Chapter 39
Triplet Imaging of Oxygen Consumption During the Contraction of a Single Smooth Muscle Cell (A7r5)

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1 Introduction

We have recently demonstrated a novel technique [1] that enables monitoring of oxygen kinetics at the cellular and sub-cellular level. The method was based on quenching by oxygen of the triplet state of a standard fluorescent compound.

Many other methods enabling oxygen sensing at the cell level exist. Previous work on this topic includes methods based on fluorescence intensity quenching [2], phosphorescence lifetime based methods [3], luminescence based methods [4], and methods working with delayed fluorescence [5].

However, most of these methods are based on low-intensity signals, which does not allow for both high-spatial and high-temporal resolution. Our method is based on a modulated excitation [6] of a standard fluorescent molecule. The resulting

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M. Wolf et al. (eds.), *Oxygen Transport to Tissue XXXIII*, Advances in Experimental Medicine and Biology 737, DOI 10.1007/978-1-4614-1566-4_39,
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signal can be detected with a standard integrating charge coupled device camera. This method is compatible with almost any fluorophore with a (non-radiative) triplet state that can be quenched by dissolved molecular oxygen. Further on, since a multitude of fluorophores can be used, a number of standard labeling protocols exist that can be used with our technique.

2 Oxygen Sensing by Triplet State Quenching

More than 60 years ago, German scientists discovered that molecular oxygen interacts with fluorescent compounds and quenches their luminescence [7]. This strong interaction is related to the particular electronic configuration of the ground state of molecular oxygen. The molecule resides in a triplet configuration, which enables it as an acceptor for energy transfers from the excited triplet state of a fluorescent molecule as described in Fig. 39.1. The relationship between the quencher (oxygen) and the triplet state lifetime can be described by the Stern–Volmer equation (see [1] for further details). Inside a cell, the molecule is exposed to varying micro-environments,

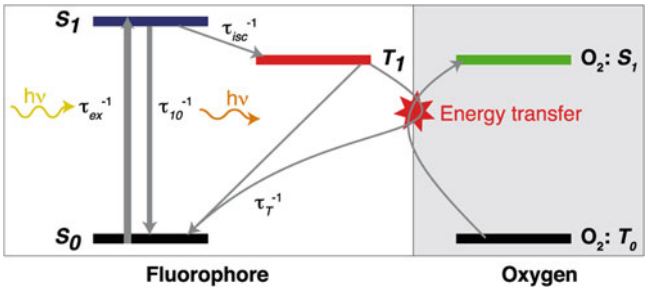

Fig. 39.1 Jablonski diagram of a fluorescent molecule illustrating the three basic energy states of the molecule. Molecular oxygen exhibits a triplet configuration in its ground state which allows it to accept energy transfers from the triplet state of the fluorescent molecule. Reprint from [1]

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which modify locally both the diffusion time as well as the triplet lifetime in the absence of the quencher because of additional relaxation pathways [8]. This makes it difficult to map oxygen concentrations to measured triplet lifetimes. But nevertheless *variations* in oxygen concentrations are directly proportional to *variations* in triplet lifetimes (see [1] for derivation of formalism). It is hence possible to look at the kinetics of oxygen consumption at the cellular and sub-cellular levels using the described quenching mechanism.

3 Triplet Imaging by Modulated Excitation

The method is based on a modulated excitation scheme, which leads to characteristic population of the triplet as well as singlet states. The resultant fluorescent signal shows a distinctive decrease in intensity for longer pulse widths of excitation. This decrease is related to the increasing population on the triplet state.

We built a custom-made excitation setup where the emission of a Millenia Pro laser ($\lambda = 532$ nm; Newport Spectra Physics) was switched with an acousto-optic modulator (rise-time of ~10 ns; Gooch and Housego). This source was used to illuminate the sample observed on a standard upright microscope (Axiovert 200, Objective: Plan-Neofluar 40×/0.75 Ph2; Carl Zeiss). The image acquisition was done with an electromagnetic charge-coupled device camera (Luca, Andor Technology). Further details can be found in [1].

A typical image of a smooth muscle cell A7r5 with good triplet state lifetime contrast can be seen in Fig. 39.2. Many sub-cellular compartments can be distinguished on the triplet lifetime image (red and yellow spots). As described in Sect. 39.2, these are most likely linked to different micro-environments and not different oxygen concentrations.

3.1 Calibration by Oxygen Scavenging System

In order to relate the triplet lifetime to oxygen concentrations, we have calibrated and validated the oxygen sensitivity of our setup, using the enzyme ascorbase (Sigma-Aldrich) for catalyzing the oxidation of L-ascorbic acid (Sigma-Aldrich) as proposed by Lo [9]. In parallel to the triplet imaging measurement, we monitored the oxygen content of the solution using a dissolved oxygen probe (SG6 Seven Go Pro with InLab 605 sensor; Mettler-Toledo). As shown in Fig. 39.3a the probe reveals the linear decrease of dissolved oxygen. The graph of the triplet state relaxation rate $k_T = 1/\tau_T$ in Fig. 39.3b also demonstrates a linear relationship, which suggests that the molecules' response to oxygen can indeed be described by the Stern–Volmer model.

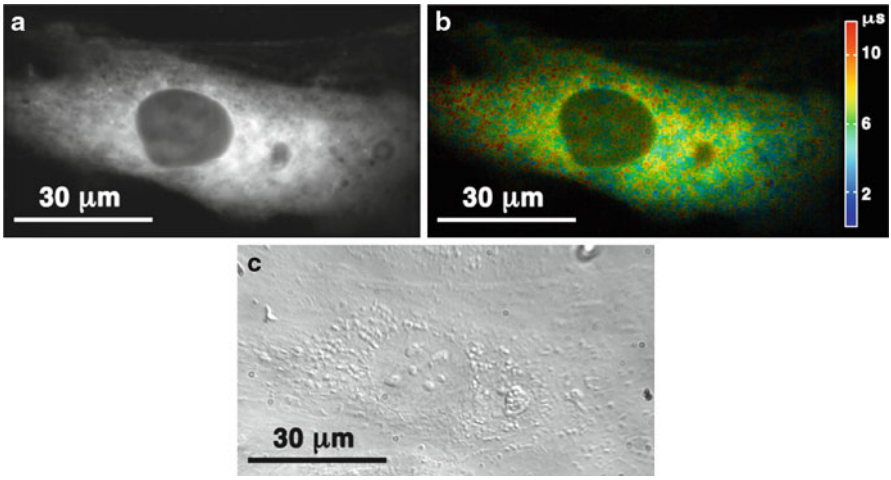

Fig. 39.2 Triplet state image of a smooth muscle cells A7r5 with transient transfection of the cytosolic fusion protein β-galactosidase (SNAP-β-gal) employed for the labeling with TMR. (**a**) Fluorescence intensity image. (**b**) Triplet lifetime image with color encoded lifetime. (**c**) Differential interference contrast (DIC) image. The acquisition settings for this image were optimized for best triplet lifetime contrast resulting in peak intensities at the center of the image of ~4 mW/mm^2. This cell has been labeled using the standard covalys labeling protocol (incubation with TMR-Star at 5 µM for 30 min)

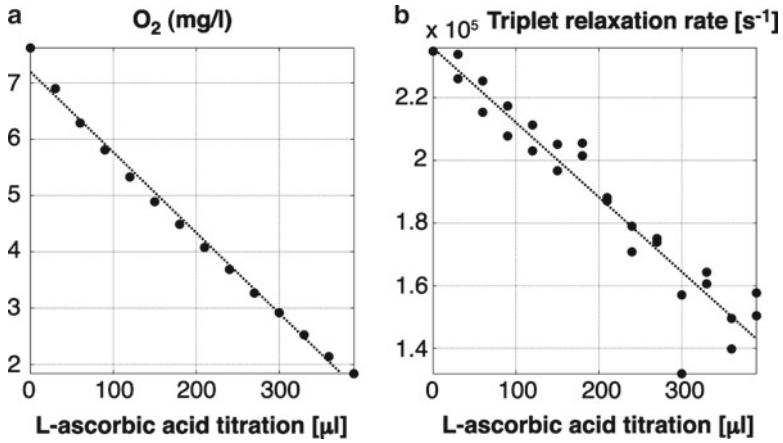

Fig. 39.3 Observation of TMR adhering to a glass cover slide in a flow cell. Titration of 25.5 mM L-ascorbic acid removes the oxygen in controlled discrete steps. (**a**) Dissolved oxygen as measured by a commercial probe. (**b**) Triplet state relaxation rate $k_T = 1/\tau_T$. Reprinted from [1]

4 Triplet Imaging of Single Smooth Muscle Cells A7r5

In a more applied experiment, we assessed the oxygen consumption of a single rat thoracic aorta smooth muscle cell A7r5 (Promochem; CRL-1444) during a [Arg^8]-vasopressin acetate salt (AVP; Sigma; V9879) stimulated contraction experiment [10]. The cells have been labeled using the SNAP-tag technique [11]. We transfected A7r5 with the SNAP-tag-β-galactosidase which is a cytosolic protein linked to a tag. Labeling the tag allowed attaching tetramethylrhodamine (TMR) as fluorophore. Further details on the experimental methods can be found in [1].

The results of three AVP stimulation experiments are shown in Fig. 39.4a. The displayed curves stem from a selected area inside the cells (usually about one-third of the whole cell size, see [1] for the corresponding images). As mentioned above, the variation in $k_T = 1/\tau_T$ is proportional to the variation in the oxygen concentration. After global stimulation with 500 nM AVP the cells undergo a contraction. This leads to an oxygen consumption inside the cell, which can be observed on the temporal evolution of the triplet relaxation rate. In Fig. 39.4b the temporal evolutions are normalized with respect to their initial and final steady states. For the negative controls, we used a factor of 0.4×10^{-5} for the normalization corresponding to a typical difference between the steady state values before and after adding AVP. The intracellular oxygen concentration shows a mono-exponential decay upon contraction. This is in good agreement with previously measured results in skeletal muscle fibers [12].

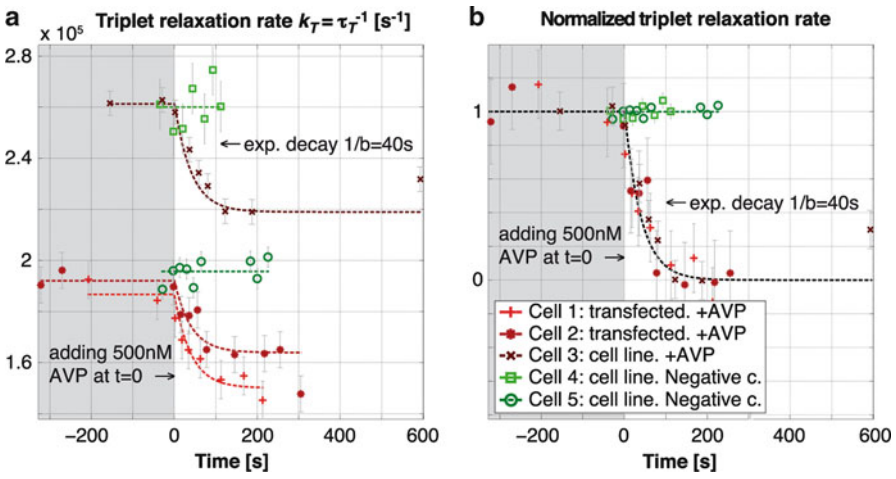

Fig. 39.4 Three experiments on [Arg8]-vasopressin (AVP) induced contraction of smooth muscle cells A7r5 with transient transfections of the cytosolic fusion protein β-galactosidase (SNAP-β-gal) employed for the labeling with TMR. Comparison with negative control (no AVP-stimulation). Reprinted from [1]

5 Conclusion

We have demonstrated a novel concept for functional wide-field microscopy that allows monitoring of oxygen kinetics with both high spatial and temporal resolutions. In a calibration experiment, the system demonstrated a linear response to the removal of oxygen following the Stern–Volmer model. In a second step we have measured the oxygen consumption of a single smooth muscle cell A7r5 upon induced contraction. The intracellular oxygen concentration shows a mono-exponential decay upon contraction.

In conclusion, our proposed triplet-state imaging concept is a novel method for investigating oxygen concentration variations at the cellular and sub-cellular levels with time resolutions in the order of a second.

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