

# Singlet Oxygen Microscope: From Phase-Separated Polymers to Single Biological Cells

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## ABSTRACT

The lowest excited electronic state of molecular oxygen, singlet molecular oxygen ( $a^1\Delta_g$ ), is an intermediate in many chemical and biological processes. Tools and methods have been developed to create singlet-oxygen-based optical images of heterogeneous samples that range from phase-separated polymers to biological cells. Such images provide unique insight into a variety of oxygen-dependent phenomena, including the photoinitiated death of cells.

## Introduction

Molecular oxygen is one of the most important molecules with respect to life on earth.<sup>1</sup> It plays a key role in the maintenance of life and in mechanisms by which life is

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extinguished and materials destroyed. Despite all of this, the study of oxygen and oxygen-dependent processes continues to present challenging problems.

We have a wide-ranging program to examine systems in which oxygen plays a role. Of special interest are systems in which the lowest excited state of oxygen is a component. This state, commonly called “singlet oxygen”, can be produced in many ways, although the most common is upon exposure of a given sample to light. Thus, it is pertinent not just to systems exposed to sunlight, but to the ever-increasing number of materials exposed to lamps and lasers.

Singlet oxygen is an important intermediate in many chemical and biological processes. It has a unique reactivity that can result, for example, in polymer degradation<sup>2</sup> or the death of biological cells.<sup>3,4</sup> The latter is exploited in photodynamic therapies, where light is used as a tool to combat cancer, and treat ocular and vascular ailments.<sup>5</sup> In complex biological and polymeric systems, the behavior of singlet oxygen is often determined by microscopic heterogeneities and phase-separated domains. Thus, much would be gained if singlet oxygen could be directly monitored with both time and spatial resolution from such systems.

We set out to create dynamic optical images of heterogeneous samples based on spectroscopic transitions that involve singlet oxygen. This was and remains a challenging endeavor because the pertinent transitions are extremely weak and occur in the infrared region of the spectrum, where signal detection can be problematic.<sup>6–8</sup> In the creation of images, we wanted to exploit the known effect of solvent on singlet oxygen signal intensities, lifetimes, and spectral shifts.<sup>6–8</sup> Such images could also be constructed on the basis of concentration gradients of ground-state oxygen in a given system.

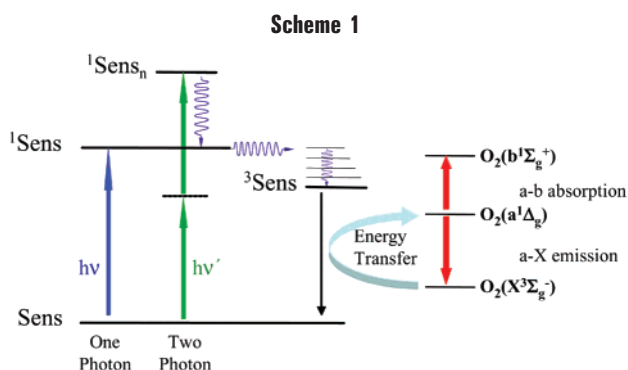
We have now developed methods that can be used to create singlet oxygen images of a range of materials, including single cells.<sup>9–12</sup> It is important to recognize that, in a program on “singlet oxygen microscopy”, many of our efforts transcend oxygen-dependent problems and are thus pertinent to the general research community. In this Account, we describe selected features of this program.

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**Photosensitized Production of Singlet Oxygen.** A diagram illustrating aspects of singlet oxygen production pertinent to this Account is shown in Scheme 1.

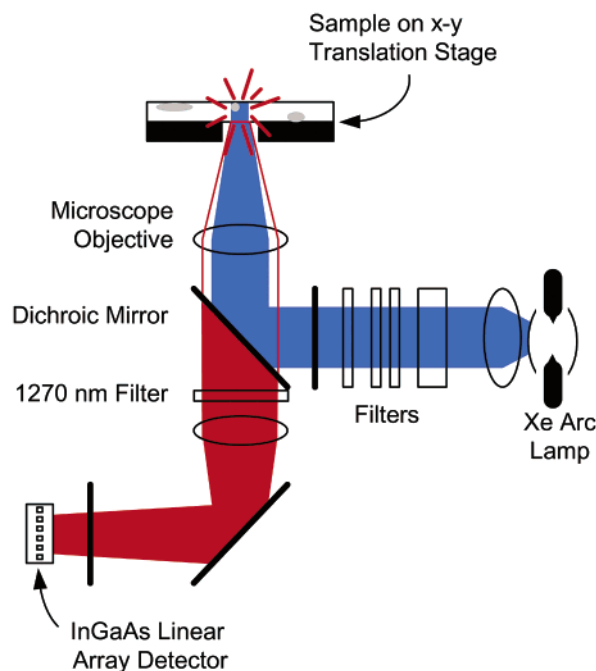


Upon irradiation of a so-called sensitizer, Sens, energy transfer from the excited-state sensitizer to ground-state oxygen,  $\text{O}_2(X^3\Sigma_g^-)$ , is one way to produce singlet oxygen. Different molecules can act as a singlet oxygen sensitizer. The sensitizer can be an inherent component of a given system (e.g., a naturally occurring porphyrin in a cell or a critical part of a polymer), which in turn, contributes to the characteristic oxygen-dependent behavior of that system. Alternatively, sensitizers can be added to a system to impart a selected and specific response, as is the case, for example, in photodynamic therapy.

Sensitizer excitation can be achieved either by absorption of a single photon or by the simultaneous absorption of two photons.<sup>13,14</sup> Depending on the sensitizer, the excited state initially populated may not be the same. Nevertheless, an important feature of a good sensitizer is the rapid and efficient production of the comparatively long-lived triplet state,  $^3\text{Sens}$ , which then, in a collision with ground-state oxygen, efficiently produces singlet oxygen. This process of energy transfer can either produce the lowest excited electronic state,  $a^1\Delta_g$ , or the second excited state,  $b^1\Sigma_g^+$ , of oxygen, depending principally on the energy of the sensitizer triplet state.<sup>7</sup> If  $\text{O}_2(b^1\Sigma_g^+)$  is produced, however, it rapidly decays to form  $\text{O}_2(a^1\Delta_g)$ , and it is this latter state that we refer to as “singlet oxygen”.

In selecting sensitizers for the creation of singlet oxygen images, it is not sufficient to only choose molecules that have a high quantum yield of singlet oxygen production. One must also consider sensitizer stability upon prolonged irradiation and solubility in a given phase-separated domain. To facilitate light absorption in thin films and/or small focal volumes, sensitizers with large absorption coefficients are desired. Finally, for experiments in which singlet oxygen is monitored by its weak phosphorescence (vide infra), it is critical to have a sensitizer with an extremely small quantum efficiency of luminescence in the near-IR. To meet such stringent criteria, we must often design and synthesize new sensitizers.

**Optical Detection of Singlet Oxygen.** Once formed,  $\text{O}_2(a^1\Delta_g)$  can be monitored optically via two approaches (Scheme 1). The pertinent transitions,  $a^1\Delta_g \rightarrow X^3\Sigma_g^-$  emission and  $a^1\Delta_g \rightarrow b^1\Sigma_g^+$  absorption, occur over discrete



**FIGURE 1.** Illustration of the inverted microscope in which singlet oxygen phosphorescence is monitored using an array detector.

and narrow wavelength ranges with well-defined band shapes.<sup>15</sup> This imparts significant advantages to our work.

The a-X phosphorescence spectrum has a band maximum centered at  $\sim 1270$  nm, the position of which depends on the solvent.<sup>16</sup> The phosphorescence quantum efficiency is extremely low, ranging from  $\sim 10^{-4}$  to  $10^{-7}$ , again depending on the solvent.<sup>7,17</sup> In media containing quenchers that can shorten the singlet oxygen lifetime into the nanosecond domain, particularly aqueous biological systems, the phosphorescence quantum efficiency can be smaller than  $10^{-7}$ . In such systems, interfering luminescence from the sensitizer can make detection of the singlet oxygen signal difficult.

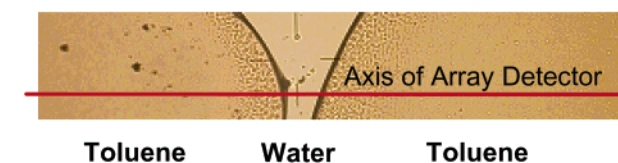
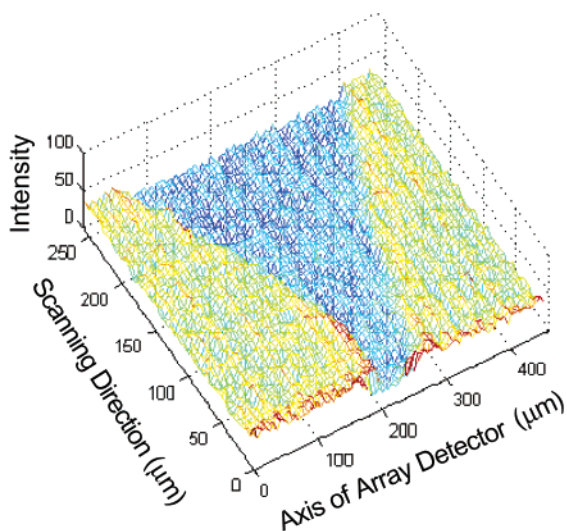
The a-b absorption spectrum has a solvent-dependent band maximum centered at  $\sim 1925$  nm.<sup>15,18</sup> This is likewise a weak transition, with a solvent-dependent molar absorption coefficient less than  $100 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>19</sup> Although competing absorption by the solvent can limit the use of this technique, this approach nevertheless complements the detection of a-X phosphorescence and, as such, provides a useful tool.

It is convenient to describe our work in terms of three microscopes, and the remainder of this Account is divided accordingly.

## Singlet Oxygen Emission Microscope

We have established that, upon irradiation of a sample containing a sensitizer, singlet oxygen can be detected via its a-X emission using an InGaAs array detector.<sup>10,11</sup> The pertinent features of this microscope are shown in Figure 1.

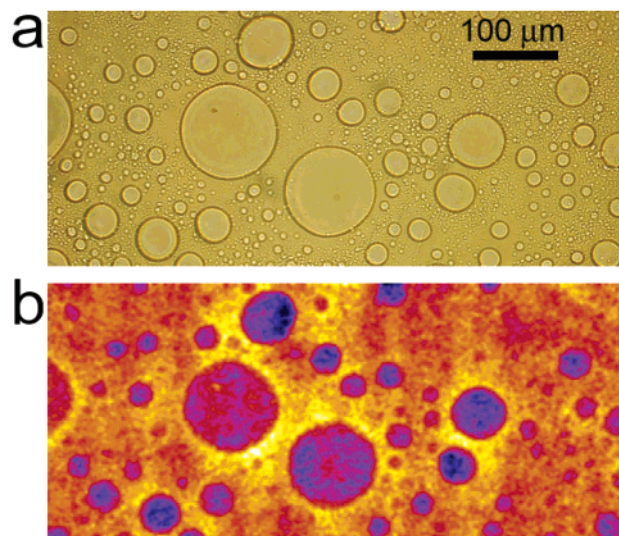
Briefly, the sample to be examined is mounted on the stage of an inverted microscope. Sensitizer irradiation is achieved using a xenon lamp, the output of which is



**FIGURE 2.** Images of a water channel between droplets of toluene. The singlet oxygen sensitizer used is only soluble in toluene. The bottom panel shows a visible image of the sample, and the top panel shows the singlet oxygen image created using data from the linear array detector.

passed through a series of filters to yield a narrow bandwidth of visible light, which in turn, is coupled onto the sample stage through the microscope objective using a dichroic mirror. Singlet oxygen phosphorescence is collected using the same microscope objective, transmitted through the dichroic mirror and spectral isolation filters, and focused onto the InGaAs detector placed at the image plane of the microscope. Thus far, data have been recorded using a linear array detector. Consequently, to obtain a two-dimensional image, it is necessary to translate the sample on the microscope stage in discrete steps and to build the image using the “slices” successively recorded by the array (Figure 2).

Our spatial resolution has thus far been limited by the Nyquist sampling criterion.<sup>20</sup> Specifically, along the axis of the detector array ( $x$  axis), the resolution is dependent on the magnification of the objective and the size of the individual InGaAs elements in the array. Along the scanning axis ( $y$  axis), the step size with which the sample is moved also contributes to the spatial resolution. On this basis, singlet oxygen images have been created with a resolution of  $5\ \mu\text{m}$  (i.e., we can resolve two point sources separated by  $5\ \mu\text{m}$ ). However, for the same objective magnification, we have been able to achieve a resolution of  $2.5\ \mu\text{m}$  using a repetitive-sampling technique, which reduces the apparent size of each element in the detector array (vide infra). Given the Rayleigh diffraction criterion,<sup>20</sup> the limiting resolution with which the  $1270\ \text{nm}$  singlet oxygen phosphorescence can be imaged is  $r_0(\text{detection})$



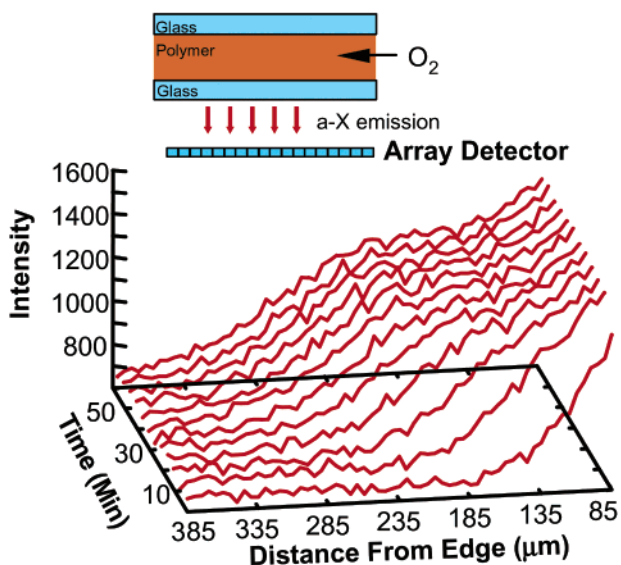
**FIGURE 3.** Images of a phase-separated blend of glassy polymers. (a) Visible image. (b) Singlet oxygen image (no singlet oxygen = black < blue < red < orange < yellow < white = high singlet oxygen concentration).<sup>43</sup>

$= 0.61\lambda/\text{NA}$ , where NA is the numerical aperture of the microscope objective and  $\lambda$  is the wavelength of light. Thus far, we have used objectives with a NA of 0.4; hence,  $r_0(\text{detection}) = 1.94\ \mu\text{m}$ .

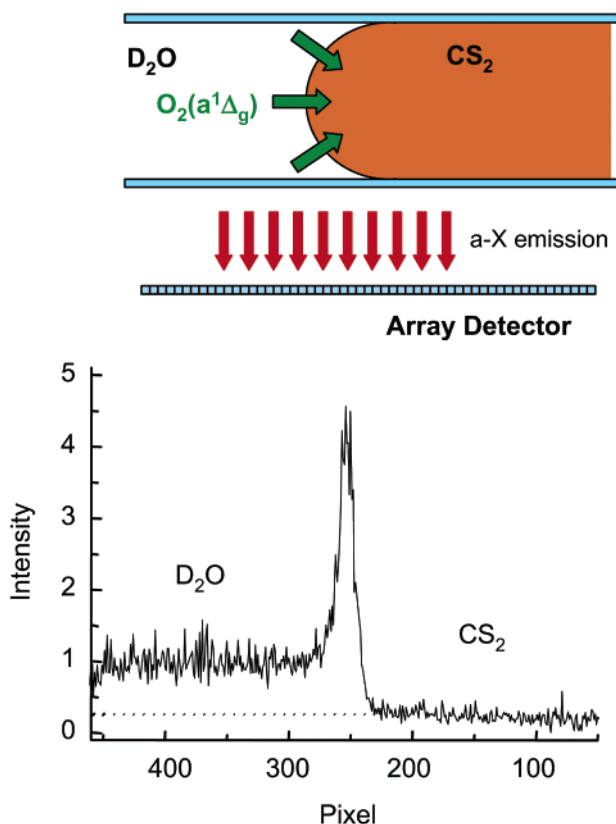
**Heterogeneous Liquids and Polymers.** With this microscope, we have made singlet oxygen images of different phase-separated liquid and polymeric samples (Figures 2 and 3).<sup>10,11</sup> We have also investigated a range of fundamental issues.

The study of oxygen diffusion in a range of materials allows us not only to address general issues associated with mass transport,<sup>21</sup> but also to contribute to the development of useful barrier materials. To this end, critical information can be obtained from spatially resolved data recorded as oxygen migrates through a sample (Figure 4). This technique is expected to be particularly useful with heterogeneous samples, where oxygen encounters phase-separated domains that either retard or accelerate the overall diffusion process. Even for the “simplest” cases of oxygen sorption in heterogeneous samples (e.g., bi- and trilayer films), the theoretical modeling and quantification of such diffusion is a challenge.<sup>22</sup> Nevertheless, the potential rewards of studying diffusion through curved surfaces and droplets far outweigh the difficulties encountered.

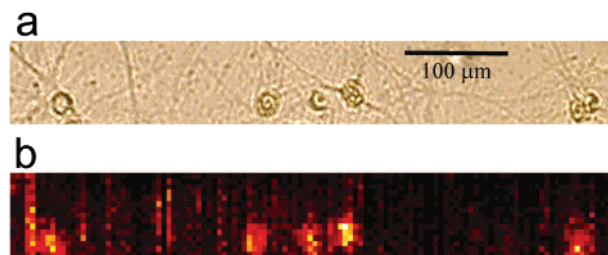
In a related study, we have mapped the steady-state concentration profile of singlet oxygen across an interfacial boundary under conditions in which singlet oxygen must diffuse from one phase-separated domain in which it is generated into a second sensitizer-free phase-separated domain.<sup>11</sup> This work was performed using  $\text{CS}_2$  droplets in  $\text{D}_2\text{O}$  (Figure 5). The results obtained, however, are directly applicable to more complicated phase-separated materials, such as biological systems, where singlet oxygen diffusion plays an important role in intra- and intercellular signaling.<sup>23</sup>



**FIGURE 4.** Spatially resolved singlet oxygen phosphorescence recorded upon exposing the edge of a 20- $\mu\text{m}$  thick polystyrene film to 100 Torr of oxygen. Each trace was recorded with the InGaAs linear array over a 30-s period at 5-min intervals upon irradiation of a sensitizer dissolved in the film.



**FIGURE 5.** Singlet oxygen profile across the liquid interface between  $\text{D}_2\text{O}$  and  $\text{CS}_2$ . Singlet oxygen was produced in the  $\text{D}_2\text{O}$  phase upon irradiation of a water-soluble sensitizer. The profile recorded shows the effects of singlet oxygen diffusion across the interface into the  $\text{CS}_2$  domain in which the solubility of oxygen is larger than that of  $\text{D}_2\text{O}$ . The data also reflect a quantum yield of singlet oxygen phosphorescence in  $\text{CS}_2$  that is greater than that in  $\text{D}_2\text{O}$ .



**FIGURE 6.** Images of nerve cells into which a sensitizer had been incorporated. (a) Visible image. (b) Singlet oxygen image.

**Single Biological Cells.** Images of single cells based on singlet oxygen would allow us to contribute in a unique way to research on mechanisms of oxygen-dependent cell death. For example, in apoptosis, one must address an intricately choreographed series of events that result in a systematic dismantling of the cell. It has been established that apoptosis proceeds through spatially resolved events that occur in or on specific subcellular components<sup>24</sup> and that, in many photoinduced processes, singlet oxygen is involved in events that trigger apoptosis.<sup>3</sup> Thus far, however, pertinent data on singlet oxygen in biological systems have come from indirect studies and experiments performed on bulk ensembles of cells.<sup>3,25,26</sup> In this context, the direct optical detection of singlet oxygen from a single cell is a required step. Only when this has been achieved can one realistically discuss the goal of creating and using singlet oxygen images with subcellular resolution.

Optical detection of singlet oxygen from a cell presents unique challenges, the most important of which is that the signal to be observed originates from an aqueous system with a high concentration of singlet oxygen quenchers (e.g., proteins). In  $\text{H}_2\text{O}$ , where the singlet oxygen lifetime,  $\tau_\Delta$ , is  $\sim 3.5 \mu\text{s}$ <sup>27</sup> and the rate constant for the  $a-X$  radiative transition,  $k_r$ , is  $0.11 \text{ s}^{-1}$ ,<sup>28</sup> the efficiency of singlet oxygen phosphorescence is low ( $\tau_\Delta k_r \sim 3.9 \times 10^{-7}$ ). In biologically significant environments, where quenchers can reduce  $\tau_\Delta$  into the nanosecond domain,<sup>25,26</sup> detection of the  $a-X$  phosphorescence becomes even more challenging.

Our experiments were performed using nerve cells into which a cationic porphyrin had been incorporated as the sensitizer.<sup>12</sup> Moreover, to increase our chances of seeing a singlet oxygen signal, intra- and extracellular  $\text{H}_2\text{O}$  was replaced with  $\text{D}_2\text{O}$ . Although the  $a-X$  radiative rate constant  $k_r$  in  $\text{D}_2\text{O}$  is the same as that in  $\text{H}_2\text{O}$ ,<sup>28</sup> the singlet oxygen lifetime in  $\text{D}_2\text{O}$  is  $\sim 68 \mu\text{s}$ .<sup>29</sup> Thus, in the absence of any quenchers that reduce  $\tau_\Delta$ , the efficiency of singlet oxygen phosphorescence from the  $\text{D}_2\text{O}$ -incubated cells should be  $\sim 19$  times greater than that from  $\text{H}_2\text{O}$ -incubated cells.

Under these conditions, we were indeed able to optically detect singlet oxygen from single cells and create crude low-resolution singlet oxygen images of the cells (Figure 6).<sup>12</sup> As expected, the intensity of the signal decreased upon exposing the cells to  $\text{H}_2\text{O}$ . However, the extent of this intensity change (a factor of  $\sim 3.8$ ) was not as large as that predicted based solely on the singlet oxygen lifetimes in quencher-free solvents (a factor of

~19). The data are thus consistent with a system in which molecules in the cell quench singlet oxygen.

The fact that we observe a solvent isotope effect indicates that, in this system, the solvent still plays a role in deactivating singlet oxygen (i.e., the quenchers present do not totally dominate the deactivation process). Thus, on one hand, singlet oxygen could have a lifetime that is consistent with diffusion over appreciable distances in the cell. On the other hand, given previous estimates for  $\tau_{\Delta}$  in cells,<sup>25,26</sup> our solvent isotope effect could also reflect a facile equilibrium between singlet oxygen in a quencher-free extracellular environment and an intracellular population of singlet oxygen, where  $\tau_{\Delta}$  is much shorter. Specifically, once formed within the cell, if singlet oxygen diffuses through the cell membrane, then some fraction of the signal that we observe could originate from a domain where  $\tau_{\Delta}$  is much greater. An example of this phenomenon is illustrated by the spatial profile shown in Figure 5.

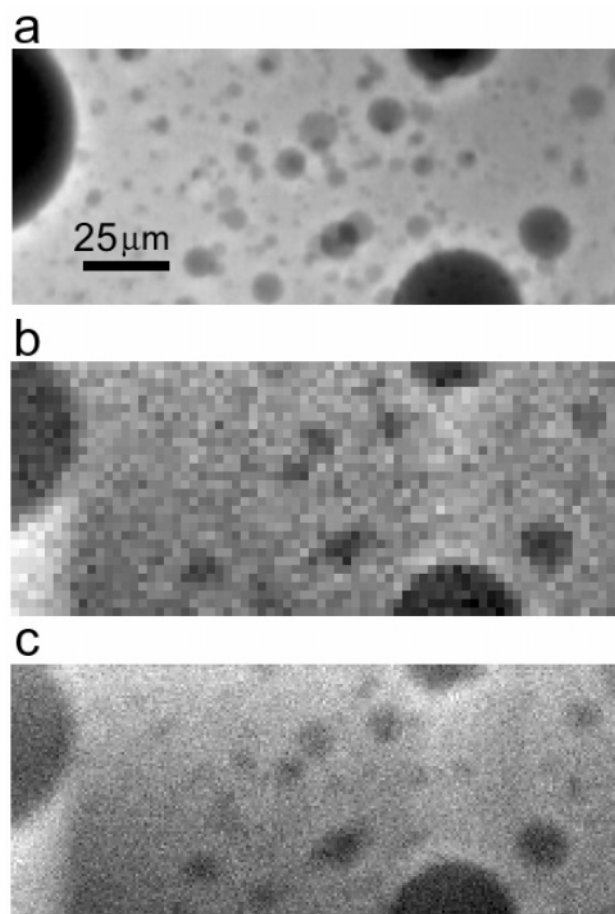
**Current and Future Efforts.** We continue to modify this technique such that higher resolution images can be acquired over shorter periods of time. To this end, the implementation of a two-dimensional array detector will have significant impact, in that it will obviate the need to scan the sample and build the image using successive “slices” of data.

We have also ascertained that, for a given image, spatial resolution can be increased by using the individual elements in the array detector in a mode of “repetitive-sampling” or “dithering” (Figure 7).<sup>30</sup> In this approach, the apparent size of each element in the array is effectively reduced by collecting phosphorescence data from a larger number of sample positions on the microscope stage. The important characteristic of each new sample position, in both the  $x$  and  $y$  directions, is that the extent to which the sample is moved relative to the previous position corresponds to some fraction of the effective pixel size.

## Two-Photon Singlet Oxygen Microscope

**Nonlinear, Two-Photon Irradiation.** Production of an excited state can also occur upon the simultaneous absorption of two low energy photons rather than the absorption of a single higher energy photon (Scheme 1). While the probability of absorbing two photons in such a process is small, it increases nonlinearly with the photon flux and can be made to occur in the small volume of a focused laser beam. One advantage of this nonlinear process is that, at the wavelengths used for the two-photon experiment, many materials only absorb light at the laser focus. Thus, depth penetration in the sample is readily achieved.

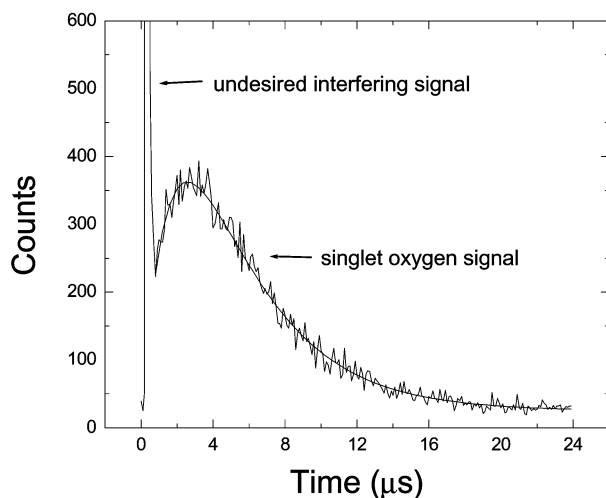
In these experiments, the volume in which excitation occurs is essentially limited by the diffraction of light.<sup>31</sup> Thus, upon irradiation in the visible or near-IR regions of the spectrum, subfemtoliter focal volumes can be obtained, which correspond to a lateral spatial resolution in the range ~300–700 nm.<sup>31</sup> The possibility to create excited states with such resolution has been exploited in



**FIGURE 7.** Images of a phase-separated blend of glassy polymers. (a) Visible image based on the fluorescence of the sensitizer incorporated in the polymers. (b) Singlet oxygen image with a “normal”, Nyquist-limited resolution of 5.0  $\mu\text{m}$ . (c) Singlet oxygen image recorded using the technique of “dithering” that yields a resolution of 2.5  $\mu\text{m}$ .

a number of significant ways. For example, two-photon fluorescence microscopy provides a useful tool in imaging biological samples.<sup>32,33</sup>

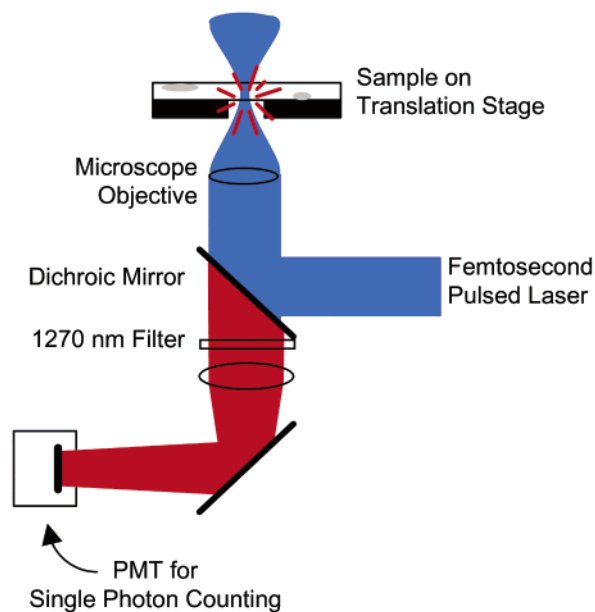
**Two-Photon Photosensitized Production and Detection of Singlet Oxygen.** We have established that singlet oxygen can be generated upon light absorption by a sensitizer in a two-photon process.<sup>13,14</sup> Most importantly, we have been able to optically detect the singlet oxygen thus generated in time-resolved a-X phosphorescence experiments performed in a range of solvents, including H<sub>2</sub>O (Figure 8). In our early work, samples were irradiated using a nanosecond pulsed laser and the singlet oxygen signal was monitored using a Ge detector with a response time of ~400 ns.<sup>13,14</sup> However, as our work has evolved, it has become necessary to use femtosecond laser systems that have spatial and temporal profiles that are more conducive to these nonlinear experiments and higher pulse repetition rates that allow rapid acquisition of data. Equally significant is the use of a near-IR photomultiplier tube (PMT) with a response time of ~2 ns, which is suited to detect singlet oxygen phosphorescence in a mode of single-photon counting.



**FIGURE 8.** Time-resolved singlet oxygen phosphorescence recorded in a single-photon-counting experiment. In this study, performed in air-saturated  $\text{H}_2\text{O}$ , sensitizer excitation was achieved via two-photon absorption. Events that result in singlet oxygen formation and decay are clearly displaced in time from events coincident with pulsed laser irradiation. For this experiment, the beam waist of the irradiating laser was  $600 \mu\text{m}$ .

**Sensitizer Development.** A key aspect of our work has been the development of sensitizers suitable for use in these nonlinear experiments. Desirable molecules must not only have a comparatively large two-photon absorption probability (i.e., the so-called two-photon absorption cross section) and a large quantum efficiency of singlet oxygen production, but also they must be soluble in the medium of choice and stable upon prolonged irradiation. The issue of sensitizer luminescence, however, is not that critical because these experiments are performed in a time-resolved mode, and it is relatively straightforward to discriminate against luminescence that might otherwise interfere with the detection of singlet oxygen. Specifically, under almost all circumstances of interest, singlet oxygen is sufficiently long-lived that the a-X phosphorescence is temporally displaced relative to phenomena coincident with the exciting laser pulse (e.g., sensitizer fluorescence and scattered laser light) (Figure 8). This is a key issue, not just for monitoring singlet oxygen but, for example, when quantifying two-photon absorption cross sections of the sensitizer.

**Two-Photon, Singlet Oxygen Imaging.** There are several ways in which spatially resolved singlet oxygen data can be recorded upon two-photon excitation of a sensitizer. Thus far, we have concentrated on the approach illustrated in Figure 9. One uses a microscope objective to focus the irradiating laser, which in turn, results in singlet oxygen formation within a small volume. The same microscope objective is then used to collect the singlet oxygen phosphorescence and transmit it to the PMT used for single-photon counting. Although kinetic data can be recorded and used (e.g., Figure 8), it is simpler to gate and integrate the time-resolved signal and record a singlet oxygen intensity. An image is then constructed by moving the sample and recording data from a series of points. Because of the depth penetration inherent to a two-



**FIGURE 9.** Illustration of the inverted microscope used to create singlet oxygen in and monitor singlet oxygen from a small focal volume upon two-photon irradiation of a sensitizer.

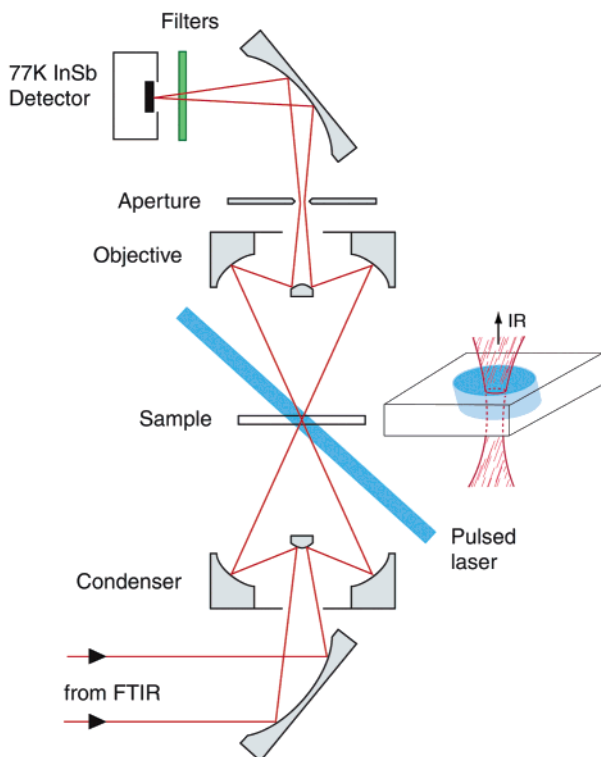
photon experiment, this process of raster scanning can be used to generate three-dimensional images.

Using the approach shown in Figure 9 and an objective with a numerical aperture of 0.9, we have been able to detect singlet oxygen from media in which the quantum efficiency of a-X phosphorescence is comparatively large (e.g.,  $\text{CS}_2$ , benzene, and polystyrene). This statement must be considered with several points in mind.

First, the two-photon singlet oxygen microscope has the distinct advantage that the data collected originate from a small excitation volume. Given the microscope objective and wavelengths used in our experiments, this volume is generally smaller than 1 fL, with a cross sectional area of excitation that has a diameter of  $\sim 500 \text{ nm}$ . Second, within its lifetime in the given medium, singlet oxygen will diffuse over a certain distance. Thus, the phosphorescence signal recorded in these experiments invariably originates from a volume that is larger than that irradiated. For example, in glassy polystyrene, the lifetime of singlet oxygen is  $\sim 20 \mu\text{s}$ .<sup>34</sup> Let us assume that we detect singlet oxygen over a period  $t$  of twice its lifetime (i.e.,  $t \sim 40 \mu\text{s}$ ). Using a coefficient  $D$  for oxygen diffusion in polystyrene of  $2 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ ,<sup>35</sup> the distance traveled by singlet oxygen in  $40 \mu\text{s}$  is  $\sim 40 \text{ nm}$  (root-mean-square linear displacement =  $(2tD)^{1/2}$ ). On the other hand, in liquid  $\text{H}_2\text{O}$ , although the singlet oxygen lifetime is shorter ( $\tau_{\Delta} = 3.5 \mu\text{s}$ ),<sup>27</sup> the oxygen diffusion coefficient is much larger ( $D = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ ).<sup>36</sup> Thus, over the period  $t$  of twice its lifetime, singlet oxygen will diffuse  $\sim 170 \text{ nm}$ .

Most importantly, the dimensions associated with singlet oxygen generation and diffusion in this two-photon experiment will generally be *smaller* than the spatial resolution accessible when using an array detector to image the 1270 nm a-X phosphorescence.

**Current and Future Efforts.** The immediate goal with this microscope is to work with aqueous systems where



**FIGURE 10.** Illustration of the microscope used to monitor singlet oxygen in a time-resolved absorption experiment following pulsed laser excitation of a sensitizer.

the quantum efficiency of singlet oxygen phosphorescence is small. Moreover, to achieve a spatial resolution exceeding that limited by diffraction, we are exploring the options of using nonlinear optical techniques modified through the use of evanescent fields<sup>37</sup> and nanoscale metal tips.<sup>38</sup> Because of metal-dependent local field effects, the latter has the potential added advantage of increasing the two-photon transition probability in the sensitizer and, thus, creating a higher concentration of singlet oxygen to be monitored.

## Singlet Oxygen Absorption Microscope

We have established that, using the a–b transition at  $\sim 1925$  nm (Scheme 1), singlet oxygen can be detected in a time-resolved absorption experiment and that this signal can likewise be detected with spatial resolution using a microscope.<sup>9</sup> This approach complements the singlet oxygen emission experiments and thus, in some cases, allows us to examine systems that might otherwise be inaccessible.

These experiments are most readily performed using a step-scan Fourier transform spectrometer that has been modified to facilitate the detection of the weak singlet oxygen signal after pulsed laser irradiation of a sensitizer.<sup>39</sup> Spatial resolution in this pump–probe experiment is achieved by taking the output of the continuous-wave IR probe source, modulated by the interferometer, and focusing it into the sample (Figure 10).<sup>9</sup> The size of a given pixel used in the construction of the singlet oxygen image is determined by the size of an aperture placed at the

image plane of the microscope. Thus, attempts to increase the spatial resolution by decreasing the aperture size are necessarily accompanied by a decrease in the flux incident on the IR detector. To date, we have been able to record data from 400- $\mu\text{m}$  thick polystyrene films in which the signal derives from a cylinder that is  $\sim 100$   $\mu\text{m}$  in diameter. Among other things, this resolution is sufficient to study oxygen diffusion in many phase-separated polymer blends.

**Current and Future Efforts.** Although somewhat useful with respect to microscopy, these absorption experiments are arguably more important in our program to investigate the effect of a given perturbation (e.g., solvent) on the spectroscopic transitions in oxygen.<sup>15,18,19,40</sup> Specifically, by looking at perturbation-dependent changes in (1) the position and band shape of both absorption and emission spectra and (2) the probabilities of both radiative and nonradiative transitions, we can monitor how processes formally forbidden in the isolated molecule are influenced by the surrounding environment.

## Conclusions

Using a variety of tools and methods, we have demonstrated that singlet oxygen can be optically detected with high spatial resolution from a range of phase-separated, heterogeneous samples, including single biological cells. Moreover, the data obtained can be used to construct singlet oxygen images of these samples at a resolution that is significant for the study of many chemical and biological processes.

Most importantly, we have demonstrated the viability of a program that can ultimately provide unique insight into a variety of oxygen-dependent and singlet-oxygen-dependent phenomena that occur in heterogeneous samples characterized by submicrometer spatial domains. In this regard, perhaps the most significant issue to be addressed focuses on the spatially dependent role(s) played by singlet oxygen in photoinduced events that result in the death of biological cells. Finally, it is not entirely unreasonable to consider that our tools could also be applied in the study of singlet oxygen endogenously generated by both plant<sup>41</sup> as well as animal cells.<sup>42</sup>

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