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Review

Concerns in the application of fluorescent probes DCDHF-DA, DHR 123 and DHE to measure reactive oxygen species *in vitro*

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ABSTRACT

Reactive oxygen species (ROS) are formed in biological systems by partial reduction of molecular oxygen. The essential role of ROS in maintaining physiological health may be corrupted into oxidative stress by their over-production or the exhaustion of antioxidant mechanisms. Many studies covering a broad range of methodologies have investigated ROS production and their toxic mechanisms of action. Of these methodologies, fluorometry has been among the preferred techniques. Three frequently used fluorescent probes for *in vitro* studies are 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA), Dihydrorhodamine 123 (DHR 123) and Dihydroethidium (DHE). Apart from the unavoidable limitations of auto-oxidation, photo-oxidation and photo-conversion, there are also concerns relating to protocol modification for the improved monitoring of ROS. This paper aims to highlight such contributing factors, including cell culture conditions and the characteristics of individual fluorescent probes in the utilization of these selected probes in *in vitro* systems.

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1. Introduction

Reactive oxygen species (ROS) may be formed by partial reduction of molecular oxygen through endogenous (physiological) or exogenous (environmental) oxidative processes in biological systems. ROS represent a broad category of molecules consisting of both radical and non-radical species. The former includes superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (HO•), peroxyl radical (RO_2•), alkoxyl radical (RO•) and hydroperoxyl radical (HO_2•). The non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen and peroxynitrite ($ONOO^-$). $ONOO^-$ can also be categorized as a reactive nitrogen species (RNS) (Gomes et al., 2005; Halliwell and Gutteridge, 2007; Sorg, 2004).

Historically, the fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA), Dihydrorhodamine 123 (DHR 123) and Dihydroethidium (Hydroethidine/HET; DHE) were often employed by researchers for monitoring the formation of ROS in cells. Keston and Brandt in 1965 were the first to use DCDHF-DA for measuring hydrogen peroxide fluorometricly in the presence of horseradish peroxidase (HRP) (Crow, 1997; Keston and Brandt, 1965). Subsequently, it became a widely used fluorophore to detect intracellular ROS in various cell-types. To the confusion of many researchers, DCDHF-DA has been referred to by many names including DCDHF-DA, DCFH-DA, DCHF-DA, $DCFH_2$-DA and H_2DCFDA (Kweon et al., 2001; Chen et al., 2010). A chloromethyl derivative of this probe, known as CM-H_2DCFD, has been developed for more precise monitoring of intracellular ROS as DCDHF-DA tends to leak out after diffusing into cells (Haugland, 1996; Kweon et al., 2001). DHR 123 was first presented in the late 1980s. It is an analog of DCDHF-DA and differs to that compound in its lack of

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diacetate (DA) and dichloro substituents of DCDHF-DA. In addition, the two hydroxyl groups of DCDHF-DA have been replaced by amino groups (Crow, 1997). DHE was first used by Gallop et al. (1984) as a redox probe. The ability of DHE to freely permeate cell membranes has resulted its wide use for monitoring ROS (Gomes et al., 2005).

Unavoidable limitations such as auto-oxidation, photo-oxidation, photo-bleaching, photo-conversion, cytotoxicity and non-specificity may interfere with the formation of fluorescence and thus the detection of ROS (Chen et al., 2010; Gomes et al., 2005). Nevertheless, better results should be possible if technical concerns in their application are taken into account. The aim of this review is to summarize some of the contributing factors, including cell culture conditions and fluorescent probe characteristics in the utilization of DCDHF-DA or its derivatives (so-called fluorescein-based probes), DHR 123 and DHE in *in vitro* systems.

2. Formation of fluorescent products

Theoretically, a photon of excitation light is absorbed by an electron of a fluorescent molecule, a so-called fluorophore, raising the energy level of the electron to an excited state. This excitation period is short and the electron returns to the ground state through the dissipation of excitation energy *via* molecular collisions or transference to a proximal molecule, and then the emission of the remaining energy as a photon (Jameson et al., 2003).

DCDHF-DA, a membrane permeable dye, passively diffuses into cells, where its acetate groups are actively cleaved by esterases to form 2′,7′-dichlorodihydrofluorescein (DCDHF). It is then oxidized by free radical compounds to the fluorescent 2′,7′-dichlorofluorescein (DCF) (Hempel et al., 1999). DHR 123, a lipophilic non-fluorescent molecule, readily diffuses across cell membranes and is efficiently oxidized by free radicals to form a fluorescent cationic and lipophilic probe rhodamine 123 (Rh 123) (Gomes et al., 2005; Kweon et al., 2001). In the case of DHE, the probe undergoes a two-electron oxidation by free radicals inside the cell resulting in the formation of ethidium (E^+), a fluorescent compound (Fig. 1). It is believed that its fluorescence increases after binding to DNA, resulting in red fluorescence (Gomes et al., 2005).

3. Cell culture conditions

Cell numbers vary from experiment to experiment and this can influence fluorometer-based assays such as oxidative stress evaluation. Cell culture densities may also fluctuate as a result either natural cell death or cell death induced by stressors, so researchers should be mindful of this when measuring ROS. There are some contradictory findings regarding links between these two parameters (Maeda et al., 1993; Saeki et al., 1997; Yasaka et al., 1996; Yasui et al., 2000). In one example, Long et al. (2003) challenged the role of oxidative stress and apoptosis in three different densities of mouse fibroblast cell lines. The results of their study revealed that spontaneous apoptosis increased with increasing cell-density, whereas H_2O_2-induced apoptosis was greater at lower densities. In contrast, Li et al. (1999) reported a linearly increasing rate of HO· production with increasing cell density. The authors investigated their hypothesis in a series of mouse epidermal cell lines exposed to the anti-cancer compound diaziquone (AZQ). Such a controversy has been linked to the use of different cell types (Long et al., 2003). The expression of ROS formation in connection with cell density can therefore be obtained by normalizing the measured ROS on the number of viable cells (Bopp et al., 2008). In addition, a preliminary study to determine the optimal cell number with regard to density-dependence of cell death and degree of yielded fluorescence linked to oxidative stress would be useful.

Fluorescent probes have been used to detect ROS in both basic systems for growing cells in suspension (non-adherent) and adherent cultures (Hempel et al., 1999; Misra and Niyogi, 2009; Oya et al., 1986; Pourahmad and O'Brien, 2000). The free-floating cells in suspension culture facilitate the immediate application of probes; while cells in adherent culture require an incubation time ranging from a few hours up to a day. This time gap allows the seeded cells to recover and attach to coated cell/tissue culture plates with an artificial substrate such as polyornithine, laminin, Poly-L-lysine, fibronectin or matrigel (Hempel et al., 1999; Hynes et al., 2006; Long et al., 2003; Nat et al., 2007; Nawaz et al., 2005b; Radice et al., 2004). This time interval in adherent cultures also helps the cells to achieve some degree of equilibrium with the *in vitro* condition (Dickson, 1971). For example, time series studies evaluating some cellular parameters in adherent primary culture of trout hepatocytes have shown changes (Ferraris et al., 2002; Yazdani et al., 2015). In addition, the resultant suspension culture from tissue perfusion procedure appeared to be a mixture of cell types including red blood cells (Bonney et al., 1974; Dickson, 1971). For removing red blood cells, nonadherent tissue cells and debris, changing the medium or washing the culture with buffers has been suggested (Hempel et al., 1999; Manzl et al., 2004).

4. Fluorescent probe characteristics and influencing factors

DCDHF-DA and its derivatives, DHR 123 and DHE have routinely been used in *in vitro* studies and some authors claim that they specifically detect intracellular hydrogen peroxide, peroxynitrite and superoxide, respectively (Bindokas et al., 1996; Bindokas et al., 2003; Kooy et al., 1994; Rico et al., 2009; Roy et al., 2009). In contrast, the non-specificity of some fluorescent probes including DCDHF-DA (HO•, H_2O_2, $RO_2^•$), DHR 123 (H_2O_2/HRP, HOCl) and DHE ($O_2^{•-}$, H_2O_2, $ONOO^-$, HOCl) has been well reviewed in *e.g.* Gomes et al. (2005), Tarpey and Fridovich (2001) and Wardman (2007).

In the fluorescent labeling procedure, one must initially determine the concentration-response relationship between the probe and the fluorescent signal (Braut-Boucher and Aubery, 2010). Manufacturers' protocols (*e.g.* Invitrogen Life Technologies) have suggested an empirical determined final working concentration of dyes of ~1–10 μM in a simple physiological buffer such as phosphate buffered saline (PBS), Hank's buffered saline solution (HBSS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) rather than a complex media for cell culture. In order to gain a stronger fluorescent signal, the application of minimal loading dose is recommended. The intracellular environment can be disturbed at high concentrations because the probes can work as either antioxidants or prooxidants. In addition, leakage and quenching can also occur at higher probe concentrations (Braut-Boucher and Aubery, 2010; Zuo and Clanton, 2002). Royall and Ischiropoulos (1993) reported >90% loss of intracellular probes in endothelial cell cultures loaded with 11 μM DCDHF-DA, 11 μM DCF or 5 μM DHR 123 for 1 h, followed by 1 hour exposure to probe-free medium. In the same study, intracellular Rh 123 decreased by only 15%. High concentrations of DHE have been linked with the formation of superoxide-independent fluorescence *via* saturation of mitochondrial nucleic acid binding sites by derived E^+, facilitating its bonding to nuclear DNA (Budd et al., 1997).

Fluorescent probes are readily available as premade solutions, but some researchers may prefer to use the powder forms. In these cases, the choice of solvent requires the careful consideration of its properties. Water is poor solvent of acetylated dye powders and thus requires the initial use of high-grade solvents such as dimethyl sulfoxide (DMSO), dimethylform-amide (DMF) or 100% ethanol prior to empirical work. The dissolved DCDHF-DA and DHR 123 are transparent solutions which, after purging with N_2, is stored in the dark at −20 °C to prevent auto-oxidation. Contrastingly, a freshly prepared DHE fluorescent probe possesses a light pink color, which darkens as it ages, eventually turning red due to auto-oxidation. This can therefore affect the sensitivity of assay through increased background fluorescence (Zuo and Clanton, 2002).