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Enabling widespread use of two-photon phosphorescence lifetime microscopy (2PLM) of oxygen in the brain

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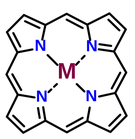
Abstract

Over the past several years we have developed a method for high resolution, real-time imaging of oxygen in the brain. This method, termed two-photon phosphorescence lifetime microscopy (2PLM) of oxygen, combines the state-of-the-art two-photon enhanced phosphorescent probes and a unique variant of two-photon laser scanning microscopy. With support from NINDS (R24-NS092986) we are in the process of setting up a resource that will promote widespread use of the 2PLM technology, making this new method available to researchers working in the are of neuroscience. We are taking proposals now for new studies involving oxygen metabolism in the brain and looking forward discussing these projects with the researchers interested in utilizing the resource.

Principles of the method: oxygen measurements by phosphorescence quenching

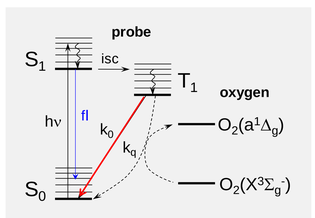


M = Pd, Pt

Pt and Pd porphyrins phosphoresce in solutions at ambient temperatures. Their phosphorescence is quenched by molecular oxygen, such that the kinetics of quenching follows the Sterm-Volmer relationship:

$$1/\tau = 1/\tau_0 + k_q \times pO_2$$

- τ - phosphorescence decay time at a partial pressure of oxygen pO_2;
- τ_0 - phosphorescence lifetime in the absence of oxygen (pO_2=0);
- k_q - bimolecular quenching rate constant.

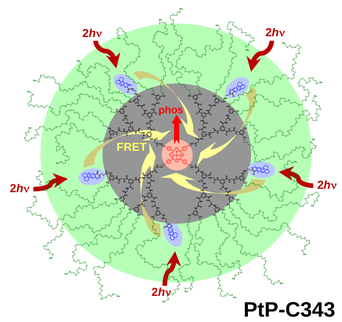


The phosphorescence decay time (τ) of a bio-compatible probe can serve as a quantitative indicator of pO_2, provided the probe is able to retain its specificity and calibration parameters in *in vivo*.

To ensure bio-compatibility and specificity **phosphorescent Pt or Pd porphyrins are encapsulated into dendrimers coated by a polyethylene glycol (PEG) layer at the periphery**. The dendrimer controls the rate of oxygen diffusion and defines the value of the quenching rate constant k_q. The phosphorescence lifetime τ_0 is a set by the nature of the porphyrin core itself. Jointly, **the values of k_q and τ_0 determine the probe's sensitivity and the dynamic range. k_q and τ_0 must stay unchanged in biological environments**.

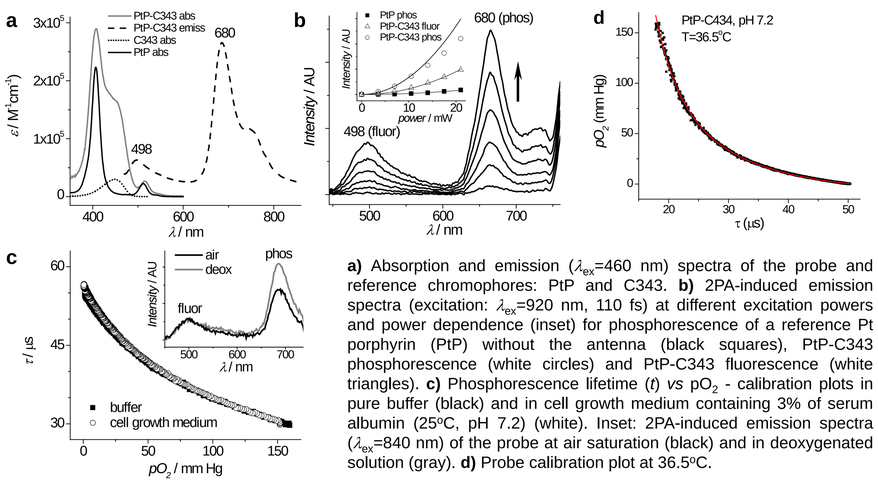
The combination of arylglycine dendrimers (AG) with PEG's (MW~1000-2000 Da) fulfill the criteria for quantitative biological oxygen sensing. The AG-PEG dendritic oxygen probes retain their calibrations *in vivo*, both in the blood and tissue interstitial space. These probes have been used in many areas of research, including oxygen measurements and imaging in the heart, muscle, liver, brain, retina of the eye, various types of tumors as well as artificial tissue constructs and simple suspensions of cells *in vitro*. Measurements by phosphorescence are minimally invasive, fast (up to 10 ms temporal resolution) and can be implemented as various forms of optical imaging: from microscopy to tomography.

Two-photon-enhanced oxygen probes



PtP-C343

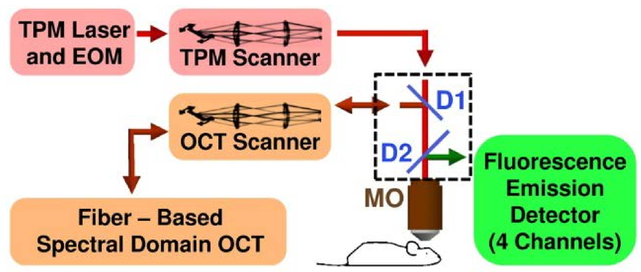
The first two-photon phosphorescent probe PtP-C343 was designed to integrate in a single molecular construct all the characteristics necessary for biological oxygen imaging. The diameter of the probe is ~5 nm in folded state. Several coumarin-343 (C343) moieties in the molecule of PtP-C343 act as a 2P antenna, channeling the excitation energy to the Pt porphyrin (PtP) core. The high fluorescence quantum yield of C343 (f_{fl}≈0.8-1.0), combined with the strong absorption of PtP in the Q-band region (λ_{max}=515 nm, ε=26,000 $M^{-1}cm^{-1}$) and large spectral overlap enable efficient FRET (~70%).

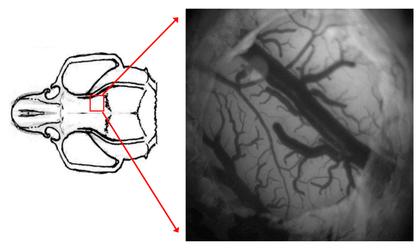


a) Absorption and emission (λ_{ex}=460 nm) spectra of the probe and reference chromophores: PtP and C343. **b)** 2PA-induced emission spectra (excitation: λ_{ex}=920 nm, 110 fs) at different excitation powers and power dependence (inset) for phosphorescence of a reference Pt porphyrin (PtP) without the antenna (black squares), PtP-C343 phosphorescence (white circles) and PtP-C343 fluorescence (white triangles). **c)** Phosphorescence lifetime (t) *vs* pO_2 - calibration plots in pure buffer (black) and in cell growth medium containing 3% of serum albumin (25°C, pH 7.2) (white). Inset: 2PA-induced emission spectra (λ_{ex}=840 nm) of the probe at air saturation (black) and in deoxygenated solution (gray). **d)** Probe calibration plot at 36.5°C.

Currently, new antenna-free two-photon oxygen probes are being designed and tested, whose performance exceeds that of PtP-C343 by ~50-100 fold!

Imaging setup and protocols





Imaging in mice and rats is performed using through a sealed cranial window, a custom-built two-photon microscope. Imaging can be performed in both anesthetized and head-restrained awake animals. In anesthetized animals, blood pressure, heart rate, expired CO_2 and temperature are monitored continuously.

For **extravascular pO_2 measurements**, phosphorescent probe is pressure-injected into the cortical tissue before sealing the cranial window.
For **intravascular pO_2 measurements**, the probe is injected intravascularly.

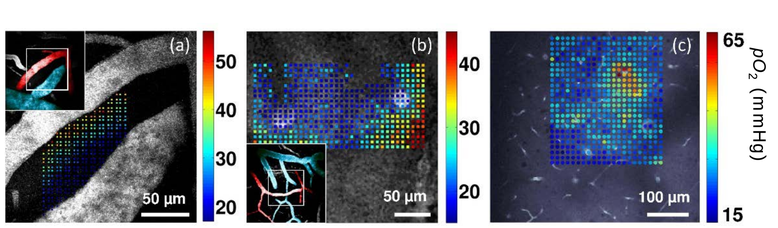
Intravasuclar measurement of pO_2 is possible in all micro vessels, including capillaries, up to a depth of 450 μm (PtP-C343) or 600 μm (new probes). So far, tissue pO_2 measurements were typically obtained up to a depth of 300 μm.

Excitation of the probe is typically performed by short trains (10-40 μs) of femtosecond pulses, followed by the phosphorescence acquisition period of about 5-6 decay times, i.e. ~300 μs for PtP-C343. The phosphorescence can be acquired either in analog or photon-counting mode.

Typical measurement parameters: excitation ~ 10 ms, decay acquisition ~ 300 μs
probe concentration (e.g. in the plasma) ~ 10-20 μM, single point dwell time ~ 150 ms.

Examples of application of 2PLM in imaging of brain oxygenation

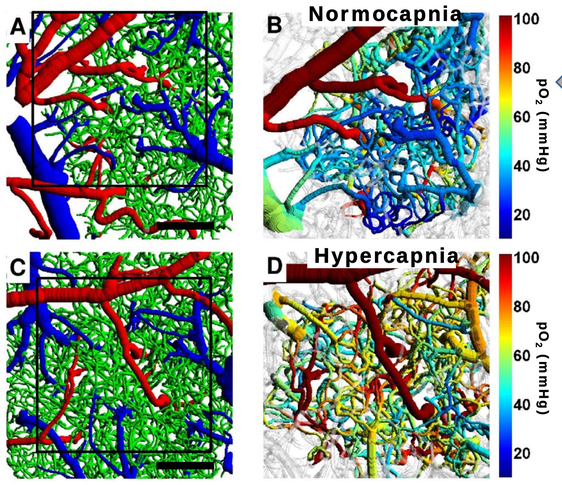
Cortical tissue pO_2 landscape is dominated by the oxygen diffusion from the arterioles



Cortical tissue pO_2 imaging in a rat. (**a**) Tissue pO_2 map (scale bar in mmHg), overlaid on FITC image of microvasculature 10 μm below brain surface. Insert shows 122 μm thick MIP of FITC labeled microvasculature. Pial artery and vein are colored with red and blue color, respectively, for easier identification. (**b**) Tissue pO_2 map 82 μm below brain surface. (**c**) Tissue and intravascular pO_2 map overlaid on FITC image of the microvasculature 200 μm below brain surface.

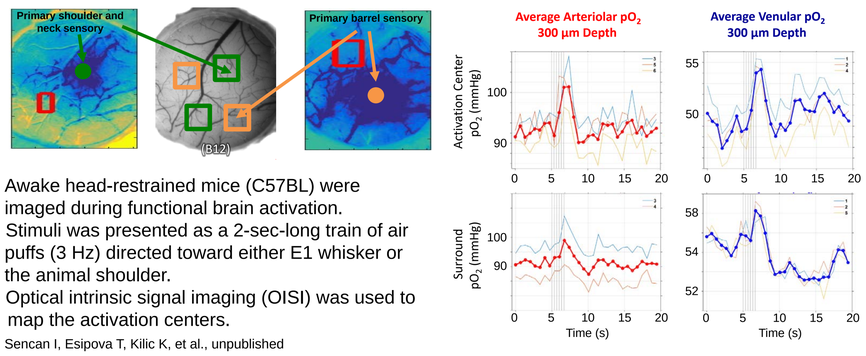
Cortical tissue pO_2 imaging in an anesthetized mouse. **A**) Microvascular angiogram with the false coloring of arterioles (red), venules (gray), and capillaries (green). **B**) Tissue pO_2 map (scale bar in mmHg), overlaid on FITC image of microvasculature 100 μm below brain surface. Pial arterioles and venules are colored with red and gray color, respectively, for easier identification. **C**) Tissue pO_2 map (scale bar in mmHg), overlaid on FITC image of microvasculature 200 μm below brain surface.

Intravascular oxygen measurements and imaging



Hypercapnia in tracheotomized and ventilated mice was induced by adding ~5% CO_2 in a mixture of air and O_2 (arterial PCO_2 = 45-51 mmHg; OCT-measured CBF increase = 30%). (**A**) and (**C**). Projections of the two microvascular stacks with the labeled microvascular segments: arterioles (red), capillaries (green), and venules (blue). (**B**) and (**D**). PO_2 measurements (color-coded) during normocapnia (**B**) and hypercapnia (**D**) overlaid over microvascular structures presented in (**A**) and (**C**), respectively. Black squares in (**A**) and (**C**) represent PO_2 measurement fields of view presented in (**B**) and (**D**), respectively. Scale bars 200 μm.

Intravascular pO_2 imaging in awake mice during functional activation



- Awake head-restrained mice (C57BL) were imaged during functional brain activation.
- Stimuli was presented as a 2-sec-long train of air puffs (3 Hz) directed toward either E1 whisker or the animal shoulder.
- Optical intrinsic signal imaging (OISI) was used to map the activation centers.

Sencan I, Esipova T, Kilic K, et al., unpublished

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