Calibration and validation of an optical sensor for intracellular oxygen measurements

Dhruv Sud and Mary-Ann Mycek*
University of Michigan, Department of Biomedical Engineering, 1101 Beal Avenue, Ann Arbor, Michigan 48109-2099

Abstract. Calibration of fluorescent optical sensors for accurate, quantitative intracellular measurements in vivo suffers from lack of a representative medium that appropriately simulates the molecular complexity of the cytosol. We present a novel protocol for accurate intracellular oxygen sensing via fluorescence lifetime imaging microscopy (FLIM) using cell lysate-FLIM measurements to correct the in vitro calibration of a fluorescent oxygen sensor, and we describe electron paramagnetic resonance (EPR) validation studies. Lysate-FLIM studies provided biochemical information, while EPR provided a “gold standard” for intracellular oxygen estimation. Oxygen levels were evaluated in living human normal squamous and adenocarcinoma esophageal epithelial cells, and good agreement was observed between oxygen levels derived from the optical protocol and EPR. The proposed protocol introduces the concept of a living cell line as a reference for estimating unknown oxygen levels in other cell lines and accounts for high degrees of variability between different cell lines. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3116714]

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Fig. 1 Intensity-overlay FLIM image of the oxygen sensor RTDP in living cells (~10–12 μm diam). RTDP lifetime maps serve as the input to the Stern–Volmer equation \[ \frac{\tau_0}{\tau} = \frac{1}{K_q} \] for estimation of oxygen levels.

their intracellular composition, any calibration of the fluorescent probe is unlikely to be universally applicable. The methods discussed here aim to provide a generic protocol for accurate intracellular oxygen sensing with any oxygen-sensitive fluorophore. The protocol developed employs a calibration correction derived from optical measurements on cellular lysate, which is representative of the intracellular environment and commonly used to recover enzymes and proteins in their active states.

Oxygen is the terminal electron acceptor of oxidative phosphorylation in mitochondria and a key indicator of metabolic function in aerobic cells and organisms. Information obtained from accurate oxygen estimation has been applied to study mitochondrial function, signaling pathways, effects of various stimuli, membrane permeability, disease differentiation, and to screen for new drugs. Various methods exist for estimating oxygen at different scales, whether in whole organs [magnetic resonance imaging (MRI) and nuclear magnetic resonance (NMR)], blood (absorption), tissues [electrochemical microelectrodes], or individual cells (phosphorescence, fluorescence, [electron paramagnetic resonance (EPR)]). Optical approaches, such as fluorescence microscopy, provide the most viable approach for intracellular oxygen sensing with high sensitivity and subcellular spatial resolution. Fluorescence lifetime-based methods additionally circumvent most complications associated with intensity-based studies, such as photobleaching, optical loss, and variable fluorophore concentration in cells.

Figure 1 shows a representative intensity-overlay fluorescence lifetime imaging microscopy (FLIM) image of the fluorescent oxygen sensor ruthenium tris(2,2’,2”-dipyridyl) dichloride hexahydrate (RTDP) in living cells. Such lifetime maps serve as the input to the Stern–Volmer equation for estimation of oxygen levels via collisional quenching. The oxygen sensitivity of RTDP was calibrated at 37 °C in PBS and applied to extracellular and intracellular oxygen measurements using FLIM. Here, for the first time, lysate-FLIM studies were performed to refine this calibration and to make it sample specific. All fluorescence-based oxygen measurements were then compared to EPR.

*Address all correspondence to: mycek@umich.edu.

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Cellular lysate of HET-1 (human normal squamous esophageal epithelial cells) and SEG-1 (human Barrett’s adenocarcinoma esophageal epithelial cells) were generated using the NP-40 buffer via the following protocol. Briefly, NP-40 was thawed on ice and 10 µL/mL of Halt Protease Inhibitor was added for inhibition of serine-, cysteine-, metallo-, and aspartic acid-proteases in addition to aminopeptidases. Because of DNA/RNA release, 50 µL/mL each of DNAse and RNAse was added to reduce viscosity. Cells grown in culture flasks were scraped off into PBS, centrifuged, and washed twice with ice-cold PBS. The NP-40 formulation was added in a ratio of 1 mL buffer/10^6 cells. The solution was placed on ice and vortexed for 30 s every 10 min. After 30 min, the solution was transferred to microcentrifuge tubes and run at 13,000 rpm for 10 min at 4 °C. This allowed the heavier debris (e.g., cell membrane components) to sediment; the clear aliquot was removed and frozen at −80 °C. For treatment with LiNc–BuO into cells, as well as removal of remaining extracellular particles, both HET and SEG were grown to 70% confluence in 35-mm dishes, then suspended in 1mL MEM. A probe sonicator was used to create particles with sizes 13,000 rpm for 10 min at 4° C. This allowed the heavier supernatant liquid was then aliquoted out. For treatment with extracellular oxygen was measured via EPR on living SEG cells and reported elsewhere.2 Given that extracellular oxygen was ~227 µM and no known mechanisms of active oxygen transport exists in cells, it is likely that [O_2]_SEG was overestimated by the uncorrected calibration. Indeed, cell lysate studies indicated that the potential lifetime difference might have been overestimated by ~13.4 ns (Table 1). When corrected by this factor, we obtain a revised average intracellular RTDP lifetime difference of 30.6 ns and corrected value of [O_2]_BLIM_SEG=225.8 µM.

EPR oximetry provided a well-established means of validating these intracellular oxygen studies. Lysate studies on the experimental probe LiNc–BuO indicated little or no effect of the intracellular environment. As shown in Table 1 data in gray boxes), intracellular oxygen levels measured via EPR on living HET cells ([O_2]_EPR_HET=180.0 µM) were very close to the values estimated via RTDP FLIM maps of living HET cells. Also (data in gray boxes), intracellular oxygen levels measured via EPR on living SEG cells ([O_2]_EPR_SEG=210.1 µM) were close to the values estimated via RTDP FLIM maps of living SEG cells that were corrected using the manufacturer’s guidelines for the spectrometer (www.bruker.com). A previously reported calibration was used to convert the spectral bandwidth to oxygen levels (0% [O_2]=210 mG line width, 8.5 mG/mmHg oxygen sensitivity for LiNc–BuO).16

Table 1 shows the analysis of results. Both HET-I and SEG-I were exposed to similar oxygen levels (227 µM in solution), yet exhibited different RTDP lifetimes. Given that both cell lysates were generated in an identical fashion and that the experiments were not affected by factors such as pH, viscosity, temperature differences, or aggregation (shown previously in Ref. 2), the differences in lysate RTDP lifetime were likely indicative of cytosolic composition and/or of greater dynamic quenching in SEG versus HET. The differences in lysate RTDP lifetime were small compared to RTDP lifetime (~13.4/300 ns, or ~4.5%) and were statistically significant (p<0.05).

Table 1: Lifetime differences Δτ=τ_{HET}−τ_{SEG} and oxygen estimates from FLIM experiments on living cells and cellular lysates. Revised values of Δτ (computed as a−b) were used to correct [O_2]_{SEG} levels. Corrected FLIM-derived oxygen estimates [O_2]_{BLIM} were in good agreement with EPR validation measurements ([O_2]_{EPR}; K_q values were estimated via the Stern–Volmer equation with known lifetime and corrected oxygen values for both HET and SEG cell lines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Δτ (ns)</th>
<th>[O_2]_EPR (µM)</th>
<th>Revised Δτ (ns)</th>
<th>[O_2]_BLIM (µM)</th>
<th>[O_2]_EPR (µM)</th>
<th>Estimated K_q</th>
</tr>
</thead>
<tbody>
<tr>
<td>HET cells</td>
<td>182.1</td>
<td>182.1±9.4</td>
<td>180.0±9.4</td>
<td>0.0044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEG cells</td>
<td>44(a)</td>
<td>260.5±30.6</td>
<td>225.8±10</td>
<td>210.1±8.5</td>
<td>0.0054</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1**: Revised values of Δτ for both HET and SEG cell lines. Corrected FLIM-derived oxygen estimates [O_2]_{BLIM} were in good agreement with EPR validation measurements ([O_2]_{EPR}; K_q values were estimated via the Stern–Volmer equation with known lifetime and corrected oxygen values for both HET and SEG cell lines.}

**Table 1**: Revised values of Δτ for both HET and SEG cell lines. Corrected FLIM-derived oxygen estimates [O_2]_{BLIM} were in good agreement with EPR validation measurements ([O_2]_{EPR}; K_q values were estimated via the Stern–Volmer equation with known lifetime and corrected oxygen values for both HET and SEG cell lines.
correction values obtained from the cell lysate studies described above. We note that the trend of \([O_2]_{seq} < [O_2]_{HET}\) was maintained, despite some expected variations due to the differences between fluorescence imaging and EPR spectroscopic methods (e.g., the use of adherent vs. suspended cell samples).

These results suggest a novel protocol for intracellular oxygen sensing in living cells by using a reference cell line with known oxygenation. In the study reported here, that purpose was served by the HET cells due to their stable oxygen levels across both fluorescence and EPR studies. However, any established cell line could be used in this role. For example, commercially available Chinese Hamster Ovary (CHO) cells have been used for EPR oxygenation studies with reproducible results ([O_2]_CHO = 180 ± 9.3 μM).\(^6\)

The protocol, extensible to any oxygen-sensitive fluorophore, would involve measuring \(\tau_0\) for the fluorophore in solution. Both the reference and the experimental cell lines would then be incubated with the probe to estimate oxygen levels, which could be corrected via lysate-FLIM studies. Finally, corrected lifetime and oxygen values could be used to compute the Stern–Volmer quenching constant \(K_q\) that is specific to the experimental cell line and valid for all future experiments without the need for any additional corrections.

Linear subtraction of lifetimes obtained from the RTDP-cell and RTDP-lysate studies is a simplified assumption made due to the unknown nature of the biochemical component that quenches RTDP lifetime. Future work in this regard could involve use of more accurate and complicated mathematical models for correcting measured lifetime values. Compared to previous calibration efforts for intracellular oxygen sensing, the validated FLIM-based approach described here has the advantages of using a living reference biological sample, accounting for specific intracellular biochemical information, and enabling accurate, quantitative mapping of intracellular oxygen \textit{in vivo}.

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References