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Highlight Article

Measurement of Singlet-Oxygen *In Vivo*: Progress and Pitfalls

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ABSTRACT

This article is a highlight of the paper by Jarvi *et al.* in this issue of Photochemistry and Photobiology as well as a brief overview of the state of the field of singlet-oxygen ($^1O_2$) detection *in vivo*. The *in vivo* detection of $^1O_2$ using its characteristic 1270 nm phosphorescence is technically challenging. Nevertheless, substantial progress has been made in this area. Major advances have included the commercial development of photomultiplier tubes sensitive to 1270 nm light, techniques for spatially resolving the location of $^1O_2$ at a subcellular level and more complex mathematical models for interpreting the kinetics of $^1O_2$ emission from living cells. It is now recognized that oxygen consumption, photosensitizer bleaching, oxidation of biological molecules and diffusion of $^1O_2$ can significantly change the kinetics of $^1O_2$ emission from living cells.

INTRODUCTION

This article is a highlight of the paper by Jarvi *et al.* in this issue of Photochemistry and Photobiology as well as a brief overview of the state of the field of singlet-oxygen ($^1O_2$) detection *in vivo* (1). Singlet-oxygen is believed to be the major mediator of the phototoxic effects of a wide variety of photosensitizers. These include phototoxic natural products, such as hypericin and synthetic photosensitizers, such as Photofrin II® and verteporfin, that have been developed for clinical use in photodynamic therapy (2,3). For this reason, major efforts have been made to develop assays for $^1O_2$ generated within cultured cells and within intact living organisms (1,4–12). For the biological scientist, quantitative assays for $^1O_2$ generation *in vivo* are powerful tools for studying the complex interactions of $^1O_2$ with cells. For the clinician, these assays may ultimately provide more accurate dosimetry for photodynamic therapy (1,4,5,7,9).

The measurement of time-resolved $^1O_2$ phosphorescence at 1270 nm is probably the most common used technique for the measurement of $^1O_2$ *in vivo* (1,4–8,10–12). Other assays, such as the measurement of photosensitizer bleaching, have been reported to be useful for the detection of $^1O_2$ *in vivo*, but these assays will not be discussed in this very brief review (9).

When used as an *in vivo* assay, the measurement of phosphorescence at 1270 nm has several advantages. First, the assay is not invasive. There is no need to add a $^1O_2$ trap that might interfere with the complex biochemistry of the cell. Second, the kinetics of $^1O_2$ emission may provide information about the photosensitizer triplet lifetime and about the $^1O_2$ lifetime within the cell (1,11,12). Third, the assay provides information in real time. This is particularly important when the assay is to be used for adjusting the fluence of light in photodynamic therapy.

Unfortunately, the measurement of $^1O_2$ phosphorescence from living cells has proven to be a technically challenging problem for two reasons. First, the $^1O_2$ emission is very weak. The low quantum yield for $^1O_2$ phosphorescence is the result of the combination of a small radiative rate for $^1O_2$ in aqueous environments and a very short lifetime for $^1O_2$ within the cell (1,12–15). The radiative rate for $^1O_2$ in water is roughly 0.11 s^{-1}, while the lifetime of $^1O_2$ within the cell is probably 0.5 μs or less (1,12–15). This would put the quantum yield for $^1O_2$ phosphorescence at no more than 5×10^{-8}. Second, it is necessary to separate the $^1O_2$ emission from other sources of light emission (1). These sources include light scattering, fluorescence from optical elements, photosensitizer fluorescence, photosensitizer phosphorescence and delayed photosensitizer fluorescence. The $^1O_2$ emission is so weak that for many experimental conditions, spectral resolution alone has not been sufficient to isolate the $^1O_2$ emission from other sources of luminescence. Consequently, many *in vivo* studies of $^1O_2$ have used time resolution in addition to spectral resolution to isolate the $^1O_2$ phosphorescence. Time resolution will often distinguish the $^1O_2$ emission from fluorescence and light scattering, but may not separate the $^1O_2$ emission from the photosensitizer phosphorescence and from delayed fluorescence.

INSTRUMENTATION

Photomultiplier tubes sensitive to near-infrared light

The commercial development of photomultiplier tubes (PMTs) that are sensitive to 1270 nm radiation has been a major

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advance (16). These PMTs have a fast response time compared to $^1O_2$ kinetics, even within cells, and have enough internal gain to be used with photon-counting electronics. Prior to the availability of these PMTs, time-resolved studies were carried out using germanium or indium–gallium–arsenide photodiodes coupled to analog amplifiers (4–6). With photodiodes operated in an analog mode, there was always a tradeoff between sensitivity and frequency response. Since time-resolved *in vivo* detection of $^1O_2$ emission requires both high speed and high sensitivity, this task was difficult with photodiode-based detection systems (4–6). Unfortunately, current near-infrared PMTs suffer from low quantum efficiency (*ca* 2%) and high backgrounds, even when appropriately cooled (16). Thus, there is still considerable room for improvement in the performance of near-infrared light detectors.

Singlet-oxygen microscopy

A second major advance in instrumentation came from Ogilby's group (8,10,11). These investigators developed methods for imaging $^1O_2$ on a subcellular level. Two systems have been developed for this purpose. Both systems use the optics of conventional microscopes. In the first system, continuous irradiation from a filtered xenon light source is focused onto a monolayer of cells using the condensing lens of a microscope. The spatial resolution for the 1270 nm emission is obtained by using a one-dimensional photodiode array that is moved in a stepwise fashion across the image focal plane. In the second system, pulsed-laser irradiation is focused to a small spot using a microscope objective. The spot is scanned over a monolayer of cells in a raster pattern. The $^1O_2$ phosphorescence is then detected using a photomultiplier sensitive to 1270 nm emission. The second system provides both spatial and temporal resolution.

THEORETICAL MODELS FOR THE BEHAVIOR OF $^1O_2$ *IN VIVO*

Simplified model for $^1O_2$ kinetics

As a starting point for data analysis, most investigators have used a simplified model of $^1O_2$ kinetics (17). Only the following reactions are considered:

$$h\nu + P \rightarrow {}^1P^* \tag{1}$$

$${}^1P^* \rightarrow {}^3P^* \tag{2}$$

$${}^3P^* + O_2 \rightarrow P + {}^1O_2({}^1\Delta_g) \tag{3}$$

$${}^1O_2 + Q \rightarrow O_2 + Q \text{ or } Q_{ox} \tag{4}$$

were $h\nu$ represents a photon of light, P is the ground-state photosensitizer, ${}^1P^*$ is the photosensitizer in an excited singlet state, ${}^3P^*$ is the photosensitizer in an excited triplet state, O_2 is ground-state oxygen (${}^3\Sigma_g^-$) and $^1O_2$ is oxygen in the excited $^1\Delta_g$ state, Q is a $^1O_2$ quencher and Q_{ox} is an oxidation product. It is further assumed that an initial concentration of excited triplet photosensitizer, $[{}^3P_0{}^*]$, is formed instantaneously from an extremely short laser pulse and that the lifetimes of both $^1O_2$ and the excited triplet photosensiter remain constant. When these assumptions are made, the time dependence of the excited triplet photosensitizer and the time dependence of the $^1O_2$ are given by Eqs (5) and (6), respectively (17).

$$[{}^3P^*] = [{}^3P_0^*]e^{-t/\tau_T} \tag{5}$$

$$[{}^1O_2] \propto \left(\frac{\tau_D}{\tau_T - \tau_D}\right)\left(e^{-t/\tau_T} - e^{-t/\tau_D}\right) \tag{6}$$

Here τ_D is the $^1O_2$ lifetime and τ_T is the lifetime of the excited triplet photosensitizer. The rise time for the $^1O_2$ emission is determined by the smaller of τ_T and τ_D, while the decay time for the $^1O_2$ emission is determined by the larger τ_T and τ_D (17). Thus, an unambiguous interpretation of the $^1O_2$ emission kinetics requires a knowledge of which lifetime is larger. This knowledge is most often provided by separate measurements of the kinetics of the photosensitizer triplet state.

The photosensitizer lifetime depends on the oxygen concentration. Within living cells, the photosensitizer lifetime is typically on the order of 3 μS (1,12). Most estimates of the lifetime of $^1O_2$ in cells are in the range of 0.5 μS or less (1,12,14,15). Thus, one would expect that the rise time for the $^1O_2$ emission would be determined by the lifetime of $^1O_2$ within the cell and the decay time for the $^1O_2$ emission would be determined by the lifetime of the photosensitizer triplet. This is the opposite of what is usually found when studies are done using dilute solutions made with deuterium oxide or with organic solvents. In these latter cases, the decay time of the $^1O_2$ emission is determined by the lifetime of $^1O_2$.

Modifications to the simplified model

For *in vivo* studies of $^1O_2$, a very large light fluence is needed to generate enough $^1O_2$ to measure from its 1270 nm phosphorescence. Under these conditions, more realistic models for $^1O_2$ kinetics may need to consider the effects of oxygen depletion, photosensitizer bleaching and oxidation of biological molecules in the cell. When these effects are taken into account, Eq. (6) no longer provides a good description for the $^1O_2$ kinetics (1,12). Further, the slow diffusion of both ground-state oxygen and of $^1O_2$ may prolong the $^1O_2$ phosphorescence (11).

An assumption often made by investigators is that the cell can be considered homogeneous. It is recognized that the lifetime of $^1O_2$ in aqueous regions of the cell may be different than the lifetime in membranes. However, the membranes are so thin that it is often assumed that there is such a rapid equilibrium of $^1O_2$ between the various phases present that the behavior of $^1O_2$ can be described by parameters that represent weighted averages from the various phases (18).

The assumption of a homogeneous cellular environment for $^1O_2$ may not be adequate to explain the kinetics of $^1O_2$ phosphorescence in some circumstances. Hackbarth *et al.* studied the kinetics of $^1O_2$ emission from photosensitizer-labeled liposomes following pulsed-laser excitation (12). They found that Eq. (6) was an inadequate description for $^1O_2$ emission kinetics. There was excess emission shortly after the laser pulse that the authors attributed to $^1O_2$ phosphorescence from the liposomes rather than from the surrounding buffer. In order to explain their experimental data, these authors

developed a more complex mathematical model that considered diffusion of $^1O_2$ into and out of the liposomes. A few past studies have also used diffusion models to rationalize $^1O_2$ kinetics in biological systems with multiple phases (19,20). The diffusion model used by Hackbarth *et al.* predicted that the kinetics of $^1O_2$ within the liposomes was slightly different than the kinetics outside the liposomes and that this difference could explain the excess emission at early times.

One of the most important conclusions from the study of Hackbarth *et al.* is that sometimes it may be necessary to consider the emission from membrane bound organelles separately emission from the aqueous portions of the cell. Some investigators have dismissed the importance of $^1O_2$ emission directly from membrane structures because the volume of these structures is small relative to the volume of the aqueous phase of the cell. However, oxygen (and presumably $^1O_2$) partitions into the lipid phase (21). Further, the radiative rate for $^1O_2$ is significantly higher in a lipid environment than in an aqueous environment (13). The combination of these two factors may intensify the $^1O_2$ emission from membrane structures by an order of magnitude or more (12).

SINGLET-OXYGEN LIFETIME WITHIN CELLS

The lifetime of $^1O_2$ within the cell is a critical parameter that is needed to understand the interactions of $^1O_2$ within cells. For example, the mean diffusion distance for $^1O_2$ within the cell can be calculated from the $^1O_2$ lifetime and the viscosity within the cell. In regard to photodynamic therapy, the lifetime of $^1O_2$ is one of the factors needed to calculate the concentration of $^1O_2$ from the intensity of the 1270 nm phosphorescence. Consequently, a number of investigators have made estimates for lifetime of $^1O_2$ within cells (1,10–12,14,15). The majority of studies have measured the lifetime of $^1O_2$ using large aggregates of cells (cell cultures, solubilized cell contents or intact animal tissue) (1,12,14,15). These studies consistently find a very short $^1O_2$ lifetime, 0.5 μs or less. This short lifetime has two implications. First, most of the $^1O_2$ is reactively or physically quenched by biological molecules rather than by water. Second, the mean diffusion distance for $^1O_2$ is very small, much smaller than the size of the average cell (14).

In contrast, experiments that use 1270 nm phosphorescence to measure the $^1O_2$ lifetime within small regions within individual cells have yielded much longer $^1O_2$ lifetimes, on the order of 3 μs (10,11). This value is close to the $^1O_2$ lifetime in water (22). Hackbarth *et al.* have looked into this matter in some detail (12). These authors concluded that the extremely high light fluence needed to measure $^1O_2$ at a subcellular level generates so much $^1O_2$ that many of the biological molecules are oxidized, leaving water as the major remaining $^1O_2$ quencher (12).

THE INITIAL EMISSION SPIKE

Most studies of $^1O_2$ emission from photosensitizer-labeled cells have used pulsed-laser excitation of the photosensitizer. A major limitation of this method is the presence of an emission spike due mainly to light scattering and photosensitizer fluorescence. The emission spike, while short in duration, can be orders of magnitude more intense than the $^1O_2$ emission. This spike can overload light detectors and their associated electronics. Most investigators have dealt with this problem by electronically gating the detection system to ignore the light emission for a short period of time after the laser pulse. In this issue of Photochemistry and Photobiology, Jarvi *et al.* demonstrate the importance of selecting the optimal gating time (1). Opening the gate too soon can cause a spillover from the emission spike. This spillover corrupts the $^1O_2$ emission data. Opening the gate too late causes a loss of the information-rich early part of the $^1O_2$ emission with the consequence of an inaccurate estimation of the amount of $^1O_2$ generated.

Jarvi *et al.* have suggested one solution to this problem, the development of a rapid internal gain control for near-infrared PMTs (1). Other solutions, such as the development of ultra fast electronic shutters, may also be possible.

CONCLUSIONS

There has been definite progress in the *in vivo* detection of $^1O_2$. At present, these measurements remain technically difficult and consequently are carried out in only a few laboratories. Hopefully this will change in the future with *in vivo* assays for $^1O_2$ becoming more standardized and more readily available.

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REFERENCES

1. Jarvi, M. T., M. J. Niedre, M. S. Patterson and B. C. Wilson (2011) The influence of oxygen depletion and photosensitizer triplet-state dynamics during photodynamic therapy on accurate singlet oxygen luminescence monitoring and analysis of treatment dose response. *Photochem. Photobiol.* **87**, 223–234.
2. Ehrenberg, B., J. L. Anderson and C. S. Foote (1998) Kinetics and yield of singlet oxygen photosensitized by hypericin in organic and biological media. *Photochem. Photobiol.* **68**, 135–140.
3. Nowak-Sliwinska, P., A. Karocki, M. Elas, A. Pawlak, G. Stochel and K. Urbanska (2006) Verteporfin, photofrin II and merocyanine 540 as PDT photosensitizers against melanoma cells. *Biochem. Biophys. Res. Commun.* **349**, 549–555.
4. Parker, J. G. (1987) Optical monitoring of singlet oxygen generation during photodynamic treatment of tumors. *IEEE Circuits Devices Mag.* **3**, 10–21.
5. Patterson, M. S., S. J. Madsen and B. C. Wilson (1990) Experimental tests of the feasibility of singlet oxygen luminescence monitoring *in vivo* during photodynamic therapy. *J. Photochem. Photobiol. B* **5**, 69–84.
6. Baker, A. and J. R. Kanofsky (1991) Direct observation of singlet oxygen phosphorescence at 1270 nm from L1210 leukemia cells exposed to polyporphyrin and light. *Arch. Biochem. Biophys.* **286**, 70–75.
7. Niedre, M., M. S. Patterson and B. C. Wilson (2002) Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells *in vitro* and tissues *in vivo*. *Photochem. Photobiol.* **75**, 382–391.
8. Zebger, I., J. W. Snyder, L. K. Andersen, L. Poulsen, Z. Gao, J. D. C. Lambert, U. Kristiansen and P. R. Ogilby (2004) Direct optical detection of singlet oxygen from a single cell. *Photochem. Photobiol.* **79**, 319–322.
9. Dysart, J. S., G. Singh and M. S. Patterson (2005) Calculation of singlet oxygen dose from photosensitizer fluorescence and

photobleaching during mTHPC photodynamic therapy of MLL cells. *Photochem. Photobiol.* **81**, 196–205.
10. Snyder, J. W., E. Skovsen, J. D. C. Lambert and P. R. Ogilby (2005) Subcellular, time-resolved studies of singlet oxygen in single cells. *J. Am. Chem. Soc.* **127**, 14558–14559.
11. Hatz, S., L. Poulsen and P. R. Ogilby (2008) Time-resolved singlet oxygen phosphorescence measurements from photosensitized experiments in single cells: Effect of oxygen diffusion and oxygen concentration. *Photochem. Photobiol.* **84**, 1284–1290.
12. Hackbarth, S., J. Schlothauer, A. Preuss and B. Röder (2010) New insights to primary photodynamic effects – singlet oxygen kinetics in living cells. *J. Photochem. Photobiol. B* **98**, 173–179.
13. Poulsen, T. D., P. R. Ogilby and K. V. Mikkelsen (1998) Solvent effects on the $O_2(a^1\Delta_g)$—$O_2(X^3\Sigma_g^-)$ radiative transition: Comments regarding charge-transfer interaction. *J. Phys. Chem. A* **102**, 9829–9832.
14. Moan, J. and K. Berg (1991) The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem. Photobiol.* **53**, 549–553.
15. Baker, A. and J. R. Kanofsky (1992) Quenching of singlet oxygen by biomolecules from L1210 cells. *Photochem. Photobiol.* **55**, 523–528.
16. Jiménez-Banzo, A., X. Ragàs, P. Kapusta and S. Nonell (2008) Time-resolved methods in biophysics. 7. Photon counting vs. analog time-resolved singlet oxygen phosphorescence detection. *Photochem. Photobiol. Sci.* **7**, 1003–1010.
17. Parker, J. G. and W. D. Stanbro (1984) Dependence of photosensitized singlet oxygen production on porphyrin structure and solvent. In *Porphyrin Localization and Treatment of Tumors* (Edited by D. R. Doiron and C. J. Gomer), pp. 259–284. Alan R. Liss, New York.
18. Rodgers, M. A. (1983) Time resolved studies of 1.27 micron luminescence from singlet oxygen generated in homogeneous and heterogeneous fluids. *Photochem. Photobiol.* **37**, 99–103.
19. Baker, A. and J. R. Kanofsky (1993) Time-resolved studies of singlet-oxygen emission from L1210 leukemia cells labeled with 5-(N-hexadecanoyl)amino eosin. A comparison with a one-dimensional model of singlet-oxygen diffusion and quenching. *Photochem. Photobiol.* **57**, 720–727.
20. Kanofsky, J. R. (1991) Quenching of singlet oxygen by human red cell ghosts. *Photochem. Photobiol.* **53**, 93–99.
21. Fischkoff, S. and J. M. Vanderkooi (1975) Oxygen diffusion in biological and artificial membranes determined by the fluorochrome pyrene. *J. Gen. Physiol.* **65**, 663–676.
22. Rodgers, M. A. J. and P. T. Snowden (1982) Lifetime of O_2 ($^1\Delta_g$) in liquid water as determined by time-resolved infrared luminescence measurements. *J. Am. Chem. Soc.* **104**, 5541–5543.