NIR area array CCD-based singlet oxygen luminescence imaging for photodynamic therapy

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Abstract: In this work, a near-infrared CCD-based singlet oxygen luminescence two-dimensional imaging method is proposed to detect singlet oxygen by its 1270nm luminescence. Two-dimensional singlet oxygen images with its near-infrared luminescence during photosensitization could be obtained with a CCD integration time of 1s, without scanning. The data presented shows a linear relationship between the singlet oxygen luminescence intensity and sample concentration. This method provides a detection sensitivity of 0.00189mg/ml (Hematoporphyrin monomethyl Ether dissolved in ethanol) and a spatial resolution better than 100µm. We applied this method in vivo to demonstrate its potential in monitoring photodynamic therapy.

1. Introduction
Photodynamic therapy (PDT) is an emerging therapy applied in area ranging from cancer treatment to some nonmalignant conditions [1-3]. In PDT, photosensitizer (PS) is incorporated into abnormal tissues and then irradiated with visible light [2,3]. Photoexcited sensitiser transfers energy to the ground-state oxygen via the type-Π photochemical pathway to produce singlet oxygen (\(^{1}O_2\)) [4]. Singlet oxygen is an important intermediate in many chemical and biological processes, and is believed to play significant roles in destructing biological cells [4-6]. In the past years, singlet oxygen has been directly detected by its weak 1270 nm luminescence (\(a^{1}\Delta_g \rightarrow X^{3}\Sigma_u^{-}\)) and most of these works have been done on bulk samples. In these measurements, significant results were acquired though the singlet oxygen luminescence signal was the average value of total sample [7,8]. Considering the inhomogeneities in PS distribution, oxygen concentration and/or the space intensity of exciting light, which can affect the production and behavior of singlet oxygen, methods have been proposed to detect singlet oxygen luminescence with spatial resolution [9-12]. In these methods, single photomultiplier tubes or linear detector arrays were used to obtain singlet oxygen luminescence images from the 1270 nm weak emission. In 2002, Lars Klemmt et al. reported a singlet oxygen image with a spatial resolution of 2.5μm detected by an
InGaAs linear array [9]. In 2004, I. Zebger et al. used a linear InGaAs device to create a singlet oxygen image of a single cell [10]. In 2005, Mark J Niedre et al. used a laser-scanning system to image singlet oxygen luminescence in an intradermal tumor model in mice [11]. In 2009, Thomas Breitenbach et al. reported singlet-oxygen-based images of a cell obtained by raster-scanned method [12]. These scanning-based researches have greatly prompted the singlet oxygen luminescence imaging, and were of significant advance in basic photobiological research and PDT dosimetry.

In the scanning-based singlet oxygen luminescence imaging methods, there is a high demand for the precision of motion of the moving stage required to load samples, as well as the edge overlap restructuring algorithm used to remove large position errors and join traces in image reconstruction, resulting in more complexity of the system and adverse effect to the measurement accuracy [13]. Moreover because the long integration time in each scan is need for the weak singlet oxygen luminescence signal and image data need to be collected sequentially, scanning image methods need a relatively long time to acquire an image. This may bring adverse influence to the final results because of the changes of parameters mentioned above that can affect the production and behavior of singlet oxygen during the data collection, as well as the time-critical in vivo detection of singlet oxygen luminescence signal [14-16]. Thus, a fast-imaging technique with relatively large imaging area and reasonable spatial resolution suitable for clinic application is necessary [17]. In this paper, we developed a two-dimensional singlet oxygen luminescence imaging method without scanning by use of a high quantum efficiency, high uniformity and low dark current infrared area CCD detector. Images of photodynamically generated singlet oxygen luminescence both in vitro and in vivo have been acquired with an integration time of 1s by this method. PS samples with different concentrations were used to test the relationship between luminescence intensity and sample concentration. The detection sensitivity of the system has been investigated and spatial resolution of the system has been tested by exciting light spots with dark lines of various widths. In in vivo experiments, we used the method to acquire singlet oxygen luminescence images from mice injected with PS.

2. Methods and materials

2.1 optical imaging system design

The schematic diagram of our experimental system is shown in Fig.1. The whole system includes three parts: 1. the semiconductor laser source for excitation, 2. optical filters/ NIR area CCD detection system, 3. a standard frame grabber carder NI-1427 as the data acquisition system. A semiconductor laser (Changchun New Industries Optoelectronics Technology Co., Ltd.) with a wavelength of 470 nm and a power of 12 mW, was used as light source. The laser light was collimated and fell on a beam expander. The expanded beam was reflected and then an aperture was used to select a spot with a suitable size for both the sample and the detect range of the system. This selected light was used to photo-excite the region of interest in the sample to produce singlet oxygen luminescence signal. An achromatic lens was used to collect the emission from the sample, which included the singlet oxygen luminescence signal, PS fluorescence, back-scattered laser light and autofluorescence from tissues in vivo. A longpass filter (LP1150, Thor labs) and a NIR bandpass filter (1270BP20, Omega optical) centered at 1270 nm with a full-width-at-half-maximum (FWHM) of
20 nm and an attenuation OD better than OD5 were used to get rid of PS fluorescence, back-scattered laser light and autofluorescence from tissues in vivo, and obtain NIR luminescence signal of the singlet oxygen centered in 1270 nm ± 10 nm. The transmitted emission from the filters was coupled by a NIR lens onto an InGaAs area detector (Xeva-CL-FPA-1.7-320-TE1-60Hz, Xenics), whose spectra response ranged from 900 nm to 1700 nm with a uniform quantum efficiency of 84%. The CCD surface was cooled at -20 °C to reduce thermal noise. We used a standard frame grabber carder NI-1427 for the image data acquisition.

Figure 1. Schematic layout of the NIR area CCD-based singlet oxygen luminescence imaging system.

2.2 Experimental sections
In the study, Hematoporphyrin monomethyl Ether (HMME, Shanghai Xianhui Phaunaccutical Co.Ltd) was dissolved in ethanol in different concentration to be illuminated with above-mentioned light source to produce singlet oxygen. All samples were held in a 10 mm × 10 mm × 5 mm quartz cuvette mounted on a sample stage, which was able to maintain the source-sample-detector geometry constant during experiments. The space intensity of the exciting light was 60 mw/cm² and images were captured at an exposure time of 1 s in these experiments.

In the study in vivo, HMME was dissolved as the introduction from producer to make PS solution with a concentration of 2 mg/ml. Two female Kunming mice (Experimental Animal Centre of San Yat-sen University of Medical Science) were used. All the mice were anesthetized with a 1.4 mg/g intra-peritoneal dose of Urethane, and then depilated on the zone of dorsal with 8% Na₂S (Guangzhou Chemical Reagent Factory). For the first mouse, HMME solution was subcutaneously injected into the hairless area with a 0.05 ml dose for photosensitization. After a waiting period of 30 min that allows for the diffusion of PS in the tissues, light exposure was performed with a density of 60 mw/cm². The second one was not
injected with HMME solution and irradiated by the same exciting light, as the control group.

3. Results and Discussions

3.1 validation of singlet oxygen luminescence signal

To demonstrate that the signal detected by our system was indeed derived from singlet oxygen, experiments were performed with three samples of HMME dissolved in alcohol at the concentration of 0.08mg/ml. The first sample was exposed to air, the second was added with 1,4-Diazabicyclo[2,2,2]octane (DABCO), an acknowledged quencher of singlet oxygen, and the last was bubbled with nitrogen gas to displace the dissolved oxygen. Fig. 2 shows the images of samples irradiated by the exciting light of the same fluence rate. The singlet oxygen luminescence signal could be detected and the average signal intensity calculated by integration method is 79.98 counts, compared with the background of 30.98 counts, besides the shapes and details of the singlet oxygen luminescence image are observed in the first sample (Fig. 2(a)). As expected for singlet oxygen luminescence, the near-IR signal disappeared in the sample to which the singlet oxygen quencher DABCO had been added (Fig. 2(b)), as well as the third case that the sample equilibrated with nitrogen (Fig. 2(c)). When nitrogen bubbling stopped and the sample equilibrated with nitrogen previously was exposed to air, the signal could appear again due to the diffusion of air back into the liquid sample (Fig. 2(d)).

![Figure 2](image)

**Figure 2.** Images of samples in different situations. (a) Sample exposed to air, (b) sample with addition of singlet oxygen quencher DABCO, (c) sample bubbled with nitrogen, (d) sample in (c) exposed to air again.

3.2 Linear sensing range and detection sensitivity

HMME dissolved in ethanol with concentrations of 0.01, 0.015, 0.02, 0.04, 0.08, and 0.16mg/ml were prepared for the test of linear sensing range of the system. A background image was recorded upon the irradiation of ethanol without PS dissolved. The singlet oxygen luminescence images were taken immediately after the start of the irradiation. In this series each pixel represents the background-subtracted signal at 1270 nm. Fig. 3(a) shows the series of singlet oxygen luminescence images of samples with different concentrations. It is clear that the singlet oxygen luminescence signal increases with the increasing of sample density for the same fluence rate. From the data in Fig. 3(a) the average singlet oxygen luminescence intensities calculated by integration method are 10.3, 14.2, 18.87, 30.60, 52.70 and 114.40 counts, as plotted in Fig. 3(b). The relationship between the average singlet oxygen luminescence intensity and the concentration of the HMME was shown in Fig. 3(b). It can be found that the
average singlet oxygen luminescence intensity shows a linear relationship with the concentration of HMME from 0.01mg/ml to 0.16mg/ml.

Figure 3. Detection of HMME samples with different concentrations. (a) Images of the HMME dissolved in alcohol with the concentration of 0.01, 0.015, 0.2, 0.4, 0.8, and 0.16mg/ml from the top left to right bottom. (b) The dependence of average singlet oxygen signal intensity on the concentration of HMME samples derived from (a), the error bars represent the standard deviation of three samples with the same concentration in each concentration.

We could calculate the detection sensitivity of the system as follow:

\[ S_d = \frac{D_s}{SNR} \]  

Where \( S_d \) is the detection sensitivity, \( D_s \) the sample density and \( SNR \) the signal-to-noise ratio. In this study, we used the 0.08mg/ml HMME solution to test the detection sensitivity and singlet oxygen luminescence image was acquired immediately after the start of the irradiation. Fig. 4(a) is the original image acquired by our system and the intensity graph of marked row A. In Fig. 4(b) and (c), the value of every pixel is replaced by the average of the intensity levels in the 3x3 and 5x5 neighborhood, as a smoothing, linear spatial filter to further enhance the signal-to-noise ratio. The definition of SNR is the ratio of signal intensity to the standard deviation of background. In Fig. 4, the singlet oxygen average intensity is
approximately 103.428 counts and the standard deviations of the background are 6.1339, 3.2792 and 2.4442, respectively. Thus the SNR achieved from Fig. 4 are 16.8617, 31.5406 and 42.3156 respectively. According to Eq. (1), as the sample density $D_s$ is 0.08mg/ml, the sensitivity of our system can achieve 0.00189mg/ml with a SNR of 42.3156 as a result of 5×5 smoothing, linear spatial filter. The cost of smoothing, linear spatial filter is the reduction of spatial resolution.

![Image](image_url)

**Figure 4.** The results of smoothing, linear spatial filter for reducing noise level. (a) original singlet oxygen luminescence image. (b) 3×3 pixels, (c) 5×5 pixels neighborhood are averaged to replace each pixel. Intensity graphs of dashed row A, across the background, are plotted on the right side of these images. The SNRs achieved from (a), (b) and (c) are 16.8617, 31.5406 and 42.3156 respectively.

### 3.3 Resolving power

The pixel size of the area CCD is 30\(\mu\)m×30\(\mu\)m and the zoom of this system is 0.762. The spatial resolution of 39.4\(\mu\)m can be calculated in theory. To investigate the spatial resolution of the system, a series of filaments with diameters (D) of 200\(\mu\)m, 150\(\mu\)m and 100\(\mu\)m were put in the center of the cross section of the collimated laser beam sequentially. Thus the exciting light was divided to form a light spot with a dark line in center. Fig. 5 shows one dimensional signal intensities across the centers of the singlet oxygen luminescence images obtained upon irradiation of HMME dissolved in ethanol. As the concavity in the last image can be distinguished, corresponding to the 100\(\mu\)m case, thus the spatial resolution of the system is better than 100\(\mu\)m.
3.4 Singlet oxygen luminescence imaging in vivo

We also used this method on mice to acquire singlet oxygen luminescence imaging in vivo. One mouse with 0.05 ml HMME and another mouse without HMME injected were used for this experiment. Fig. 6 shows the results of the in vivo experiments. Fig. 6(a) shows a white-light image of an animal taken by commercial digital camera (Olympus, E300), and the area irradiated which was marked by a circle. Fig. 6(b) is one of the images obtained from the mouse without HMME during irradiation. Fig. 6(c) shows a singlet oxygen luminescence image series acquired from the mouse injected with HMME during the light exposure time. Compared with the weak light acquired in the mouse without PS, shown in Fig. 6(b), the singlet oxygen luminescence images could be obtained clearly from the mouse injected with PS in vivo, as shown in Fig. 6(c). The image time series of HMME case shows a decreasing singlet oxygen luminescence signal during the long exposure time, compared with the weak light intensity and no intensity depletion in the case without HMME. We deduce that the data without PS mainly represents the scattered background light. The causes of the decrease of singlet oxygen signal are likely to be PS photobleaching, the photochemical depletion of molecular oxygen in tissues [18,19].
4. Conclusions

We have proposed a newly developed NIR area CCD-based singlet oxygen luminescence two-dimensional imaging method for photosensitization. Using this method, we demonstrate that singlet oxygen luminescence image could be acquired with an integration time of 1s, without scanning. The experimental system has a detection sensitivity of approximately 0.00189mg/ml (HMME dissolved in ethanol) and a resolving power better than 100µm. We also obtained photodynamically generated singlet oxygen luminescence images in vivo. We believe that the proposed NIR area CCD-based singlet oxygen luminescence two-dimensional imaging method is promising for in vivo monitoring of singlet oxygen produced in photosensitization.

Acknowledgements

This research was made possible with the financial support from the 863 project, China (grant 2006AA06Z402), NSFC China (30770592).
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