On the use of fluorescence probes for detecting reactive oxygen and nitrogen species associated with photodynamic therapy

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Abstract. Fluorescent probes are frequently employed for the detection of different reactive oxygen and nitrogen species formed during the irradiation of photosensitized cells and tissues. Investigators often interpret the results in terms of information provided with the different probes without examining specificity or determinants of fluorogenic reactions. We examine five fluorescent probes in a cell-free system: reduced 2′,7′-dichlorofluorescein, dihydroethidine, dihydrorhodamine, 3′-(*p* aminophenyl) fluorescein (APF), and 4′,5′-diaminofluorescein. Of these, only APF demonstrates a high degree of specificity for a single reactive species. There is a substantial influence of peroxidase activity on all fluorogenic interactions. The fluorescence of the photosensitizing agent also must be taken into account in evaluating results. [DOI: 10.1117/1.3484258]

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The literature contains numerous reports on the use of fluorescent probes for detection of reactive oxygen (ROS) and nitrogen (RNS) species. Invitrogen/Molecular Probes provides a collection of such probes for which the only indication of specificity is provided by the reproduction of a table derived from Ref. 1. This compares the fluorescence of two new fluorescein analogs, APF and 3′-(p-hydroxyphenyl) fluorescein (HPF), with the fluorescence of DCF in a cell-free system containing different reagents.

In the present study, we examined fluorogenic reactions associated with the exposure of five fluorogenic probes to reactive oxygen and nitrogen species, also in a cell-free system, following procedures reported in Ref. 1. The species examined were $\cdot O_2^-$ [100 μM KO_2 in anhydrous DMSO], H_2O_2 (100 μM), and ·OH [formed from 20 μM $Fe(NH_4)SO_4$ +100 μM H_2O_2]. Fluorogenic effects of reactive nitrogen species were estimated using diethylamine nitric oxide [DEANO, 100 μM]. In aqueous media, NO is released from this compound at pH 7. NO is then spontaneously oxidized to the nitrosonium cation.

Tests were carried out using 5 μM concentrations of each probe in 3 ml of HEPES buffer pH 7. Fluorescence was measured 30 min after addition of the reagents specified above. Fluorescence excitation was provided by a 100-W quartz-halogen lamp with the wavelength selected by a monochromator. The fluorescence signal was monitored using an Instaspec IV (Oriel Corp, Stratford, Connecticut) CCD system. Excitation wavelengths were 485 nm [4′,5′-diaminofluorescein (DAF)], 500 nm [2′,7′-dichlorofluorescein (DCF) and dihydroethidine (DHE)], and 490 nm [dihydrorhodamine (DHR) and 3′ (*p*-aminophenyl) fluorescein (APF)]. The fluorescence intensity at the emission optimum was recorded. All probes were obtained from Invitrogen/Molecular Probes, Eugene, Oregon, except for DEANO (Cayman Chemical Co., Ann Arbor, Michigan). Horseradish peroxide (HRP, 50 μg/ml) was present where specified. In studies involving DHE, DNA (50 μg/ml) was added since the long-wavelength fluorescence signal depends on binding of the oxidation product(s) to DNA.

Results are summarized in Table 1. The fluorogenic response by DCF was elicited by $\cdot OH \gg H_2O_2 > \cdot O_2^-$, with enhanced promotion when peroxidase was present. DHE and DHR also responded to these ROS, but no substantial degree of selectivity for any ROS was observed. It has been reported that DHE can be selective for $\cdot O_2^-$ detection if fluorescence (in the presence of DNA) is monitored[2] at 570 nm, but this probe cannot be used for an unambiguous detection of superoxide without an HPLC analysis of products.[3] The presence of HRP also led to a strong promotion of probe fluorescence. HRP can promote probe oxidation by a variety of mechanisms including by direct interactions and via conversion of H_2O_2 to $\cdot O_2^-$ and ·OH.[4,5] While APF was selective for ·OH, especially in the presence of HPR, we have reported[6] that this probe can also detect $^1O_2$ to a greater extent than was suggested by Ref. 1. DAF was converted to a fluorescent product $NO > \cdot OH \gg H_2O_2$ and $\cdot O_2^-$; there was also an increase in fluorescence when HRP was present. It has been reported[1] that APF can

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Table 1 Probe:ROS interactions.

	H_2O_2		•OH		$•O_2^-$		NO	
Probe	–HRP	+HRP	–HRP	+HRP	–HRP	+HRP	–HRP	+HRP
DHE	170	1882	3802	3906	1372	4241	104	124
DHR	424	6474	3571	12327	51	504	1776	1726
DCF	775	2768	10122	23966	427	1806	739	4115
APF	260	1030	5960	6945	42	62	51	210
DAF	226	3288	805	7375	104	1422	2430	5434

Fluorogenic interactions between selected fluorescence probes (5 μM) and reactive oxygen or nitrogen species generated as defined in the text. Numbers represent the mean fluorescence emission intensity on excitation at 490 to 510 nm. In four replicate determinations, the variation was less than ±3% of the values shown.

readily detect peroxinitrite ion ($ONOO^-$). The lack of response of APF to NO shown in Table 1 indicates that this species is not being produced during release of NO from the diethylamine derivative.

While the studies reported in the table do not provide unambiguous information on fluorescence yields, i.e., fluorescence per mole of ROS or RNS, they do provide a comparison of the relative sensitivity of each probe to a given species, along with information on effects of peroxidases. In an attempt to improve specificity, Xu et al.[7] has described a naphthofluorescein derivative that emits fluorescence at 670 nm on exposure to $•O_2^-$. It might be preferable to prepare this agent starting with 2′,7′-difluorofluorescein, since naphthofluoresceins are nonpolar and are difficult to work within aqueous environments. Moreover, fluorescence emission from naphthofluorescein is highly pH dependent, with a pKa of ~7.5. This will complicate fluorescence measurements, especially if the probe accumulates in subcellular regions of low pH.

Maeda et al.[8] described another potentially useful probe for $•O_2^-$ with only a minor response to •OH. This reagent is based on a nitrobenzenesulfonyl ester structure that can be cleaved by—SH reagents. This is noted in the report, but in a critical test, only a 50 μM concentration was used; this is perhaps 1% of the expected intracellular GSH concentration.

These examples illustrate the problems associated with attempts to translate results obtained in cell-free systems into corresponding procedures in cell culture. Other commonly encountered problems may relate to ability of fluorescent probes to penetrate the plasma membrane, spontaneous oxidations, pH of subcellular compartments, and the presence of fluorogenic enzymes, e.g., peroxidases. We propose that if a fluorogenic ROS or RNS probe cannot clearly delineate among different reactive species in a cell-free system, using such a probe to draw conclusions concerning the appearance of such species in culture system may be unrealistic.

As a further example of difficulties in interpretation of data obtained with fluorescent probes, we reported that the Bc1-2 antagonist HA14-1 promoted the apoptotic response to photodynamic therapy[9] (PDT). When a report[10] appeared indicating that HA14-1 could cause the spontaneous production of ROS, we considered that the latter effect might explain, at least in part, the synergistic effect. The ability of HA14-1 to evoke formation of ROS was based on studies[10] involving DCF. The fluorescence observed when HA14-1 was added to cell cultures was actually derived from a fluorogenic reaction between HA14-1 and serum albumin that mimicked[11] the excitation and emission properties of DCF.

An additional consideration in the use of fluorescent probes in the context of PDT is illustrated by Fig. 1. Murine leukemia P388 cells were incubated in medium containing 2 μM benzoporphyrin derivative (Verteporfin, BPD) for 60 min, with a 5 μM concentration of the RNS probe DAF added during the final 30 min. The cells were then resuspended in fresh medium and irradiated at 690 nm (90 mJ/cm^2), conditions we have found capable of killing 50% of the cell population. Fluorescence microscopy was used to assess the resulting fluorogenic interactions using 450- to 490-nm excitation and monitoring fluorescence at 500 to 550 or at 500 to 700 nm. Experimental conditions included probe alone [Figs. 1(a)–1(c)], probe+photosensitizer in the dark [Figs. 1(d)–1(f)], and after irradiation [Figs. 1(g)–1(i)].

Images obtained with broadband (500- to 700-nm) acquisition indicated a substantial fluorogenic response when BPD was present, but this occurred whether or not the photosensitized cells were irradiated [compare Figs. 1(e) and 1(h)]. The

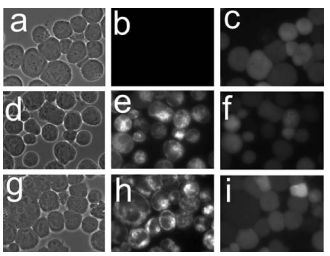

Fig. 1 Phase contrast and fluorescence images of murine leukemia P388 cells incubated with BPD+DAF and irradiated as described in the text: (a) to (c) control cells containing only DAF, (d) to (f) cells containing DAF and BPD but not irradiated, and (g) to (i) irradiated cells loaded with DAF+BPD. Note (a), (d), and (g) are phase contrast images; (b), (e), and (h) are fluorescence images acquired at 500 to 700 nm for 100 ms; and (c), (f), and (i) are fluorescence at 500 to 550 nm acquired for 2000 ms.

fluorescence signal appeared to derive from mitochondria, the site where BPD is localized.[12] These results illustrate the fact that photosensitizing agents also fluoresce, so that care must be taken to exclude such fluorescence from the probe detection parameters. Fluorescence images obtained with narrowband fluorescence acquisition [Figs. 1(c), 1(f), and 1(i)] revealed that there was no significant fluorogenic response by DAF, hence no significant formation of RNS on irradiation. The relative intensities of the fluorescence can be estimated by the time needed for image acquisition: 100 ms for Figs. 1(b), 1(e), and 1(h) and 2000 ms for Figs. 1(c), 1(f), and 1(i).

Based on studies in a cell-free system, together with additional experiments that will be reported elsewhere, we conclude that DHR can be used to distinguish H_2O_2 from $\cdot O_2^-$, but that peroxidase activity or presence of ·OH can complicate interpretation of results. A prior report had arrived at a similar conclusion.[13] DHE is indeed more responsive to $\cdot O_2^-$ than to H_2O_2, but can be oxidized[3] by other ROS.

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