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FRET excited ratiometric oxygen sensing in living tissue

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Abstract

Dynamic analysis of oxygen (O₂) has been limited by the lack of a real-time, quantitative, and biocompatible sensor. To address these demands, we designed a ratiometric optode matrix consisting of the phosphorescence quenching dye platinum (II) octaethylporphine ketone (PtOEPK) and nanocrystal quantum dots (NQDs), which when embedded within an inert polymer matrix allows long-term pre-designed excitation through fluorescence resonance energy transfer (FRET). Depositing this matrix on various glass substrates allowed the development of a series of optical sensors able to measure interstitial oxygen concentration [O₂] with several hundred millisecond temporal resolution in varying biological microdomains of active brain tissue.

The relationship between oxygen consumption and neuronal activity is critical for understanding the basis for normal and pathological brain functioning. To date this assumption has relied on various methodologies that either indirectly monitor hemodynamic (Ogawa et al. 1990, Vanzetta and Grinvald 1999, Wolf et al. 2007) and metabolic changes (LaManna et al. 1987, Lakowicz et al. 1992), or more direct sensing of O₂ using polarography (Clark 1956), Silver 1973), binding of nitroimidazole-based compounds (Koch 2002), and electron paramagnetic resonance methods (Swartz and Clarkson 1998). To improve spatial and temporal resolution, more recent advances in direct [O₂] sensing have relied on phosphorescence quenching dyes that linearly and reversibly change luminescence by single-photon (Kautsky and Hirsch 1931, Vanderkooi et al. 1987, Papkovsky and O'Riordan 2005) or two-photon excitation (Sakadzic et al. 2010), allowing intracellular (Xu et al. 2001, Koo et al. 2004, Park et al. 2005, O'Riordan et al. 2007, Zhdanov et al. 2008, Dmitriev et al. 2010, Koo Lee et al. 2010, Wang et al. 2011) or extracellular (Babilas et al. 2008, Finikova et al. 2008, Hynes et al. 2009, Lebedev et al. 2009, Ceroni et al. 2011, Esipova et al. 2011) measurements in biological samples. Conventionally, such dyes have been coupled with two-photon enhancing dendrimers (Brinas et al. 2005) or O₂ insensitive organic reference dyes that are restricted by low luminescence lifetimes, dissimilar bleaching curves, and the requirement that each dye must be closely matched to the excitation and emission spectrum when using a single excitation source (Xu et al. 2001). To design a ratiometric sensor suited for greater functionality and ease of fabrication for accurate O₂ sensing, we coupled the oxygen sensing dye platinum (II) octaethylporphine

*First authorship shared

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ketone (PtOEPK) with highly photostable nanocrystal quantum dots (NQDs) in an inert polymer matrix.

Fluorescent NQDs are excellent candidates for constructing biosensors due to their high quantum yields, photostability, broad excitation profiles, multiphoton capability, and color tuneability (Michalet et al. 2005). The NQDs we have chosen not only act as an O₂ insensitive reference light emitter, but their narrow emission provides perfect spectral overlap at the absorption peak of PtOEPK, allowing optimized FRET excitation (Figure 1 a, 1 b). Previously, FRET O₂ sensing has become promising for quantifying O₂ in solution (McLaurin et al. 2009, Amelia et al. 2011), but this methodology has yet to show feasibility within biological tissue. In order to utilize FRET O₂ sensing within a complex biological system, we entrapped the highly toxic dye mixture within a biocompatible and O₂ permeable polyvinyl chloride (PVC) polymer matrix (Papkovsky 2004). This matrix could then be deposited on various glass substrates to simultaneously allow the interstitial monitoring of O₂ and electrical recordings over various biological microdomains during spontaneous states of neuronal hyperexcitability including seizures.

Methods

Animals

Experiments were performed on male Sprague-Dawley rats (P21-P35) with Pennsylvania State University Institutional Animal Care and Use Committee approved protocol.

Electrophysiology

Animals were anesthetized using diethyl-ether and decapitated, brains were removed, the left hippocampus was isolated, and transverse 350 – 400 μ m sections were cut in cold dissection buffer (in mM: 2.6 KCL, 1.23 NaH₂PO₄, 24 NaHCO₃, 0.1CaCl₂, 2MgCl₂, 205 sucrose, 20 glucose, 4°C) using a vibratome. Slices were then incubated for a minimum of 1 hour in artificial cerebral spinal fluid (ACSF: in mM: 130 NaCl, 2 MgSO₄, 3.5 KCl, 2 CaCl₂, 10 glucose, 2.5 NaH₂PO₄, 24 NaHCO₃ at pH 7.3, 30°C) oxygenated with 95% O₂: 5% CO₂. Recordings were taken using (ACSF: in mM: 130 NaCl, 0.6 MgSO₄, 3.5 KCl, 1 CaCl₂, 10 glucose, 2.5 NaH₂PO₄, 24 NaHCO₃ at pH 7.3, 32–34°C) oxygenated with 95% O₂: 5% CO₂. Extracellular recordings were performed using pulled borosilicate glass micropipettes (1–3 M Ω 0.9% NaCl) and current clamp recordings (4–7 M Ω) containing (in mM: 116 K gluconate, 6 KCl, 0.5 EGTA, 20 HEPES, 10 phosphocreatine, 0.3 NaGTP, 2 NaCl, 4 MgATP, and 0.3% Neurobiotin at pH 7.25, 295 mOsm). Electrical recordings were performed using an Axoclamp 2B MCC700 (Molecular devices) amplifier filtered (5 kHz whole cell, 1 kHz extracellular) and digitized at 10 kHz (Digidata and Pclamp#7, Molecular Devices).

Seizures were initiated by bath application of the potassium channel blocker 4-aminopyridine (4-AP, 100 – 200 μ M) and raising the bath temperature to 32–34°C (Perreault et al. 1991). Alignment of seizure onset was determined electrically by the identifiable fast positive extracellular shift (FPES, 1–5 mV, Figure 4.3). Termination was determined by the cessation of spike activity and a return to baseline membrane potential. Single cell recordings were performed on cells showing electrical signatures (step and ramp current) and identifiable morphology of pyramidal neurons. Optical and electrical recordings were performed on an Olympus IX71 inverted microscope (2D thin film experiments), Zeiss Axioskop2 FSPlus (optode coated microelectrode experiments), and an Olympus BX51WI (backfilled micro-optode experiments), equipped with a dual emission beam splitter (Optical Insights, Santa Fe, New Mexico) and Sensicam EM camera (Cooke Instruments, Inc.) driven with IPLab software (BioVision Technologies) at an acquisition rate of 5 frames/second.

Optical and electrical data were taken simultaneously by triggering electrical activity to start with each optical recording.

Two-dimensional optode sensor

An O₂ sensitive optode matrix was fabricated by dissolving a 2:1 ratio of polyvinyl chloride (PVC) and bis(2-ethylhexyl)sebacate (DOS) with a 3:1 ratio of PtOEPK and CdSe/ZnS NQDs (6nm, Ocean NanoTeach LLC) dissolved in tetrahydrofuran (THF). The optode matrix was prepared by slowly dissolving 22 mg of PVC and 44 mg of DOS in 1.5 mL THF. Then 10 ng of PtOEPK and 3.3 ng of NQDs was added, thoroughly mixed, and the matrix was evaporated to 1 mL. Thin films (~40 nm) were deposited on microscope cover glass (24×60 no.1) by spin coating 20 µl of matrix at 3000 rotations per minute for 30 seconds. Films were then thoroughly rinsed with deionized (DI) water and placed in a desiccator overnight to remove any excess THF. Before each use the optode coated cover slips were mounted and sealed within a perfusion-type electrophysiology platform. Hippocampal slices (350 µm) were placed directly on top of the thin film and perfused with 95% O₂: 5% CO₂ saturated artificial cerebral spinal fluid (ACSF). Epifluorescence was focused on the thin film coating using an Olympus IX71 Inverted Microscope equipped with Hg bulb illumination and an UPlanSApo (4 ×/0.16 w) objective (Olympus).

Optode coated microelectrodes

Using a previously described dip coating method (Park et al. 2005) with the optode matrix as described above, pulled borosilicate microelectrodes (1–3 MΩ, 1–2 µm tip diameter) were mounted onto a micromanipulator and submerged 10× within the dye and polymer matrix to deposit a thin (<50 nm) homogeneous coating on the distal portion of the electrode (Barker et al. 1998). To ensure the tip remained unclogged and functional for local field potential recordings, each electrode was prefilled with 0.9% NaCl prior to dipping. The electrode was then thoroughly rinsed with distilled water and dried under a stream of N₂ to ensure complete removal of THF. The optode coated microelectrode was mounted onto a micromanipulator and placed at a depth of ~100 µm into extracellular matrix of the CA1 stratum pyramidale and simultaneous optical and electrical recordings were taken. Luminescence was monitored using a Zeiss Axioscope 2 upright microscope with Hg bulb illumination and an Achroplan (10 ×/ 0.30 w) objective (Zeiss).

Backfilled micro-optodes

Using a 4:1 ratio of PtOEPK:NQDs and 2:1 ratio of PVC:DOS in THF, pulled borosilicate micropipettes (tip diameter 3 µm) were submerged into the optode matrix to allow capillary action to backfill each micro-optode. Using a syringe, positive pressure was applied to the filled micro-optode to ensure the matrix was concentrated to the tip. Filled micro-optodes were then dried under a stream of N₂, thoroughly rinsed in distilled water, dried again, and stored in a desiccator until use. To allow measurements around single cell microdomains, a ratiometrically balanced backfilled micro-optode was first placed juxtacellular to the target cell of interest using differential infrared contrast optics. A second intracellular electrode was used for whole cell patch clamping. Optical measurements were taken from a 3 µm×3 µm region of interest from the micro-optode tip using an Olympus BX51W1 equipped with Hg bulb illumination and a LUMPlanFI (40 ×/ 0.80 w) objective (Olympus).

Time-resolved photoluminescence

Femtosecond laser pulses with temporal duration of 80 fs from an optical parametric amplifier (TOPAS-C, Coherent) pumped by a regenerative Tksapphire amplifier (Libra HE, Coherent) were employed to excite the sensor sample for dynamical studies of the emission.

The temporal traces were recorded with sub-10 ns resolution by a photomultiplier (PMT, Hamamatsu) and fast oscilloscope.

Results

FRET excitation of phosphorescence quenching dye

In order to induce FRET, we utilized a PVC polymer matrix to promote short distance (~10 nm) dipole-dipole interactions. The average donor-acceptor separation distance between NQDs and OSD molecules was determined by their concentrations embedded in the polymer matrix. FRET excitation was confirmed by time-resolved photoluminescence (TRPL) studies where NQDs were excited using a femto-second laser and the emission relaxation rate increased with an increase in OSD concentration [supplementary data]. FRET was also confirmed with single and two-photon excitation of thin film optode coatings with varying NQD and PtOEPK concentrations (Figure 1 c, d). At the same absorption intensity, PtOEPK emission was dependent of the presence of NQD.

Quantitative calibration of the thin film optode sensors was performed before and after every experiment by measuring the ratiometric luminescence intensity in varying $[O_2]$ within artificial cerebral spinal fluid (ACSF) at 34°C. Varying $[O_2]$ were obtained by adding mixtures of 95% O_2 : 5% CO_2 and 95% N_2 : 5% CO_2 within the ACSF perfusate. The ratio (R) of the emission luminescence from NQDs and OSDs was calculated with the form $R = I_{NQD} / I_{OSD}$ Where I_{NQD} and I_{OSD} are the NQDs and OSD luminescent intensities at a given $[O_2]$. From 0 to 100% oxygen saturation at 34°C, the OSD phosphorescence quenching is reversible and approximately linearly dependent on dissolved O_2 level as described by the Stern-Volmer equation:

$$I_o/I_o = 1 + K_{sv}[O_2]$$

where I_o is the emission of the OSD in N_2 saturated solution, I_c is the emission in a given dissolved $[O_2]$, and K_{sv} is the Stern-Volmer quenching constant determined by the slope of luminescence vs. $[O_2]$ (Stern and Volmer 1919)(Figure 1 e, 1 f).

The response time of our sensors was estimated by the Einstein diffusion equation:

$$R_2 = 2D\tau$$

where R_2 is the diffusion length, D is the diffusion constant (which is estimated to be $2 \times 10^{-9} m^2/s$ from the diffusion constant of O_2 in water), and τ is the response time (Einstein 1905). This produces a theoretical response time of <100 μs for a ~ 100 nm thick film and <200 ms for a 3 μm^2 backfilled micro-optode. The actual response times from our FRET optodes were evaluated manually by switching 95% N_2 : 5% CO_2 saturated ACSF buffer with 95% O_2 : 5% CO_2 saturated buffer and measuring the time to achieve 90% maximum intensity. This method confirmed an upper limit response time of less than 200 ms.

The reproducibility of each optode sensor was determined using a constant excitation intensity and recording similar emission intensities with the same camera settings. The same recipe was followed for each set of experiments and errors <5 % were found between calibration tests of fresh sensors. Before each experiment, the luminescence of each optode was checked to ensure a balanced OSD and NQD emission intensity. Optodes where balanced luminescence was not observed were discarded.

Probing interstitial oxygen during spontaneous SLE

We first monitored O₂ dynamics by recording the ratiometric intensity of an optode film in direct contact with an entire hippocampal slice (Figure 2 a). In the absence of tissue, the NQD and PtOEPK luminescence was balanced when continuously perfused with saturated 95% O₂: 5% CO₂ ACSF. The addition of a hippocampal slice produced a marked increase in PtOEPK luminescence while NQD luminescence showed no intensity change (Figure 2 b). This initial increase represents a marked decrease in [O₂] due to the metabolic activity of the tissue and O₂ diffusion limitations. For a 350 μm thick slice, the [O₂] gradually decreased horizontally from ~18 mg/L to ~5 mg/L 250 μm from the slice edge even though 31 ± 1 mg/L [O₂] perfused over the slice at a rate of 1 mL/min at 34°C (Figure 2 c).

To induce spontaneous hyperexcitability, 200 μm of the potassium channel blocker 4-aminopyridine (4-AP) was administered in the bath solution. Electrical activity was recorded using a local field potential electrode within the CA1 st. pyramdale. Within minutes spontaneous seizure-like events (SLEs) resulted in focal increases of O₂ consumption localized primarily within the CA1 st. pyramdale (Figure 2 d, e, Movie [supplementary data]). In all observed slices (n=33), a decrease in [O₂] occurred prior to the fast positive excitatory spike (FPES), an activity marker associated with seizure onset (Ziburkus et al. 2006), and the hypoxic duration outlasted the electrical seizure termination. Each hypoxic episode would then slowly return back to baseline values if not interrupted by a subsequent SLE (Figure 2 f).

To ensure luminescence changes were derived from changes in PtOEPK and not intrinsic factors (i.e. cell swelling), the OSD was replaced with the oxygen insensitive dye HITC iodide (excitation 590 nm, emission 750 nm). During spontaneous SLEs, no change in HITC iodide luminescence was observed [supplementary data].

To measure more localized changes in [O₂] associated with hyperexcitability, we then inserted optode coated local field potential electrodes within the CA1 st. pyramdale (Figure 3 a, c). The ratiometric luminescence from a 25 μm×25 μm region of interest was recorded from the microelectrode tip that again revealed a decrease in [O₂] during spontaneous SLEs (Figure 3 c). Similar to the 2D films, an increase in O₂ consumption was found prior to FPES onset within the st. pyramdale followed by prolonged hypoxic periods that outlasted the duration of the SLE.

To further improve the spatial limitations of biological O₂ sensing we positioned a backfilled micro-optode (Figure 4 a) juxtacellular to an outlying CA1 pyramidal neuron outside the dense cell body layer. A second intracellular electrode was then inserted to allow whole cell recordings from the same target cell (Figure 4 c). Excitation at 450nm generated NQD and OSD emission that was recorded from a 3 μm×3 μm region of interest within the distal portion of the electrode tip (Figure 4 b). In all stable recordings, spontaneous SLEs resulted in an increase in OSD luminescence (decrease in [O₂]) around individual CA1 pyramidal cells (Figure 4 d), while no change in NQD luminescence was observed. Control experiments were recorded from backfilled micro-optodes placed at least 50 μm from any specific cell in the CA1 st. oriens with a local field potential electrode in the st. pyramdale. During a seizure event, no change in luminescence was observed unless the micro optode was positioned juxtacellular within 50 μm to a single cell [supplemental data].

Discussion

Real-time monitoring of O₂ is of great interest due to its fundamental role in virtually every aspect of cellular metabolism. Here we present a ratiometric and multifunctional O₂ sensing technology to allow simultaneous optical and electrical recordings from large scale neural

population networks to individual extracellular microdomains. To show functionality and biocompatibility, we monitored the fast interstitial $[O_2]$ fluctuations during spontaneous states of hyperexcitability in living tissue. These real-time metabolic responses offer a critical insight into the cellular demands associated with hyperexcitability and its associated hypermetabolism.

Although previous methods of measuring $[O_2]$ have extensively been used, the development of fast quantitative optical methods will improve the spatiotemporal resolution for measuring $[O_2]$ and cellular activity within living tissue. Our single photon excitation FRET based methodology offers several advantages. First, the sensor is self-referencing, as the ratio of the reference to the sensing dye represents a parameter that is independent of dye concentration or variations with instrumentation. Second, coupling the OSD and NQD mixture within a biologically inert and O_2 permeable polymer matrix improves luminescent stability and provides possibilities for alternative sensing modalities using multiphoton microscopy techniques (McLaurin et al. 2009, Achatz et al. 2011, Amelia et al. 2011). Third, in contrast with polarographic techniques, our molecular sensor does not consume the O_2 it seeks to measure. Lastly, this alternative sensing method can be easily fabricated and deposited on various glass substrates depending on the desired application.

Although our FRET sensors were designed to provide limited interactions with biological macromolecules, a detailed investigation of long-term biocompatibility and toxicity was beyond the scope of this study. A potential safety concern with any plasticized PVC matrix is leaching (Tickner et al. 2001). Although we did encounter a baseline luminescence change ($<20\%$ over 200 s) in the backfilled micro-optode experiments, the drift is most likely due to photobleaching, as an increased light intensity was required to excite the microliter amounts of OSD/NQDs/plastizier. Even if leaching were to occur, we were able to reliably record the electrical activity from a single cell for tens of minutes, even though a plasticized probe was located juxtacellular to the same cell.

As with all types of sensors, selectivity is also a potential concern. Oxygen sensing dyes may also be quenched by nitric oxide (NO) (Koo et al. 2004). In our experimental setup we cannot rule out the possibility of NO production, but the nanomolar concentrations reported within the literature (Brown 1995) are minimal in comparison to the millimolar oxygen changes we observe at rest and during individual seizures. Another possible contributor to changes in OSD signaling is the production of other oxygen species, as intense hyperactivity undoubtedly generates some forms of reactive oxygen species (ROS), especially from within the mitochondrial electron transport chain (Murphy 2009). The rapid production of ROS in the brain can be catalyzed with a variety of enzymes (superoxide dismutase, catalase, glutathione peroxidase) or antioxidants to neutralize the unpaired electrons. These free radical scavengers are usually sufficient to deal with the toxic threat of ROS production but we cannot rule out the possibility that ROS are generated and contribute to our measurements during seizure activity (Schiff and Somjen 1985).

Conclusions

To improve the spatiotemporal resolution, selectivity, and functionality of direct O_2 measurement techniques, we have designed a FRET based oxygen sensor to allow real-time extracellular O_2 measurements in living tissue. Using new developments in molecular imaging combined with intracellular and extracellular recordings, we were able to localize, quantify, and assess the interstitial oxygen demands associated with changes in neuronal activity. This ratiometric and multifunctional O_2 sensor allows simultaneous optical and electrical recordings from larger-scale brain structures to smaller-scale microdomains localized around single cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We report how to fabricate a series of FRET based optical oxygen sensors.
- FRET sensing can monitor extracellular oxygen dynamics in living tissue.
- Seizure events are associated with layer specific O₂ changes in the hippocampus.

Figure 1.

(a) Scheme of FRET transfer between NQDs and the phosphorescence quenching oxygen dye PtOEPK. (b) Excitation and emission spectra of NQDs and PtOEPK. Excitation at 450 nm caused narrow NQD emission at the same peak absorption of PtOEPK (590 nm). (c) Single-photon excitation of NQDs, PtOEPK, and dye mixture. 480 nm excitation of a PVC matrix containing only PtOEPK caused minimal emission intensity (*black trace*) unless coupled with NQDs (*blue trace*), (d) Excitation of an optode thin film using two-photon excitation at varying NQD:OSD ratios. 860 nm excitation and a mass ratio of 1:3 of QD:PtOEPK produced a balanced emission intensity ratio at 590 nm and 757 nm. e)

Emission response of NQDs and PtOEPK in varying [O₂]. **(f)** Linearity and reversibility (*inset*) of FRET matrix in various [O₂] at 34°C, ($R^2=0.9983$).

Figure 2.

(a) Experimental setup used to record luminescence from a FRET based optode. Absorption at 450 ± 30 nm was used to excite a 40 nm thin film optode matrix in direct contact with a hippocampal slice. The emission was then split using a dual emission beam splitter and a ratiometric image was captured in the camera, (b) Optode emission from NQDs (600 ± 30 nm) and OSD (750 ± 30 nm) as oxygen saturated ACSF perfused the slice, (c) Calibrated ratiometric optode film under a slice perfused at a rate of 1 mL/min at 34°C . (d) Simultaneous local field potential and $[O_2]$ recorded from a $50 \mu\text{m} \times 50 \mu\text{m}$ region of interest within the CA1 st. pyramidal during a spontaneous SLE (seizure duration indicated by red

bar), (e) Calibrated ratiometric images were overlaid with a bright field image to visualize real-time spatiotemporal changes in O_2 dynamics over the entire slice. Changes in O_2 were localized primarily within the CA1 that preceded FPES onset and outlasted the electrical seizure duration. X = local field potential electrode position, $\square = 50\ \mu\text{m} \times 50\ \mu\text{m}$ region of interest, (f) Ratiometric imaging within the CA1 st. pyramidale during single or multiple SLEs. Seizure onset was associated with a decrease in I_{NQD}/I_{OSD} that returned to baseline values if not interrupted by a subsequent SLE. Seizure onset denoted by *.

Figure 3.

(a) Schematic of an optode coating on the tip of a functional local field potential microelectrode. The electrode was first prefilled with 0.9% NaCl and the distal portion of the electrode was dipped into the optode matrix to deposit a homogeneous coating on the tip. (b) Luminescence changes from OSD emission (750 ± 30 nm) recorded during a SLE. FPES onset occurred starting at timeframe 40 s. Scale bar $100\mu\text{m}$ (c) An optode coated microelectrode allowed simultaneous $[O_2]$ and electrical activity to be monitored within the CA1 st. pyramidal. During an individual SLE (red bar) a marked decrease in $[O_2]$ was observed prior to FPES onset that outlasted the duration of the electrical seizure event.

Figure 4.

(a) Pulled glass electrodes were dipped into the FRET optode matrix and capillary action concentrated a small amount of dye and polymer at the tip. Scale bar 10 μm. (b) Excitation at 450 ± 30 nm resulted in bright OSD and NQD emission from the tip of the backfilled electrode. Optical recordings were taken from the $3 \mu\text{m} \times 3 \mu\text{m}$ region of interest (■) at the distal portion of the electrode. Scale bar 10 μm. (c) Bright field image of backfilled juxtacellular optode (*top*) and whole cell patch clamp electrode (*bottom*) attached to a pyramidal neuron just outside the dense cell body layer of the stratum pyramidale. Scale bar 10 μm. (d) Intracellular electrical and juxtacellular optode recordings during an individual SLE. A backfilled electrode placed juxtacellular to a pyramidal neuron (*red trace*) increased luminescence during SLE onset while NQDs (*black trace*) revealed no change in luminescence with the SLE.