50%) 6h after TBHP exposure, and >90% cell death 18h later (Kweon et al., 2004).

For over 40 years DCFDA has been used extensively as a fluorescent probe, and it has been assumed that DCFDA does not affect cell metabolism or viability even though few studies have confirmed the absence of toxicity. Bass et al., (Bass et al., 1983) pre-incubated human leukocytes with 5-12.5μM DCFDA for 15min, then examined chemotactic, degranulation and oxidative metabolic responses. Directional migration of leukocytes was not significantly affected by DCFDA, and neither was lysozyme release. Similarly, Mehanna

et al., (Mehanna, Baudouin, & Brignole-Baudouin, 2011) found that a 20min exposure to 20μM DCFDA did not affect the viability of HTM-3 cells. In contrast, Andoh et al., (Andoh et al., 2006) found that DCFDA seemed to contribute significantly to cellular anti-toxicity defence. In this study, HeLa cells were incubated with 10μM sodium arsenite, 1μM cadmium chloride and 10μM hemin in the absence or presence of 20-40μM DCFDA for 24h. Cell viability was monitored by measuring the activity of lactate dehydrogenase, and in a trypan blue exclusion assay. DCFDA protected the cells against the cytotoxic effects of hemin, arsenite and cadmium (Andoh et al., 2006).

In the crystal violet staining assay (CV), dye dissolved in methanol stains the nuclei of live cells and fixes cells to the culture plates (Saotome, Morita, & Umeda, 1989). After solubilisation with acetic acid, the optical density can be measured with a spectrophotometer. The crystal violet assay is an excellent alternative to enzymatic assays (Bechtel & Bonavida, 2001; Reid, Lang, Froscio, Humpage, & Young, 2015; Saotome et al., 1989; Siddiqui, Shabbir, Mikhailidis, Mumtaz, & Thompson, 2006), as it does not rely on the metabolic function of specific enzymes (Saotome et al., 1989). Measuring cell viability after ROS assay would not only give more information about the effects of DCFDA, but also would aid in the correlation of cell response after being challenged with a ROS inducer.

Therefore the aim of this study is to monitor real time production of ROS in the same cells using a DCF assay and to assess the number of viable cells on the same microplates after exposure to DCFDA and/or TBHP.

Materials and methods

Chemicals and Reagents

All chemicals and reagents used in this study were obtained from Sigma-Aldrich (Australia), unless specified otherwise. The DCFDA cellular ROS detection assay kit was purchased from Abcam (Melbourne, Australia).

Preparation of solutions

100 mL 1X DCFDA kit dilution Buffer (Abcam, Australia) was prepared by mixing 10 mL 10X dilution buffer with 90 mL ddH$_2$O. DCFDA was diluted in 1X kit dilution buffer to generate a stock solution of 50μM, which was further diluted in 1X kit dilution buffer to give 5, 10 and 25μM on the day of use. Ter-butyl hydrogen peroxide (TBHP) dilutions were prepared in complete RPMI with 10% FCS without phenol red, to give final concentrations of 12.5, 25 and 50μM. The 0.5% crystal violet stain was prepared in a 50% diluted methanol solution. 100% Acetic acid was diluted to 33% with demineralised water and used as a destain solution in the crystal violet assay.

Cell culture

The MCF-7 human epithelial breast adenocarcinoma cell line was obtained from the America Type Culture Collection (ATCC) and maintained in RPMI media, supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10mg/mL streptomycin. Media were replaced every 2-3 days and cells were harvested with 0.1% trypsin/EDTA solution and subcultured twice a week. Cell culture flasks containing 80% confluent cells in exponential growth phase were used for all experiments.

Cell-free DCFDA fluorescence

Aliquots of 50μM DCFDA or aliquots of 100μM TBHP were added to either 1X kit dilution buffer or to phenol red-free RPMI with or without 10%FCS to obtain a concentration of 10μM DCFDA or 20μM TBHP. 100μL of each solution was added to wells in dark, clear bottom 96-well microplates in triplicate (Greiner CELLSTAR®, Sigma-Aldrich Australia). Fluorescence readings were taken using a plate spectrofluorometer (VICTOR X multilabel, Perkin Elmer, Australia) after 1h incubation at 37°C in a humidified 5% CO$_2$. Fluorescence was measured using the following settings: excitation at 495nm, emission at 530nm; temperature 37°C; reading mode: bottom; number of reads: 3. Wells with only 1X buffer or RPMI with or without 10%FCS were included as background negative controls. Each

experiment was repeated on three separate occasions (n=3). Relative fluorescence units (RFU) were calculated by subtracting blank readings from all measurements. Plates were kept at 37°C in a humidified 5% CO_2 incubator in the dark between readings.

DCFDA concentration range study

MCF-7 cells (20,000 cells per well) were added to dark, clear bottom 96-well microplates according to DCFDA kit manufacturer's instructions and incubated at 37°C in 5% CO_2 for 24h to allow adherence. Cells were washed with 1X warm phosphate buffered saline (PBS) and exposed to 100µL of 0, 5, 10, 25 or 50µM DCFDA in 1X kit dilution buffer for 45min at 37°C in a humidified 5% CO_2 incubator in the dark. The DCFDA solution was replaced with RPMI and 10% FCS and the basal ROS production detected by recording fluorescence according to three different schedules: every hour for a 6h incubation period, only once after a single 6h continuous incubation (6h+), and after 24h continuous TBHP exposure (24h+).

TBHP- stimulated ROS generation; Dose-response curve

Additional MCF-7 cells (20,000 cells per well) were added to dark, clear bottom 96-well microplates according to DCFDA kit manufacturer's instructions and exposed to a range of concentrations of DCFDA 0-50µM (described above). DCFDA solutions were removed, and cells washed with 1X PBS. MCF-7 cells were then exposed to 100µL 0, 12.5, 25 or 50µM TBHP in complete RPMI with 10% FCS without phenol red. Fluorescence was detected according to the 6h, 6h+, 24h+ schedule described above.

Controls were cells in media only (background negative control), cells loaded with DCFDA but not exposed to TBHP (basal ROS production), wells containing DCFDA and TBHP but no cells, and cells exposed to ranging concentrations of TBHP (0-50 µM) without DCFDA (autofluorescence control). RFU were calculated by subtracting background readings (cells in media only), from all measurements and determining the fold change. Each concentration of DCFDA and TBHP was examined in two replicate wells. Each experiment was repeated on three separate occasions (n=3).

Image acquisition

Cell morphology was examined using an inverted light microscope (Olympus CKX41) and camera (Olympus DP22). Four digital images of each well were taken with a 20X (0.40php) objective using an image acquisition system (DP2- SAL, Olympus).

Cell viability study after DCFDA- cellular reactive oxygen species detection assay:

The crystal violet (CV) assay was performed on the same MCF-7 cells that were examined in the DCFDA – ROS assay (described above). Media containing TBHP were removed and replaced with 50μL of Crystal Violet stain (0.5%). The cells were stained and fixed for 10min at room temperature. Excess stain was rinsed away with demineralised water, and cells were left to air dry overnight. 50μL of destain solution was added for 10min. The optical density was read at 570nm with correction at 630nm (Reid et al., 2015). A separate crystal violet standard curve was produced in each replicate experiment in which cell densities ranged from 0 – 80,000 cells per well in replicates of 6 for each cell density. To generate standard curves, absorbance readings were plotted against cell densities with an average linear correlation of R^2 = 0.99 (n=3 replicate standard curves). Numbers of viable cells after exposure to DCFDA and/or TBHP were determined by comparison with the CV standard curve generated for the same replicate experiment.

Statistical analysis

A mixed analysis of variance (ANOVA) was performed to test the dose-dependence effect of DCFDA and TBHP and pair-wise comparisons with Bonferroni post-hoc adjustment for multiple comparisons were used to investigate statistical significance. To examine the effect of the three different time schedules on ROS production, an ANOVA was conducted that used time, DCFDA and TBHP concentrations as independent factors. Statistical significance was assessed by Tukey HSD and Bonferroni post-hoc tests. A one-way ANOVA with Tukey HSD post-hoc was conducted to examine the fluorescence signal in MCF-7 when loaded with varying concentrations of DCFDA, and to examine the fluorescence signal in the cell-free system. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Australia). Statistical significance was set at p ≤ 0.05.

To examine the effects of the DCFDA and TBHP on MCF-7 cell viability a two-way ANOVA with Tukey HSD post-hoc was conducted. All experiments were performed as three independent replicates, and all data expressed as mean ± standard deviation.

Results

In cell free controls, 1h incubation of 10µM DCFDA in 1X ROS assay kit buffer generated the same background fluorescence as DCFDA-free buffer (Figure 1). The same concentration of DCFDA in phenol-red free RPMI cell culture medium, either with or without 10% FCS, generated significantly higher levels of fluorescence than DCFDA in buffer (Figure 1), but the presence or absence of 10% FCS had no significant effect on fluorescence. In the absence of DCFDA, the ROS generator TBHP (25µM) did not cause fluorescence in cell free buffer or in RPMI medium (Figure 1).
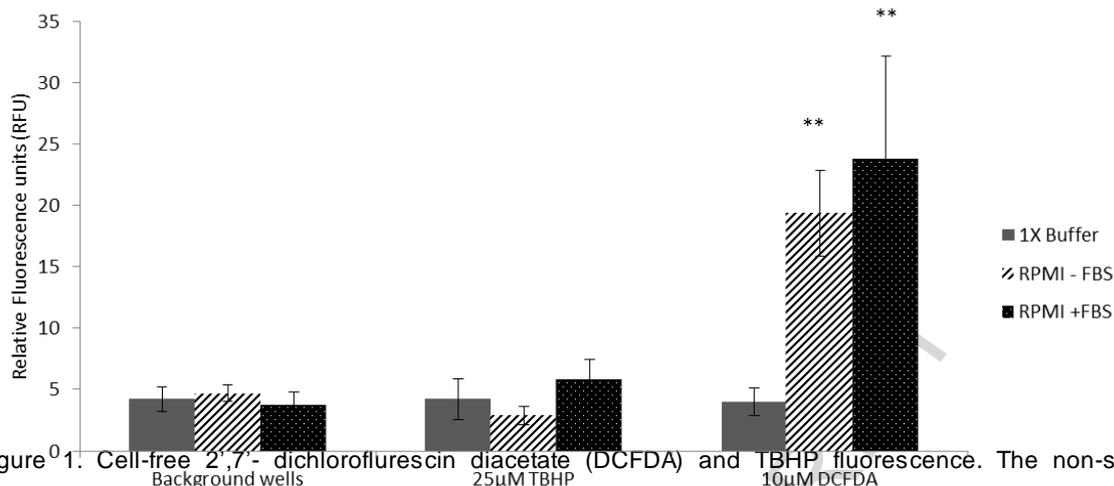
Figure 1. Cell-free 2',7'- dichloroflurescin diacetate (DCFDA) and TBHP fluorescence. The non-specific background fluorescence of 10μM DCFDA or 25μM TBHP was assessed in wells containing either 1X ROS assay kit buffer or Phenol red-free RPMI cell culture medium with or without 10% foetal calf serum (FCS) after 1h incubation at 37°C in a humidified 5% CO_2. Background wells contained only 1X buffer, or RPMI with or without 10%FCS. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to background control.

MCF-7 cells generated ROS in the absence of TBHP (Figures 2, 3). Background (or basal) fluorescence values after 6h continuous incubation were significantly higher in cells loaded with 50μM DCFDA (55 ± 13.02 RFU per well) than in cells loaded with 5- 25μM DCFDA (Figure 3). The addition of TBHP caused a significant dose-dependent increase in ROS generation at each time point compared to the background values from the same time-point (Figure 2).

A time-dependent, significant increase in fluorescence was detected up to 3h compared to pre-incubation readings (0h, p<0.05, Figure 2). After 3h there was no significant increase in fluorescence compared to the 0h levels.
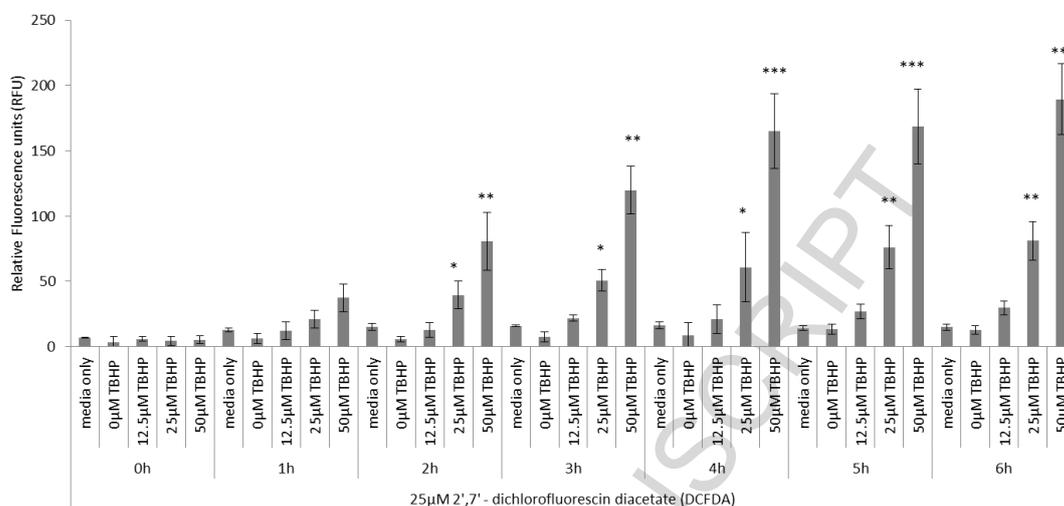
Figure 2. TBHP-stimulated ROS generation by MCF-7 cells loaded with 25µM 2',7'- dichloroflurescin diacetate (DCFDA). DCFDA fluorescence was assessed every hour for 6h in cells exposed to 0, 12.5, 25 and 50µM TBHP. Cells in phenol red-free RPMI without DCFDA or TBHP were used as background negative control. Means ± SD of 3 independent experiments shown. Data analysed by mixed ANOVA within factor of time with pair-wise post-hoc test. *p ≤ 0.05; ** p ≤ 0.01, *** p ≤ 0.0001 compared to 0µM TBHP vehicle control within each time of detection.
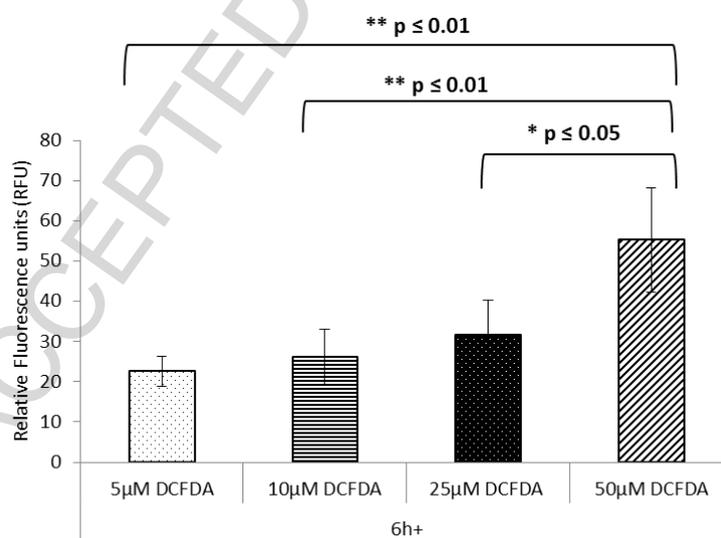

Figure 3. ROS generation by MCF-7 cells loaded with 5-50µM 2',7'- dichloroflurescin diacetate (DCFDA). Background (or basal) DCFDA fluorescence in cells that were cultured for 6h culture in phenol red-free RPMI without TBHP. Means ± SD of 3 independent experiments shown. Data analysed by one-way ANOVA with Tukey HSD post-hoc test. *p ≤ 0.05; ** p ≤ 0.01.

Reading the fluorescence every hour for 6 hours did not affect the emission signal, because there was no significant difference between the final 6th hour OD result and those generated by cells that were read only once, after continuous 6h incubation (Figure 4).

Although exposure to TBHP for 6h generated the same amount of ROS as a shorter 3h exposure (Figure 2), an extended 24h exposure to TBHP generated significantly higher levels of ROS than the shorter 6h exposure (Figure 4), even after accounting for the increase in background ROS production.

DCFDA was required to be able to detect TBHP generated ROS, because cells exposed to 0-50µM TBHP in the absence of DCFDA showed no increase in fluorescence after 6 or 24h (data not shown).
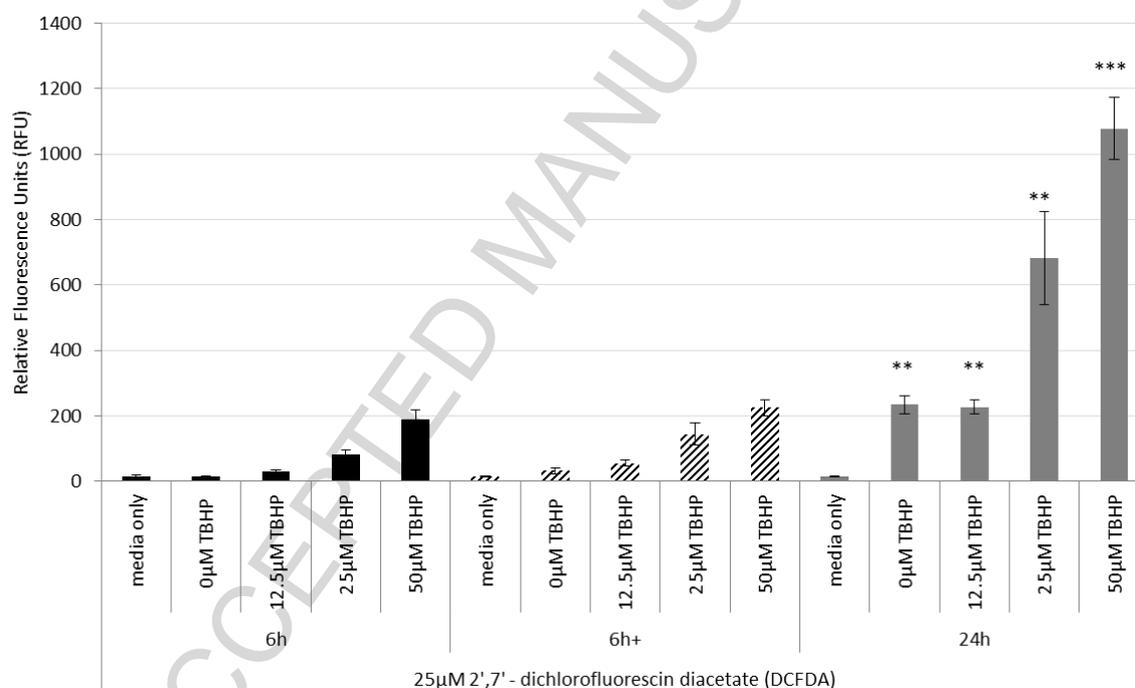

Figure 4. Effect of continuous real time monitoring on DCFDA assay. MCF-7 cells were loaded with 25µM 2',7'-dichloroflurescin diacetate (DCFDA) for 45min, then exposed to 0, 12.5, 25 or 50µM TBHP for 6 or 24h. The DCF fluorescence was read every hour for 6h, or after 6h+ or 24h+ uninterrupted in vitro cultures. Means ± SD of 3 independent experiments shown. Data analysed by three-way between factors ANOVA with Pair-wise post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$ significant difference from +6h continuous exposure at same TBHP concentration.

MCF-7 Cell viability after DCFDA and TBHP exposure

When DCFDA concentrations were 0-25µM, the addition of 0 - 25µM TBHP had no significant effect on cell viability (Figure 5) but there was a TBHP concentration-dependent

decrease in cell viability, compared to the medium control, when cells were loaded with 50µM DCFDA (Figure 5).

Exposing 20,000 MCF-7 cells to the combination of 50µM DCFDA and 50µM TBHP seemed to be more toxic after 24h (11,655 ± 6753 cells per well) than exposing the cells to 50µM TBHP alone (22,032 ± 1388 cells per well) (Figure 6); however the difference in viability was not statistically significant.

Figure 5. Effect of DCFDA and TBHP on MCF-7 Cell viability. Cells were loaded with 0-50μM 2',7'-dichloroflurescin diacetate (DCFDA) for 45min then washed and exposed to 0, 12.5, 25 and 50μM TBHP for 6 or 24h. Fluorescence was read every hour for 6h or after 6h+ or 24h+ uninterrupted in vitro culture. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were subjected to 2-way ANOVA with Tukey post-hoc test, and difference from media control from same incubation period shown. * $p \leq 0.05$.
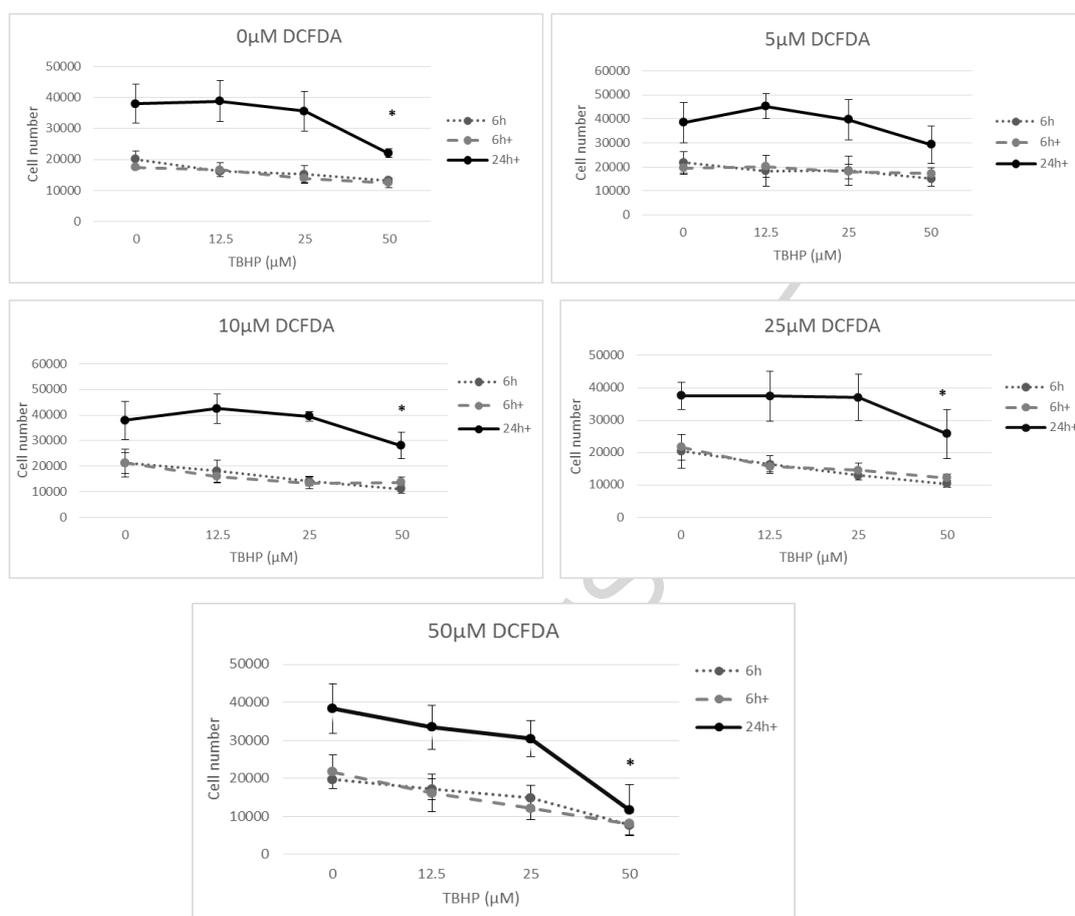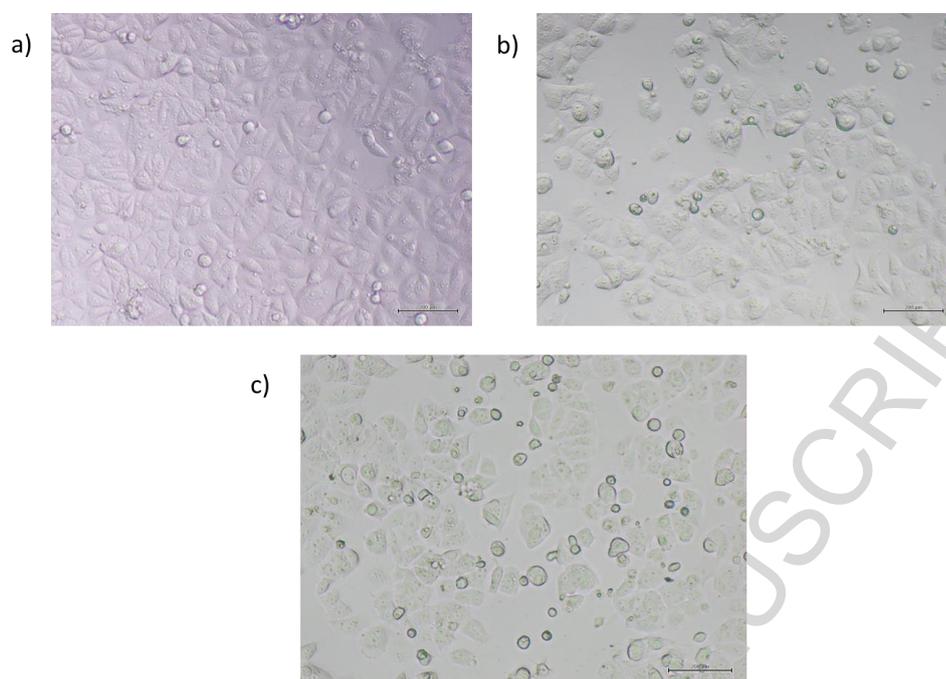
Figure 6. Effect of DCFDA and TBHP on MCF-7 Cell viability. Cells were loaded with 0-50µM 2',7'-dichloroflurescin diacetate (DCFDA) for 45min then washed and exposed to 0, 12.5, 25 and 50µM TBHP for 24h. Cell morphology was examined using an inverted microscope; digital images were taken with DP2-SAL, Olympus acquisition system of **a)** Medium only control; **b)** DCFDA- free cells exposed to 50 µM TBHP; and **c)** cells loaded with 50µM DCFDA and exposed to 50µM TBHP for 24h.

Discussion:

Reactive oxygen species are difficult to measure because they are present in low concentrations intracellularly, and they have short lifetimes (Gomes et al., 2005). The majority of published studies report measurement of the ROS detection signal at only one time-point (Chen et al., 2010) but this single snapshot is unlikely to capture the peak or nadir of ROS levels in cells, and this approach is also sensitive to assay conditions such as the DCFDA loading concentration (Wan et al., 2003; Wan et al., 2005). This is the first study to characterise ROS generation in the same cells at hourly intervals for 6h, and which additionally describes the effects of a range of concentrations of DCFDA on ROS detection and cell viability. To our knowledge, this report is the first to explore a DCFDA-based assay's potential for real time ROS detection in breast cancer cells.

Under normal physiological conditions, cancer cells generate high levels of ROS to activate different signaling pathways that are crucial for cell proliferation and survival (Benhar, Engelberg, & Levitzki, 2002; Jackson & Loeb, 2001; Storz, 2005); and also express high levels of antioxidant molecules to regulate ROS and prevent cell damage or death (Cadenas, 2004; Simon, Haj-Yehia, & Levi-Schaffer, 2000). In our study, increasing levels of ROS in MCF-7 cells were detected in the absence of TBHP, suggesting an imbalance of the intracellular Redox state that could be a consequence of insufficient antioxidant molecules in medium supplemented with 10% FCS or insufficient ROS scavenging inside the cells (Chen et al., 2010), spontaneous deacetylation of DCFDA to DCF (Royall & Ischiropoulos, 1993; Zhou, Diwu, Panchuk-Voloshina, & Haugland, 1997), artefactual signal due to light-induced oxidation (Winterbourn, 2014) or competitive inhibition of antioxidant molecules by DCFDA (Wrona, Patel, & Wardman, 2005). High fluorescence levels were also detected in a cell-free model with RPMI with or without FCS. Brubacher and Bols (2000) observed that tyrosine combined with ubiquitous metal contaminants of physiological buffers can result in high levels of oxidation, which may be wrongly interpreted as cellular ROS (Brubacher & Bols, 2001). RPMI and other common use media contain different amino-acids, including tyrosine, which could potentially influence fluorescence intensity; therefore we recommend testing the reaction of culture media with DCFDA, as part of ROS assay establishment. Basal ROS production by MCF-7 cells loaded with 5 – 50μM DCFDA (in the absence of TBHP) was detected after 6h culture, with the apparent ROS levels dependent on the loading concentration of DCFDA. DCF assay results are often expressed as the net increase of cellular fluorescence over that of the background control group (Wan et al., 2005), but subtracting the basal ROS had no effect on the DCFDA dose-dependent increase in ROS levels. Adding TBHP increased ROS generation to levels that exceeded the capacity of 5μM DCFDA to metabolise the ROS (Bass et al., 1983; Chen et al., 2010; Royall & Ischiropoulos, 1993; Wrona et al., 2005) and ROS detection after loading cells with 25μM DCFDA and stimulating with TBHP was significantly higher than with 5μM DCFDA. The higher 25μM

DCFDA concentration was not limiting after 6h culture because ROS detection values were higher after the extended 24h culture period, demonstrating the capacity of 25μM DCFDA for detecting higher ROS levels.

Two factors seemed to affect MCF-7 cell viability; the amount of ROS stimulated by TBHP and the concentration of DCFDA. 50μM DCDFA was not cytotoxic to MCF-7 cells grown for 24h, but the addition of 50μM TBHP caused an increase in ROS and a consequent decrease in cell viability. The same conditions of 24h exposure and 50μM TBHP however were less toxic to MCF-7 cells in the absence of DCFDA, suggesting that there was an interaction between DCFDA, TBHP (ROS), and the culture conditions; hence we recommend using the lower 25μM concentration of DCFDA in order to prevent non-specific ROS generation.

Real time monitoring every hour for 6h did not significantly reduce the DCFDA fluorescent signal; suggesting that for MCF-7 cells, the dose-dependent increase in fluorescent signal was not compromised by exposing the cells every hour to the detecting light beam of the fluorescence plate reader; validating our proposal that the DCFDA assay can be used to measure ongoing ROS production in real time. This is important because reactive oxygen species are highly reactive and extremely short-lived molecules, which can be generated within seconds of stimulation. ROS have also the tendency toward chain reaction and generation of other RO molecules (Maneesh & Jayalekshmi, 2006), triggering an amplified cascade of intracellular events and activation of different pathways (Liou & Storz, 2010). Therefore, it is possible that shorter incubation periods could be applicable to the detection of intracellular ROS, with a fluorescent signal that extends over the hours as more detectable ROS are generated within the cells. It's important to highlight that because of the non-specific oxidation of DCFDA and the multiple pathways that can lead to DCF fluorescence, this assay must be utilised as a qualitative marker for total ROS production, rather than a precise indictor of any specific kind of ROS (Tarpey & Fridovich, 2001).

ROS detection is dependent on the concentration of DCFDA that maximises assay sensitivity and ROS detection, whilst minimising dye-induced cytotoxicity. In this study that balance lay between 10- 25µM DCFDA combined with a loading exposure of 45min. Cells loaded with 10-25µM DCFDA and exposed to 0-50µM TBHP yielded a more precise estimation of ROS, with a significant and dose-dependent increase in fluorescence signal in all three independent experiments. We conclude that MCF-7 cells can be loaded with 10-25µM DCFDA for 45min, and that a 6h exposure to 0-50µM TBHP is sufficient to generate a sensitive and reproducible standard curve. The finding that the DCF fluorescent signal can be measured repeatedly in the same cells could potentially increase the utility of the assay for future research applications.

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