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REVIEW

2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy

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Abstract

Reactive oxygen species (ROS) are critically important chemical intermediates in biological studies, due to their multiple physiologically essential functions and their often pathologically deleterious effects. Consequently, it is vital that their presence in biological samples has to be quantifiable. However, their high activity, very short life span and extremely low concentrations make ROS measurement a scientifically challenging subject for researchers. One of the widespread methods for ROS detection, based on the oxidation of the non-fluorescent probe 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) to yield the highly fluorescent 2′,7′-dichlorofluorescein (DCF), was developed more than 40 years ago. However, from its initial application, argumentative questions have arisen regarding its action mechanisms, reaction principles and especially its specificity. Herein, the authors attempt to undertake a comprehensive review: to describe the basic characteristics of $DCFH_2$; to discuss the present views of the mechanisms of its fluorescence formation; to summarize the fluorescence formation interferents; to outline its application in biological research; and to underline its advantages and disadvantages in ROS detection as well as for the methodological considerations that arise during analysis.

Keywords: *$DCFH_2$-DA, $DCFH_2$, DCF, ROS, fluorescent probe*

Abbreviations: *3-AT, 3-amino-1,2,4-triazole; BSO, buthionine sulphoximine; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DCF, 2′,7′-dichlorofluorescein; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; $DCFH_2$-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DDC, diethyldithiocarbamate; DHR, dihydrorhodamine; DMSO, dimethylsulphoxide; GSH, glutathione; HE, hydroethidine; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidise; L-NAME, NG-nitro-L-arginine methyl ester; L-NMMA, NG-methyl-L-arginine; $MB^{\bullet 2+}$, photoinduced methylene blue di-cation-radical; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\bullet -}$, superoxide anion; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; PMA, phorbol 12-myristate 13-acetate; PMNLs, polymorphonuclear leukocytes; RNS, reactive nitrogen species; RO·, alkoxy radical; ROO·, peroxy radical; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase; XO, xanthine oxidase*

Introduction

The term reactive oxygen species (ROS) describes a variety of active small molecules derived as a consequence of metabolism of molecular oxygen. In biological systems, most of the cellular ROS is formed as an ‘unintended’ by-product (like the superoxide formed by electrons escaping the mitochondrial respiratory chain) or as an ‘intended’ product (like the superoxide released by oxidases such as xanthine oxidase or NADPH oxidase). The ROS designation encompasses a series of very small and highly reactive molecules such as hydroxyl radical (·OH), superoxide

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anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydrochlorous acid, hypobromous acid, hydroperoxides, alkoxy radical (RO·) and peroxy radical (ROO·), among others [1–3].

Although ROS are formed as natural byproducts of the normal metabolism of oxygen, recent studies have revealed that they are also involved in a variety of cellular processes ranging from cell proliferation to cell adaptation to hypoxia or from apoptosis to carcinogenesis. They also serve to maintain or re-establish redox homeostasis, act as intracellular second messengers and modulate signal transduction pathways [4–6]. Under physiological conditions, the deleterious effects of ROS are minimized by antioxidant defense mechanisms, which scavenge ROS and prevent their accumulation in excess and/or aid in the repair of their damage. However, any imbalance in the pro-oxidant/antioxidant equilibrium that favours the pro-oxidants will result in a deleterious state called oxidative stress (uncontrolled ROS production) [3]. Oxidative stress is now implicated in the pathogenesis of a number of diseases, including cancer [7–9], diabetes mellitus [10,11], atherosclerosis [12,13], hypertension [14,15], neurodegenerative diseases [16,17] and many others.

A number of direct or indirect analytical methods are currently available for monitoring and quantification of ROS generation in biological samples [18,19]. Fluorescent probes have been proven particularly useful in this respect, due to their high sensitivity, simplicity and reproducibility, and many have been synthesized and used for ROS measurement [20–22]. However, only a few of these probes have been systematically evaluated and widely accepted. One of the most popular, 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), was initially developed in the 1960s for H_2O_2 detection in cell-free systems [23]. However, even after 40 years of application in research, its full chemical mechanism is still not completely elucidated.

Unification of the confusing nomenclature

Like many drugs that have multiple designations, such as chemical name, common name and various brand names (giving physicians headaches), the multiple names for many compounds chemically similar to $DCFH_2$-DA, including $DCFH_2$ and DCF, have little difference in either pronunciation or spelling. This generates confusion among many researchers, especially for those newcomers to the field. Actually, these names have varied over time and among different research groups or labs. A summary of the most prevalent full names and abbreviations that have appeared in the literature for $DCFH_2$-DA, $DCFH_2$ and DCF is presented in Table I. As can be seen, there has been little consensus or consistency in the nomenclature of this fluorescent probe and its cohorts. In light of their

Table I. The different names for $DCFH_2$-DA, $DCFH_2$ and DCF.

$DCFH_2$-DA	$DCFH_2$	DCF	Ref
Diacetyldichlorofluorescin (LDADCF)	Dichlorofluorescein (LDCF)	2′,7′-dichlorofluorescein (DCF)	[23]
2′,7′-dichlorofluorescin-diacetate (DCFH-DA)	2′,7′-dichlorofluorescin (DCFH)	2′,7′-dichlorofluorescein (DCF)	[25]
2,7-Dihydrodichlorofluorescein diacetate (DCF-DA)	2,7-dihydrodichlorofluorescein (DCF-H)		[26]
2,7-dichlorodihydrofluorescein	2,7-Dichlorodihydrofluorescein (DCDHF)	2,7-dichIorofluorescein (DCF)	[27]
2,7-dichlorofluoresceindiacetate (DCFH-DA)	Dichlorofluorescein (DCFH)	Dichlorofluorescein (DCF)	[29]
2′,7′ -dichlorodihydrofluorescein diacetate (DCFH2-DA)	Dichlorodihydrofluorescein (DCFH2)	2′,7′-dichlorofluorescein (DCF)	[54]
2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA)	2′,7′-dichlorodihydrofluorescein (H_2DCF)	2′,7′-dichlorofluorescein (DCF)	[64]
2′,7′-Dichlorofluorescin diacetate (DCFDA)	Dichlorofluorescin	Dichlorofluorescein (DCF)	[77]
Dichlorodihydrofluorescein diacetate (H2DCFDA)	DCFH	Dichlorofluorescein (DCF)	[79]
Dichlorofluorescein diacetate (DCF)			[83]
2′,7′-dichlorofluorescein diacetate (DCFH)		2′,7′-dichlorofluorescein (DCF)	[97]
Leuco-2′,7′-dichlorofluorescein diacetate	Leuco-DCF	2′,7′-dichlorofluorescein (DCF)	[100]
	2,7-dichlorofluorescin (H2DCF)	Dichlorofluorescein (DCF)	[51]
2,7-Dichlorodihydrofluorescein diacetate (DCDHF-DA)	Dichlorodihydrofluorescein (DCDHF)	Dichlorofluorescein (DCF)	[122]
2′,7′-dichlorodihydrofluorescein Diacetate (DCFH-DA)	2′,7′-dichlorodihydrof!uorescein (DCFH)	2′,7′-dichlorofluorescein (DCF)	[123]
2′,7′-Dichlorodihydrofluorescein diacetate (DCFH2-DA)	2′,7′-dichlorodihydrofluorescein (DCFH2)	2′,7′-dichlorofluorescein (DCF)	[124]
2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA)	2′,7′-dichlorofluorescin (DCHF)	2′,7′-dichlorofluorescein (DCF)	[125]

chemical structures, however, and holding to common naming rules, we propose to use the abbreviation $DCFH_2$-DA to designate 2′,7′-dichlorodihydrofluorescein diacetate (where H_2 refers to dihydro), $DCFH_2$ for 2′,7′-dichlorodihydrofluorescein and DCF for 2′,7′-dichlorofluorescein. Hence, in this review, these abbreviations are used to avoid ambiguity.

The chemical characteristics and pharmacological effects

General chemical characteristics

Structurally $DCFH_2$-DA, $DCFH_2$ and DCF are very similar to other fluorescent probes, such as fluorescein and dihydrorhodamine (DHR) (Figures 1 and 2). In their native state, both $DCFH_2$-DA and $DCFH_2$ are non-fluorescent and colourless, while the yellow DCF shows intense fluorescence upon excitation, with a wavelength of ~485–500 nm, and it emits a wavelength at ~515–530 nm. In methanol and aqueous solution, the maximum UV absorption of $DCFH_2$-DA is 256 nm and 320 nm, respectively [24,25], while for $DCFH_2$ it is ~30 nm higher, at 270~285 nm [24,26]. The absorption peak for DCF is at 488~503 nm in phosphate buffer (pH 7.2 and 6.8) [23,25,26] with an extinction coefficient at 500 nm of 59 500 $M^{-1}cm^{-1}$ [27].

DCF exists in two tautomeric forms, depending on pH (Figure 2, bottom). Under basic conditions, the dominant form is the open form (3b), which is fully conjugated across the tricyclic system and which possesses a distinctive maximum absorption at 500 nm. Under neutral or acidic conditions, ring closure occurs and the lactonic form (3a) is generated; this form is not fully conjugated and lacks any typical UV absorption or fluorescence [24].

A semiquinone radical intermediate, $DCFH^{\cdot}$, could be generated by oxidizing $DCFH_2$ or by reducing DCF with radiolytically generated radicals. It has an absorption at 350~400 nm and decays over ~10 ms to form DCF. Detailed properties of this intermediate have been summarized by Wrona and Wardman [28].

Auto-oxidation and photo-oxidation

In the solid state or in ethanolic solution, the auto-oxidation (spontaneous deacetylation) of $DCFH_2$-DA to $DCFH_2$ will be negligible (less than 0.2% after 3 months in ethanol in the dark). In contrast, alkaline activation and dilution can significantly increase the auto-oxidation rate [23], which occurs in a linear fashion over time. Similar rates of auto-oxidation are observed in room air and under culture conditions (e.g. M199 medium), with ~20% of $DCFH_2$-DA undergoing deacetylation in 1 h. Interestingly, the

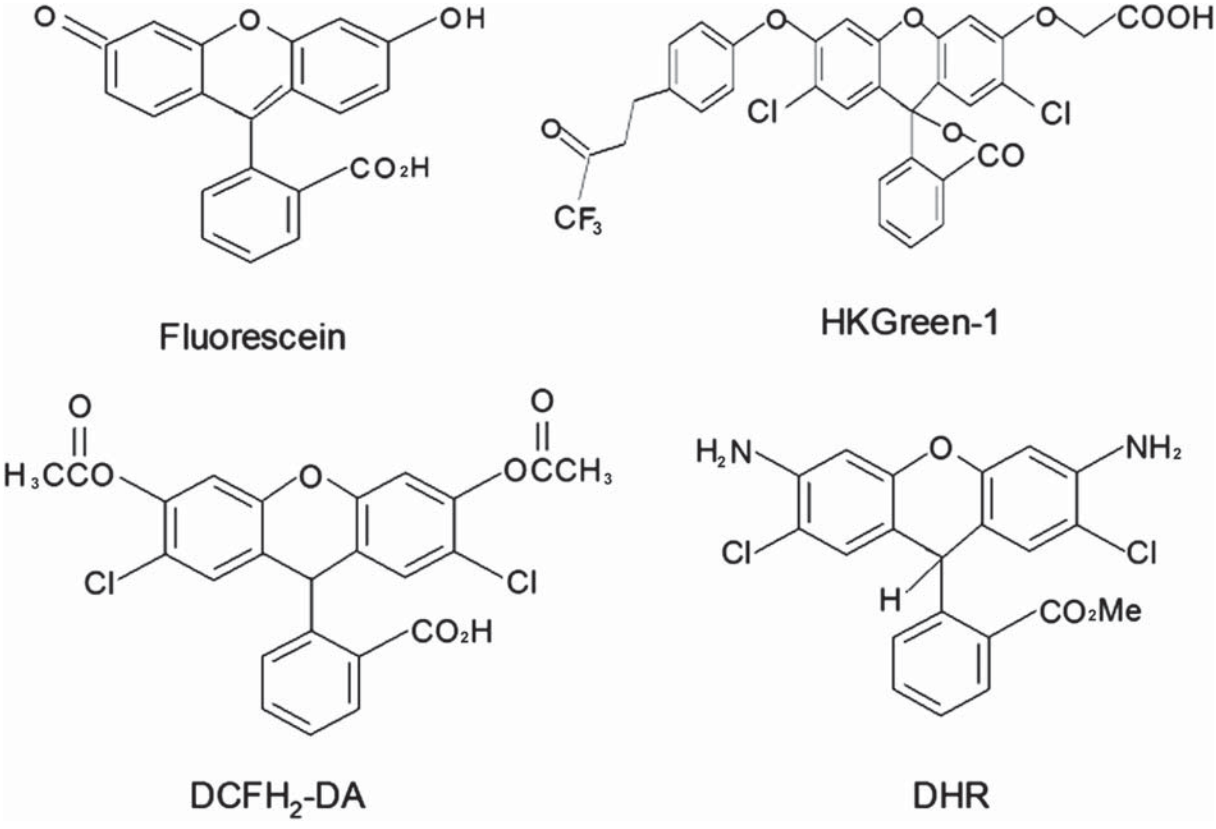

Figure 1. Chemical structure of fluorescein, HKGreen-1, $DCFH_2$-DA and DHR.

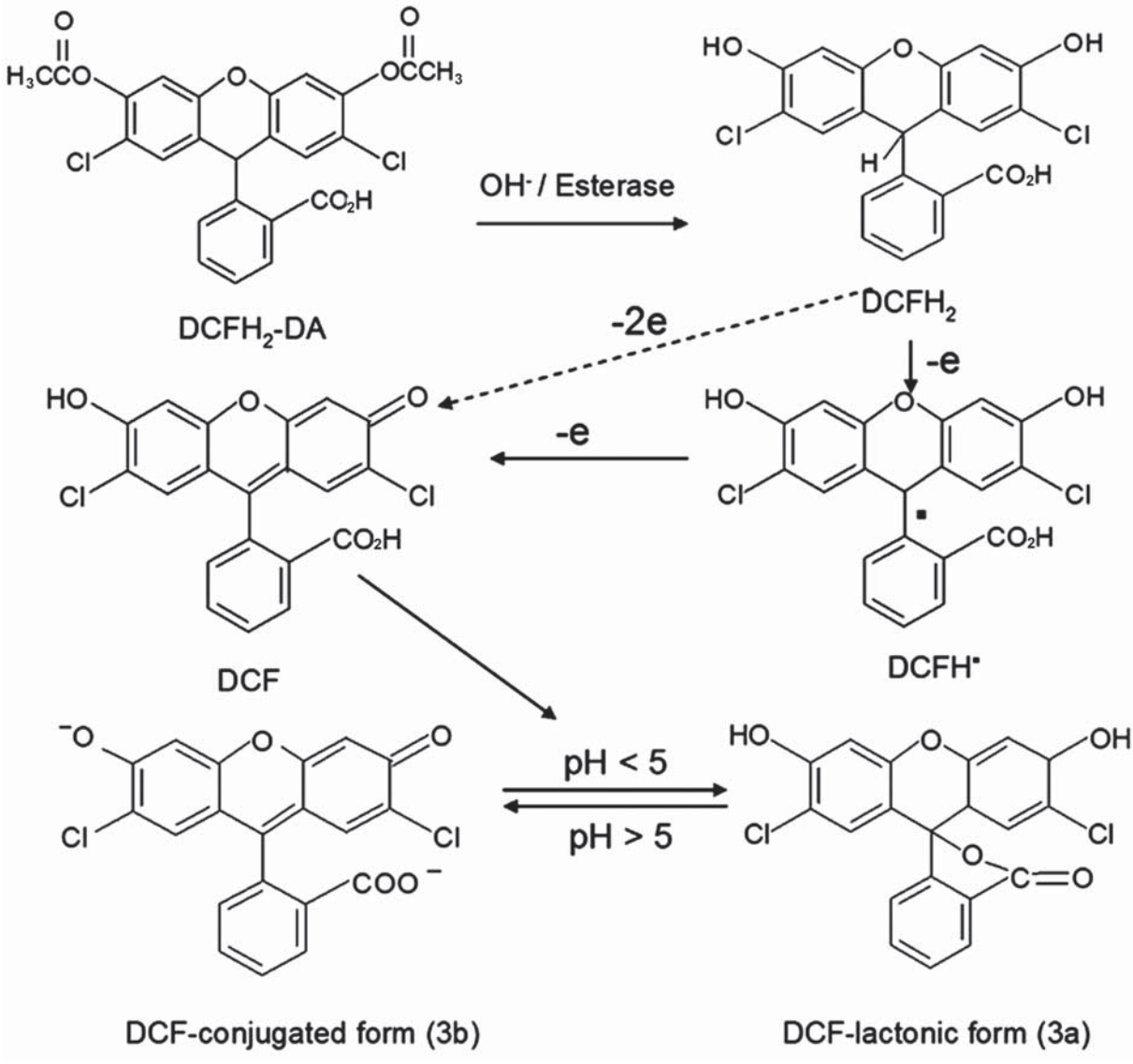

Figure 2. The formation of DCF from $DCFH_2$-DA.

spontaneous oxidation of $DCFH_2$ to DCF under either of these two conditions is also linear, but the rate in the room air was reported as ~10-times faster than that in culture medium [29]. In Tris buffer, more than 85% of $DCFH_2$ is spontaneously oxidized to DCF within 24 h [30]. The reasons for the observed differences are not clear: one possible factor maybe a pH effect, while another may be the presence of anti-oxidants in the culture medium, which might prevent the oxidation of $DCFH_2$ to DCF.

Photo-oxidation of $DCFH_2$ can also be observed in UV-induced cell-free systems and in keratinocytes irradiated with visible light [31,32]. Again, the mechanisms are not very clear and some controversies in the detailed processes have appeared [32–34].

Pharmacological effects

$DCFH_2$-DA has been widely used as a probe for more than 40 years, but its pharmacological effects have been essentially ignored by most researchers. Only a few papers are available that discuss potential pharmacological issues. For example, Bass et al. [35] showed that incubation of human leukocytes with 5–12.5 μM $DCFH_2$-DA for 15 min did not appear to elicit any toxic effects. In contrast, Andoh et al. [36] found that $DCFH_2$-DA could dose-dependently inhibit arsenite, cadmium and hemin-induced heme oxygenase-1 (HO-1) expression at transcriptional level in HeLa cells. Similarly, $DCFH_2$ abolished the phosphorylation of extracellular signal-regulated kinase (ERK) and prevented the nuclear translocation of a transcriptional activator Nrf2. Furthermore, the presence of $DCFH_2$-DA led to an increase in cell viability following exposure to sodium arsenite, cadmium chloride and hemin, which indicated that $DCFH_2$ might contribute significantly to cellular defense mechanisms against toxic insults [36]. Hence, it becomes important to minimize the effect of the fluorescent probe itself during ROS measurement.

For this reason, pharmacological effects need to be carefully evaluated in order to avoid misinterpretation of results.

To further complicate these issues, $DCFH_2$ has also been reported to be a substrate for xanthine oxidase (XO). Kinetic studies of the oxidation of $DCFH_2$ by XO indicated a Km(app) of 0.62 mM, while hypoxanthine competed with $DCFH_2$ with a Ki(app) of 1.03 mM [37].

Fluorescence formation mechanisms

Commonly accepted mechanisms

Generally speaking, the ideal fluorescent ROS probe for biological assays should meet the following criteria:

1) *Stability*: Stability means that there will be minimal auto-oxidation and photo-oxidation in the culture/assay buffer/cell system being investigated and that the probe will not react with them.
2) *Permeability*: Permeability means that the probe can easily cross/diffuse through the cell membrane and will build up a certain intracellular concentration that can be maintained for a certain period.
3) The parent compounds should be non-fluorescent or have low fluorescence so as to give low background, while the oxidation products should have intense fluorescence and be trapped in the cells.
4) *Homogeneous distribution of the oxidation products inside the cells*: This would be perfect when the total intracellular ROS is to be evaluated. However, sometimes a specific distribution within a cellular compartment or organelle is desired, especially when there is a need to determine the role of ROS formation by a specific organelle or to study the mechanisms of action of cell compartment antioxidants.
5) *Cytotoxicity*: The probes should have little or no cytotoxicity or pharmacological effects and should not induce significant cellular

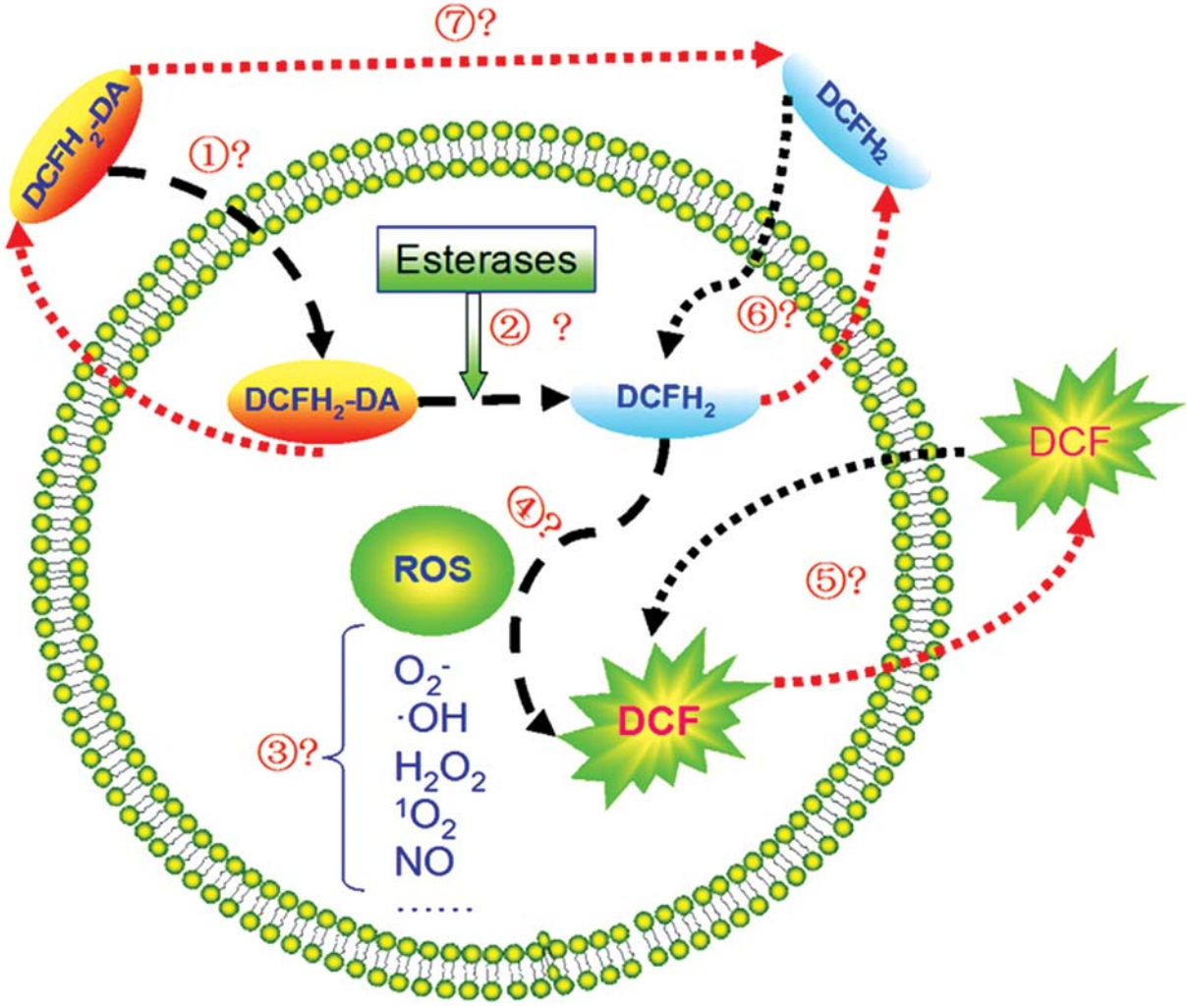

Figure 3. The generally accepted formation mechanism of DCF in cells.

effects that would result in ROS generation, either directly or indirectly.

6) The probe should be easy to prepare and its analysis should be simple, quick and robust. However, it must be pointed out that no probe yet meets all of these requirements. Indeed, perhaps there might be no such 'ideal' fluorescent probe existing.

The commonly accepted mechanism for $DCFH_2$-DA/$DCFH_2$ acting as a ROS probe is simple and is described schematically in Figure 3. (1) The non-fluorescent lipophilic $DCFH_2$-DA diffuses and crosses the cell membrane; (2) Under the action of intracellular esterases, $DCFH_2$-DA deacetylates to form $DCFH_2$, which is also non-fluorescent but is now membrane-impermeable; and (3) $DCFH_2$ reacts with intracellular ROS to give the fluorescent compound DCF [23,38–40].

However, as this probe has been applied to more and more studies, this concept has been challenged by many researchers and there are controversies involving nearly every step of the putative mechanism. Some questions, also shown in Figure 3, include: (4) Which ROS species is responsible for $DCFH_2$ oxidation? (5) Could DCF diffuse freely across the cell membrane? (6) Is the $DCFH_2$ truly trapped in the cells? and (7) Could $DCFH_2$-DA deacetylate to form $DCFH_2$ prior to entry into the cells?

Where do these probes locate?

The cellular distribution of the probe is of critical importance, since this determines which kind of ROS will be measured. However, there is disagreement and even confusion regarding the cellular location of $DCFH_2$ and DCF. Most early papers maintained that $DCFH_2$, which bears two phenolic groups and a carboxylic acid moiety, is sufficiently polar to enter the aqueous compartment of the cell, where it is converted to DCF by intracellular ROS [38,41,42].

However, a recent study into the location of $DCFH_2$-DA, $DCFH_2$ and DCF within a liposomal bilayer model [24] challenged this traditional view: (1) The flat $DCFH_2$-DA molecule was found to lie between the lipid chains, in a plane parallel to them, and was in constant rapid rotation in this plane. (2) The flat $DCFH_2$ molecule, just like $DCFH_2$-DA, also lays in between the lipid chains, again in a plane parallel to them, and was also in constant rapid rotation. It was somewhat above $DCFH_2$-DA, but still far from the interface. (3) The location of DCF (3a) within the liposomal bilayer is somewhat above $DCFH_2$-DA and $DCFH_2$, but still well within the liposomal bilayer. (4) It is difficult to be exact about the location of DCF (3b), but it is clear that this molecule indeed lies parallel to the lipid chains (perpendicular to the interface), with the carboxyl group anchored not far from the interface.

Actually, as early as in 1988, Scott et al. [43] had already noted the possibility that $DCFH_2$-DA might deacetylate at or near the cell membrane surface and that the resulting $DCFH_2$ remains surface-associated during its subsequent oxidation. Some fluorescence microscopic observations support the accumulation of DCF in or near the cell membrane. For example, copper-1,10-phenanthroline treated liver carcinoma Bel-7402 cells clearly showed a much higher DCF fluorescence in or near the membrane than in the cytoplasm [44]. Recently, Swift and Sarvazyan [45] reported that $DCFH_2$ and DCF localized mainly in the mitochondria and not in the cytosol in cardiac myocytes.

Although Afri et al.'s [24] results suggested that all of these probes are located within the lipid bilayer, it is obvious that biological membranes are far more complicated than a liposomal bilayer. Experimental evidence has revealed that some probes derived from $DCFH_2$-DA (such as $DCFH_2$, DCF) can indeed cross the cell membrane and enter the cytoplasm (see below).

How is $DCFH_2$-DA converted to $DCFH_2$?

In cell-free systems, the deacetylation of $DCFH_2$-DA to form $DCFH_2$ could be achieved generally in one of two ways:

1) *The chemical* '*activation*' *process*: By addition of some form of strong alkaline solution, normally NaOH, to $DCFH_2$-DA solution [46].
2) *The biological* '*catalysis*' *process*: By the action of specific esterase activity that would hydrolyse $DCFH_2$-DA [34]. In cell systems, it is widely accepted that $DCFH_2$-DA is hydrolysed by intracellular esterases to form $DCFH_2$ after entry into the cytoplasm.

It is still unclear exactly which esterase is responsible for the hydrolysis of $DCFH_2$-DA. Its identity is still unknown and there are no papers available that discuss this. However, acetylesterase, an enzyme that catalyses acetic ester and H_2O to form alcohol and acetate, is a potential candidate, since Moffat and Snell [47] found that a single C esterase (acetylesterase) was responsible for metabolism of fluorescein diacetate, an analogue of $DCFH_2$-DA [48], in *B. plicatilis*. Lipase, which could hydrolyse dibutylfluorescein [23], another analogue of $DCFH_2$-DA, increases $DCFH_2$-DA oxidation, suggesting that it might be another possible candidate.

Are $DCFH_2$ and DCF trapped within the cells?

Rotman and Papermaster [49] were first to suggest that $DCFH_2$ could accumulate inside cells, but that the intracellular concentration was dependent on the integrity of the cell membrane. Bass et al. [35] declared that $DCFH_2$-DA is clearly trapped within human polymorphonuclear leukocytes (PMNL) and estimated that a concentration of 5 μM $DCFH_2$-DA would, by simple diffusion, yield an intracellular accumulation of 0.67 attomoles $DCFH_2$-DA per cell [35]. Royall and Ischiropoulos [29] demonstrated that $DCFH_2$-DA established stable intracellular concentrations of $DCFH_2$ and DCF within 15 min (~2.2 nmol/mg protein) in bovine aortic endothelial cells (BAECs), which could remain stable for at least 2 h as long as there was continued presence of the probe in the extracellular medium. However, this stable level decreased by more than 90% if cells were exposed to culture medium without probe for a further 1 h. These results strongly suggested that $DCFH_2$ was not completely trapped in endothelial cells, nor was DCF [29]. Similar to what was observed in BAECs, both $DCFH_2$ and DCF reached a steady state within 10 min after $DCFH_2$-DA addition to cardiomyocytes, but both accumulated mostly in mitochondria [45], which suggested that mitochondria were one of the places where conversion of $DCFH_2$ to DCF was occurring. Furthermore, the amount of fluorescent probe was constant for loading times up to 1 h in cardiomyocytes, which is quite different from the rapid leakage seen from human glomerular endothelial cells [45] and BAECs [29]. In *Saccharomyces cerevisiae*, this leakage also seems to be substantial [50].

It is reasonable to assume that a certain amount of $DCFH_2$-DA/$DCFH_2$ could be trapped in the cells and that a temporary equilibrium would build up between the intracellular and extracellular concentrations. However, different cell types show different kinetics for probe accumulation and retention, which affects the availability and utility of the probe in different systems. Experiments with $DCFH_2$-DA/$DCFH_2$ therefore require prior knowledge of probe behaviour in the specific cells or tissue type to be studied.

What is the mechanism of formation of DCF from $DCFH_2$?

The mechanism of formation of DCF from $DCFH_2$ is rather more complicated than previously conceived. Initially, it was presumed that $DCFH_2$ loses two electrons at a time to form DCF [51] (Figure 2). Further study has since revealed that $DCFH_2$ oxidation to DCF involves two single-electron oxidation steps: $DCFH_2$ loses one electron to form the obligatory intermediate DCFH·, while the latter loses another electron to form DCF. In addition, photo-excitation ($\lambda > 300$ nm) can

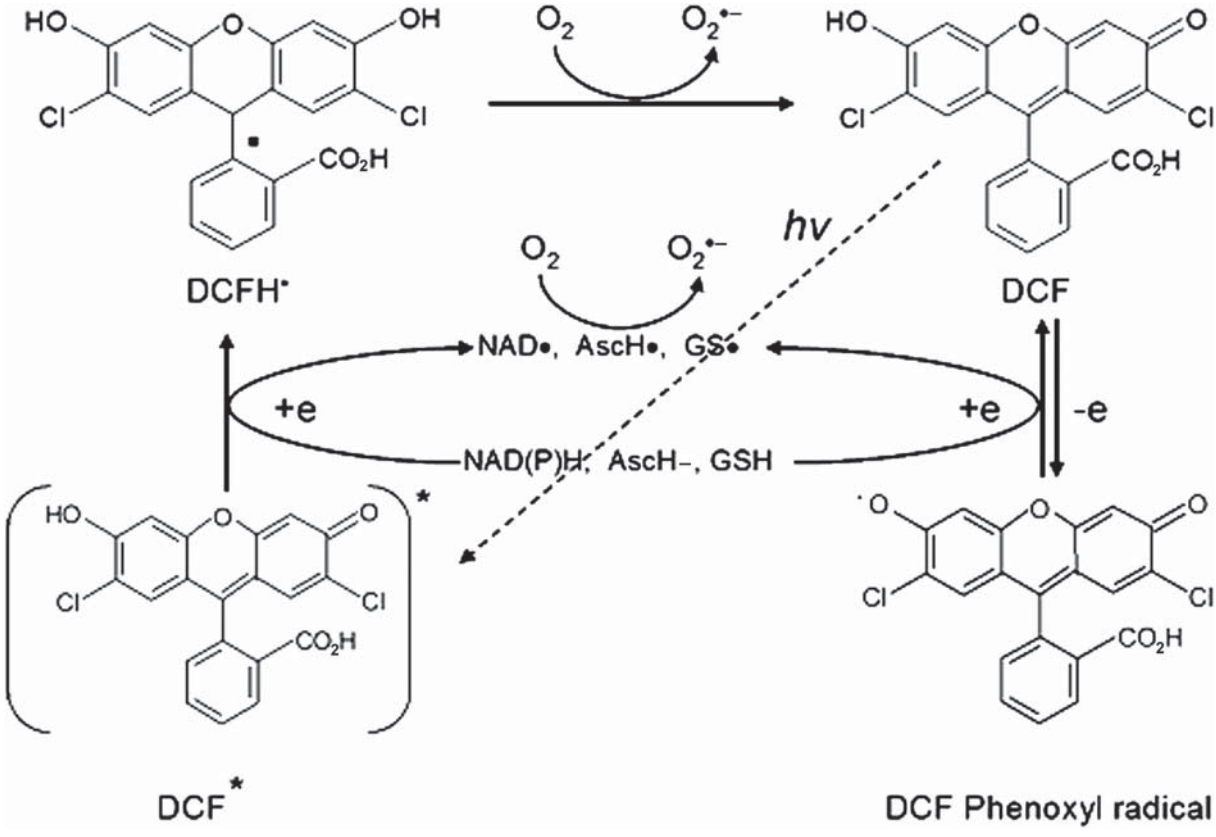

Figure 4. Formation of DCF phenoxyl radical.

transform DCF to its excited state(s) (DCF*), which can oxidize glutathione (GSH), NADH and ascorbic acid (AscH-) to form glutathione thiyl radical (GS·), NAD· and ascorbate anion radical (AscH·), respectively [33] (Figure 4). DCF could be oxidized either by horseradish peroxidase (HRP)-compound I or -compound II with the obligatory generation of the DCF phenoxyl radical (DCF·), which could also oxidize GSH, NADH and ascorbic acid, again generating GS·, NAD· radical and AscH·, respectively. Simultaneously, O_2 would be reduced to superoxide [40] (Figure 4). The detailed oxidation process for $DCFH_2$ was recently reviewed in detail by Wardman [22]. A novel fluorescent product termed monochlorofluorescein was also reported following photo-irradiation of $DCFH_2$ [52].

Interferents in fluorescence formation

Superoxide dismutase (SOD) and catalase (CAT)

The effect of SOD and CAT on $DCFH_2$ oxidation is controversial, both in cell-free and intact cell systems.

In cell-free systems, Fe^{2+}/H_2O_2-induced $DCFH_2$ oxidation was inhibited by CAT or by the ·OH scavenger dimethylsulphoxide (DMSO), while SOD had no effect. In a superoxide generating system consisting of hypoxanthine/XO, oxidation of $DCFH_2$ was unaffected by either SOD or CAT [37]. In the HRP/peroxynitrite ($ONOO^-$) system, both SOD and CAT were able to inhibit $DCFH_2$ and $DCFH_2$-DA oxidation [53].

In XO + acetaldehyde stimulated human PMNLs, CAT showed a dose-dependent inhibitory effect while SOD showed no effect on $DCFH_2$ oxidation [35]. In LLC-PK1 renal epithelial cells, both CAT (0.1 mg/ml) and SOD (0.1 mg/ml) markedly decreased cellular $DCFH_2$-DA oxidation, by 68% and 84%, respectively [43]. The SOD inhibitory effects were also observed in LNC, HeLa, AH-70, HL-60, CGC, SY5Y, A-10 and HUVEC cells or cell lines, while no effects were seen in lymphoid cells or hepatocytes. The inhibitory effects of CAT have been reported in macrophages, LNC, Hela, hepatocyte, HL-60, CGC, A-10 and HUVEC cells or cell lines, while no effects were seen for lymphoid cells (Table II). However, DCF fluorescence was intensified in CuZn SOD-transfected HaCaT and RAW 264.7 cells [54].

Diethyldithiocarbamate (DDC), an inhibitor of SOD, greatly reduced the intensity of DCF fluorescence in neurons [42,55], human neutrophils [56], rat alveolar macrophages [57] and endothelial cells [58], making the interpretation of the effect of SOD difficult. The effect of DDC on $DCFH_2$ oxidation is also complicated and may be interpreted as follows: (1) SOD inhibition by DDC results in increased accumulation of $O_2^{\cdot-}$, which results in increased H_2O_2 and ·OH formation. This process then has the potential to increase $DCFH_2$ oxidation. (2) DDC was also found to have antioxidant potential and could directly react with H_2O_2 and $O_2^{\cdot-}$ [59], which would decrease $DCFH_2$ oxidation. (3) Kim et al. [54] compared the peroxidase activity of CuZn-SOD, cytochrome *c*, HRP, Cu^{2+} and Fe^{3+} under various conditions and found an enormous increase in the rate of $DCFH_2$ oxidation in response to bicarbonate and were able to distinguish CuZnSOD from cytochrome *c* and HRP. DCF fluorescence was also intensified in CuZnSOD-transfected HaCaT and RAW 264.7 cells. These results suggested that CuZnSOD is a potential intracellular catalyst for the H_2O_2-dependent oxidation

Table II. The DCF fluorescence formation interferents.

Cell-free system	→(Iron/H_2O_2) [37] →(X/XO) [37] ↓(SIN-1) [56] ↑(photoreaction) [34] →(pyocyanin) [89] →(HRP/$DCFH_2$-DA) [53]	↓(Iron/H_2O_2) [37] →(X/XO) [37] →(photoreaction) [34] →(pyocyanin) [89] ↓(HRP/$DCFH_2$-DA) [53]	↑(SIN-1) [56] →(SNAP) [56] ↓(Iron/H_2O_2)DMSO [37]	-(X/XO)DFO [37] ↓(Iron/H_2O_2)DFO [56] ↑(photoreaction) azide [34]
Cell system	→(PMNL) [35] ↓(LLC-PK1) [43] →(Macros) [64] ↓(LNC) [126] ↓(HeLa) [88] ↓(AH70) [73] →(lymphoid) [127] →(hepalocyte) [80] ↓(HL-60) [128] ↓(CGC) [74] ↓(SY5Y) [130] ↓(A-10) [129] ↓(HUVEC) [76]	↓(PMNL) [35] ↓(LLC-PK1) [43] ↓(Macros) [64] ↓(LNC) [126] ↓(HeLa) [88] →(lymphoid) [127] ↓(hepatocyte) [80] ↓(HL-60) [128] ↓(CGC) [74] l(A-10) [129] ↓(HUVEC) [76]	↑(PC12)SNP [69] →(Macros)LNMMA [64] ↓(AH70)LNMMA [73] ↓(CGC)LNAME [69] ↓(A-10)LNAME [75] ↓(Oat leaves) cPTIO [70] ↓(HUVEC)LNAME [76] →(LLC-PKl)MO [43] ↓(Macros) DMSO [64] ↓(hepatocyte)DMSO [80] ↓(HUVEQDMSO [76] ↓(LLC-PK1)DMS0[43]	↑(PMNL) azide [35] →(neurons)3-AT [42] ↑(neurons)NEM [42] ↓(Macros) azide [64] →(Macros) DFO [64] ↓(LNC) GSH/GSH-Px [126] ↓(hepatocyte)DFO [80] ↑(HL-60) [128] DFO [128] ↓(neuronspDC [42] ↓(neutrophil) DDC [56] →(neutrophil) SHA [56]

X/XO, hypoxanthine/xanthine oxidase; MO, mannitol; DFO, desferoxamine; NEM, N-ethylmaleimide, inhibits gluthatione peroxidise; 3-AT, 3-amino-1,2,4-triazole, inhibits CAT; DDC, N,N-diethyldithiocarbamate, inhibits SOD; Macros, macrophages; SHA, myeloperoxidase inhibitor SHA, which inhibits the formation of HOCl; CGC, cerebellar granule cells; SNAP, NO donor, *S*-nitroso-*N*-acetylpenicillamine; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ↓, decrease; ↑, increase; →, no effect.

of $DCFH_2$. Therefore, inhibition of SOD will significantly decrease the DCF formation rate. The effect of (2) and (3) is much more potent than (1), which accounts for the decreased intracellular DCF fluorescence in the presence of DDC (see Figure 5, right).

The mechanism underlying the inhibitory effect observed following addition of exogenous SOD to cell media might be quite different from that of endogenous SOD expression or inhibition. Since exogenous SOD could not ordinarily enter the living cells [43], external SOD could only scavenge the radicals that could diffuse freely across the membrane, such as H_2O_2, $O_2^{\cdot -}$ and $HO_2^{\cdot}$ (hydroperoxyl radical) [60] (Figure 5, (5)). SOD acts on $O_2^{\cdot -}$ to form H_2O_2 (Figure 5, (4)), which further promotes extracellular $DCFH_2$ to form DCF (Figure 5, (6)). Furthermore, the extracellular SOD concentration will be much higher than the intracellular concentration, which means the $O_2^{\cdot -}$ could be rapidly and completely consumed, resulting in an accelerated external flow of $O_2^{\cdot -}$ (Figure 5, (5)). The overall result might be a decrease in intracellular DCF fluorescence and an increase in extracellular DCF fluorescence (see Figure 5, left). This hypothesis is consistent with a report indicating that extracellular fluorescence was much higher than intracellular in $DCFH_2$-DA loaded endothelial cells [29].

The effect of sodium azide, an inhibitor of CAT, on DCF fluorescence is also controversial. In PMNLs, granulocytes, cardiomyocytes or HeLa cells, azide increased intracellular $DCFH_2$ oxidation [35,61–63], while in mononuclear phagocytes, it significantly reduced $DCFH_2$ oxidation in both unstimulated and PMA stimulated cells [64]. In macrophages, sodium azide showed no effect at all [65]. In a cell-free system, $DCFH_2$ oxidation induced by beta-amyloid was strongly inhibited by sodium azide [66], while in isolated rat liver mitochondria, sodium azide significantly increased $DCFH_2$ oxidation [67]. Another CAT inhibitor, 3-amino-1,2,4-triazole (3-AT), also showed no inhibitory effect on resting neurons [42].

Nitric oxide (NO) donors and nitric oxide synthase (NOS) inhibitors

Both 3-morpholinosydnonimine (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP) are widely used as NO donors. However, in a cell-free system, SIN-1 induced a robust and fast increase in DCF formation. This reached a steady state after ~10 min, while SNAP

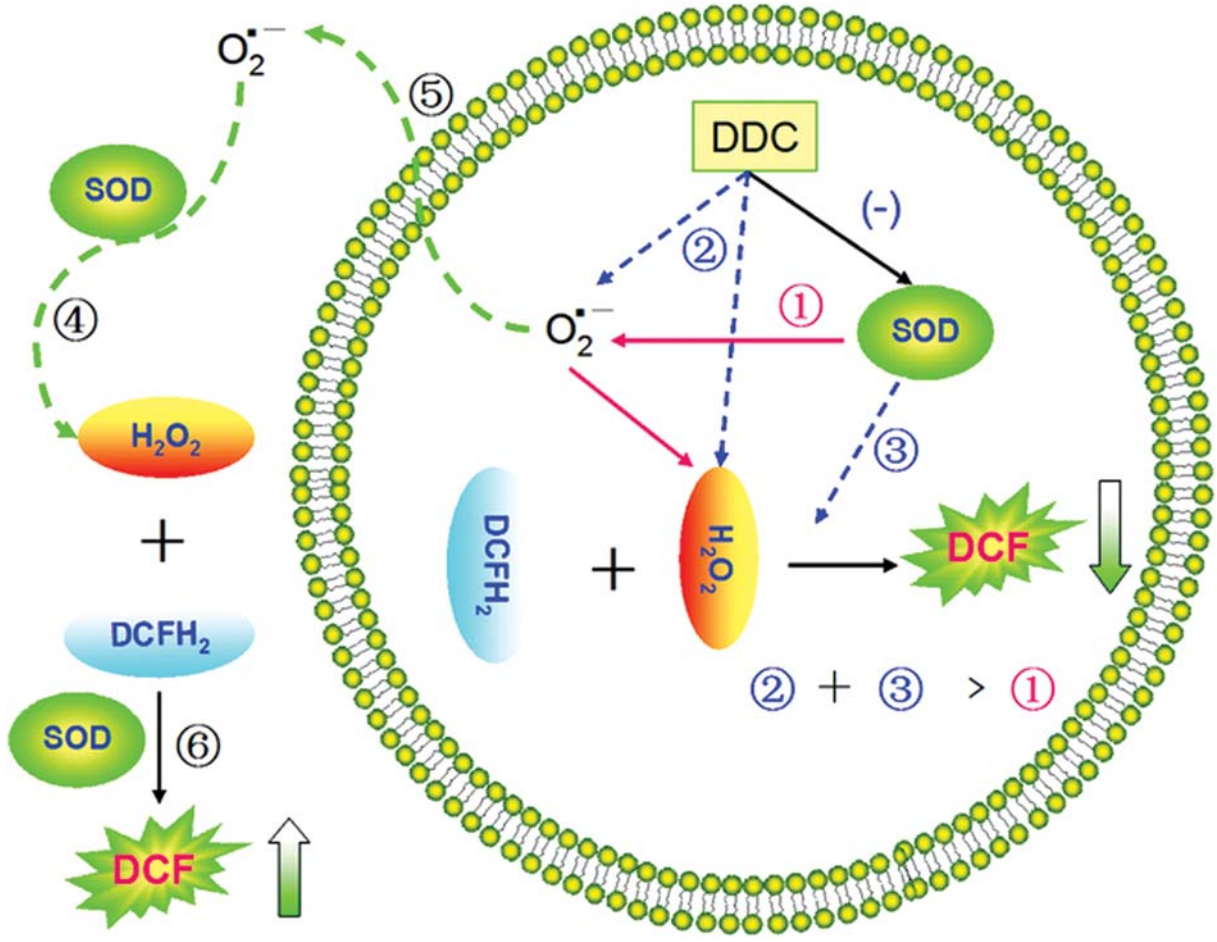

Figure 5. Effect of SOD on ROS formation.

failed to do so [56]. In addition to NO, SIN-1 also releases $O_2^{\cdot-}$ and induces peroxynitrite formation, which is absent in SNAP [68]. This might account for the observed differences, since NO would not directly oxidize $DCFH_2$ to DCF. The NO donor, sodium nitroprusside (SNP), induced $DCFH_2$ oxidation in PC12 cells and this could be inhibited by reduced haemoglobin [69]. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) markedly enhanced $DCFH_2$ oxidation in oat leaves [70], which might be due to the formation of nitrogen dioxide by cPTIO [71,72]. The NOS inhibitor NG-methyl L-arginine (L-NMMA) showed no effect on $DCFH_2$ oxidation in PMA activated mononuclear phagocytes [64], while it successfully inhibited $DCFH_2$ oxidation in AH-70 cells [73]. Another NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) showed inhibitory action in cerebellar granule cells [74], smooth muscle cell line A-10 [75] and HUVEC cells [76].

Others

Some chemicals were found to quench DCF fluorescence in cell-free systems. These include: ethanol, *n*-butyl alcohol, isopropanol, DMSO and chloroform. Methanol decreases the yield of DCF fluorescence to only 10% of that in water [77]. On the other hand, 2,4-dichlorophenol, diethyl ether, tetrahydrofuran [46], dihydroxyacetone, ADP/Pi, ADP, Pi, proline and myxothiazol (mitochondrial complex III inhibitor) increase DCF fluorescence, while lactate/pyruvate, glucose, glutamate/malate, succinate/malate, octanoate, antimycin A, rotenone and digitonin showed no effect in Tris buffer [78]. It is interesting to note that bovine serum albumin (BSA) showed no effect on $DCFH_2$-DA fluorescence in Tris buffer [78], but strongly suppressed H_2O_2 induced DCF fluorescence formation in HBSS buffer [79]. NADH showed no effect on $DCFH_2$ fluorescence [78], but was able to reduce the DCF phenoxyl radical to DCF and generate $NAD^{\cdot}$, which reduced oxygen to $O_2^{\cdot-}$ in the presence of HRP [40].

DMSO inhibits Fe^{2+}/H_2O_2 induced $DCFH_2$ oxidation [37]. It is also effective in inhibiting PMA-induced $DCFH_2$ oxidation in respiratory burst activity in mononuclear phagocytes [64], LLC-PK1 renal epithelial cells [43], hepatocytes [80] and vascular endothelial cells [76], which might be due to its ·OH scavenging activity. However, in many studies, the effect of DMSO or ethanol on $DCFH_2$ oxidation has been totally neglected. Actually, as two strong ·OH scavengers, both compounds have very high rate constants with ·OH (6.6×10^9 $M^{-1}s^{-1}$ for DMSO and 1.2×10^9 $M^{-1}s^{-1}$ for ethanol [81]). Therefore, when these are used as solvents for $DCFH_2$-DA, they could potentially compete with $DCFH_2$ to react with ·OH (the rate constant is 1.3×10^{10} $M^{-1}s^{-1}$ [82]) since the generally used $DCFH_2$ concentration is ~10 μM, while even less than 1‰ DMSO or ethanol is equivalent to more than 10 mM.

The effect of SOD, CAT, etc. on $DCFH_2$ oxidation in cell-free and cell system is summarized in Table II.

Cytochrome *c* is a potent catalyst of DCF formation. Early studies using $DCFH_2$-DA showed that apoptosis of GT1–7 neural cells induced by GSH depletion was accompanied by increased intracellular ROS generation. This increase was prevented by over-expression of the anti-apoptotic proto-oncogene *bcl-2*, suggesting that Bcl-2 suppresses the production of ROS [83]. However, Burkitt and Wardman [84] found, in a cell-free model, that the rate of DCF formation was extremely sensitive to nanomolar concentrations of cytochrome *c*, suggesting that cytochrome *c* was a potent catalyst of DCF formation. Since cells undergoing apoptosis release cytochrome *c* from mitochondria [85], the suppression of DCF formation by the anti-apoptotic oncoprotein Bcl-2 could result from its prevention of mitochondrial cytochrome *c* release, rather than from direct inhibition of ROS production. Although over-expression of Bcl-2 could abrogate cytochrome *c* release and apoptosis in monocytes [86], these results should be interpreted with caution since the depletion of intracellular GSH by buthionine sulphoximine (BSO), the specific and irreversible inhibitor of γ-glutamylcysteine synthetase, would also result in the production of ROS [87] and increase $DCFH_2$ oxidation. Furthermore, in view of the extremely high concentration of GSH in cells and its potent antioxidant activity, GSH depletion might also facilitate the formation of intracellular ROS.

A number of other substances might also directly interact with $DCFH_2$. For example, exogenous hemin and metalloporphyrins were able to rapidly oxidize $DCFH_2$ in a time- and dose-dependent manner in HeLa cells, a reaction that was independent of the generation of ROS [88]. Furthermore, the *Pseudomonas aeruginosa* secretory product, pyocyanin, the quinonoid anti-cancer drugs mitoxantrone and ametantrone and phenazine methosulphate all could directly oxidize $DCFH_2$ to DCF without a need for generation of ROS intermediates [89].

Biological reactivity and application

As a fluorescent probe for the respiratory burst

The respiratory burst (sometimes called the oxidative burst) describes a rapid release of ROS from immune cells (e.g. neutrophils and macrophages) as they come into contact with different bacteria or fungi. The respiratory burst plays an important role in killing pathogens that invade the human body. $DCFH_2$-DA has been used for decades for measuring

the respiratory burst in PMNL cells [35], human neutrophils [90], PMA stimulated alveolar macrophages and monocytes [91], leukocytes [92], bovine neutrophils [93], murine macrophage cell line, J774, human monocytic cell lines, Mono Mac 6 [94], Asian elephant (*Elephas maximus*) heterophils, Holstein cattle (*Bos taurus*) neutrophils [95] and hard clam (*Mercenaria mercenaria*) hemocytes [96].

The respiratory burst, as measured by $DCFH_2$-DA/$DCFH_2$, shows several distinct characteristics:

1) The kinetics of the respiratory burst in PMA-treated macrophages, when measured using $DCFH_2$-DA, show a slow and steady increase in fluorescence while the kinetic model of $DCFH_2$ under the same conditions is much more similar to that of cytochrome *c*, which shows a burst of fluorescence [64].
2) The overall sensitivity of $DCFH_2$ is much higher than that observed with $DCFH_2$-DA, but with higher background fluorescence [64].
3) Cytochrome *c* is a membrane impermeable molecule. Its reduction by $O_2^{\cdot-}$ can only measure extracellular oxidative stress, while the membrane permeable molecule nitroblue tetrazolium is generally applied to evaluate intracellular $O_2^{\cdot-}$. Compared with these probes, $DCFH_2$ oxidation occurs both intracellularly and extracellularly and could be oxidized by quite a few ROS species, which is one advantage of $DCFH_2$ as a respiratory burst probe [64].
4) $DCFH_2$, DHR and hydroethidine (HE) are presently considered to be fluorescence probes for detecting respiratory burst activity. Walrand et al. [97], in a comparison of these probes, showed that $DCFH_2$ (5 μM), DHR (1 μM) and hydroethidine (HE) (10 μM) exhibited the same increase in fluorescence intensity after activation of PMNs with PMA. However, $DCFH_2$ also reacts with reactive nitrogen species (RNS); while DHR is specifically responsive to H_2O_2 accumulation and HE seemed to be preferentially oxidized by $O_2^{\cdot-}$. Hence, the choice of probe to be used depends on the reactive species of interest.

As a fluorescent probe for ROS

In 1990 Swann and Acosta [98] applied $DCFH_2$-DA as a fluorescent probe for quantifying intracellular generation of ROS in primary cultured renal cortical epithelial cells. At present, increasingly more researchers prefer to use $DCFH_2$-DA/$DCFH_2$ as the fluorescent probe for ROS measurement, due to its non-discriminatory oxidation of many kinds of ROS. Actually, as discussed below, the probe could also be oxidized by RNS. Therefore, the ROS level measured by this probe might be over-estimated, since it is difficult to distinguish between the contribution of ROS and RNS in its oxidation process.

Recently, $DCFH_2$-DA/$DCFH_2$ was also used to determine the ROS production in specific organelles such as isolated mitochondria [67,99].

Reactivity with superoxide anion

$DCFH_2$ showed little or no reactivity with $O_2^{\cdot-}$. In the $O_2^{\cdot-}$ generating xanthine/XO system, $DCFH_2$ oxidation was inhibited by SOD, a potent $O_2^{\cdot-}$ scavenger [37,41]. Reduction of cytochrome *c* induced by potassium superoxide (KO_2) does not increase DCF fluorescence, suggesting that $O_2^{\cdot-}$ does not directly oxidize $DCFH_2$ [37]. A steady-state radiolysis of oxygenated formate solutions (a highly specific, well-quantified and controllable source of $O_2^{\cdot-}$) generated $O_2^{\cdot-}$ also shows very weak reactivity to $DCFH_2$ [82]. Chemically speaking, it is reasonable that $DCFH_2$ could not be easily oxidized by $O_2^{\cdot-}$ since, under standard state conditions, $O_2^{\cdot-}$ is a better reductant than oxidant with a half cell reduction potential of –330 mV [37].

Reactivity with H_2O_2

$DCFH_2$-DA/$DCFH_2$ is the most widely used fluorescent probe for H_2O_2 quantitative analysis in early studies. Keston and Brandt [46] first applied $DCFH_2$-DA to H_2O_2 measurement in the presence of HRP in cell-free systems, which could detect as little as 10 nM H_2O_2. Later, Black and Brandt [51] calculated that 1 M H_2O_2 generated 2 M DCF in cell-free systems, while others reported that 1 M H_2O_2 generated 1 M [77] or 5.3 M DCF [100]. The different catalytic efficiency between hematin and HRP might account for the observed yield difference for DCF. Furthermore, the mitochondrially derived ROS might also contribute to the oxidation of $DCFH_2$ and result in the high DCF yield rate in the latter study [67,99].

Now it is clear that H_2O_2 could not directly oxidize $DCFH_2$, while other biological substances, such as peroxidase, hematin, cytochrome *c*, free iron, haemoglobin and myoglobin, CAT, Cu/Zn SOD, XO, lipoxygenase and prostaglandin H synthase could all work as efficient catalysts [22].

Reactivity with ·OH

The oxidation of $DCFH_2$ by ·OH raises no doubt. However, the exact mechanisms by which this takes place remain unclear. LeBel et al. [41] found that for a Fe^{2+}/H_2O_2 system, ·OH scavengers such as DMSO, ethanol, mannitol and Tris could only

partially inhibit $DCFH_2$ oxidation. Furthermore, when $DCFH_2$ was used as a scavenger in non-chelated H_2O_2/Fe^{2+} systems (e.g. benzoate hydroxylation and deoxyribose oxidation), $DCFH_2$ moderately, but significantly, inhibited the oxidation of deoxyribose in a concentration-dependent manner, while benzoate hydroxylation was unaltered. These data suggested that free ·OH was not involved in the oxidation of $DCFH_2$, while the site-specific ·OH formed by the binding of $DCFH_2$ carboxyl group to Fe^{2+} can oxidize $DCFH_2$ [41]. However, subsequent studies revealed that Fe^{2+}/H_2O_2-induced formation of ·OH (indicated by the oxidation of salicylic acid to 2,3-dihydroxybenzoic acid) was proportional to $DCFH_2$ oxidation and could be inhibited by DMSO and mannitol, suggesting that ·OH might be responsible for the Fe^{2+}/H_2O_2-mediated oxidation of $DCFH_2$ [37]. Since salicylic acid contains the vicinal heteroatom needed for site-specific Fenton chemistry and the DMSO and mannitol concentrations used in the latter study were much higher than the former (32 mM vs 5 mM and 70 mM vs 5 mM for DMSO and mannitol, respectively), it is difficult to distinguish between the contribution of free ·OH or a hydroxyl radical intermediate in this process. In any case, DMSO has been observed to inhibit $DCFH_2$ oxidation in several kinds of cells or cell lines (see above). Recently, in another ·OH generating system, ·OH was shown to oxidize $DCFH_2$ with a high efficiency and the calculated rate constant was $1.3\times10^{10}M^{-1}s^{-1}$ [82].

Reactivity with $^1O_2$

UVA irradiation induced a dose-dependent increase in intracellular fluorescence in $DCFH_2$-DA loaded human bladder carcinoma line cells and keratinocytes. This was enhanced by elevation of intracellular levels of the photosensitizer protoporphyrin IX, a $^1O_2$ generator, suggesting the oxidation of $DCFH_2$ by $^1O_2$ [101]. In a cell-free system, the prevention of β-amyloid protein induced $DCFH_2$ oxidation by two $^1O_2$ scavenging species, sodium azide and His, also supports this reaction [66]. $DCFH_2$ was able to efficiently quench $^1O_2$. The calculated rate constants in methanol and in a methanol/D_2O mixture at pH 7.4 were $(1.4\pm0.2)\times10^8$ $M^{-1}s^{-1}$ and $(2.6\pm0.4)\times10^8$ $M^{-1}s^{-1}$, respectively. However, this process was physical in nature and did not contribute to fluorescence production. $DCFH_2$ is actually oxidized by secondary radicals derived from $^1O_2$ and not by $^1O_2$ itself; thus, it cannot be regarded as a specific probe for $^1O_2$. In addition, DCF appeared to be a weak $^1O_2$ photogenerator in both aqueous and organic phases [32]. However, a more recently paper [102] has revealed that $DCFH_2$ can be directly oxidized by $^1O_2$ and the photoinduced methylene blue di-cation-radical ($MB^{\bullet 2+}$) and that the efficiency of the reaction of $DCFH_2$ with $^1O_2$ was greater than that with $MB^{\bullet 2+}$, suggesting that $^1O_2$ reacted with $DCFH_2$ and contributed to the formation of DCF.

Reactivity with NO

Some researchers have attempted to use $DCFH_2$-DA/$DCFH_2$ for analysis of NO production in human neutrophils [103], neuronal cells [69] and cerebellar granule cells [104]. Rao et al. [103] suggested the use of a $DCFH_2$ assay for measurement of NO in those cells where production of $O_2^{\cdot-}$ was non-existent or minimal (such as neural or endothelial cells) and in conjunction with W-13 in those cells that produced $O_2^{\cdot-}$. Gunasekar et al. [69] reported that at least 50% of the oxidation of $DCFH_2$ in cerebellar granule cells stimulated by glutamate was attributed to intracellular generation of NO and recommended the use of Hb or SOD when $DCFH_2$ was employed to determine intracellular NO concentrations. Gabriel et al. [104] differentiated $DCFH_2$ oxidation derived from NO and ROS by using NO scavengers or NOS inhibitors. Imrich et al. [105] investigated the utility of $DCFH_2$ for measurement of NOS activity in rat alveolar macrophages activated by IFN-γ.

The key experimental support for use of $DCFH_2$ as a NO probe is the observation that pure NO is capable of oxidizing $DCFH_2$ in solution [103]. However, subsequent studies have revealed that the oxidative effect was not due to NO directly but either to NO_2 and/or possibly N_2O_3 under some non-physiological conditions [22]. NO_2 is a known contaminant of NO gas and could either have been introduced via continuous bubbling or was formed by reaction of NO with traces of oxygen [106].

Apart from its non-specificity, the low sensitivity (the detection limits of NO for cell-free system and cell system were 8 μM and 16 μM, respectively [69]) strongly excludes the usefulness of $DCFH_2$ for real-time NO measurement, taking into account the relatively low levels of NO (~10 nM) under normal physiological conditions.

Reactivity with peroxynitrite

Peroxynitrite ($ONOO^-$), a highly reactive oxidant that is generated through the spontaneous reaction of NO and $O_2^{\cdot-}$, is a potent and short-lived species that promotes oxidative damage and which has attracted much attention by biological researchers recently. However, the qualitative and quantitative measurement of $ONOO^-$ in biological systems has been an ongoing challenge for the past decade [107]. DHR was assumed as a sensitive and efficient fluorescent probe for $ONOO^-$ production in cell free systems [108]. Due to its structural similarity, some researchers explored the possibility of using $DCFH_2$ as a probe for $ONOO^-$.

Table III. Comparison of oxidation of $DCFH_2$ and DHR by $ONOO^-$.

	$DCFH_2$	DHR
UV spectra of oxidized compounds	DCF A=500 nm ε=59,500 m^{-1} cm^{-1} [106]	Rhodamine (Rho) A=500 nm ε =78800 M^{-1} cm^{-1} [106]
Efficiency	38% (pH7.4) [106] 48–60% (pH8.5) [110]	44% (pH7.4)[106] 43–51% (pH8.5) [110]
Maximal molar yields	0.54 ± 0.06 MDCF/M (pH8.5) [110] 0.37+0.02 MDCF/M [109]	0.47 ± 0.04 M Rho/M (pH8.5) [110]
Speed	$DCFH_2$=DHR[1] [26]	
Fluorescence intensity	$DCFH_2$ >>DHR[2] [26]	
Effect of SIN-1	linear rates of oxidation 0.81 mM DCF /min [106]	linear rates of oxidation 0.97 mM Rho/min [106]
Effect of CAT	Abolished the HPR/H_2O_2- mediated oxidation 0.86 mM/min [106]	Abolished the HPR/H_2O_2-mediated oxidation 0.31 mM/min [106]
Effect of NO	not quench $DCFH_2$ oxidation [106]	quenching DHR oxidation [106]
Reactivity to Hypocblorous acid	8-fold more efficient at oxidizing DHR than $DCFH_2$ [106]	
Effect of L-cysteine	$DCFH_2$ = DHR[3] [109]	
Effect of urate	$DCFH_2$<<DHR[4] [109]	

1. 2. Comparison of the $ONOO^-$ mediated fluorescence increase of $DCFH_2$ with that of DHR revealed that the rate of oxidation by $ONOO^-$ was similar for both compounds, but that $DCFH_2$ displayed a 4-fold greater increase in fluorescence intensity.
3. L-cysteine shows similar inhibitory effect on $ONOO^-$ mediated oxidation of $DCFH_2$ and DHR.
4. Urate was much less efficient at inhibiting peroxynitrite-mediated oxidation of $DCFH_2$ compared to efficient inhibition of peroxynitrite-mediated oxidation of DHR.

In a cell-free system, $ONOO^-$ mediated oxidation of $DCFH_2$ is much more rapid and efficient than oxidation by H_2O_2, NO or $O_2^{\cdot-}$. $ONOO^-$ mediated oxidation of $DCFH_2$ reached a steady state after 2 min, whereas H_2O_2 and NO continued to oxidize $DCFH_2$ for at least 10 min, implying that $DCFH_2$ is much more sensitive to oxidation by $ONOO^-$ than by H_2O_2 and NO [26]. These results demonstrated that $DCFH_2$ might be an excellent indicator of $ONOO^-$ formation in living cells [26]. Simultaneous generation of NO and $O_2^{\cdot-}$ induced the oxidation of $DCFH_2$ to DCF, while NO alone under aerobic conditions could not [106,109]. Furthermore, it is clear now that both $O_2^{\cdot-}$ and NO are released in the decay of SIN-1 and that $ONOO^-$ is the primary oxidant formed from SIN-1 decomposition [106].

In vitro oxidation of $DCFH_2$ mediated by $ONOO^-$ was not inhibited by the •OH scavengers mannitol or DMSO and was not dependent upon metal ion-catalysed reactions. $DCFH_2$-DA was not susceptible to oxidation by $ONOO^-$ [109].

$ONOO^-$ could readily oxidize both $DCFH_2$ and DHR, but their reactivity with $ONOO^-$ *in vitro* showed different efficiencies [106] (Table III). Glebska and Koppenol [110] demonstrated that these processes were zero-order between pH 3–10 and that the yield of DCF and rhodamine was as a function of pH (significantly increased at pH values>). Kooy et al. [109] reported that chemically synthesized $ONOO^-$ induced the oxidation of $DCFH_2$ to DCF in a linear fashion, with a calculated molar yield of 0.37±0.02 mole DCF formed per mole $ONOO^-$ at saturating $DCFH_2$.

The details of the mechanism of $DCFH_2$ oxidation by $ONOO^-$ remain largely unknown. Radi et al. [107] presumed that $ONOO^-$ itself may mediate the oxidation of $DCFH_2$, while Glebska and Koppenol [110] concluded that fluorescent probes like DHR and $DCFH_2$ were less suitable for reporting $ONOO^-$ formation *in vivo* and were not specific detectors of $ONOO^-$. Under limiting concentrations of $ONOO^-$, over the range of 1.1–10 μM of $DCFH_2$, the oxidation rate of $DCFH_2$ by $ONOO^-$ was independent of the initial concentration of $ONOO^-$, which suggested that $DCFH_2$ was not a $ONOO^-$ scavenger. Furthermore, the reaction of $DCFH_2$ with $ONOO^-$ was zero-order; thus, there could be no direct reaction between $ONOO^-$ and $DCFH_2$. In addition, the increase in yield of oxidized products as a function of pH indicates that the $ONOO^-$ anion might arise from an adduct with the indicator, followed by protonation and oxidation of the indicator. Nitrogen dioxide and trioxocarbonate were determined as the reactive species that oxidized $DCFH_2$ and DHR.

Recently, Yang and colleagues synthesized several HK derivatives as fluorescent probes for hypochlorous acid [111] and peroxynitrite [112,113]. Among these, one has been designated as HKGreen-1 and possesses a ketone unit linked to a DCF moiety through an aryl ether linkage (Figure 1). NO, $^1O_2$, $O_2^{\cdot-}$, ·OH, ROO· and ·OCl show little reactivity with HKGreen-1 compared with $ONOO^-$ in cell free systems. Primary cultured neuronal cell results showed that HKGreen-1 was a highly selective fluorescent probe for the detection of $ONOO^-$ in living cells [112].

Other biological applications

In early studies, $DCFH_2$-DA/$DCFH_2$ was also used to determine the monoamine oxidase activity [99], serum uric acid and glucose concentration [114] and to identify spermine in seminal stains

[115]. However, these early applications nearly all, without exception, utilize the reaction of $DCFH_2$ with H_2O_2. Due to the development of new technology, these analytical methods now rarely appear in publications.

Methodological considerations

Before applying $DCFH_2$-DA/$DCFH_2$ to biological systems, several practical guidelines and tips need to be emphasized in every step of the analytical process:

1) Several techniques have been employed for $DCFH_2$-DA/$DCFH_2$ assay, including flow cytometry [35,61,92], spectrofluorometry [46,51,67,105,116], fluorescence microscopy [26,44,67,117], HPLC [26,34,52] and confocal laser scanning microscopy [118,119]. Proper technique selection is important; for example, to determine the intracellular ROS, flow cytometry might be the best choice.
2) Stock solutions of $DCFH_2$-DA/$DCFH_2$ should be made in a light-protected vessel that is purged with nitrogen or helium and the deacetylation of $DCFH_2$-DA should be performed in a light-protected vial. Working solutions of $DCFH_2$ must be stored in light-protected vessels and kept on ice and adjusted to biological pH [27]. We strongly recommend that the working solution be prepared fresh and used only once.
3) Bear in mind that the assay buffer and culture media, such as HEPES buffer, medium L-15, Dulbecco's Modified Eagle's Medium and Medium 199, have potential influences on $DCFH_2$-DA or $DCFH_2$ oxidation [64,120].
4) Generally, 10 μM of $DCFH_2$-DA or $DCFH_2$ are added to the cell media and incubation is carried out for 1 h. However, considering the strong competition between $DCFH_2$ and intracellular antioxidants, a higher concentration might be recommended to establish appropriate intracellular concentration.
5) Although typically only 15 min is needed to achieve a stable intracellular concentration when $DCFH_2$-DA is chosen as the probe in endothelial cells [29], the incubation time in other cells should be somewhat longer since too brief a loading time may limit the availability of $DCFH_2$, resulting in an underestimation of ROS levels [64]. Furthermore, the influence of esterase activity in the assay system should be estimated [121]; this has been overlooked by most researchers.
6) When the fluorescence is observed with a microscope, the exposure time and light intensity should be strictly controlled to minimize the self-amplification of the fluorescence signal [33].
7) Interpretation of results obtained from cellular systems is much more difficult than for cell-free systems. As discussed above, oxidation of $DCFH_2$ is non-specific, which means several or even dozens of antioxidants could compete with it for reaction with ROS. Whether $DCFH_2$ could react with ROS, or to what extent it could be oxidized by ROS, would be largely determined by the rate constants, the relative intracellular concentrations of $DCFH_2$ and other antioxidant concentrations. Since nearly all kinds of ROS are very active and have very short half-lives (less than few seconds) and are present at low concentrations (pM~nM under physiological conditions), their bioavailability should also be considered. Although the $DCFH_2$ levels might vary widely with cell type, taking into account the initial incubation concentration, esterase activity and subsequent leakage, the intracellular concentration may be less than ~mM level [29,35,82], while some endogenous antioxidants such as GSH, ascorbate and urate are nearly or above mM level. Therefore, competitive effects of endogenous antioxidants could occur [82], which means that the intracellular ROS production will be under-estimated. This type of methodological inaccuracy and these technical pitfalls should be borne in mind, to avoid artifactual conclusions.

Conclusion

In conclusion, although there are still many controversies, especially about its specificity, $DCFH_2$-DA/$DCFH_2$ are useful probes for oxidative study in cell-free and biological systems. However, the oxidation of $DCFH_2$ is non-specific and is in competition with endogenous antioxidants. It might be used as a probe for a specific kind of ROS under certain conditions, but is more suitable as a marker for the total ROS production. Hence, Tarpey and Fridovich's [19] remark hits the nail on the head: 'Because of the multiple pathway that can lead to DCF fluorescence and inherent uncertainty relating to endogenous versus artifactual oxidant generation ,this assay may best be applied as a qualitative marker of cellular oxidant stress, rather than a precise indicator of rates of H_2O_2 formation'.

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