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# What does the commonly used DCF-test for oxidative stress really show?

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## SYNOPSIS

Dihydrodichlorofluorescein ($H_2$DCF-DA) is widely used to evaluate "cellular oxidative stress". After passing through the plasma membrane, this lipophilic and non-fluorescent compound is de-esterified to a hydrophilic alcohol ($H_2$DCF) that may be oxidized to fluorescent DCF by a process usually considered to involve reactive oxygen species (ROS). It is, however, not always recognized that, being a hydrophilic molecule, $H_2$DCF does not pass membranes, except for the outer, fenestrated mitochondrial ones. It is also not generally realized that oxidation of $H_2$DCF is dependent either on Fenton-type reactions or on unspecific enzymatic oxidation by cytochrome *c*, for neither superoxide, nor hydrogen peroxide, directly oxidizes $H_2$DCF. Consequently, oxidation of $H_2$DCF requires the presence of either cytochrome *c* or of both redox-active transition metals and hydrogen peroxide. Redox-active metals exist mainly within lysosomes, while cytochrome *c* resides bound to the outer side of the inner mitochondrial membrane. Following exposure to $H_2$DCF-DA, weak mitochondrial fluorescence was found in both the oxidation-resistant ARPE-19 cells and the much more sensitive J774 cells. This fluorescence was only marginally enhanced following short exposure to hydrogen peroxide, showing it by itself being unable to oxidize $H_2$DCF. Cells that were either exposed to the lysosomotropic detergent MSDH, exposed to prolonged oxidative stress, or spontaneously apoptotic showed lysosomal permeabilization and strong DCF-induced fluorescence. The results suggest that DCF-dependent fluorescence largely reflects relocation to the cytosol of lysosomal iron and/or mitochondrial cytochrome *c*.

**SHORT TITLE:** DCF and oxidative stress

**KEYWORDS:** DCF, lysosomes, oxidative stress, ROS, transition metals.

## ABBREVIATIONS

AMD, age-related macular degeneration; AO, acridine orange; CP22, 1-propyl-2-methyl-3-hydroxypyrid-4-one; DCF, dichlorofluorescein; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; FAC, ferric ammonium citrate; FBS, fetal bovine serum; $H_2$DCF, dihydrodichlorofluorescein; $H_2$DCF-DA, dihydrodichlorofluorescein diacetate; $H_2O_2$, hydrogen peroxide; HBSS, Hank's buffered salt solution; LMP, lysosomal membrane permeabilization; MMP, mitochondrial membrane permeabilization; MSDH, *O*-methyl-serine dodecylamide hydrochloride; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester.

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## INTRODUCTION

The molecular mechanisms behind age-related macular degeneration (AMD) are much debated but far from understood, even though it is the most common cause of visual impairment among the elderly in the western world [1-3]. However, based on recent evidence, oxidative stress within retinal pigment epithelial (RPE) cells, with the ensuing accumulation of lysosomal lipofuscin and thereby depressed phagocytosis/autophagocytosis, is considered a major etiological factor behind AMD [4-8]. Consequently, in relation to various experimental studies on cultures of RPE cells, the extent of cellular oxidative stress is often being evaluated.

A method that is frequently relied upon for this purpose is the DCF-test [9-11]. Dihydrodichlorofluorescein diacetate ($H_2$DCF-DA) is then added to cells in culture and the intracellular oxidation of $H_2$DCF to DCF documented over time. $H_2$DCF-DA is a non-fluorescent lipophilic ester that easily crosses the plasma membrane and passes into the cytosol, where it is rapidly cleaved by unspecific esterases [12]. One of the reaction products is the non-fluorescent alcohol $H_2$DCF. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence when excited with blue light. The brightness of this fluorescence is usually considered to reflect the extent to which 'reactive oxygen species' (ROS) are present, unfortunately without any further definition of what kind of ROS may give rise to the oxidation [13, 14]. Furthermore, it is often wrongly assumed that $H_2$DCF is evenly distributed in the cell following the intracellular cleavage of the added diacetate ester, neglecting the inability of hydrophilic molecules to traverse membranes. Moreover, the evaluation of the DCF-test commonly involves plate readers or flow cytofluorometers, which do not allow any careful morphological analysis of individual cells that, if undertaken, might have disclosed unexpected cellular variations. Thus, the DCF-test often seems to be somewhat uncritically used.

We previously showed that oxidation of $H_2$DCF to DCF is not a result of exposure to reactive oxygen species (ROS) in general, but rather indicates the specific impact of hydroxyl radicals formed during Fenton-type reactions [15]. Here we also stress that cytochrome *c,* as has been previously pointed out [16-18], operates as an unspecific peroxidase with $H_2$DCF as a target. It is, therefore, plausible to suppose that lysosomal membrane permeabilization (LMP), with release to the cytosol of redox-active iron, and/or mitochondrial release of cytochrome *c* is required for the induction of strong cytosolic DCF-mediated fluorescence. We also point out that in the absence of LMP and apoptosis/necrosis, $H_2$DCF only occurs in the cytosol and the mitochondrial inter-membranous space, where it gives rise to weak cytosolic and somewhat stronger mitochondrial fluorescence that indicates oxidation. Most probably, this faint fluorescence, that regularly seems to be considered background and to be over-looked, results from the normal mitochondrial production of hydrogen peroxide that diffuses all over the cell, the presence of cytochrome *c* in the mitochondrial inter-membranous space as well as of minute amounts of labile iron under transport in the cytosol. Consequently, if lysosomal membrane permeabilization (LMP) and related mitochondrial damage with relocation of cytochrome *c* has taken place, a positive DCF-test might be interpreted as a sign of oxidative stress even if that would not necessarily be the case. Similarly, a low or undetectable level of DCF fluorescence may not necessarily indicate the absence of ROS but rather designate stable lysosomal and mitochondrial compartments.

## MATERIALS AND METHODS

### Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 medium, fetal bovine serum (FBS), penicillin and streptomycin were from Invitrogen (Paisley, UK). Acridine orange base (AO) was from Gurr (Poole, UK), 1-propyl-2-methyl-3-hydroxypyrid-4-one (CP22) and *O*-methyl-serine dodecylamide hydrochloride (MSDH) were kind gifts from Prof. Robert Hider, University of

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London, (UK) and Dr. Gene N. Dubowchik (Wallingford, CT, USA), respectively. All other reagents were from Sigma (St. Louis, MO, USA).

**Cells and culture conditions**

ARPE-19 (human immortalized retinal pigment epithelial) cells and murine J774 macrophage-like histiocytic lymphoma cells (both obtained from ATCC, Manassas, VA, USA) were grown at 37°C in humidified air with 5% $CO_2$ in DMEM and F12 (1:1) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were sub-cultivated twice a week, seeded in 6-well plates with or without cover slips at a concentration of $5x10^5$ cells/well (J774), while the much larger ARPE-19 cells were seeded at $2x10^5$ cells/well with or without cover slips. Cells were subjected to experiments 12, 24 and 48 h following subcultivation.

**Conditions for basic studies with $H_2$DCF-DA only**

Small amounts of stock solutions, containing 1 to 10 mM $H_2$DCF-DA in DMSO, were added to fresh complete growth medium. The carefully blended solution was added to the culture dishes following removal of the old medium. Cells on cover slips were in that way exposed to 3-30 µM $H_2$DCF-DA in medium with 0.03-3% DMSO for 30 min at otherwise standard culture conditions. The cells were then rinsed in HBSS, directly mounted in HBSS, and within 5-6 min subjected to laser scanning confocal microscopy using a Nikon Eclipse C1 laser scanning confocal microscope (Tokyo, Japan).

Following tests with varying concentrations of $H_2$DCF-DA and DMSO, a stock solution of 10 mM $H_2$DCF-DA in DMSO was selected for both cell types and added to medium in such a way that the final concentrations of $H_2$DCF-DA and DMSO was 10 µM and 0.1%, respectively. The cells were observed and photographed using the above confocal microscope, r and the Nikon EZ-C1 V3.70 software for image acquisition. DCF fluorescence was detected using a 515/30 nm band pass filter. Since the DCF fluorescence of control cells is rather weak, the medium size pinhole and an electronic gain of 6.5 were applied. All photographs were taken at a resolution of 2 Mpixels. To allow comparison between experiments, these settings were then kept the same for all experiments involving evaluation of DCF-induced fluorescence, even when the intensity of fluorescence was much enhanced due to the conditions being applied and, consequently, a small pinhole and less amplification would have given better resolution and prevented over-exposure of strongly fluorescent cells.

**Localisation of mitochondria using the mitochondria-specific dye TMRE**

Mitochondria were demonstrated using the cationic and lipophilic dye tetramethylrhodamine ethyl ester (TMRE), which accumulates in the matrix of normal mitochondria. Cells were incubated with TMRE in complete culture medium (100 nM; 15 min; 37°C) and observed using the above-mentioned Nikon confocal microscope. Since mitochondrial TMRE fluorescence is strong, optimal documentation conditions were used (the smallest pinhole and a low gain).

Overlays of DCF/TMRE pictures could not be produced because TMRE is a metachromatic fluorophore that provides both red and green fluorescence when activated by a 488 nm laser and the green one is much stronger than that of DCF in control cells. TMRE fluorescence was detected using a 590/50 nm band pass filter.

**Studies aiming at varying cytosolic labile iron concentrations before exposure to $H_2$DCF-DA**

To evaluate the influence on DCF-induced fluorescence of different concentrations of labile iron in the cytosol, as well as in the mitochondrial inter-membranous space, cells were initially exposed to $H_2$DCF-DA as above and then mounted in HBSS with 500 µM ferric ammonium citrate (FAC) to enhance labile iron. Separately, cells were incubated with $H_2$DCF-DA together with 100 µM of the iron chelator 1-propyl-2-methyl-3-hydroxypyrid-4-one (CP22) to ligate labile iron and then mounted

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in HBSS in the continuous presence of CP22. CP22 (MW 203.7) is an effective, water-soluble bidentate iron chelator with a complex constant of ~$10^{36}$ [19]. When complexed with CP22, iron is prevented from redox-cycling and unable to support Fenton-type reactions.

**Studies aiming at evaluating DCF-fluorescence in relation to oxidative stress**

*1. Exposure to hydrogen peroxide.* In order to induce oxidative stress, ARPE-19 and J774 cells were initially exposed to $H_2$DCF-DA as above and then mounted in HBSS with 500 µM $H_2O_2$. To induce apoptosis of the ARPE-19 (very resistant to oxidative stress) and J774 (sensitive) cells, they were exposed to 15 mM and 100 µM $H_2O_2$ in HBSS, respectively, for 30 min and then kept at standard culture conditions for about 6 h when they were incubated with $H_2$DCF-DA and studied as described above. In order to further emphasize the differences in lysosomal membrane stability between cell types, both of them were exposed to 100 µM $H_2O_2$ in HBSS for 30 min and then kept at standard culture conditions for about 6 h, when they were exposed to the lysosomotropic, metachromatic fluorochrome acridine orange (AO) as described before [20-24]. A 488 nm argon laser was used for excitation and AO fluorescence was detected using 515/30 nm (green) and 590/50 nm (red) band pass filters.

*2. Exposure to a lysosomotropic detergent.* In order to induce lysosomal rupture by other means than oxidative stress, ARPE-19 cells were initially exposed to $H_2$DCF-DA as above. They were then mounted in HBSS with 100 µM of the lysosomotropic detergent *O*-methyl-serine dodecylamide hydrochloride (MSDH), which is known to induce lysosomal labilization [25], before being studied over a 15 min period of time. In separate experiments, designed to verify the LMP-effect of MSDH, cells were initially exposed to AO as above and then mounted in HBSS with MSDH and followed over time.

**Evaluation of DCF-induced fluorescence in apoptotic ARPE-19 cells**

Apoptotic and post-apoptotic necrotic cells are always present in cell cultures, especially shortly after subcultivation, because a number of cells do not survive that procedure. Such apoptotic ARPE-19 cells were identified, 12 h following seeding, based on their characteristic morphology that includes chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and formation of apoptotic bodies. DCF-induced fluorescence was evaluated following exposure to $H_2$DCF-DA as described above.

**Assessment of the capacity of cytochrome *c* to oxidize $H_2$DCF**

Using a modification [15] of a technique described by Myhre et al. [26], $H_2$DCF was exposed to cytochrome *c* +/- the small iron chelator CP22 (at 1 and 20 µM, respectively). Briefly, a standard consisting of ferric iron (10 µM) was reduced to its ferrous form by cysteine (100 µM) in a HEPES buffer (pH 7.0; 150 mM). Hydrogen peroxide (100 µM) was added to initiate oxidative conversion of non-fluorescent $H_2$DCF (5 µM) to fluorescent DCF [$H_2$DCF was obtained by hydrolyzing its diacetate ester ($H_2$DCF-DA)]. DMSO (10%) and CP22 (20 µM) were used to demonstrate the involvement of $HO^{\bullet}$ and iron, respectively.

The capacity of cytochrome *c* to oxidize $H_2$DCF was then compared to that of the Fe(II) standard. Fluorescence was measured in a FL600 Microplate Fluorescence Reader (Bio-Tek, Winooski, VT, USA) at $\lambda_{ex}$ 485 nm and $\lambda_{em}$ 530 nm.

**Statistics**

DCF-induced fluorescence intensity was measured using the NIH ImageJ software v. 1.42q. Following delineation of each cell, their mean fluorescence was calculated and analysis performed using Student's t-test.

**RESULTS**

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**Normal cells show a weak cytosolic and a somewhat stronger mitochondrial-type DCF-fluorescence pattern, while apoptotic cells demonstrate strong diffuse fluorescence**

As mentioned in the Methods section, initial experiments indicated that a final concentration of 10 μM $H_2$DCF-DA in complete culture medium, obtained by using a stock solution of 10 mM $H_2$DCF-DA in DMSO, gave acceptable results and allowed reasonable documentation of both normal and apoptotic ARPE-19 and J774 cells using the same confocal settings. It should be pointed out that the fluorescence obtained from normal cells was low (particularly in J774 cells). In studies using flow- or plate-reader fluorometry, it would have been considered non-specific background. The final concentration of DMSO did not influence the results as long as it was below 1%. Normal ARPE-19 and stretched-out J774 cells showed a mitochondrial DCF-induced weak fluorescence in combination with a very faint cytosolic fluorescence (Figures 1A and 2A), while apoptotic ARPE-19 and J774 cells presented strong DCF-induced fluorescence that was 6-14 times higher than that of the control cells (Figures 1C, 2B and 2D) whether the apoptosis was spontaneous (ARPE-19 cells; Figure 1C) or due to exposure to oxidative stress (Figure 2, B and D). It needs to be emphasized that in opposition to the ARPE-19 cells that adhere firmly to their substratum, the J774 cells tend to round up and easily detach, especially when becoming apoptotic. Therefore, the rinsing steps that are needed for the $H_2$DCF-DA-exposure makes it very difficult to find more than occasional apoptotic cells in the mounted cultures that are studied in the confocal microscope (see further legends to Figure 2).

The initially weak mitochondrial-type DCF fluorescence pattern from normal cells became slightly stronger after a few seconds of exposure to blue light, which is considered an oxidative effect and probably due to formation of singlet oxygen. Due to the low intensity of fluorescence, the smallest pinhole and a low electronic gain, normally chosen in order to obtain the sharpest possible confocal microscopy pictures, could not be used. Nevertheless, it was clear that the DCF-mediated fluorescence of non-apoptotic cells originated in organelles that morphologically looked like mitochondria. Using the mitochondria-specific stain TMRE on cells not exposed to $H_2$DCF-DA, we found an identical pattern of fluorescence, proving that, apart from the weak cytosolic fluorescence, the DCF-induced fluorescence of normal cells originates in mitochondria (Figure 1B).

Both cell types showed a slight, but significant, increase in DCF-mediated fluorescence intensity when mounted in 500 μM hydrogen peroxide shortly before microscopy, suggesting the presence of small amounts of labile iron in the cytosol (Figure 1D and 2C).

**In normal cells, DCF-induced fluorescence is a function of cytosolic Fenton-type reactions and mitochondrial enzymatic oxidation by cytochrome *c***

As already mentioned, the main portion of the DCF-induced fluorescence of normal cells of both types showed a distinct mitochondrial pattern, although better visualized in the thin and flat ARPE-19 cells than in the thicker and more rounded up J774 cells (Figures 1A and 2A). This was confirmed by the identical DCF- and TMRE-induced fluorescences (although overlay pictures could not be produced because of the green TMRE-induced fluorescence following excitation with blue light – TMRE is a metachromatic fluorophore). Since hydrophilic molecules do not penetrate cellular membranes, it seems safe to assume that the DCF-induced fluorescence originates in the mitochondrial intermembranous space where cytochrome *c* is present, together with traces of low mass labile iron compounds under transport to the mitochondrial matrix.

Following exposure to FAC, the fluorescence from both compartments was substantially enhanced (Figure 3A), indicating the presence of $H_2$DCF all over the cytosol as well as in the mitochondrial intermembranous space. This finding shows that oxidation of $H_2$DCF requires the presence of low mass iron in redox-active form, which was confirmed by the observation that cells

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exposed to the small iron chelator CP22 presented a clear depression of both cytosolic and mitochondrial DCF-induced fluorescence (Figure 3B).

To demonstrate the capacity of cytochrome *c* to catalyze oxidation of $H_2$DCF to DCF in the presence of hydrogen peroxide, we applied the *in vitro* DCF test as it was described before [15]. As shown in Figure 4 (A and B), cytochrome c, as well as hydroxyl radicals, readily induce such oxidation, which is in accordance with previous findings of other groups [16-18].

**Strong cytosolic DCF-induced fluorescence is a function of LMP-dependent relocation to the cytosol of redox-active iron, as well as of release of cytochrome *c* following MMP**

As pointed out before, short exposure to hydrogen peroxide (100 µM) is insufficient to induce LMP, as assayed by the AO-uptake method, in the very oxidative-stress-resistant ARPE-19 cells [21]. The J774 cells are much more sensitive and apoptosis can easily be initiated by exposure for 30 min to 100 µM hydrogen peroxide (Figure 5). However, no immediate LMP occurs, not even if cells are exposed to higher concentrations, because peroxidation and ensuing fragmentation of the lysosomal membranes require some time [20]. We hypothesized, based on the results following the addition of FAC to the cells (see above), that LMP might induce DCF fluorescence by allowing lysosomal transition metals (mainly Fe) to relocate to the cytosol and meet $H_2$DCF and catalyze its oxidation. LMP also results in MMP with release to the cytosol of cytochrome *c* with ensuing enzymatic oxidation of $H_2$DCF (see further below).

Following exposure of cells to the metachromatic and lysosomotropic chromophore acridine orange (AO), normal ARPE-19 and J774 cells show a large number of distinct and bright red lysosomes when activated with blue light, while the cytosol simultaneously shows moderate green fluorescence (Figures 5 A and C).

As pointed out above, many J774 cells that were exposed to 100 µM hydrogen peroxide started to show signs of early apoptosis (nuclear pycnosis and plasma membrane blebbing). They also showed a reduced number of lysosomes and enhanced cytosolic green fluorescence following exposure to AO (Figure 5D). Meanwhile the ARPE-19 cells, which are resistant to oxidative stress, remained normal when similarly treated (Figure 5B). As mentioned earlier, apoptotic and necrotic cells show a reduced number of intact lysosomes [21, 22, 27].

**LMP induced without oxidative stress also causes strong diffuse DCF-induced fluorescence**

The above-described results, obtained by exposing the cells to hydrogen peroxide (see also Figures 1D and 2C), suggested that oxidative stress *per se* does not necessarily give rise to more than a slight and almost insignificant increase in DCF-induced fluorescence. In order to further substantiate that hypothesis, we wanted to induce LMP by other means than oxidative stress. As shown before, lysosomotropic detergents induce LMP [25, 28]. By exposing ARPE-19 cells to MSDH at 100 µM and then comparing the resulting time-dependent increase in DCF-fluorescence intensity with the decreased number of intact lysosomes, as visualized with the AO-uptake test, it became clear that LMP and enhanced DCF-induced fluorescence were strongly linked (Figure 6, A and B). This finding confirms that a high DCF-induced fluorescence is not necessarily a consequence of oxidative stress but rather a function of relocation to the cytosol of lysosomal redox-active iron and mitochondrial cytochrome *c*. However, it should be kept in mind that lysosomal rupture, with release to the cytosol not only of low mass iron but also of cathepsins, certainly secondarily induces enhanced production of superoxide and hydrogen peroxide from damaged mitochondria.

## DISCUSSION

The findings of this study show that significant cytosolic oxidation of $H_2$DCF to DCF depends on the combined effect of Fenton-type reactions and enzymatic activity of cytochrome *c*.

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Consequently, it should not be considered a result of the presence of unspecified 'ROS'. Rather, the reaction is apparently dependent on the relocation of transition metals in redox-active form from lysosomes and/or of cytochrome *c* from mitochondria. Moreover, because $H_2$DCF is a hydrophilic alcohol, it would not under normal conditions be expected to traverse cellular membranes, apart from the fenestrated mitochondrial outer membrane, which is known to allow molecules up to 5,000 Da to pass [29].

The observed mitochondrial fluorescence from normal cells, albeit low, was also observed by at least one other group of investigators [30]. It reflects the combined presence of redox-active iron, hydrogen peroxide and enzymatically active cytochrome *c* in the intermembranous space. Heme-proteins, such as cytochrome *c* are iron-containing proteins that act as pseudoperoxidases due to their content of compound I, which consists of a ferryl Fe(IV) species and a porphyrin radical state of heme [16]. Since the water soluble, low molecular weight iron chelator CP22 was found to somewhat depress both the weak mitochondrial as well as the even weaker cytosolic fluorescence of normal cells, it is likely that some low mass iron in labile form is responsible for the formation of DCF in the cytosol of normal cells and, partly, in the mitochondria. Most probably, such iron is under transport from lysosomes, where it has been liberated following degradation of autophagocytosed ferruginous macromolecules [31], for storage in ferritin or for synthesis of iron-containing macromolecules. As is well known, many metabolic synthetic steps that involve iron take place both in the cytosol and, above all, in the mitochondrial matrix.

When cells were initially exposed to FAC, the cytosolic and mitochondrial DCF-induced fluorescence was clearly enhanced. This observation indicates that following exposure to $H_2$DCF-DA, the cleaved reaction product $H_2$DCF is evenly distributed in the cytosol and also enters the mitochondrial intermembranous space. The finding that the cytosolic fluorescence of cells not exposed to FAC is weak, even after a short exposure to hydrogen peroxide, suggests that the amount of labile cytosolic iron is normally minute and that iron under transport from the lysosomal compartment may well be carried in a non-redox-active form [22].

Only when $H_2$DCF interacts with significant amounts of redox-active low mass transition metals or with cytochrome *c* may we expect a more pronounced general cellular DCF-induced fluorescence. It is now well established that the only cellular organelle that contains such metals (mainly iron) in significant amounts, is the lysosomal compartment [22, 23, 31]. We have previously shown, using the calcein method for detection of 'labile iron', that when lysosomes rupture, low mass iron is relocated to the cytosol [32]. Thus it is reasonable to assume that LMP, rather than oxidative stress *per se,* would be the major mechanism behind any obvious DCF-induced fluorescence. Therefore, it was no surprise to find that the exposure of cells to the lysosomotropic detergent MSDH in amounts that induce LMP and ensuing apoptosis/necrosis initiated strong DCF-induced fluorescence. Under normal conditions, hydroxyl radicals are regularly produced intralysosomally because the hydrogen peroxide that is normally present in small amounts in the cell permeates lysosomes and initiates Fenton-type reactions. These radicals are, however, not detected by the DCF-method because $H_2$DCF is a hydrophilic, non-permeating molecule. Following LMP and ensuing relocation of lysosomal redox-active iron to the cytosol, the very same amount of iron now gives rise to DCF-fluorescence since occurred LMP allows $H_2$DCF, redox-active iron, and hydrogen peroxide to meet. Consequently, at the very same degree of 'oxidative stress' it is the stability of the lysosomal membranes that influences the outcome of the DCF-test. Importantly, when the test is positive no higher 'oxidative stress' necessarily needs to be present than when it is negative.

Importantly, oxidative stress in the form of a brief exposure to hydrogen peroxide only slightly changed the fluorescence of both ARPE-19 cells, which are highly resistant to oxidative stress, and J774 cells that are much more sensitive. The J774 cells, however, following a 30 min period of oxidative stress and a several hours long period at standard culture conditions, showed both LMP and a strong DCF-induced fluorescence. This shows that hydrogen peroxide by itself does not

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induce oxidation of $H_2$DCF, unless small amounts of labile iron are present, but also that peroxidation/fragmentation of lysosomal membranes following oxidative stress is a time-dependent process [20]. Since both the studied cell types degrade hydrogen peroxide to a similar degree [21], it is reasonable to assume that their large differences in lysosomal stability against oxidative stress reflect much different intralysosomal concentrations of redox-active transition metals.

In the literature, we find many examples of the DCF-test being used to demonstrate 'oxidative stress' when in fact it most probably reflects LMP. A typical example is [33], where it is shown (Figure 1 of the paper) that transgenic islet cells overexpressing metallothioneins are significantly "less sensitive to oxidative stress" than control cells. Importantly, in this paper the authors found that DCF-induced fluorescence engendered in normal cells following the addition of $H_2O_2$ was nearly absent in cells overexpressing metallothionein, despite the fact that the same concentration of peroxide was present. We previously showed that overexpression of metallothioneins or heat shock protein 70 (HSP70) gives rise to increased autophagy of these proteins that, in turn, creates a temporary reduction of intralysosomal redox-active iron that lasts as long as the influx of metallothioneins persists. That makes lysosomes less prone to undergo LMP following exposure to various forms of oxidative stress [15, 34].

Another example is [35] where it is shown that radiation of cultured human prostatic cancer cells in combination with exposure to hydrogen peroxide strongly enhances formation of ROS, as evaluated with the $H_2$DCF-DA method. Pretreatment with ammonium chloride abolished the formation of DCF fluorescence, although cells were then exposed to the same concentration of hydrogen peroxide. Moreover, it was demonstrated that radiation plus hydrogen peroxide ruptures most lysosomes, while they were preserved in cells pre-treated with ammonium chloride. The results strongly suggest that the appearance of cytosolic DCF-induced fluorescence requires lysosomal rupture with relocation of redox-active iron that in turn mediates oxidation of $H_2$DCF to DCF. Such oxidation may very well take place without any enhanced oxidative stress at all, but rather rely on normal cellular concentrations of hydrogen peroxide for the necessary formation of hydroxyl radicals.

A clear understanding of the factors that determine DCF-induced fluorescence is required in order to appreciate what DCF-induced fluorescence really means. Presently, and based on the results of this study, we suggest that it should be considered to reflect LMP and MMP with ensuing relocation to the cytosol of redox-active iron and cytochrome *c* rather than to be the result of some incompletely defined 'ROS or oxidative stress'.

In summary, strong DCF-fluorescence seems to require the simultaneous presence in the cytosol of either $H_2$DCF, hydrogen peroxide and Fe(II) or of $H_2$DCF and cytochrome *c*. Relocation to the cytosol of Fe(II) or cytochrome *c*, in turn, requires LMP or detachment of cytochrome *c* from the outside of the inner mitochondrial membrane. In apoptosis LMP is often an upstream event that results in MMP with release of cytochrome c [22, 31]. Consequently, induction of DCF-fluorescence may indicate LMP and/or MMP, stemming from whatever cause and not just from oxidative stress.

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## LEGENDS FOR FIGURES

**Figure 1, A - E. Exposure to $H_2$DCF-DA induces weak mitochondrial and hardly noticeable cytosolic DCF-induced fluorescence in control ARPE-19 cells that only slightly changes following exposure to hydrogen peroxide, while spontaneously apoptotic cells show strong cytosolic fluorescence.**

(**A**) ARPE-19 cells were exposed to $H_2$DCF-DA (10 µM; 30 min) in complete medium 48 h after seeding, rapidly rinsed and mounted in HBSS for confocal laser scanning microscopy that was initiated within 5-6 minutes. Note the mitochondrial-type green fluorescence pattern, which was confirmed (**B**) by the appearance of an identical red fluorescence following exposure to the mitochondria-specific fluorochrome TMRE. Figure (**C**) depicts a few spontaneously apoptotic cells known to contain ruptured lysosomes (membrane blebbing typical for apoptosis are arrowed) from a culture early after subcultivation when apoptotic cells are common, while (**D**) shows cells a few min after being mounted in 500 µM hydrogen peroxide in HBSS, but otherwise treated as in (**A**). Note the small difference in fluorescence intensities between (**A**) and (**D**). In Panel (**E**), mean fluorescence intensity values are given for (**A**), (**C**) and (**D**). Note the almost 9-fold increase in DCF-fluorescence in apoptotic cells as compared to normal ones, whereas the increase in fluorescence of cells exposed to hydrogen peroxide is marginal.

The medium sized pinhole and a gain of 6.5 was used for (**A**, **C** and **D**), which caused considerable over-exposure of the apoptotic cells in (**C**), while for (**B**) the small pinhole was applied in combination with gain 5.0. Due to the disturbing green fluorescence from the metachromatic fluorophore TMRE, it was not possible to construct overlays.

**Figure 2, A - E. Normal J774 cells show DCF-induced fluorescence similar to that of the ARPE-19 cells, while oxidative-stress-induced apoptotic cells display strong cytosolic fluorescence.**

As for Figure 1, cells were exposed to $H_2$DCF-DA (10 µM; 30 min). (**A**). Normal J774 cells. (**B**). J774 cells 6 h after a 30 min long exposure to initially 100 µM hydrogen peroxide. Due to the unavoidable loss of the rounded-up apoptotic cells during the rinsing processes, few such cells remain. They are also in another plane and barely attached. Therefore, (**B)** is an overlay from two slightly different focal planes (the apoptotic cell is arrowed). (**C**) shows J774 cells mounted in HBSS with 100 µM hydrogen peroxide (compare with 1D). Note that exposure to hydrogen peroxide only causes a minor increase of fluorescence intensity. (**D**). To induce oxidative stress-dependent apoptosis in ARPE-19 cells they had to be exposed for 30 min to 15 mM hydrogen peroxide. Following an additional 6 h period at standard culture conditions, they showed a number of apoptotic cells with strong, diffuse DCF-induced fluorescence. Mean cellular fluorescence values are displayed in Panel (E). Conditions for confocal microscopy were as for Figure 1A.

**Figure 3, A - C. Exposure to low mass iron somewhat enhances the cytosolic as well as the mitochondrial DCF-induced fluorescence of ARPE-19 cells, while exposure to an iron-chelator has the opposite effect.**

Cells were prepared as described for Figure 1A, except that they were mounted in HBSS with 500 µM ferric ammonium citrate (**A**) or pre-incubated for 30 min (together with $H_2$DCF-DA) with 100 µM of the potent, water-soluble iron-chelator CP22 and then mounted in HBSS in the continuous presence of CP22 (**B**). Cells were studied and documented under the confocal microscope within 5-6 min. Compared to control ARPE-19 cells (Figure 1A), exposure to low mass iron enhanced the fluorescence, while exposure to the iron-chelator depressed it. Mean cellular fluorescence values are displayed in Panel (**C**). Conditions for confocal microscopy were as for Figure 1A.

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**Figure 4, A and B**. **Cytochrome *c* oxidizes $H_2$DCF in the presence of hydrogen peroxide.**
(**A**)**.** Cytochrome *c* (upper line), acts as a potent unspecific peroxidase that is not significantly inhibited by CP22-mediated iron-chelation (results not shown). $H_2O_2$ does not by itself significantly oxidize $H_2$DCF (results not shown). The hydroxyl radical-dependent $H_2$DCF oxidation (Fenton reaction – middle line), is preventable by CP22-mediated iron-chelation (lower line).
(**B**). Normalization to the cytochrome *c* values of Δ (10 min) DCF fluorescence. Mean +/- STD (n=3). If error bars are not visible (as in the middle line for **A**), they are smaller than the symbol. Importantly, the magnitudes of the enzymatic cytochrome *c* reaction and the Fenton-reaction cannot be directly compared because the reducing agent cysteine, needed to reduce Fe(III) to Fe(II) for the Fenton reaction, is also an effective acceptor of hydroxyl radicals.

**Figure 5, A - D. ARPE-19 and J774 cell lysosomes are differently sensitive to oxidative stress**.
The two cell-types were exposed to initially 100 μM hydrogen peroxide for 30 min (during the incubation a major part of the hydrogen peroxide was degraded by the cells), followed by another 6 h at standard culture conditions, and then exposed to AO as described in the Methods section. Control ARPE-19 and J774 cells (not exposed to hydrogen peroxide) are shown in (**A**) and (**C**), respectively. The ARPE-19 cells remained unaffected with intact lysosomes (**B**), while the J774 cells largely rounded up and often detached (compare with Figure 2B). Remaining J774 cells often showed decreased numbers of intact lysosomes in parallel with increased cytosolic and nuclear green fluorescence (due to the relocation from lysosomes to the cytosol of the metachromatic fluorophore AO), although yet no apoptotic morphology (arrow-heads) (**D**). A few still not detached apoptotic cells showed nuclear fragmentation (long arrow), while occasional cells had phagocytosed apoptotic cells or fragments (short arrow). The confocal laser scanning microscopy settings were the small pinhole and gains 5.0 (red detector) and 5.6 (green detector).

**Figure 6, A - C. Lysosomal rupture induced without oxidative stress greatly enhances cytosolic DCF-mediated fluorescence in ARPE-19 cells.**
(**A**). Cells were prepared as described for Figure 1A, except that they were mounted in HBSS with 100 μM of the lysosomotropic detergent MSDH. Note increasing green DCF-induced fluorescence over a 7 min period [0 (Start), 3, 5 and 7 min]. (**B**). Cells were pre-loaded with AO (as for Figure 5) and then mounted in HBSS with MSDH. When followed over time the number of intact lysosomes with distinct red fluorescence diminished, while the cytosolic green-yellowish fluorescence from relocated AO increased. Panel (**C**) shows mean cellular fluorescence values of cells in (**A**). The results suggest that relocation of redox-active iron and cytochrome *c* from lysosomes and mitochondria, respectively, gives rise to strong DCF-induced fluorescence. The confocal laser scanning microscopy settings were for (**A**) the medium sized pinhole and a gain of 6.5 and for (**B**) the small pinhole and gains 5.0 (red detector) and 5.6 (green detector).

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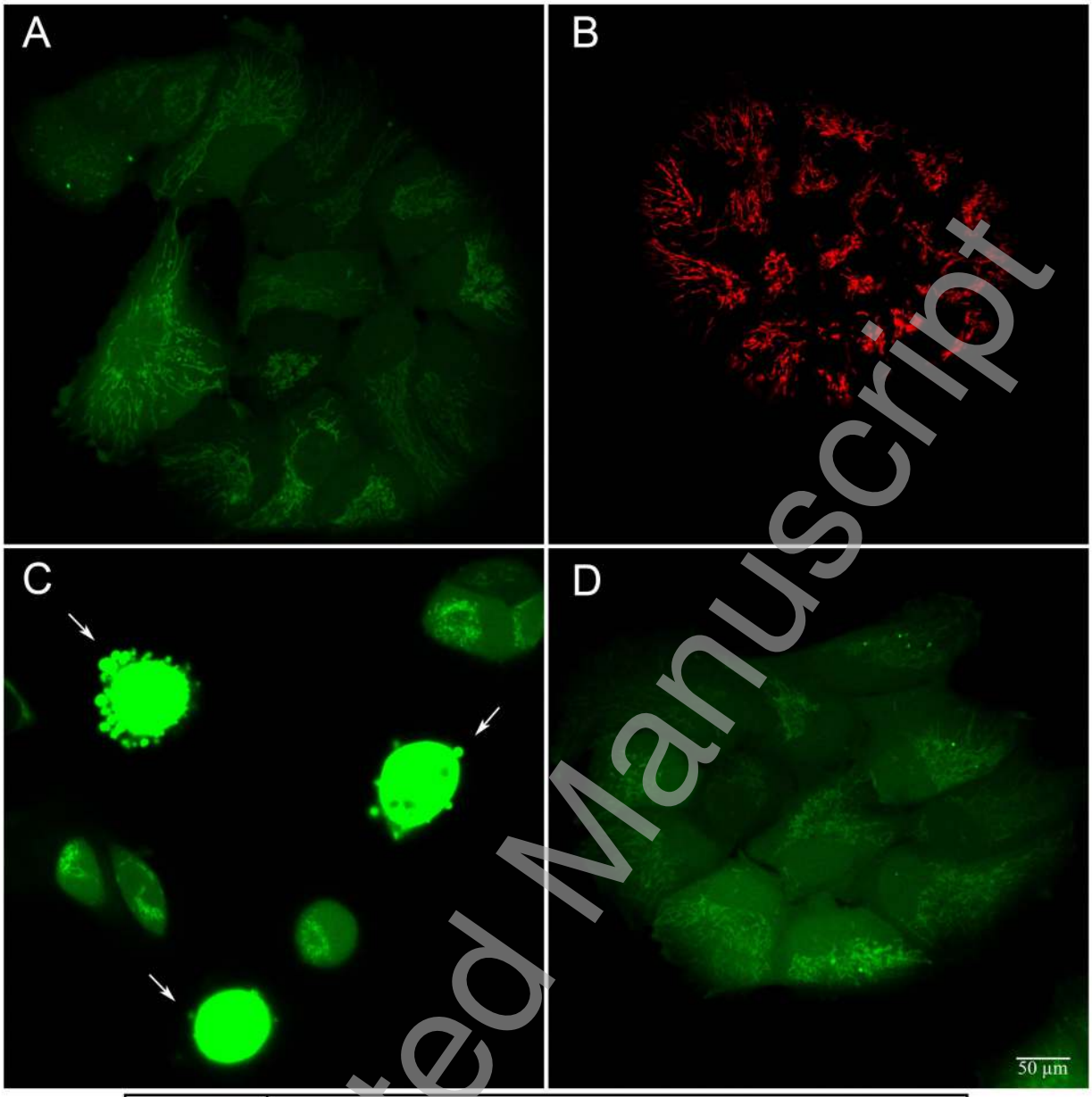


| E | A | C | D |
|---|---|---|---|
| | n = 14 | n = 3 (arrowed) | n = 12 |
| Fl. mean | 9.8 | 84.7 | 14.0 |
| Fl. span | 6 - 18 | 84 - 85 | 8 - 24 |
| p-value | | < 0.001 (vs Fig. 1A) | <0.05 (vs Fig. 1A) |

Figure 1

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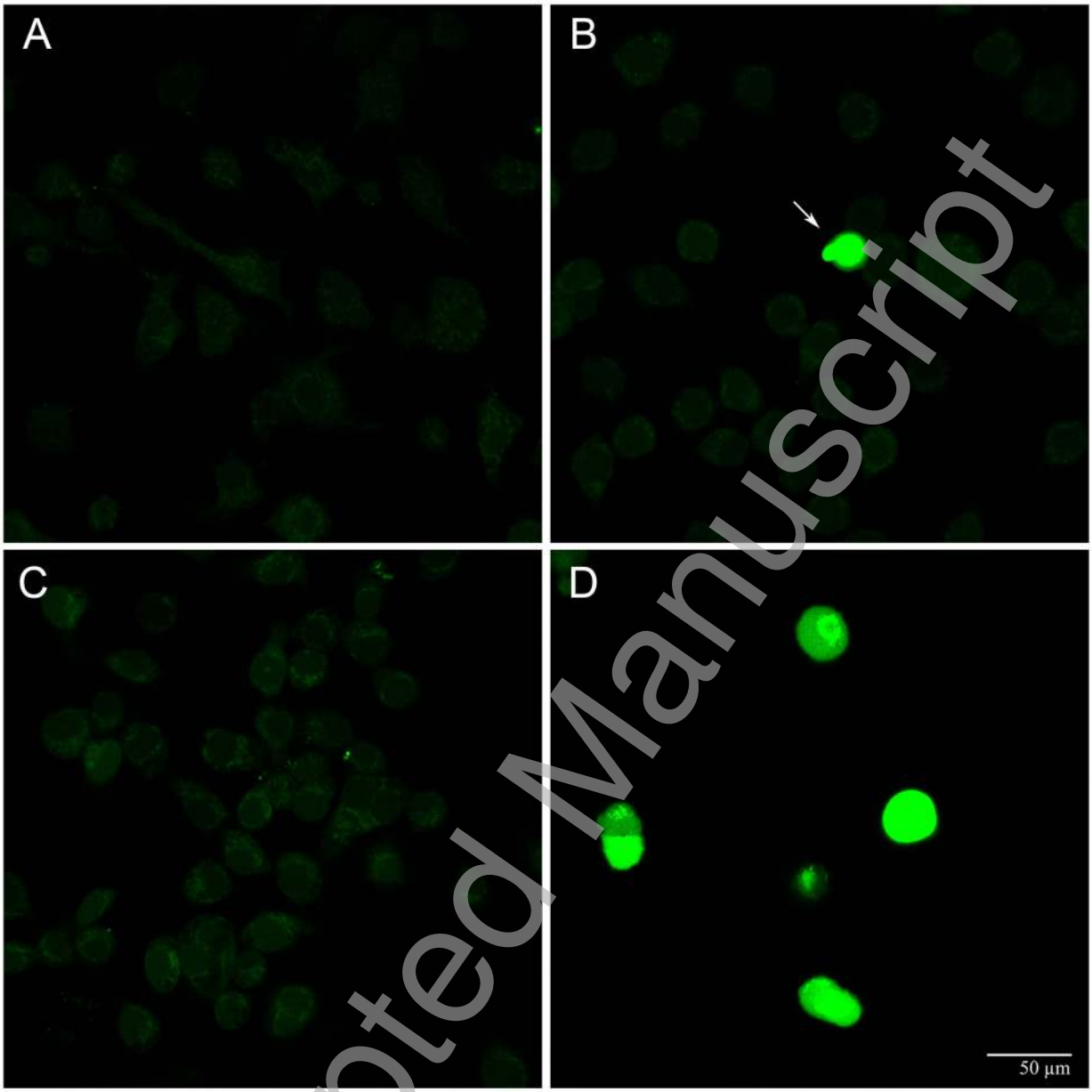


| E | A | $B_{apoptotic}$ | $B_{remaining}$ | C | D |
|---|---|---|---|---|---|
| | n = 28 | n = 1 | n = 36 | n = 43 | n = 6 |
| Fl. mean | 4.5 | 62 | 7.1 | 6.8 | 56.2 |
| Fl. span | 2 - 12 | - | 4 - 8 | 4 - 10 | 17 - 85 |
| p-value | | | <0.05 (vs Fig. 2A) | < 0.001 (vs Fig. 2A) | <0.001 (vs Fig. 1A) |

Figure 2

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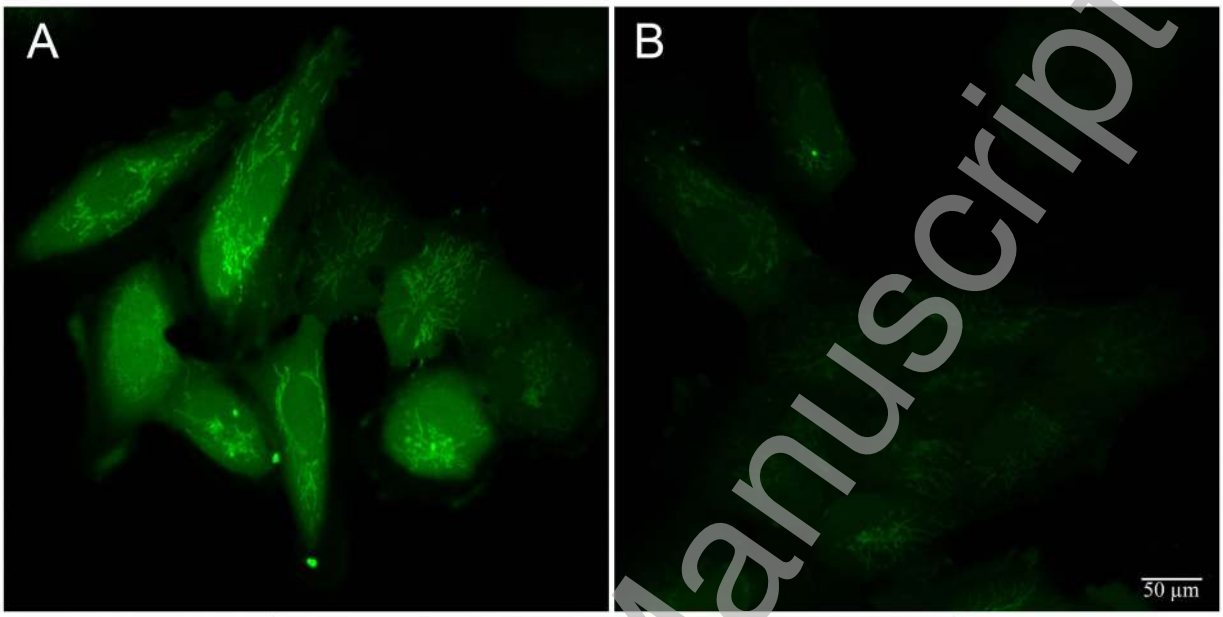


| C | A | B |
|---|---|---|
| | n = 9 | n = 15 |
| Fl. mean | 15.8 | 5.0 |
| Fl. span | 8 - 22 | 2 - 7 |
| p-value | <0.01 (vs Fig. 1A) | <0.001 (vs Fig. 1A) |

Figure 3

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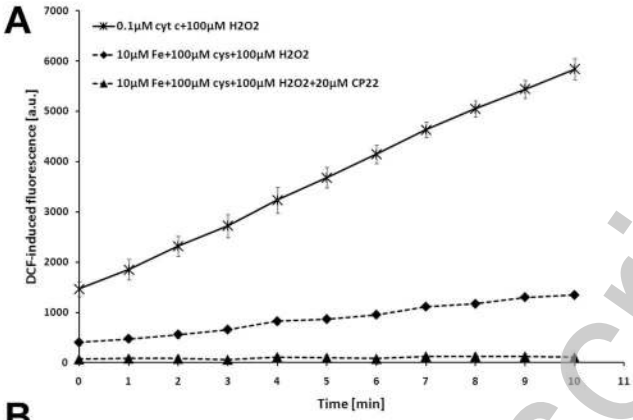


| | | | | | |
|---|---|---|---|---|---|
| 10 μM Fe | - | - | + | + | + |
| 0.1 μM cyt c | + | + | - | - | - |
| 100 μM cysteine | - | - | + | + | + |
| 100 μM $H_2O_2$ | + | - | - | + | + |
| 20 μM CP22 | - | - | - | + | - |
| 10% DMSO | - | - | - | - | + |
| 5 μM $H_2$DCF | + | + | + | + | + |

Figure 4

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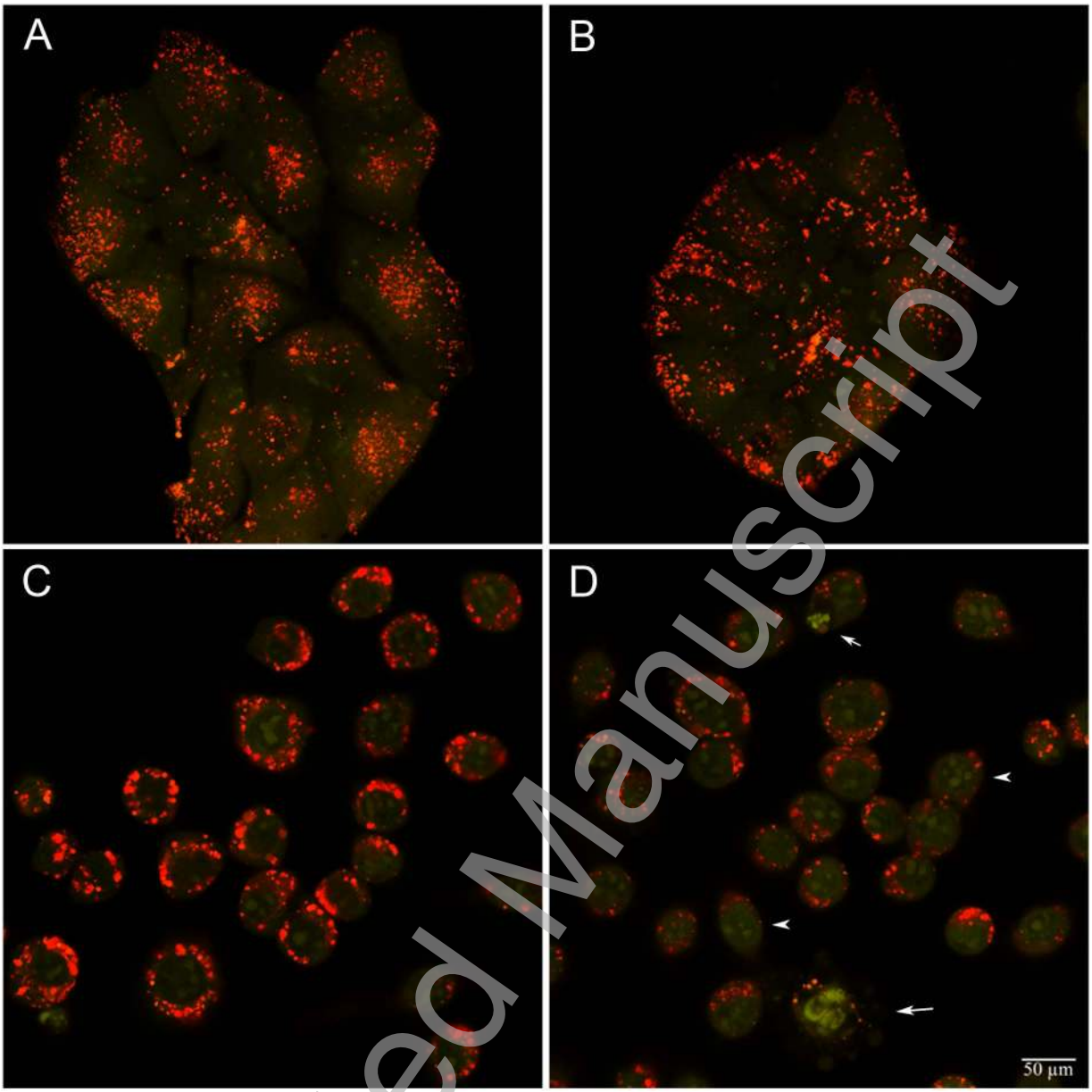


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Figure 5

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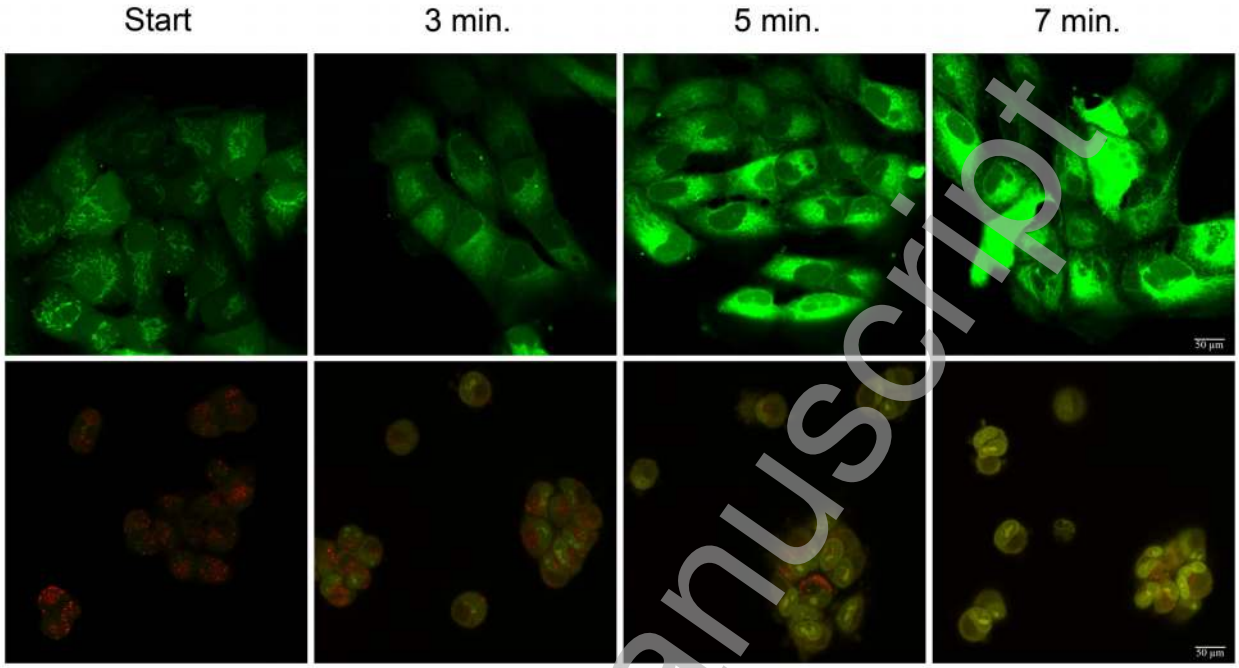


C

| | Start | 3 min. | 5 min. | 7 min. |
|---|---|---|---|---|
| | n = 24 | n = 14 | n = 24 | n = 20 |
| Fl. mean | 12.8 | 14.1 | 23.8 | 33.3 |
| Fl. span | 3 - 26 | 5 - 24 | 5 - 39 | 5 - 73 |
| p-value | | n.s. (vs Fig. 6A$_{Start}$) | <0.001 (vs Fig. 6A$_{Start}$) | <0.001 (vs Fig. 6A$_{Start}$) |

Figure 6

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