Journal of Fluorescence, Vol. 7, No. 1, 1997

Fluorescence Lifetime Imaging of Oxygen in Living Cells

H. C. Gerritsen,[1,3] R. Sanders,[1,2] A. Draaijer,[2] C. Ince,[1] and Y. K. Levine[1]

Received June 14, 1996; accepted August 23, 1996

The usefulness of the fluorescent probe ruthenium tris(2,2'-dipyridyl) dichloride hydrate (RTDP) for the quantitative imaging of oxygen in single cells was investigated utilizing fluorescence lifetime imaging. The results indicate that the fluorescence behavior of RTDP in the presence of oxygen can be described by the Stern–Volmer equation. This shows that fluorescence quenching by oxygen is a dynamic quenching process. In addition, it was demonstrated that the fluorescence lifetime of RTDP is insensitive to pH, ion concentration, and cellular contents. This implies that a simple calibration procedure in buffers can be used to quantify oxygen concentrations within cells. First fluorescence imaging experiments on J774 macrophages show a nonuniform fluorescence intensity and a uniform fluorescence lifetime image. This indicates that the RTDP is heterogeneously partitioned throughout the cells, while the oxygen concentration is constant.

KEY WORDS: Fluorescence lifetime imaging; oxygen imaging; confocal microscopy; macrophages.

INTRODUCTION

Much effort has been spent on the development of oxygen sensors for the quantification of oxygen concentrations in chemical and biological systems. To this end, macroscopic electrochemical (Clark-type electrodes) and fluorescence-based sensors were developed.[1–5] The fluorescence-based sensors rely on quenching of the fluorescence by collisions with oxygen. The collisions introduce an extra decay channel for the excited molecules, which results in a reduction both of the fluorescence intensity and of the fluorescence decay time. The Stern–Volmer equation [Eq. (1)] relates the relative change of the fluorescence decay time and fluorescence intensity to the quencher (oxygen) concentration.

$$\frac{\tau_0}{\tau} = \frac{I_0}{I} = 1 + k_{sv}[O_2] \qquad (1)$$

with $[O_2]$ the oxygen concentration, I_0 and I the intensities, τ_0 and τ the fluorescence decay times in the absence and presence, respectively, of quenching and k_{sv} the quenching coefficient. k_{sv} depends on the temperature, viscosity of the surrounding medium, collision radius, and quenching efficiency of the fluorescent molecule.[6]

Equation (1) shows that the oxygen concentration can easily be related to the ratio of the unquenched and quenched fluorescence intensities or fluorescence lifetimes. Both approaches can be implemented in imaging systems for the quantitative imaging of oxygen concentrations in, e.g., living cells. However, the intensity ratio determination is prone to a number of experimental difficulties. In particular, it requires the acquisition of an unquenched calibration image to correct for inhomogeneous probe partitioning. This necessitates the recording of an image from an oxygen-free sample, which is complicated when working on living samples. The fluorescence lifetime, on the other hand, is insensitive to partitioning effects of the probe in the sample and τ_0 can simply be determined in an oxygen-free buffer. Other benefits of employing the lifetime rather than the inten-

[1] Department of Molecular Biophysics, Utrecht University, P.O. Box 80.000, 3508 TA Utrecht, The Netherlands.
[2] TNO, Institute of Environmental Sciences, Group Sensors, P.O. Box 6011, 2600 JA Delft, The Netherlands.
[3] To whom correspondence should be addressed.

1053-0509/97/0300-0011$12.50/0

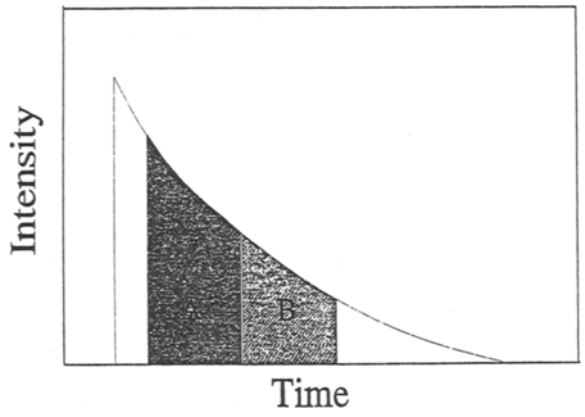

Fig. 1. The principle of time-gated detection. After pulsed excitation the fluorescence is collected in the two time windows A and B.

sity are its insensitivity to, e.g., photo bleaching and absorption/scattering effects.

Fluorescence lifetime imaging has been implemented by several groups in wide-field[7–9] as well as in confocal[10] microscopes. The advantage of the confocal arrangement is that only fluorescence from a small volume element in the sample is observed. This significantly improves the contrast of the fluorescence lifetime images compared to wide-field imaging systems. An additional advantage of the confocal approach is that only single-channel detection is required, which simplifies the design of the fluorescence lifetime detection system significantly.

Here we describe the fluorescence lifetime properties of the oxygen-sensitive probe ruthenium tris(2,2'-dipyridyl) dichloride hydrate (RTDP). The potential of this probe for the quantitative imaging of oxygen concentrations using fluorescence lifetime imaging is illustrated by an imaging study of oxygen in single cells (J774 macrophages).

MATERIALS AND METHODS

Confocal Fluorescence Lifetime Imaging

The fluorescence lifetime imaging experiments described here are carried out with a modified conventional confocal laser scanning microscope (CLSM) equipped with a low-power cw argon-ion laser.[11] The modifications required for fluorescence lifetime imaging have been described by Buurman *et al.*[10] Here we limit ourselves to a brief description of the main modifications of the CLSM.

A fast electrooptical chopper is positioned between the Ar-ion laser and the microscope. This combination produces 2-ns pulses with a repetition frequency of up to 25 MHz at a wavelength of 488 nm that are used to excite the sample. The fluorescence emission is detected sequentially in two time-gated windows, each delayed by a different time with respect to the excitation pulses (see Fig. 1). The window widths (>2 ns) and delays can be adjusted independently. The fluorescence lifetime is a function of the ratio of the accumulated fluorescence intensities.[12] For a monoexponential decay of the fluorescence intensity and equal gate widths, the fluorescence lifetime τ is given by

$$\tau = \frac{\Delta t}{\ln (I_A/I_B)} \tag{2}$$

where Δt is the time delay between the two (adjacent) windows and I_A and I_B are the corresponding fluorescence intensities. In the case of a fluorescence decay showing more than one decay component, only a single effective lifetime can be determined using this method. The fluorescence intensity is acquired for every excitation pulse in the two windows A and B sequentially. This is repeated about 1000 times, after which the intensities accumulated in the two windows are read by a frame-grabber. This procedure is repeated for all pixels (voxels) in the image.

All the measurements presented in this paper were recorded using an Olympus oil-immersion objective 100×/NA 1.25. The fluorescence emission in the lifetime experiments was selected by means of a dichroic beamsplitter (510 nm) in combination with a 520-nm long-pass filter. The unquenched RTDP probe exhibits a comparatively slow monoexponential decay (τ_0) of about 765 ns[3]. This forced us to use the low repetition frequency of 555 kHz for the excitation light source. Despite this low repetition rate, it turned out to be possible to record a 256 × 256 image of single cells in about 100 s. Acquisition times of 400 s for a 512 × 512 image were used for calibrations on buffers. Fluorescence lifetime images were acquired on setting the leading edge of window A several nanoseconds after the excitation pulse. The leading edge of window B started about 300 ns after the leading edge of window A. Both windows had an identical width of 300 ns.

Calibration and Sample Preparation

We have determined here the lifetime response of the microscope as a function of the oxygen concentration experimentally. This was achieved by recording the win-

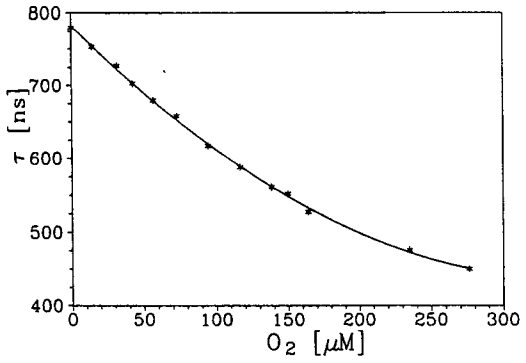

Fig. 2. The fluorescence lifetime of RTDP in buffers containing oxygen concentrations in the range <002 to 300 μ*M*.

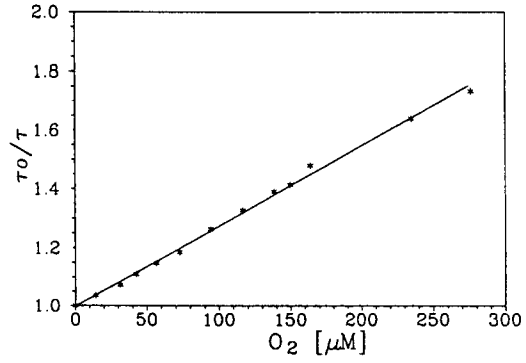

Fig. 3. The ratio of the unquenched and quenched lifetimes of RTDP as a function of the buffer oxygen concentration.

dow ratios using a series of probes dissolved in buffers containing known oxygen concentrations and converting them to lifetimes using Eq. (2). The oxygen concentration in an RTDP (Molecular Probes, Eugene, OR) solution of 3.5 mg/ml in water was varied by bubbling nitrogen gas through the buffer solution for different times. The oxygen concentration in the solution was measured with a macroscopic oxygen sensor (Oxi91 sensor; WTW, Weilheim, Germany) after a 3-min equilibration period. The solutions were then transferred to an airtight sample holder[13] for imaging. The sample compartment was flushed with the buffer solution for 3 min, prior to measuring the window ratio.

The environmental effects on RTDP fluorescence were studied using water and phosphate buffers of pH 7, 7.5, and 8.0 at 3.5 mg RTDP/ml. The phosphate buffers contained 0.2 *M* Na_2HPO_4 and 0.2 *M* NaH_2PO_4.

Macrophages of the cell line J774 were grown for 24 h on glass coverslips and used within 3 days. They were stained with RTDP using the following procedure. Ninety microliters of the RTDP stock solution (27.5 mg/ml RTDP in water) was added to 3 ml of the growth medium. Two hundred microliters of a solution with 250 m*M* $CaCl_2$ and 200 μl of a pH 7.05 buffer containing 42 m*M* Hepes, 275 m*M* NaCl, 10 m*M* KCl, 1.4 m*M* NaH_2PO_4, and 10 m*M* glucose was added. The macrophages were then incubated for 2 to 4 h at 37°C. After the incubation period the macrophages were washed three times with a KR–Hepes buffer, pH 7.4, containing NaCl (130 m*M*), KCl (4.7 m*M*), $CaCl_2$ (1.3 m*M*), NaH_2PO_4 (0.44 m*M*), $MgCl_2$ (1.1 m*M*), $NaHCO_3$ (20 m*M*), glucose (0.2%, w/v) and Hepes (10 m*M*).

The imaging experiments were carried out immediately after the staining procedure. The imaging experiments as well as the calibration of the microscope response were all carried out at room temperature.

RESULTS AND DISCUSSION

Figure 2 shows the fluorescence lifetime of RTDP in buffers containing oxygen concentrations in the range of <0.02 to 300 μ*M*. This covers the concentration ranges of physiological interest (0–60 μ*M*) as well as the venous to arterial range (90–260 μ*M*). The calibration curve in Fig. 2 was used in subsequent experiments to convert fluorescence lifetimes into oxygen concentrations. From the accuracy in the window ratio of about 1%, an accuracy of about 2% is found for the oxygen concentration calibration curve.

To verify the validity of the Stern–Volmer relation for describing the fluorescence quenching of RTDP, the ratio of the unquenched and quenched lifetimes was plotted as a function of the oxygen concentration. Figure 3 shows the dependence of this ratio on $[O_2]$ in buffers. An excellent linear relation was found over the entire O_2 concentration range used, from 0 to 300 μ*M*: $\tau_0/\tau = 0.998 + 2.73\ 10^{-3}\ [O_2]$, with $R = 0.999$. As predicted by the Stern–Volmer equation the intercept of the curve with the *y*-axis was virtually 1.

The usefulness of RTDP for quantitative O_2 imaging in cells is also determined by the sensitivity of its fluorescence behavior to environmental factors such as pH, ion concentrations, and other cell components. The effects of pH and ion concentrations were mimicked in imaging experiments using buffers of pH 7, 7.5, and 8 with no added NaCl or 150 m*M* NaCl at a constant oxygen concentration. No changes in the fluorescence lifetime within the experimental errors were observed.

The fluorescence lifetime of a probe is, in general, independent of intensity effects including fading due to photobleaching. However, besides photobleaching, photodegradation may take place. The latter can introduce

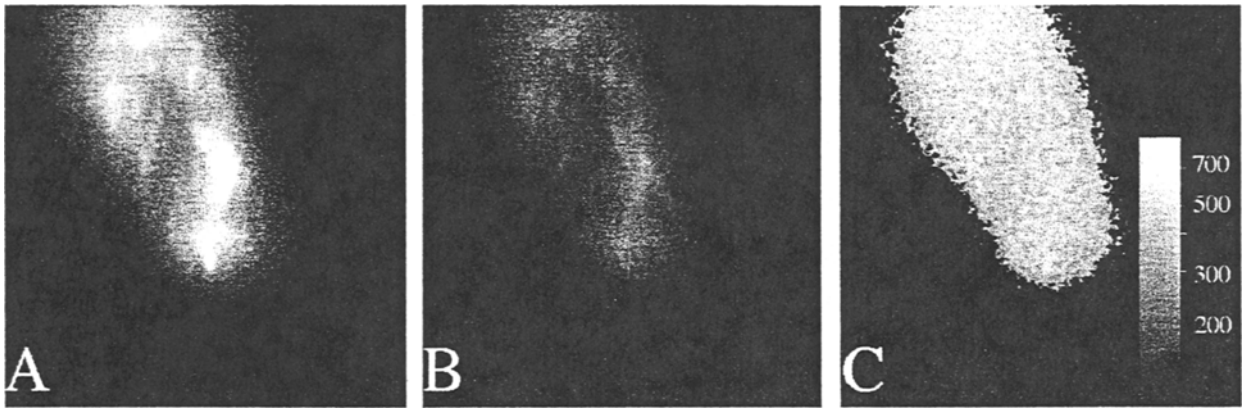

Fig. 4. Intensity images corresponding to windows A (A) and B (B) of a RTDP-stained J774 macrophage. The fluorescence lifetime image (C) shows that the lifetime and thus the oxygen concentration are constant throughout the cell. The scale of the calibration bar is nanoseconds.

photoproducts whose fluorescence emission bands overlap that of RTDP and exhibit a different fluorescence lifetime. To investigate the sensitivity of RTDP to photodegradation, we followed the variation of the window ratio of RTDP dissolved in a buffer at pH 7 for 2 h. No significant changes were found in the fluorescence lifetime, even though the fluorescence intensity decreased by about 10% (data not shown). We thus conclude that illumination of the sample does not cause any significant photodegradation on the time scale of the experiments described here.

Finally, we used the information obtained above to carry out quantitative O_2-imaging experiments on RTDP stained J774 macrophages. Typical intensity images of a macrophage corresponding to windows A and B are shown in Figs. 4A and B, respectively. The images show a nonuniform fluorescence intensity across the cell, which can be due either to a inhomogeneous partitioning of the probe across the cell or to differences in oxygen-concentration within the cell. The former possibility is ruled out by the corresponding fluorescence lifetime image, Fig. 4C, which shows that the lifetime is constant throughout the cell. In Fig. 4C the lifetime is calculated from the window ratio using Eq. (2). Pixels with intensities below a certain threshold were set to zero. The constant lifetime indicates a uniform oxygen concentration across the cell. Therefore, the heterogeneities in the fluorescence intensity image can be attributed to a differential partitioning of the probe into cell organelles in the cytoplasm.

We noted that during the imaging experiments, leaking of probe out of the sample took place. This problem can be solved by esterizing RTDP. However, probe leaking was slow on the time scale of the experiments. Moreover, fluorescence lifetime imaging is not sensitive to intensity effects such as probe leaking.

CONCLUSIONS

We have investigated the usefulness of the fluorescent probe RTDP for the quantitative imaging of oxygen concentrations using fluorescence lifetimes. The results indicate that the fluorescence behavior of RTDP in the presence of oxygen can be well described as a dynamic quenching process, satisfying the Stern–Volmer equation. In addition, it was demonstrated that the fluorescence lifetime of RTDP is insensitive to pH, ion concentration, and cellular contents. This implies that a simple calibration procedure in buffers can be used to quantify oxygen concentrations within cells.

ACKNOWLEDGMENTS

This research is supported by the Technology Foundation (STW), Grant UNS00.2225, under the auspices of The Netherlands Organization for Scientific Research (NWO).

REFERENCES

1. H. W. Kroneis and H. J. Marsoner (1983) *Sensors Actuators* **1**, 587.
2. E. R. Carraway, J. N. Demas, B. A. DeGraff, and J. R. Bacon (1991) *Anal. Chem.* **63**, 337–342.
3. M. E. Lippitsch, J. Pusterhofer, M. J. P. Leiner, and O. S. Wolfbeis (1988) *Anal. Chim. Acta* **205**, 1–6.

4. O. S. Wolfbeis, L. J. Weis, M. J. P. Leiner, Leiner, and W. E. Ziegler (1988) *Anal. Chem.* **60**, 2028–2030.
5. S. B. Bambot, R. Holavanahali, J. R. Lakowicz, G. M. Carter, and G. Rao (1994) *Biotech. Bioeng.* **43**, 1139.
6. J. R. Lakowicz (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
7. C. G. Morgan, A. C. Mitchell, and J. G. Murray (1990) *Trans. Roy. Microsc. Soc.* **Micro 90**, 463–466.
8. X. F. Wang, T. Uchida, D. M. Colaman, and S. Minami (1991) *Appl. Spectrosc.* **45**, 360–366.
9. J. R. Lakowicz, H. Szmacinski, and K. Nowaczyk (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1271–1275.
10. E. P. Buurman, R. Sanders, A. Draaijer, H. C. Gerritsen, J. J. F. Van Veen, P. M. Houpt, and Y. K. Levine (1992) *Scanning* **14**, 155–159.
11. A. Draaijer and P. M. Houpt (1988) *Scanning* **10**, 139–145.
12. R. J. Woods, S. Scypinski, L. J. Cline Love, and H. A. Ashworth (1984) *Anal. Chem.* **56**, 1395–1400.
13. C. Ince, R. E. Beekman, and G. Verschagen (1990) *J. Immunol. Methods* **128**, 227–234.