A modular luminescence lifetime imaging system for mapping oxygen distribution in biological samples

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Abstract

We developed a new modular luminescence lifetime imaging system (MOLLI), that enables the imaging of luminescence lifetimes in the range of 1 μs to 1 s. The system can easily be adapted to different experimental applications. The central parts of the system are a recently released CCD-camera with a fast electronic shutter and gated LED (light emitting diode) or Xe excitation light sources. A personal computer controls the gating and image acquisition via a pulse delay generator. Here we present the new imaging system and give examples of its performance when used for measuring two-dimensional oxygen distributions with planar optodes. Furthermore, future applications of the system in biology are discussed. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Luminescence lifetime imaging; Fluorescence lifetime imaging; Oxygen optode; Planar optode

1. Introduction

Imaging of two-dimensional solute distributions with luminescent indicators has become an important tool in medicine, biology and physics. Most of the described image measuring systems and experimental setups were designed for specific applications like measurements of oxygen distribution in tissue [1–5], pH and Ca2+ distribution in cells [6,7], oxygen partial pressure on skin surfaces or oxygen flux into skin [8,9], oxygen distribution across the water-sediment interface [10] and in biofilms [11]. These setups were optimised for the corresponding experimental situation, but they lack versatility and many of them were e.g. confined to microscope setups [12–17]. Furthermore, imaging systems for time- and frequency-domain measurements often consist of complicated and rather bulky setups, using lasers as light sources, optical modulators, and sensitive slow scan CCD-cameras combined with fast gatable image intensifiers.

New bright semiconductor light sources, light emitting diodes (LED), that emit in the blue and blue-green part of the spectrum, offer a much cheaper and simpler alternative for a fast modulated or gated light source as compared to lasers. Furthermore, a recently developed sensitive and fast gatable CCD-camera simplifies lifetime imaging as it allows fast gating directly on the photosensitive chip. The image is digitised in the camera, and can be read out with the camera control board. An additional frame grabber is thus obsolete. We combined these new technologies with a trigger controller and a personal computer (PC) to develop a modular imaging system, which can easily be adapted to various applications. Here we describe the new imaging system and show its performance in applications where two-dimensional O2 distributions are mapped via lifetime imaging of planar O2 optodes.
Table 1
Overview of different luminescence lifetime imaging methods

<table>
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<tr>
<th>Lifetime detection</th>
<th>Procedure</th>
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<tr>
<td>Frequency domain method</td>
<td>Sinusoidal modulation and phase angle shift</td>
<td>Better separation of different species with similar lifetimes</td>
<td>Complex setups (that need an image intensifier for sinusoidal modulation of gain), good optical filtering necessary</td>
<td>[26,25,28]</td>
</tr>
<tr>
<td>Ratioing method</td>
<td>Rectangular modulation and ratioing</td>
<td>Higher signal-to-noise ratio than time-domain and fast calculation</td>
<td>Background luminescence cannot be separated, good optical filtering necessary</td>
<td>[9]</td>
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<tr>
<td>Time-domain method</td>
<td>Pulse and gate</td>
<td>Simple separation of high background with short lifetimes, simple optical filtering</td>
<td>Species with similar lifetimes are difficult to separate, background with long lifetimes is difficult to separate</td>
<td>[15,3,5,27]</td>
</tr>
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</table>

2. Theory

2.1. Oxygen sensing [2,6]

The dynamic quenching of luminescence by oxygen [1,7–10,3,4,18,5,19–24,11] is the basis for the measurement of oxygen distributions in various systems. The applied sensors have a planar structure with the luminescent indicator embedded in a polymer matrix that is spread on a transparent support foil. The sensor area is imaged through an optical emission filter in the case of intensity images or directly in the case of lifetime images by lenses or imaging fibres coupled to a photosensitive CCD-chip of the camera. Each pixel on the CCD now monitors the light intensity either as the absolute luminescence light emission, or, with proper timing, a part of the luminescence decay curve.

The oxygen optodes were calibrated with a two component model of the Stern–Volmer equation [22]:

$$\frac{\tau}{\tau_0} = \frac{I}{I_0} = \frac{\tan(\Phi)}{\tan(\Phi_0)} = \frac{1}{1 + K_{SV}[O_2]} + (1 - \text{frac})$$

(rotation)

\(\tau, I, \Phi = \) decay time, intensity or measured phase angle in presence of oxygen, \(\tau_0, I_0, \Phi_0 = \) decay time, intensity or measured phase angle in absence of oxygen, \(\text{frac} = \) fraction of quenchable luminophore, \(K_{SV} = \) bimolecular quenching coefficient, \([O_2] = \) oxygen concentration.

2.2. Image detection

Luminescence lifetime imaging has two major advantages over intensity based imaging. It allows a good contrast enhancement and background suppression of unwanted luminescence contributions in the image. If this background luminescence has a different decay time than the luminophore of interest it is possible to separate the two signals by lifetime imaging. Further, lifetime imaging does not depend on intensity variations due to photobleaching (if it does not take place faster than the image acquisition) or variable indicator concentrations, and calibration free sensing applications [5] are possible. Table 1 gives an overview of the different possible methods for lifetime imaging, their inherent advantages and disadvantages.

The presented camera can not be modulated sinusoidally for frequency-domain evaluation, which has an advantage if images of different luminophores with similar lifetimes should be separated [25,13,14]. The phase delay ratioing method by Hartmann et al. [8,9] is possible with the new camera. However, it is not suitable for our applications because there can be an enormous background luminescence in the natural systems investigated with the new imaging system.

The new system, therefore, measures luminescence in the time-domain via a so called pulse-gate method [15,3–5]. Fig. 1 shows the detection principle applied for each pixel. For excitation the light source is
This intensity is, assuming a monoexponential decay curve with the apparent lifetime \( \tau \), described by the following equation:

\[
I_t = I_0 \cdot \tau \cdot \exp \left( \frac{t}{\tau} \right) \cdot \left[ 1 - \exp \left( -\frac{\Delta t}{\tau} \right) \right]
\]  

with the unknown intensity \( I_0 \) at time \( t_0 \) when the excitation light is switched ‘off’ (see Fig. 2). Instead of fitting the values \( I_t \) to \( I_0 \) and \( \tau \) we generate the new values:

\[
W_1 = -\ln \left( \frac{I_{t_1}}{I_{t_2}} \right)
\]

\[
W_2 = -\ln \left( \frac{I_{t_3}}{I_{t_2}} \right)
\]

If the image collection happens with the condition:

\[
\Delta_{t_1} = \Delta_{t_2} = \Delta_{t_3} = \Delta = \text{const.}
\]

the new values give the following relations:

\[
W_1 = \frac{t_{t_1} - t_{t_2}}{\tau}
\]

\[
W_2 = \frac{t_{t_3} - t_{t_1}}{\tau}
\]

Therefore the lifetime per pixel can be directly calculated with the known delay times \( t_i \):

\[
\tau = \frac{(t_{t_1} - t_{t_2})^2 + (t_{t_3} - t_{t_2})^2}{W_1 \cdot (t_{t_1} - t_{t_2}) + W_2 \cdot (t_{t_3} - t_{t_2})}
\]  

This method is not limited compared to a general fit to all values \( I_t \). The generation of each value \( W_i \) (Eqs. (7) and (8)) with subsequent fit (Eq. (9)) do not yield to a principal deviation from a general fit as long as Eq. (6) is valid.

The calculation of the values (Eqs. (7) and (8)) has, by division of the average values, the smallest error accumulation and is numerically stable. A variation of the timing window interval \( \Delta_t \), that is constant for all images, principally gives the opportunity to detect a non-limited number, \( n \), of values to evaluate \( \tau \) and, therefore, an error regression in the size of \( 1/\sqrt{n} \). This can be an advantage in the case of very noisy signals.

3. Experimental

3.1. Planar optode

The sensing layers for the comparison of luminescence intensity versus lifetime images were made of three different luminophores. The background or ‘noise’ layer was a commercial luminescent paint (Feuerrot, Conrad Electronics, Hirschau, Germany) with a luminescence decay time < 1 ns. This paint was spread on a microscope slide. The layers that should be
identified were made of two different oxygen indicators. One indicator was a Tris (4,7-diphenyl-1,10-phenantrrolin)-ruthenium(II) perchlorate [8,9,22–24], dissolved in an organically modified sol-gel (ormosil) with dispersed titanium dioxide scattering particles. The other example was a platinum-octaethyl-porphyrine (Porphyrin Products, Utah, USA), dissolved in polystyrene that also can be used for oxygen determination. Both oxygen indicators were spread on microscope slides. For the oxygen measurement the ruthenium based sensor was knife-coated on a polyester foil that was cut to a size of 28 × 40 mm² to fit into the test setup.

3.2. Imaging system and application

The imaging system (Fig. 3) consists of an electrically cooled CCD-camera (SensiMod, PCO Computer Optics, Kehlheim, Germany) with a direct fast electronical shutter feature, due to a new and fast charge carrier transport from the light detection cell to the shift register, where the electrons can be accumulated until the picture frame is read out. Additionally, the camera has a special modulation input to control directly the fast shutter (t_{on}=500 ns and t_{off}=500 ns, maximum frequency = 1 MHz). The camera (dynamic range = 12 Bit, resolution = 640 × 480 pixel) is connected via a serial fibre-optical link to a camera control PCI-board in a Pentium based PC. The PC controls image acquisition, storage, display and the timing. For the precise timing of the excitation light source switching and image acquisition, the PC is connected via a GPIB interface to a delay pulse generator (DG535, SRS Stanford Research Systems, Sunnyvale, USA). Timing control and primary image acquisition was programmed in C (Watcom C/C++ + 10 Compiler, Sybase, Emeryville, USA), while the image calculations and visualisation were programmed in a special software language made for the handling of large amounts of data (IDL 5.0, Research Systems, Boulder, USA).

For the experimental applications, the camera was equipped with a macro lens (Tevidon, F1.6 × 35 mm, PCO) that imaged an area of 28 × 40 mm² onto the CCD-chip. This corresponds to a theoretical resolution of 50 μm pixel⁻¹. Two different light sources were applied for illumination, respectively excitation of the planar optodes. For lifetimes > 6 μs and luminophores with very low quantum efficiency, a Xenon flash lamp with adjustable output power and a maximum repetition rate of 30 Hz (A0021F, Oxygen Enterprises, Philadelphia, USA) was connected to a fibre-optical ring light, that was specially designed for the applications, i.e. a homogenous illumination of a circle of 50 mm diameter in a distance of 50 mm (Schöllý Fiberoptic, Denzlingen, Germany). The second light source was developed for lifetime measurements from 100 ns upward and consists of up to 12 light emitting diodes (λ_p = 470 nm, BP280CWP1K, DCL Components, Hungerford, UK), that are coupled into the same fibre-optical ring light. To enable fast switching of the LEDs, a special driving circuit with adjustable current was designed. The camera and the ring light were fixed in a special light tight housing, that can easily be applied to a vertically mounted sensing layer.

3.3. Experimental setups

For testing the luminescence background suppression, microscope slides were mounted in front of the camera setup (Fig. 4). The first strongly luminescent layer was the slide with the commercial paint, followed
by the slides with the two different oxygen indicators. To be able to compare the situations, the optical emission filter was kept at its position in front of the camera. The images were recorded with different timing between excitation light and camera.

For oxygen mapping, a special multichannel setup was designed with six different flow channels, that were connected to a peristaltic pump. Water with different oxygen concentrations can be guided through these channels, while the whole system is surrounded by a thermostating housing to keep the temperature constant. The planar optodes were pressed by a plastic window onto these channels and an outer o-ring (Fig. 5). Therefore, the system was closed towards the camera. Water was pumped from water bottles immersed in water with the same temperature as the test setup. The test water in the bottles was constantly flushed with nitrogen (Fig. 5, ox4), oxygen (Fig. 5, ox2) and room air (Fig. 5, ox1, ox3), and while channels 5 and 6 were filled with tap water and closed. In the results channel 5 (Fig. 5, ox5) cannot be seen because it was out of the camera field of view, which was adjusted for 50 µm pixel⁻¹ resolution. Images were taken at 2 pixel vertical and horizontal binning, so the spatial image resolution was reduced to 100 µm pixel⁻¹.

4. Results and discussion

Fig. 6 shows the advantage of luminescence lifetime imaging in general. The pure intensity images (Fig. 6a, c) show a nearly constant grey distribution emphasising that the background luminescence is larger then the signal. Additionally, a lot of inhomogeneities can be
seen (dark and white dots in Fig. 6a, c), that are due to the applied spreading procedure of the luminescence paint and some dirt on the microscope slide. When now the images are delayed by a certain time, $t_d$, the fluorescence of the paint is decayed while the two stripes forming a ‘X’ of the ruthenium indicator (Fig. 6b) as well as the drawn ‘M’ of the porphyrine compound (Fig. 6d) clearly can be detected.

The ruthenium based optode had a decay time at room air of about 2.8 $\mu$s, therefore, the delay of the image in Fig. 6b was $t_d = 1 \mu$s. The drawn ‘M’ with the porphyrine had a decay time of $\approx 30 \mu$s, but the image taken with a delay time $t_d = 4 \mu$s (Fig. 6d) is not as high in contrast as the ‘X’. This is due to the fact that the porphyrine has a lower quantum efficiency, a higher quenching coefficient compared to the ruthenium, and the sensing layer did not contain any additional scattering particles.

The results with the oxygen mapping setup clearly demonstrated that the test chamber needs further improvement (Fig. 7a to c). The images show no sharp channel structure. Obviously, oxygen diffused from the 100% channel (Fig. 5 ox2, the black one in Fig. 7a and b, the white one in Fig. 7c) into neighbouring channels (flow direction in Fig. 7 was from the left to the right), while the channel with zero oxygen (Fig. 5 ox4, the white one in Fig. 7a and b, the black one in Fig. 7c) received oxygen from the channels above and below (Fig. 5 ox3, ox6), respectively. Nevertheless, the lifetimes in the middle of the channels were in the same range as determined with single point measurements based on a phase modulation technique (i.e. $\tau_0 = 4.8 \pm 0.05 \mu$s, $\tau_{20} = 3.2 \pm 0.05 \mu$s, $\tau_{100} = 2.1 \pm 0.05 \mu$s for oxygen saturation of 0, 20, 100%, respectively).

Fig. 7 shows the luminescence intensity image with all associated imperfections, like texture within the sensing foil due to the knife coating process and the fast and difficult to control evaporation of the solvent of the ormosil. Additionally, some shadows can be seen, that are caused by water between the transparent window and the polyester support foil of the optode. Both phenomena are gone in the lifetime image (Fig. 7b), and the converted image of oxygen values (Fig. 7c), which represents an inversion of Fig. 7b because now the location of the most oxygen is white and the absence is black. The lifetime image was calculated both with a set of three delayed images and with four delayed images with a window at a width of $\Delta = 2 \mu$s and delay times of $t_1 = 500$ ns, $t_2 = 1 \mu$s, $t_3 = 2 \mu$s and $t_4 = 3 \mu$s, respectively. Additionally, a background blank image was subtracted with the same time window but with lights ‘off’, because the CCD-chip has a specific locally fixed noise distribution. Both fitting calculations resulted in the same lifetime distribution. This indicates the stability of the fitting procedure as well as the quality of the information gained within the

images. A 2 pixel vertical and horizontal binning was used for the oxygen mapping to increase the absolute signal range. We also took images without binning at full spatial resolution of 50 $\mu$m pixel$^{-1}$. Here the calculations gave the same lifetimes but resulted in
higher noise levels. The correspondence between the spatial image resolution per pixel, given by the camera chip and the lenses (either 50 or 100 μm pixel⁻¹ in the demonstrated results), and the possible measuring resolution of the planar optodes has to be further investigated with specific test setups. This is necessary because of possible oxygen diffusion processes parallel to the optode surface, which could smear the spatial information.

5. Conclusion

A modular lifetime imaging system (MOLLI) based on a new, fast gateable CCD-camera was developed. This camera reduces the amount of equipment needed for lifetime imaging systems and allows for an easy adaption of the imaging system to different applications like optode based oxygen mapping in sediments and biofilms, around cells and aggregates.

Although the test setup for application of defined oxygen concentrations to the planar oxygen optodes was not optimum, the imaging system demonstrated the advantages of luminescence lifetime imaging in background signal suppression and less noise sensitive signal readout of planar optodes. This combined with the possibility of the measurement of two-dimensional distributions of solutes at high spatial resolutions instead of single profiles with microsensors the new system is a powerful tool for the investigation of natural biological systems.

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References

Biographies

Gerhard Holst, born 1962, studied electronical engineering at the RWTH University in Aachen, Germany, where he received the diploma in 1990, with a final work about reflectance pulse oximetry with electro-optical and hybrid fibre-optical sensors. From 1991 to 1994, he completed his Ph.D. in the group of Professor D.W. Lübbers at the Max-Planck-Institute (MPI) for Molecular Physiology, Dortmund, Germany, about a new optical chemical sensing principle, the oxygen flux optode and its phase modulation based measuring system. From 1994 he joined the microsensor research group of the MPI for Marine Microbiology, Bremen, Germany, as a post doc, where he is currently working on the development of new fibre-optic microsensors, microoptodes, their time resolved measuring schemes, and systems for laboratory and field applications.

Oliver Kohls, born 1965, graduated in technical chemistry at the University of Hannover, Germany, in 1992. In 1995 he received a Ph.D. at the University of Hannover. His work was about optical oxygen sensors, their measuring systems and their application in biotechnological process monitoring. Since 1995, he works in the MPI for Marine Microbiology in Bremen, Germany. His scientific interest is the development of optical microsensors and their application in the natural environment, especially marine systems.

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Michael Kühl, born 1964, studied biology at the University of Aarhus, Denmark, where he received a M.Sc. degree in microbial ecology in 1988 with a thesis on the development of fibre-optic microprobes and a measuring system for microscale light measurements in sediments and biofilms. From 1988 to 1992, he completed his Ph.D. at the Department of Microbial Ecology, University of Aarhus, Denmark. His Ph.D. work involved the use of both optical and electrochemical microsensors to study the microenvironment in compact microbial communities. From 1992, he joined the MPI for Marine Microbiology, Bremen, Germany, as a post doc to build up microsensor research at the institute. From 1995 to 98 he was the head of the microsensor research group at the MPI in Bremen. Currently he holds a special research associate professorship at the Marine Biological Laboratory, University of Copenhagen, Denmark. His scientific interests are the development and use of fibre-optic and electrochemical microsensors in microbial ecology.

Thomas Richter, born 1962, graduated in mathematics (automorphic forms) and physics (negatively charged cluster ions) at the University of Freiburg, Germany, in 1987. In 1994 he received his Ph.D. at the University of Freiburg (Faculty of Biology). His work was about light propagation in higher plant leaves with a combined experimental and theoretical approach, including fibre-optic light measurements inside the leaf. He is working at the University in Freiburg on leaf photosynthesis and was a visiting scientist in microsensor research at the MPI from October 1997 to June 1998. His scientific interest concerns light driven processes in connection with ecological studies of photosynthetic performance in higher plants.