Time resolved sub-cellular singlet oxygen detection – ensemble measurements versus single cell experiments

To cite this article: S Hackbarth et al 2012 Laser Phys. Lett. 9 474

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Abstract: Systematic in vitro studies on singlet oxygen luminescence kinetics have been carried out reconciling recently published contradictory results reported for sub-cellular singlet oxygen kinetics obtained with two different approaches: single cell - and cell ensemble measurements.

The singlet oxygen luminescence kinetics in two cell lines were investigated after incubation with three different photosensitizers. In each case a strong dependency of the singlet oxygen luminescence kinetics from the applied illumination already at very low doses was observed. Analysis of the obtained results allows judging the usability of the two approaches for detailed investigation of sub-cellular singlet oxygen kinetics. Intrinsically existing detection limits as well as local heating during the measurement will be discussed.

Time resolved sub-cellular singlet oxygen detection – ensemble measurements versus single cell experiments

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Received: 28 October 2011, Revised: 21 November 2011, Accepted: 3 January 2012
Published online: 11 May 2012

Key words: singlet oxygen; photodynamic therapy; luminescence kinetics; quencher; phototoxicity

1. Introduction

1.1. Time-resolved detection of singlet oxygen (1O2)

Today there is common agreement [1–11], that singlet molecular oxygen (1O2), is the key agent in photodynamic therapy. Yet, its interactions with different molecules on the cellular level are not fully understood. Especially the correlation between light dose, oxygen concentration and the nature of possible quenching molecules on one side and the singlet oxygen kinetics on the other side has not been investigated until now on a quantitative level since it needs high end technique and strong efforts. Therefore it is of growing interest to detect the kinetics of 1O2 generation and consumption with cellular and sub-cellular resolution.

There is also common agreement that the only realistic option of a direct detection of 1O2 is given by observation of its tiny but significant luminescence around 1270 nm [1–5]. Although characteristic, the detection of this emission in vitro or even in vivo is technically challenging [12]. The rate constant of radiative deactivation of 1O2 in water is very low, just 1 out of 2.6×106 1O2 molecules emits a photon [13] and in the presence of quenchers even less. An additional serious problem is the low signal to noise ratio (SNR) of most detection systems at this wavelength region and/or their low sensitivity. Therefore the use of statistical detection systems (multiscaler or time-resolved multi pho-
Luminescence, a.u.

Laser Physics Letters

1.0

Time, µs

0.6

Time, µs

0.8

Time, µs

1.0

Time, µs

0.4

Time, µs

0.2

Time, µs

0.0

0

5

10

15

Figure 1 (online color at www.lasphys.com) Theoretical \(^{1}\)O\(_{2}\) luminescence kinetics for a membrane localized PS using the one-dimensional diffusion model described in Sec 2.4. The \(^{1}\)O\(_{2}\) luminescence components from inside (a) and outside (b) the membrane are shown in arbitrary units and normalized (insert). Calculations are based on the following parameters: membrane thickness \(d = 10\) nm [19], \(\tau_T = 3\) µs, \(\tau_O, \text{outside membrane} = 1\) µs, \(\tau_O, \text{inside membrane} = 1\) µs (blue) or 14 µs (red). The \(^{1}\)O\(_{2}\) decay time inside the membrane has only little influence on the temporal shape of the kinetics, as long as quenching is much slower than diffusion out of the membrane.

1.2. \(^{1}\)O\(_{2}\) kinetics of membrane localized photosensitizers

In general there is common agreement [1–5], that the kinetics of singlet oxygen in homogeneous environment can be described by the following equation:

\[ n_O(t) = \frac{A}{\tau_T - \tau_O} \left[ \exp \left( -\frac{t}{\tau_T} \right) - \exp \left( -\frac{t}{\tau_O} \right) \right], \tag{1} \]

where \(A\) is a positive constant, \(\tau_T\) – the triplet decay time of the photosensitizer (PS), and \(\tau_O\) – the decay time of singlet oxygen.

However, for PS localized in membranes, this simple two-exponential model was found to be not sufficient to describe the \(^{1}\)O\(_{2}\) kinetics. The rate constant of radiative \(^{1}\)O\(_{2}\) deactivation is higher in membranes compared to water [5, 17]. If \(^{1}\)O\(_{2}\) is generated in the membranes, its diffusion into an environment with much lower radiative rate constant appears like an additional quenching in the luminescence kinetics. But this “quenching” only affects the fraction of \(^{1}\)O\(_{2}\) molecules located inside the membrane. As a consequence the luminescence signals from inside and outside the membrane follow different kinetics [5]. First experimental evidence therefor was given in 1999 by Oelckers et al. [18].

In Fig. 1 the theoretical kinetics of the luminescence signal components based on the one-dimensional diffusion model (see also Sec. 2.4) from in- and outside the membranes are shown for a membrane-localized PS with 3 µs triplet decay time while the singlet oxygen lifetime outside the membrane is assumed to be 1 µs. As can be seen, both signal components are in the same order of magnitude, although the cellular membranes have a thickness of only a few nanometers [19]. This has mainly two reasons, the higher radiative rate constant of \(^{1}\)O\(_{2}\) in the membrane (see Sec. 2.4) and the small diffusion length of \(^{1}\)O\(_{2}\). Reported decay times of \(^{1}\)O\(_{2}\) in cells range from 0.4 µs [5, 16, 20] over 1.0 µs [21] and 1.5 – 1.7 µs [2] to 3.2 µs [4, 13]. If only diffusion of \(^{1}\)O\(_{2}\) perpendicular to the membrane is assumed (one-dimensional case), the diffusion length is between 50 and 120 nm. For the example shown in Fig. 1 it is about 60 nm, while the membrane thickness was estimated as 10 nm [19]. Further explanation will be given in Sec. 2.4.
1.3. The effective light dose (ELD)

Oxidative stress for a cell caused by an excited PS mainly depends on the singlet oxygen quantum yield. On the other side – due to the short diffusion range of \( ^1O_2 \) in cells – it also depends on the intracellular localization of the PS. However, only light that is absorbed by the PS can cause generation of \( ^1O_2 \). Therefore we suggest that the common description for illumination experiments with just giving the illumination dose of the whole sample is not appropriate. The geometry of the sample, the spatial shape of the light distribution as well as efficiency of light transport and the number of cells in the sample influence the local impact of a PS on a cell. To become independent from such parameters the effective light dose (ELD) was introduced [5]. ELD is defined as the average light dose directly penetrating each cell. For ENM, cells in suspension or tissue, it thus can be calculated as the product of the average light intensity applied and the geometric cross section of a single cell. In SCE where illumination is focused onto one single cell the ELD is the same as the overall illumination dose. The ELD allows comparing the different types of experiments. However, it is still subject to restrictions.

Focused illumination using very short pulses (sub-nanosecond) may result in PG ground state depletion due to the high local light intensity. This reduces the fraction of the ELD that is absorbed and thus may reduce the impact on the cell(s) under investigation. While non- or weakly focused illumination with focal spots much larger than the cells results in a nearly homogenous light dose distribution in the cell, any focus smaller than the cell like used for sub-cellular measurements in SCE results in variation of the local light dose. In this case the majority of \( ^1O_2 \) is generated in a small fraction of the cellular volume. Recently we reported, that illumination of Jurkat cells incubated with Pheo results in changes of the concentration of chemical \( ^1O_2 \) quenchers and of local oxygen concentration due to chemical reactions after generation of \( ^1O_2 \) [5,16]. The same ELD under conditions of sub-cellular light focusing will result in even faster local changes of these parameters compared to non-focused illumination. If all \( ^1O_2 \) is generated in a small fraction of the cellular volume, due to the limited diffusion range of \( ^1O_2 \), any chemical interaction of \( ^1O_2 \) with cellular components will happen in this fraction of the cellular volume.

2. Materials and methods

2.1. Photosensitizers

Pheo was isolated from spirulina Arthrospira platensis based on the extraction method from Willstätter and Stoll [22]. Chlorin e6 (Ce6) was purchased from Orpegen and used without further purification. Methyl tetrahydroxopyphenyl chlorin (mTHPC) was kindly delivered from Prof. M. Senge (TCD, Ireland).

2.2. Cell cultivation and PS incubation

Culture media, buffers, sera and penicillin/streptomycin were purchased from PAA. Jurkat cells (human acute T cell Leukemia DSMZ:ACC 282) and LNCaP cells (DSMZ: ACC 256) were cultured in RPMI 1640 medium with L-glutamine (PAA) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 \( \mu \)g/ml of streptomycin without phenol red. Cells were cultivated at 37°C in 100% humidity and 5% CO2.

Cells were incubated with concentrations of 3 \( \mu \)M (Pheo, mTHPC) or 10 \( \mu \)M (Ce6) PS in the culture medium for 5 h (mTHPC) or 24 h (Pheo, Ce6).

LNCaP cells were trypsinized before washing.

In each case cells were washed three times in PBS. For \( ^1O_2 \) luminescence measurements both cell types are resuspended to reach a final concentration of \( 1 \times 10^6 \) cells/cm\(^3\), 3 ml of this suspension are filled in cuvettes and investigated right after preparation.

2.3. Time-resolved detection of \( ^1O_2 \) luminescence

The setup is based on the NIR H-10330-45 (Hamamatsu) and a custom made time correlated multi photon counting device (TCMPC) as has been described previously [5,16]. For the measurements shown here the laser was adjusted to an average excitation power of 6 mW at 660 nm, so the pulse energy was 500 nJ/pulse at 12 kHz with a diameter of about 3 mm. For identification of the signal kinetics and independent confirmation the PSs triplet decay time was determined with flash photolysis [5].

2.4. Numerical analysis/fitting with diffusion model

Models to describe the kinetics of \( ^1O_2 \) including a one-dimensional diffusion have already been used before [5,23]. In this work fitting of the measured \( ^1O_2 \) luminescence kinetics uses the approximation, that the lipid bilayer in which the \( ^1O_2 \) is generated is planar in the x-y plane. The bilayer is assumed to be homogeneous in x and y direction, so only \( ^1O_2 \) diffusion in z-direction has to be taken into account as described in [5]. The diffusion constants of molecular oxygen were taken as \( 1 \times 10^{-5} \) and \( 2 \times 10^{-5} \) cm\(^2\)/s [1] inside and outside the membrane, respectively. The ratio between the radiative rate constants inside and outside the membrane was estimated as 11±4 as found for lipid bilayers of plain digital plain paper copiers (DPPC) [5], assuming that the additional compounds in a native membrane do not significantly alter this ratio. This assumption is based on the fact that the majority of the luminescence emission from inside the membrane will originate from areas with mainly lipids as the permeability of
proteins for oxygen is low [24]. In [25] it was shown, that films of b-lactoglobulins may completely inhibit oxygen diffusion towards embedded molecules of erythrosine B.

However, in direct vicinity of a lipoprotein the very efficient quenching by some amino acid side chains will locally shorten the $^{1}$O$_{2}$ decay time and thus reduce emission [26]. Knowing this, one can already see, that the one dimensional fit is really only an approximation, since there will be diffusion also inside the membranes towards the lipoproteins. On the other hand, due to the small distances the diffusion equilibration is still faster than any chemical reaction with amino acids, so the resulting lifetime in the membrane can be approximated by an average value, probably lower than the reported 14 $\mu$s for pure DPPC [1].

For a rough estimation of the $^{1}$O$_{2}$ quenching by proteins in membranes we used the rate constants for $^{1}$O$_{2}$ quenching by amino acids given by Davies [27]. These values were multiplied with the relative abundance of amino acids in the lipid region of membrane proteins [28]. This gives an average quenching constant for membrane localized amino acids of $4 \times 10^9$ M$^{-1}$ s$^{-1}$ ($\pm$50%). The average density of proteins is $\sim 1.4$ g/cm$^3$ [29]. Therefore the concentration of amino acids within a protein can be estimated as $\sim 11$ M. However, only a fraction of the amino acid side chains is located at the protein surface and thus accessible, which reduces the effective concentration. (Note: The low permeability of the proteins for oxygen reduces not only the amount of amino acids but also the volume of the membrane for calculation of the average amino acid concentration in the membrane in this estimation.)

If proteins cover 20 to 50% of the membrane area and 10–25% of the amino acids in the membrane located part of these proteins are accessible, the resulting rate constants for quenching in the membrane can be roughly estimated as $1 \times 10^6$ -- $1 \times 10^7$ s$^{-1}$. We chose $1 \times 10^6$ s$^{-1}$ for our fitting procedure, which corresponds to a decay time of 1 $\mu$s. This value was used, since at all times the proteins inside the membrane face the highest local concentration of $^{1}$O$_{2}$ and will thus be consumed much faster than any chemical quencher outside the membrane. At higher irradiation doses the decay time in the membrane will approach the value for a quencher-free membrane or a plain DPPC bilayer.

Up to now it was impossible to determine the $^{1}$O$_{2}$ decay time in such bilayers, but for films of DPPC $^{1}$O$_{2}$ luminescence decay times of 14 $\mu$s were reported [1].

However, above 1 $\mu$s the $^{1}$O$_{2}$ decay time in the membrane has only little influence on the signal kinetics. For illustration theoretical calculations for values of 1 and 14 $\mu$s based on the model described above are shown in Fig. 1. It is assumed, that the PSs are located inside a membrane of 10 nm thickness [19]. The chosen fit parameters are representative values – PS triplet decay time: 3 $\mu$s and $^{1}$O$_{2}$ decay time outside the membrane: 1 $\mu$s. The amplitudes of both signal components from inside and outside the membrane are slightly bigger for the longer $^{1}$O$_{2}$ decay time in the membrane, but the ratio of the amplitudes remains nearly unchanged. Also the shapes (normalized kinetics) are nearly identical for both chosen values (Fig. 1 insert).

For the sake of a faster fitting procedure (random walk algorithm) therefore the $^{1}$O$_{2}$ decay inside the membrane was fixed and 1 $\mu$s appears to be a good choice for it.

The PS triplet decay and the $^{1}$O$_{2}$ decay outside the membrane were taken as variable parameters. Due to the SNR the minimum step width of both parameters was chosen as 0.05 $\mu$s.

3. Results and discussion

In ENM many cells share the illumination dose. Cell numbers between $3 \times 10^6$ to $10^7$ per sample are reported in literature [1–3,5]. If both types of experiments (ENM and SCE) are performed to reach a comparable overall count rate consequently the ELD in SCE is easily a thousand times higher than in most reported ENM. On the upside, the SCE allows a better collection of the few photons emitted by $^{1}$O$_{2}$. A microscope objective collects about 13%, when using a numerical aperture (NA) of 0.9, like reported in [30,31], while any measurement in suspension faces aperture limitations due to the much bigger sample volume and the limited size of the detector. The here used setup collects about 5% of all emitted photons. However, the ELD for a comparable count rate is much higher in SCE compared to ENM. Consequently, the upper limit of the illumination dose for a “realistic” measurement – meaning before the actual changes in $^{1}$O$_{2}$ decay times exceed the error margin of the experiment – will be much smaller for SCE, possibly too small to perform any time-resolved measurement of $^{1}$O$_{2}$.

3.1. Changes in $^{1}$O$_{2}$ kinetics under on-going illumination

To analyze the changes of the observables in dependence on the ELD we performed series of up to 250 consecutive time-resolved $^{1}$O$_{2}$ luminescence measurements of two cells types incubated with three different PSs. Using the TCMPC setup described in [16] each of these measurements caused an ELD of about 32 nJ/cell (measured with LabMax/J 10MT-10 kHz, Coherent). This was the lowest possible dose that yields in $^{1}$O$_{2}$ luminescence signals with sufficient SNR to allow fitting of the two decay times with an accuracy of 0.1 $\mu$s. With every new measurement consequently the ELD increases by 32 nJ/cell.

The most important result is, that for all investigated samples the singlet oxygen decay time is at least twice as high after an ELD of about 400 nJ/cell compared to an ELD of 32 nJ/cell. While Pho is very photostable during the experiments, Ce6 and mTHPC suffer from bleaching, reducing the luminescence signals and limiting the number of possible consecutive measurements. In LNCaP cells this bleaching is more pronounced compared to Jurkat cells. However, fitting of the series of $^{1}$O$_{2}$ kinetics reveals increasing values for the $^{1}$O$_{2}$ decay times for all three PS in
both cell types – Jurkat and LNCaP (Fig. 2). Before and after measurement the PS triplet decay times were determined separately with flash photolysis. They are comparable to those obtained by fitting the $^1$O$_2$ kinetics as shown in Fig. 2. The increasing $^1$O$_2$ decay times at higher ELD can be explained by consumption of $^1$O$_2$ quenchers like proteins and lipids or other endogenous quencher molecules. While physical quenching does not change the quencher and molecular oxygen concentration, chemical quenching yields in consumption of both. Obviously at low ELD a high percentage of the generated $^1$O$_2$ interacts with chemical quenchers. The influence of the quenchers on the $^1$O$_2$ luminescence signal decreases with rising ELD due to ongoing quencher consumption. All $^1$O$_2$ decay times determined at ELDs above 400 nJ/cell are at least twice as big as for the first measurement. Such results can’t be called “realistic” as they exceed the error margin of the first measurement by far.

Adopting this result to SCE and taking into account the very high ELD compared to ENM the question arises: Is the measurement of “realistic” $^1$O$_2$ kinetics in SCE possible?

To answer this question we estimate the number of $^1$O$_2$ molecules that are generated in one cell during illumination with 400 nJ in the above reported ENM experiments. The PSs used in this work have extinction coefficients of less than 50,000 M$^{-1}$cm$^{-1}$ at excitation wavelength, $^1$O$_2$ quantum yields of 50 – 60% and the intracellular concentration reached about 0.2 mM [32]. Consequently the maximum fraction of the ELD absorbed by the PSs can be estimated as 1.5% under the assumption of a “spherical cell” with 10 $\mu$m diameter. This yields a maximum of $4.4\times10^{10}$ generated singlet oxygen molecules in one cell for an ELD of 400 nJ/cell.

It is known [13], that in water just 1 out of $2.6\times10^6$ molecules undergoes radiative deactivation. The presence
3.2. Local heating – an important side effect

Whenever a photon is absorbed inside a cell, a certain amount of heat is about to be produced. Cellular components like endogenous chromophores will absorb a small fraction of the incoming light and generate heat independent from the presence of exogenous PS. Nevertheless, in this paragraph only the heat production caused by the non-radiative deactivation of photoexcited PS molecules in a cell during measurement of $^{1}\text{O}_2$ luminescence will be estimated.

After absorption of a photon by PS different major pathways of deactivation are possible (Fig. 3). However, most of them end up in a complete conversion of the excitation energy into heat. This is most obvious in case of internal conversion, but also valid in case of $^{1}\text{O}_2$ generation. The latter one comprises internal conversion, intersystem crossing (ISC) and energy transfer to the molecular oxygen; all of them are non-radiative processes. Once generated, the $^{1}\text{O}_2$ will be deactivated by physical or chemical quenching. Physical quenching is a direct conversion of the excitation energy into heat. Chemical quenching in most cases will even result in additional heat production, as oxidations of organic substances are exothermic. Nevertheless, in the following estimations the reaction enthalpy will be neglected.

For fluorescence and phosphorescence, where a photon is emitted during deactivation, the amount of heat produced is given by the difference of the photon energy of the absorbed and emitted photon. To keep the estimation simple we neglect the phosphorescence since for a good PS the triplet quantum yield and the singlet oxygen quantum yield are nearly identical. To sum it up, the fluorescence quantum yield $\Phi_{F1}$ of the PS allows to estimate its heat efficiency, meaning the percentage of absorbed energy, that is converted into heat. Most of nowadays used porphyrins and chlorins have a $\Phi_{F1} < 0.3$ resulting in a minimum heat efficiency of 70% when excited in the energetically lowest Q-band. Excitation at shorter wavelengths further increases the heat efficiency.

In our experiments (see Sec. 3.1) about 1.5% of the ELD is being absorbed by the PS. As other groups use very similar protocols [1–3] this should be a representative value. When the PS are excited at 660 nm hence about 1% of the ELD is converted into heat. So during one measurement (ELD 32 nJ) around 0.3 nJ heat are produced in one cell, which causes an average temperature rise in the cell of about 0.1 K during a measurement of 20 s. The overall amount of heat generated is in all cells during this time in the sample is about 1 mJ.

In a SCE all the heat is generated in the single cell under observation. However, the higher aperture in SCE compared to ENM reduces the necessary illumination dose a bit. But even using a 4π-microscope, where nearly all emitted photons can be funneled onto the detector a comparable result gained with SCE would require an ELD more than a 100,000 times bigger than during one measurement in our ENM setup. The heat generated in the cell during this measurement would be enormous and probably enough to cause serious damage like denaturation of proteins. In [30] during 3 minutes SCE an ELD of about 1 mJ was applied to one cell, using excitation at 420 nm. Even though cells are small and heat exchange with the surrounding medium will be fast, a non-negligible temperature rise in the cell will happen. The authors observed changes in the cell viscosity during the measurement and

![Figure 3](online color at www.lasphys.com) Jablonski diagram illustrating the energy pathways after PS excitation. All dotted arrows indicate generation of heat according to the energy difference of the involved states (in case of chemical quenching the reaction enthalpy has to be counted as well). The rate constant for $^{1}\text{O}_2$ luminescence is negligible in that respect. The same is valid for the phosphorescence of most PS in presence of oxygen.
interpreted it as a result of reactions of the \( {^1\text{O}_2} \) with cellular quenchers. Taking into account the amounts of heat generated during this measurement on cellular scale it appears much more probable, that the viscosity increase is mainly caused by a strong increase in temperature and chemical reactions related to it together with a loss of intracellular water.

4. Conclusions

The above presented results reveal that it is impossible to measure \( {^1\text{O}_2} \) kinetics in a single cell with sub-cellular resolution using state of the art technology. Any presence of \( {^1\text{O}_2} \) in cells results in a consumption of naturally abounding chemical quenchers and thus an increase of the \( {^1\text{O}_2} \) decay times. Since it is impossible to detect \( {^1\text{O}_2} \) without starting this process, the overall dose applied to the cells during a measurement has to be limited. While ensemble measurements distribute the light dose among many cells, a cell under a microscope is illuminated to an extent that causes nearly complete depletion of chemical quenchers near the PS. Another unavoidable side effect of any such measurement is the local generation of heat. In a single cell measurement the cell under investigation therefore faces a non-negligible temperature increase, which also will affect the results of the measurement.

References