Optical probes and techniques for O₂ measurement in live cells and tissue

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Abstract In recent years, significant progress has been achieved in the sensing and imaging of molecular oxygen (O₂) in biological samples containing live cells and tissue. We review recent developments in the measurement of O₂ in such samples by optical means, particularly using the phosphorescence quenching technique. The main types of soluble O₂ sensors are assessed, including small molecule, supramolecular and particle-based structures used as extracellular or intracellular probes in conjunction with different detection modalities and measurement formats. For the different O₂ sensing systems, particular attention is paid to their merits and limitations, analytical performance, general convenience and applicability in specific biological applications. The latter include measurement of O₂ consumption rate, sample oxygenation, sensing of intracellular O₂, metabolic assessment of cells, and O₂ imaging of tissue, vasculature and individual cells. Altogether, this gives the potential user a comprehensive guide for the proper selection of the appropriate optical probe(s) and detection platform to suit their particular biological applications and measurement requirements.

Keywords Molecular oxygen · Cellular oxygen · Phosphorescence quenching · Sensors · Nanoparticle probes · Live cell oxygen imaging

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CPP          | Cell-penetrating peptides |
| EC           | Extracellular |
| ccO₂         | Extracellular O₂ |
| FLIM         | Fluorescence/phosphorescence lifetime imaging microscopy |
| IC           | Intracellular |
| icO₂         | Intracellular O₂ |
| NP           | Nanoparticle(s) |
| OCR          | Oxygen consumption rate |
| PEG          | Poly(ethyleneglycol) |
| RLD          | Rapid lifetime determination |
| TR-F         | Time-resolved fluorescence/phosphorescence |

Introduction

It is hard to overestimate the significance of the measurement of molecular oxygen (O₂), particularly in biological samples containing respiring cells and tissues. Detailed understanding of the biological roles of O₂ is of major fundamental and practical importance [1, 2]. Being the key metabolite and the source of energy for mammalian cells, O₂ is used to produce ATP through the electron transport chain and oxidative phosphorylation process. It is also a substrate of numerous enzymatic reactions within the cell which are vital for its normal function, and can serve as a signal for genetic adaptation to hypoxia, for example through hypoxia-inducible factor (HIF) pathway [3, 4]. O₂ is a small, gaseous, non-polar analyte which has moderate solubility in aqueous solutions (pO₂ = 160 mmHg, or ~200 μM at 37°C). It is supplied to cells and tissues by passive diffusion and, in higher multicellular organisms, by convectional transport via vasculature, red blood cells and haemoglobin [3].

On the measurement side, the main parameters that require monitoring are (1) in situ oxygenation, (2) O₂ consumption rate (OCR), and (3) localised O₂ gradients within respiring samples, as well as the dynamics of these...
parameters as a result of changes in cellular function. Under normal homeostatic conditions in vivo, these parameters are maintained within the defined physiological limits, with significant fluctuations seen in exercised muscles, brain tissue, and excitable cells [3]. OCR reflects the respiratory activity of the sample and, together with the other markers, such as ATP content, mitochondrial membrane potential, ion and metabolite concentrations and fluxes, the bioenergetic status of the cell. Deviations of the OCR from the norm are indicative of perturbed metabolism, mitochondrial dysfunction or disease state [5]. Similarly, changes in cell or tissue oxygenation are associated with many common pathological conditions including ischemia/stroke, cancer, neurological and metabolic disorders. Short-term and sustained hypoxia is known to induce rearrangement of cell metabolism, and ultimately lead to cell death or protection and adaptive responses (e.g. via Warburg effect, hypoxia-induced expression of genes and proteins such as HIF-1α, PGC-1α [4, 6, 7]). All this highlights the importance of O₂ measurement in biological samples containing respiring cells and tissue, and the need for corresponding tools and measurement methodologies to perform this in many different ways.

The area of O₂ measurement has been under active development over many years. Initially, it was boosted by the introduction of Clark-type oxygen electrodes [8], photometric (myoglobin) [9, 10] and EPR systems [11], which have been very useful in basic bioenergetic, metabolic, cell biology and toxicological studies with rather simple, macroscopic models. These techniques have been used with isolated mitochondria, suspension cells, and in point measurements in vivo [8]. More recently, they have been extended by microelectrodes [12–14], systems for adherent cells [15, 16], EPR probes [17, 18], ¹⁹F MRI [19, 20], pimonidazole staining [21], and fibre-optic sensing [22], and enhanced by the technological advancements in analytical instrumentation [23].

Biological O₂ sensing techniques based on phosphorescence quenching and soluble probes, pioneered by David Wilson et al. [24], have opened new opportunities in the area. In the last decade, these systems have been revolutionised with a number of advanced sensor chemistries, measurement methodologies and instrumentation, thus enabling new analytical tasks and applications. In addition, several new optical sensing methods have been introduced, including the measurement of O₂-dependent fluorescence of GFP constructs [25] and imaging of delayed fluorescence [26]. While becoming more available and affordable for ordinary biomedical users, the existing platforms for optical sensing and imaging of O₂ in biological samples possess a number of special features and technical challenges, which require careful consideration in their selection and use.

Here, we review the available range of optical O₂ probes and detection platforms designed for biological samples and applications, with particular focus on quenched-luminescence O₂ sensing and imaging techniques. The main types of O₂ sensing materials, detection modalities, and biological applications are analysed and cross-compared. This is aimed at giving potential users a comprehensive guide for how to select an appropriate measurement platform for their particular biological object, instrumentation available and analytical task pursued.

**Principles of O₂ sensing by photoluminescence quenching**

Dynamic quenching of luminescence by O₂ involves a collisional interaction of O₂ molecules with the luminophore in its lowest excited state, resulting in radiationless deactivation and return to the ground state. As a result, luminescence intensity (I) and lifetime (τ) are both reduced in the presence of O₂ according to the Stern–Volmer equation [27]:

\[ \frac{I_0}{I} = \tau_0/\tau = 1 + K_{s-v} \times [O_2] = 1 + k_q \times \tau_0 \times [O_2], \]

where \( I_0 \) and \( \tau_0 \) are unquenched intensity and lifetime at zero O₂, respectively, \( K_{s-v} \) is the Stern–Volmer quenching constant, and \( k_q \) the bimolecular quenching rate constant, which depends on the immediate environment of the reporter dye, temperature and sterical factors. Each O₂-sensitive luminescent material has a characteristic relationship between \([O_2]\) and \( \tau \) (or \( I \)). Luminescence lifetime is an average time which the luminophore stays in the excited state before emitting a photon. It is equal to the reciprocal of the excited state deactivation rate constant. This characteristic is independent of the concentration, and therefore lifetime measurements are the preferred luminescent parameter for sensing, from which O₂ can be quantified as follows:

\[ [O_2] = (\tau_0 - \tau) / (\tau \times K_{s-v}) \]

For an ideal situation (solution-based systems), the Stern–Volmer plot of \([O_2]\) versus \( \tau^{-1} \) is linear, and this allows for simple two-point calibration. However, many existing O₂-sensitive materials show heterogeneity and a non-linear Stern–Volmer relationship [28].

Since the analytical relationship between the primary luminescent parameter (\( I \) or \( \tau \)) and O₂ concentration is hyperbolic, maximal sensitivity to O₂ (\( dI/dO_2 \) or \( d\tau/dO_2 \)) is always at zero O₂. To achieve good measurement resolution in a given O₂ range (for in vitro studies, this range is usually 0–250 \( \mu \)M, while in vivo –0 to 50 \( \mu \)M), probe luminescence should be moderately quenched (approximately 2- to 5-fold) [29]. This requirement sets the following optimal values for the \( K_{s-v} \): approximately
10 mM$^{-1}$ at 200 μM O$_2$ or 100 mM$^{-1}$ at 20 μM O$_2$. Probes with much higher or much lower $K_{a-v}$ values will have a reduced performance due to small signal changes by O$_2$.

A calibration experiment involves measurement of the probe signal ($\tau$ or $I$) at several known O$_2$ concentrations, usually at 37°C for animal cell samples or 30°C for microbial, environmental samples, and fitting the data points to determine the $[O_2] = f(\tau)$. Under equilibrium conditions, the concentration of O$_2$ in solution and solid films is related to the partial pressure in the gas phase according to Henry’s law.

A noteworthy factor is that a major by-product of the quenching process is singlet oxygen—a reactive, but rather short-lived form of O$_2$ which mostly returns back to the ground (triplet) state O$_2$. However, singlet oxygen can also react with nearby molecules, including the dye and sample components (lipids, proteins, nucleic acids), and thus affect the sensor, sample and measurement [30].

### Oxygen-sensitive materials

The luminescent O$_2$ sensing materials described so far can be classified according to a number of criteria.

The reporter dye

Phosphorescent Pt(II)- and Pd(II)-porphyrins are among the most popular in O$_2$ sensing [24, 29, 31, 32]. These dyes exhibit phosphorescence lifetimes in the range of 40–100 μs for Pt-porphyrins and 400–1,000 μs for Pd-porphyrins, which provide them moderate to high quenchability by O$_2$. They have intense absorption bands in the UV (370–410 nm) and visible (500–550 nm) regions, and bright, well-resolved emission (630–700 nm) which is retained at ambient temperatures and in aqueous solutions [32]. This makes Pt-porphyrins well suited for the ambient O$_2$ range (0–200 μM), and Pd-porphyrins—for the low O$_2$ range <$50$ μM. Quenchability by O$_2$ can be tuned by changing the micro-environment of the dye, for example by conjugating it to a macromolecular carrier [33], surrounding it with a dendrimeric shell [31] or embedding it in a suitable polymer. A number of related dye structures have been developed, including the phosphorescent Pt and Pd complexes of benzoporphyrins and porphyrin-ketones [31, 34, 35], which show longwave-shifted excitation bands (590–650 nm), emission at 730–900 nm (better suited for tissue imaging), and greater brightness [34, 36]. The highly photostable PtPFPP dye is actively used in a number of O$_2$ sensing probes [37]. Unlike the highly phototoxic PDT drugs and photosensitizers of porphyrin origin (e.g. photofrin, chlorins, precursor 5-aminoluvulenic acid), the porphyrin dyes used in existing extracellular and intracellular oxygen probes rarely show significant phototoxic action on mammalian cells and tissue under standard (optimised) measurement conditions [38–41]. This, however, needs to be carefully monitored in each case.

Besides the porphyrins, phosphorescent Ru (II) complexes have been used in biological O$_2$ sensing and imaging systems [42, 43]. These cationic dyes have good photostability and moderate brightness (lower than the porphyrins), while their lifetimes are significantly shorter (1–5 μs). The latter results in a lower sensitivity to O$_2$, thus requiring special polymeric matrices. On the other hand, shorter lifetimes provide faster signal acquisition rates in TR-F detection and fluorescence imaging (see below). Cyclometallated complexes of Ir(III) also show convenient spectral characteristics, high brightness and medium range lifetimes, compared to Pt-porphyrin and Ru(II) dyes [44, 45]. However, modest photostability limits their use in O$_2$ sensing and imaging systems. The characteristics of some common indicator dyes used in biological O$_2$ sensing systems are given in Table 1.

### Table 1 Phosphorescent characteristics of some common dyes used in O$_2$ sensing

| Indicator dye | Excitation optimum (nm) | Emission optimum (nm) | Lifetime $\tau_0$ (μs) | Quantum yield/solvent | Refs. |
|---------------|-------------------------|-----------------------|------------------------|----------------------|-------|
| PtTCPPPP     | 415, 524                 | 690                   | ~800                   | ~0.1/water           | [31]  |
| PtTCPCTBP    | 442, 632                 | 790                   | ~240                   | 0.12/water           | [31]  |
| PtCP         | 380, 535                 | 650                   | ~60                    | 0.28/water           | [46]  |
| PtTBP        | 416, 609                 | 745                   | 50                     | 0.50/DMF             | [47]  |
| PtCPK        | 400, 596                 | 767                   | 24                     | 0.01/water           | [36]  |
| Ir(III)(C$_5$)$_2$(acac) | 472, 444 | 563                   | 11.3                   | 0.54/CHCl$_3$        | [45]  |
| [Ru(bpy)$_2$(picH$_2$)]$^{2+}$ | 460                  | 607                   | 0.7–0.9                | 0.07/water           | [42, 48] |

*PtTCPPPP: Pt(II)-meso-tetra-(4-carboxyphenyl)porphyrin, PtTCPCTBP: Pt(II)-meso-tetra-(4-carboxyphenyl)tetrabenzoporphyrin-dendrimer, PtTCPk: Pt(II)-coproporphyrin, PtTBP: Pt(II)-meso-tetraporphyrin, butyl octaester, PtCPK: Pt(II)-coproporphyrin-ketone, Ir(III)(C$_5$)$_2$(acac): cyclometallated Ir(III) μ-chloro-bridged dimer coumarin complex, [Ru(bpy)$_2$(picH)$_2$]$^{2+}$: [Ru(bpy)$_2$(2-(4-carboxyphenyl)imidazo-[4,5-f][1,10]phenanthroline)H$_2$]$^{2+}$
Probe structure

The main forms of sensor materials are the solid-state coatings and probes (water-soluble reagents). These two are bridged by the particulate sensors which in the context of this review we also regard as probes. Solid-state sensors are usually used in O₂ microsensors [12], sensor coatings [49] and integrated systems [50]. They provide reliable and accurate measurement of extracellular O₂ (see below), but have limited flexibility. These systems have been reviewed extensively [29, 51, 52] and are outside the scope of this review.

Within the group of O₂ probes, several categories can be defined. The small molecule probes, represented by PdTPCPP [53, 54], Ru(II) dyes [42, 48] and PtCP derivatives [55], comprise hydrophilic dye structures having good solubility in aqueous media provided by the carboxylic and sulfonate groups. However, such probes have a tendency to non-specifically bind to proteins, cells and surfaces (due to hydrophobic regions in their structure), migrate to different locations within the sample and/or show cross-sensitivity to sample composition (pH, ionic strength, protein content).

These issues were partly addressed by developing the supramolecular probes in which several distinct functionalities are covalently linked together in one chemical entity. For example, conjugations of an O₂ sensitive dye to a hydrophilic macromolecular carrier such as protein or PEG [32, 54, 56] help make the probe cell-impermeable and keep it in solution, tune the sensitivity to O₂ and stabilise the calibration. MitoXpress™ probe [33] from this category is now actively used in respirometric screening assays with isolated mitochondria, adherent and suspension mammalian and microbial cells [57], small organisms, and with compound libraries and environmental samples [58, 59].

The cell-impermeable dendritic probes have been developed by the Vinogradov group for imaging of tissue and vasculature [31]. In such structures, the central moiety of meso-substituted Pd/Pt-porphyrin or benzoporphyrin is modified via four peripheral carboxylic groups with dendritic polyglutamic chains that shield the phosphor from interferences (quenchers, pH, ionic strength) and reduce its quenchability by O₂. Incorporation of an additional hydrophilic shell by PEGylation of these dendrimeric structures improves their water solubility. Thus far, several such probes have been produced which span the excitation wavelengths from 400 to 700 nm and emission wavelengths from the red to near-infrared, and have different sensitivities to O₂ [35].

Another group of supramolecular probes are conjugates of the phosphorescent dyes with cell-penetrating peptides (CPP) for sensing intracellular O₂ [42, 60–62]. In this case, the luminescent moiety [a Ru(II) dye or Pt-coproporphyrin (PtCP)] is linked to a targeted delivery vector, such as polyarginine or proline-rich peptides which escort the whole structure into the cell by passive means and retain it inside [63]. The conjugation improves the functional properties of the probe with the possibility of sensing intracellular O₂ gradients, and allows simple analysis of cell populations on a commercial fluorescent reader. Modest photostability of the existing conjugates (mostly PtCP based) makes them difficult to use in O₂ imaging. In the future, this limitation can be addressed by developing new conjugates based on more photostable and/or brighter dyes (e.g. PtCPK and PtTCPTBP derivatives [36, 47]), and nanoparticle formulations with highly photostable non-functionalised dyes such as PtTFPP and targeted delivery features (see below).

Micro- and nanoparticle (NP) based probes are now under active development [64–67]. These probes with covalent or non-covalent incorporation of the dye usually have larger size (polymeric) and variable composition (distribution). Fabrication methods include impregnation of commercial polymeric beads (e.g. polystyrene), inclusion of indicator dyes during emulsion polymerisation [68] and formation of core–shell nanostructures by a precipitation method [67]. The NP technology provides greater flexibility with indicator dyes: hydrophobic structures lacking functional groups (i.e. not suitable for the other probe types) and pairs of dyes (ratiometric or FRET based O₂ sensing) can be used in such systems [69, 70]. A number of new and well-known biocompatible polymers and copolymers have been tested, such as polystyrene, polyfluorene and hydrogels. Furthermore, surface modification, for example, altering the surface charge of the NPs or adding a special peptide coat, allows optimisation of their specificity and penetration into or exclusion from the cell [64, 67, 69, 71, 72]. The advantages of the majority of NP O₂ probes are high specific brightness, photostability, relative ease of fabrication and up-scaling. The challenges are the significant size (usually >40 nm, i.e. larger than the other probes), fabrication processes to achieve reproducible composition and structure, stability under prolonged storage (drying and sterilization can be problematic), biocompatibility and toxicity in in vivo applications.

Endogenous O₂-sensitive probes are comprised of the photoluminescent structures which are encoded genetically and/or produced in living cells. A well-known approach is the imaging of respiration activity of cells by ratiometric analysis of fluorescent electron donors and redox indicators NADH and FAD [73]. The advantage is that these indicators are present in all cell lines; however, they possess low quantum yields of fluorescence (<1%) and require intense illumination of the sample. Protoporphyrin IX is another important fluorescent intermediate involved in heme biosynthesis. It can be overproduced in cells in the presence of increased amounts of 5-aminolevulinic acid.
(ALA) and used as an O₂ probe by measuring its delayed fluorescence [74]. This methodology has been tested with cultured cells, isolated hepatocytes and in vivo with intact hearts [26, 74, 75]. At the same time, the limitations of this probe are the low yield of delayed fluorescence along with strong short-lived fluorescence in the same spectral region, rather low photostability and photosensitisation activity of protoporphyrin IX. For the fluorescent redox indicators and other endogenous probes, including NAD(P)H and FAD, protoporphyrin IX. For the fluorescent redox indicators and other endogenous probes, including NAD(P)H and FAD, there are good opportunities for multiplexing with the other endogenous probes; however, such studies are still rather scarce [74, 76, 77].

Some fluorescent proteins of GFP family produce an O₂-dependent red shift in fluorescence upon their photo-activation with blue light [25]. Although this process only occurs at around 0–2% O₂, this methodology was applied successfully to study iO₂ gradients in Hep3b cells with mitochondria-targeted GFP [78].

For effective use in physiological experiments with complex biological samples, possible toxic effects of the selected O₂ probe on the cells, conditions, working concentration and time of use must be assessed and optimised at the start. For simple biological models, such as cell cultures or tissue slices, effects on cell viability, proliferation, energy production pathways and mitochondrial function are usually examined [5, 74]. For in vivo applications [3], factors such as systemic and organ toxicity (e.g. kidney, liver), probe distribution, metabolism and clearance of its components should be considered.

Measurement modalities

The main detection modalities in O₂ sensing are: (1) intensity measurements at a single wavelength; (2) ratio-metric intensity measurements with a reference (O₂-insensitive) dye; and (3) lifetime based sensing by phase modulation technique [29, 79] or by time-gated fluorometry under pulsed excitation. Since emission lifetimes of phosphorescent O₂ probes lie in the microsecond range, implementation of lifetime-based O₂ sensing is technically more easy than for conventional (nanosecond) probes and luminophores [29, 80].

Simple photoluminescence intensity measurements are generally prone to optical interferences, especially by complex biological samples producing high light scattering and autofluorescence that may affect probe calibration and lead to incorrect O₂ readings. Phase fluorometry can also be affected by these factors, unless special signal processing algorithms are applied (see, e.g., [52]). Time-resolved fluorometry (TR-F) in the microsecond time domain allows better signal-to-noise ratio and more reliable and accurate O₂ quantification via measurement of probe emission decay [3, 81]. In a simplified format called rapid lifetime determination (RLD) [80, 82], emission intensity signals (F₁, F₂) are collected at two different delay times (t₁, t₂) after the excitation pulse, from which lifetime is calculated as:

\[ \tau = \frac{(t_2 - t_1)}{\ln(F_1/F_2)} \]  

A number of commercial instruments—multi-label readers originally developed for sensitive detection of lanthanide chelate labels—have TR-F and RLD capabilities and can operate with existing O₂ probes on different assay substrates (96- and 384-well plates) [57]. One should keep in mind that such instruments have PMT detectors which are usually insensitive above 700 nm (or even above 650 nm), and their time resolution is limited by the Xe-flashlamp used (pulse width of ≥ 20 μs). Furthermore, instrument sensitivity, signal-to-noise ratio, accuracy of lifetime determination, temperature control of the sample and software capabilities may vary greatly depending on the make and model. Having tested many different instruments and probes, we consider Victor® (PerkinElmer, Finland) and FLUOstarOmega™ (BMG, Germany) TR-F reader families among the best in terms of their sensitivity and selectivity of probe detection (S/N ratio > 100), accuracy and resolution in RLD mode and temperature control. These instruments work well with PtCP and PtPFPP based probes including MitoXpress™, peptide conjugates and nanoparticles, and are now used in the high-throughput assessment of O₂ in cell populations and other sample types [57, 60, 83–86]. Representative profiles of respiration and cellular O₂ generated using both extracellular and intracellular O₂ sensing probes are shown in Fig. 1. Many standard instruments are not very compatible with the short-decay emitting probes based on Ru(II) complexes and/or with the longwave O₂ probes emitting in the very-near infrared. On the other hand, custom-built instruments tailored to the particular O₂-sensitive probes demonstrate good performance in O₂ measurement [31, 80].

Besides the macroscopic, or ‘cuvette’ formats, the above detection modalities can also be integrated with live cell/tissue imaging (LCI) platforms to implement O₂ imaging. Thus, relatively simple and inexpensive wide-field fluorescence microscopes allow two-dimensional (2-D) visualisation with sub-cellular spatial resolution of respiring objects loaded with an O₂ probe. Using conventional intensity-based mode, standard LCI systems allow monitoring of relative changes in cell oxygenation and respiration activity over time. With a proper calibration (e.g. measuring probe signal at several known O₂ levels), fluorescence intensity images can be converted into [O₂] maps [81]. However, intensity calibrations are rather unstable due to significant probe photobleaching and signal drift under illumination (which should be minimised by all
means), and affected by sample distortion (manipulation with cells or effector addition). Using a more complex probe with two reporter dyes (1 is O$_2$-insensitive) and ratiometric intensity imaging mode [69, 70], it is possible to stabilise the O$_2$ calibration if the two dyes photobleach at approximately the same rate. For high-resolution O$_2$ imaging, probe photostability becomes one of the main selection criteria and many current probes are not quite optimal in this regard. In addition, many O$_2$-sensitive dyes are effective sensitisers of singlet oxygen production [87]. Their use in imaging experiments needs thorough optimisation to ensure sufficiently high, reliably measurable luminescent signals and low phototoxicity and cell damage [30, 53].

**Laser-scanning microscopy**, represented by confocal and multi-photon luminescence LCI systems, allow visualisation of complex objects with sub-micron spatial resolution, and generation of corresponding O$_2$ maps in 3-D and 4-D (in time-lapse experiments) [87]. Multi-photon systems, which employ high-power NIR lasers, provide deeper tissue penetration (several hundred microns) and better spatial resolution; however, they are currently expensive, require indicator dyes with large cross-section of two-photon absorption and special tuning of hardware and software for the measurement of long-decay emission of O$_2$ probes. A number of dedicated O$_2$ probes with two-photon and FRET antennae, imaging systems and applications on their basis have been described recently [77, 88–92], and this area continues to develop rapidly.

**Phosphorescence and fluorescence lifetime imaging microscopy (FLIM)** enables visualisation of O$_2$ distribution in complex biological samples, and accurate quantification of O$_2$. On a microscope with wide-field illumination, 2-D O$_2$ imaging can be realised using pulsed excitation with a suitable LED or laser delivering trains of ns-µs pulses at kHz frequency, and gated CCD camera operating in the microsecond time range [81]. Following each excitation pulse and a time delay (variable), emitted photons are collected by the camera over the measurement window time and integrated over a number of pulses to generate an

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**Fig. 1** Different signal profiles revealed with the O$_2$ sensing probes. 

a Microbial respiration/growth of *E. coli* measured in phosphorescence intensity mode. The number of cells in the original sample (cfu/g) is determined from the time required to reach signal threshold [121].

b Respiration profiles of eukaryotic cells measured in RLD mode: wild-type (WT) mouse embryonic fibroblasts, knock-out (KO) cells deficient with Krebs cycle enzyme and blank control. OCRs are calculated from the slope of probe signal [57].

c Profiles of oxygenation for the respiring and non-respiring PC12 cells under mild atmospheric hypoxia (8% pO$_2$). 

d Changes in icO$_2$ in respiring PC12 cells upon the addition of uncoupler (FCCP), inhibitor (AntA) and mock control (DMSO), at 20.9% pO$_2$. Arrow indicates the time of effector addition.

e Oxygenation of MEF cells grown at different densities in a microfluidic chip Ibidi$^{®}$, measured under static conditions. Flushing the chamber with fresh medium (at the start and at arrow) causes reoxygenation and subsequent deoxygenation of the cells. 

a, b were generated with ecO$_2$ probe MitoXpress$^{TM}$ [80]; c–e with pre-calibrated icO$_2$ probes [61, 71].

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intensity frame. This is repeated at several delay times, and from these frames emission decay is reconstructed and lifetime is determined for each pixel of the CCD matrix. By applying a probe calibration function (determined in a separate experiment), lifetime images of the sample can be converted into an O₂ concentration map. For the laser-scanning systems, emission lifetimes are measured sequentially for each pixel with a PMT or photodiode detector, processed by the software to generate 2-D images of Z-stacks which are then assembled together.

Many existing O₂ probes are spectrally compatible with modern LCI systems which usually contain several light sources (lamps, LEDs, lasers) and detectors (red-NIR sensitive PMTs, photodiodes or CCD cameras). One should keep in mind that off-the-shelf LCI systems are designed to operate in intensity and ratiometric (multi-colour) modes and with nanosecond fluorophores. They have to be custom-tuned for use with long-decay emitting O₂ probes, preferably upgraded to support FLIM mode and calibrated carefully for quantitative O₂ imaging [91, 93]. Due to long-decay emission of the O₂ probe, generation of detailed 2-D and 3-D O₂ maps on FLIM systems requires relatively long signal acquisition time. So, even for the very sophisticated custom-built O₂ imaging systems, temporal resolution is not as good as for the imaging systems operating with nanosecond probes. Different LCI and FLIM systems have different signal acquisition settings, so that several key parameters require basic optimisation for each particular O₂ probe and application.

A number of alternative, specialised techniques have been described recently, which are not discussed here; for example, non-radiative triplet quenching of the lumino-phores under high-power pulsed laser excitation [26] and photoacoustic sensing [94].

**Probe localisation and measurement formats**

Depending on the measurement task, the O₂ probe needs to be introduced in a particular compartment of the sample, cell or tissue, or applied to measure a particular pool of sample O₂ such as extracellular or intracellular (see Table 2).

For imaging of tissue O₂, extracellular, cell-impermeable probes are usually employed, which are injected into blood stream or bulk tissue. Such a probe is expected to stay in the vasculature without penetrating the cells, and to have low cyto- and organ toxicity (e.g. kidney or liver damage), especially in prolonged in vivo experiments [91]. For the measurement of OCR, and related in vitro applications with cultured cells, extracellular probes dissolved in the medium are used [57] (solid-state O₂ sensitive coatings may also be suitable for these applications [50]).

**Intracellular probes**

In some applications and measurement tasks, it is necessary to introduce the probe directly into the cell and maintain it inside during the measurement without damaging the cell or affecting its normal function. Cell loading is relatively easy to achieve with phagocytic cells (e.g. macrophages [69]) using particle-based probes; however, loading of the majority of other cells is more challenging. Facilitated transport systems, such as microinjection, electroporation [66], facilitated endocytosis [80, 93], liposomal transfer and gene gun [95], have been used with some success, but these techniques require additional reagents/steps, and are tedious, invasive and stressful for the cells. They rarely provide high and/or uniform loading of cell populations, and show a high degree of cell specificity, with dependence on the conditions used (medium, additives, temperature, cell type). These techniques are difficult to use routinely.

A more efficient strategy for cell loading is to develop O₂ probes with cell-penetrating ability [42, 60–62] or to introduce ‘delivery vectors’ in existing probe structures [65]. This has been demonstrated with several supramolecular- and nanoparticle-based probes, for which fast (6–16 h) and efficient cell loading comparable with the delivery of conventional small molecule cargo or cell transfection with genetically-encoded biosensor probes [25, 96] was achieved. A significant degree of cell specificity can still be seen as no universal cell-loading vector currently exists [63]. Rational design potentially allows for targeted delivery of an O₂ probe to a particular location within the cell, by altering molecular charge or peripheral groups, or by incorporating special functionality and additional ‘vectors’ responsible for the delivery of the cargo (e.g. mitochondria targeting peptide sequences [97]). As with many other probes and drugs, a well-defined intracellular localisation is desirable. However, this is not so critical for the O₂ probes because O₂ is not contained in a specific location within the cell and diffuses quickly across and through the biological membranes. Phototoxicity and cell damage depend on the location of the probe within the cell. For example, photosensitisers of porphyrin origin used in photodynamic therapy of cancer show maximal photodamage when they are targeted to cell membranes or the nuclei [98, 99]. Based on this, one can predict [87] that for O₂ sensing the probe should be targeted to a different part of the cell, for example to the cytoplasm.

**Applications and biological uses**

Common phosphorescence based O₂-sensitive probes and their biological applications are summarised in Table 2.
Table 2 An overview of O$_2$ sensing probes tested in biological applications

| Probe name and type | Application, probe location | Equipment, detection mode | $K_{	ext{on}}$, $\mu$M$^{-1}$/ms | Status, comments | Refs. |
|---------------------|-----------------------------|---------------------------|---------------------------------|-----------------|-------|
| **Extracellular probes** | | | | | |
| PdTTPCPP (conjugated to BSA) SM | O$_2$ mapping in tissues | Ex 416, 523 nm Em 690 nm | 0.382/700 | Quantitative. Point measurement Used by several labs | [54, 101–106] |
| | Probe in blood/vasculature | Scanning phosphorescence quenching microscopy | | | |
| Oxyphor R2 (PdTTPCPP dendrimer) SM | O$_2$ mapping in tissues | Phase fluorometry: Ex 524 nm Em 690 nm 4 mm light guide; Two-photon microscope | 0.343/640 (38°C, pH 7.4) | Quantitative. Point measurement Used by several labs | [31, 105] |
| | Probe in blood/vasculature | | | | |
| Oxyphor G2 (PdTCTBP dendrimer) SM | Tissue O$_2$ gradients in vivo. | Wide field FLIM: Ex 440/632 nm, Em 790 nm | 0.086/251 (38°C, pH 7.4) | Quantitative. Used by several labs and with different models. Validated | [31, 106] |
| | Probe in blood/vasculature | | | | |
| MitoXpress (Ptcp conjugate) SM | OCR by cells, mitochondria, enzymes. Assessment of cell bioenergetics. Probe added to the medium | TR-F reader, RLD: Ex 340–420 nm Em 640–660 nm | ~0.011/80 | Quantitative. Used by many labs. Validated in drug screening | [19, 33, 57, 107] |
| PtP-C343 (Ptaop- Coumarin 343 dendrimer) SM | Tissue O$_2$. In vivo O$_2$ gradients. | Two-photon FLIM: Ex 840 nm Em 682 nm | >0.11/60 | Quantitative. Require special equipment and set-up. Used in several labs | [91, 93, 108] |
| | Probe in blood/vasculature | | | | |
| Oxyphors R4 and G4 | Tissue O$_2$ gradients in vivo. Tumor imaging. EC probe in blood/vasculature/interstitial space | Wide-field FLIM R4: 0.098/681 (37°C, pH 7.2) G4: 0.083/218 (38.2°C, pH 7.2) | Quantitative. Require special equipment and set-up | [109] |
| | | | | | |
| PS-NP (polystyrene NP doped with PdTPTBP and DY635, reference) | Targeted tumor in vivo imaging EC probe | Ratiometric-based Lifetime-based Ex 635 nm Em 670 nm (reference); 800 nm (O$_2$ sensitive) | ND | Quantitative. May be used for intracellular measurement with modified coating | [72] |
| **Intracellular probes** | | | | | |
| O$_2$ PEBBLEs (PtoepK and OEP, omosil) NP | Cell oxygenation Impermeable probe loaded with gene gun | Ratiometric wide field imaging Ex 568 nm, Em 620/750 nm | 0.032/NA | Semi-quantitative (relative). Stressful loading. 1 cell type tested | [95] |
| Probe name and type | Application, probe location | Equipment, detection mode | $K_{s-v}$, $\mu$M$^{-1}$/s | Status, comments | Refs. |
|---------------------|-----------------------------|---------------------------|-----------------------------|-----------------|-------|
| MitoXpress (PtCP conjugate), SM | Cell oxygenation, Metabolic responses | TR-F reader, RLD: Ex 340–420 nm Em 640–660 nm | $\sim 0.006/70$ | Quantitative. Facilitated loading required (16 h); 6 cell types tested (cell-specific) | [80, 83–86] |
| Ru(II)-(py)$_3$R$_8$ (peptide conjugate), SM | O2 mapping in cells Cell-permeable, self-loading probe | Wide field FLIM: Ex 460 nm Em 607 nm | ND | Semi-quantitative. 1 cell type tested | [42] |
| Cell penetrating PtCP peptide conjugates: PEPP0, 3, PtCPTE-CFR$_6$, SM | Cell oxygenation, Metabolic responses | TR-F reader, RLD: Ex 340–420 nm Em 640–660 nm Intravital confocal imaging was also demonstrated | $\sim 0.006/70$ | Quantitative. >6 cell lines tested | [60–62, 110] |
| PtOEP/PDH and PFO-NP | O$_2$ mapping in cells Probe uptake by macrophages | Ratiometric wide field imaging: Ex 350 nm Em 440/650 nm | ND | Semi-quantitative. One cell line tested (macrophages). Particle variability, require UV excitation | [69] |
| Near infrared PAA NPs (Oxyphor G2 probe in PAA gel, with peptide coat) NP | Cell oxygenation Cell permeable, self-loading probe | Wide field and confocal imaging: Ex 633 nm Em 790 nm | 0.034/ND (37°C) (without cells) | Quantitative. Several cell lines tested. High probe concentrations used. Cross-sensitivity to pH | [65] |
| RGB NPs (PtTFPP and BCPN in aminated polystyrene) NP | Cell oxygenation | Wide-field RGB imaging: Ex 330–380 nm Em Red, Green | $\sim 0.0083/NA$ | Semi-quantitative. 1 cell line tested. NP variability, long loading 48 h | [70] |
| NANO2 (PtPFPP in RL100 polymer) NP | Cell oxygenation Metabolic responses, cell bioenergetics | TR-F reader: RLD: Ex 340–420 nm Em 640–660 nm Wide-field FLIM and confocal O$_2$ imaging | $\sim 0.006/67$ | Quantitative. >5 cell lines tested. High brightness and photostability | [71] |

$K_{s-v}$ constants were calculated based on published data with assumption that at normal atmospheric pressure O$_2$ has 160 mmHg with dissolved concentration $\sim 200$ $\mu$M or 4,950 ppm PAA polyacrylamide hydrogel, PDHF poly(9,9-dihexylfluorene), PFO poly(9,9-dioclylfluorene), SM supramolecular, NA not applicable, ND no data reported, NP nanoparticle-based, BCPN butyl-N-(5-carboxypentyl)-4-piperidino-1,8-naphthalimide.
Average OCR or O₂ concentration can be measured using an extracellular O₂ probe added to the sample. Such measurements can be conducted in cuvettes or microplates on a conventional fluorescent spectrometer or TR-F reader [5, 19]. To measure absolute OCR values, a sealed, gas-impermeable vessel should be used [58]. If the sample is heterogeneous and contains precipitating matter (e.g. suspension of cells), stirring should be provided to eliminate the formation of local O₂ gradients which may lead to incorrect results. To assess relative OCRs (e.g. treated vs. untreated cells), the set-up can be simplified, for example using standard microtiter plates, which facilitate cell growth, liquid handling and up-scaling, and partial sealing of samples with mineral oil added to the wells prior to the measurement [58]. The oil forms a barrier for ambient O₂ diffusion, and leads to the development of an O₂ gradient in the sample which can be monitored with the O₂ probe and related to the OCR [57, 84]. This is a convenient format for analysing large number of samples of similar type, e.g. when screening compound libraries for mitochondrial and cytotoxicity, or analysing panels of transformed cells or microbial cultures. Simple fluorescence intensity measurements can be used in these applications, with proper controls for possible optical interferences and measurement artefacts [19]. Representative respiration profiles of bacterial and mammalian cells are shown in Fig. 1a, b.

In vivo O₂ imaging is of high fundamental and practical importance. Measurement of actual oxygenation in live respiring tissue (e.g. brain or muscle), localised O₂ gradients in the vasculature (blood vessels, capillaries) or tumour oxygenation can be realised using extracellular O₂ probes and phosphorescence lifetime-based O₂ imaging [106, 111–113]. This was also realised with a fibre-optic probe and point-by-point measurements [54], and in plant cells [79]. In recent years, wide-field FLIM systems and high-resolution confocal and two-photon laser-scanning systems [114] for imaging tissue O₂ were successfully used in complex in vivo and ex vivo studies.

Thus, a new dendrimeric probe with coumarin antennae, for which a detailed synthesis protocol was published [115], was applied to measure local oxygenation in rodent brain on a custom-built two-photon FLIM LCI system [91]. The cell-impermeable PtP-C343 probe was injected into the blood stream and measured in the brain tissue at different distances from arterial regions [31, 93]. This system showed a spatial resolution of 100 µm, stable O₂ calibration practically unaffected by the environment, and probe retention in the body (half-life) of about 2 h.

Other in vivo studies include: O₂ mapping of rodent retina where the application of anaesthetics was shown to decrease venous O₂ tension [54, 106]; O₂ dynamics in individual frog skeletal muscle fibres showing that faster frequency of muscle stimulation leads to higher drops in pO₂ in adaptive manner [116]; measurement of pO₂ in microcirculation [100, 101], tumour oxygenation [56, 72]; and FLIM of cortical extravascular O₂ in the ischemia–reperfusion model [91].

The development of icO₂ probes has extended the capabilities of O₂ sensing, particularly with respect to the in situ oxygenation of respiring samples. Thus, adherent cells in their native differentiated state can be analysed in open microplates or sealed samples (perfusion cell or culture flask). In such assays, the main parameters that require control and optimisation are cell density and metabolic activity, diffusion and mass exchange characteristics of the sample (volume of medium, viscosity, temperature) and external pO₂ [84]. icO₂ probes can be used in conjunction with TR-F readers (described above) providing simplicity, convenience, high sample throughput and good analytical performance. This sensing methodology has been applied to monitor changes in cell respiration and metabolism, responses of cells to stimulation with effectors (with treatment applied during the measurement) and to hypoxia [80, 84]. It has been used in several mechanistic studies with complex biological models [83, 85, 86]. Representative profiles of icO₂ produced with mammalian cells are shown in Fig. 1c–e.

The icO₂ probes were also used in microscopy imaging formats, to perform semi-quantitative intensity-based assessment of cellular O₂ or more accurate measurement by FLIM [81]. For example, they were used in situ respirometry with skeletal muscles [53, 102, 116], and in ex vivo imaging experiments with carotid body where cell oxygenation was correlated with the other parameters of cellular function [110]. Furthermore, perfusion chambers, microfluidic devices and 3-D tissue cultures are becoming increasingly popular in biological experiments [117]. For such systems, reliable control of sample oxygenation is critical, and it can be implemented by means of icO₂ probes and contactless measurements. And in OCR measurements with adherent cells and tissue under non-stirring conditions (e.g. on a microplate under oil), icO₂ probes can provide better sensitivity than conventional extracellular probes.

Finally, one can consider combining different O₂-sensing probes and techniques to control O₂ in complex biological experiments, for example in cell culture, tissue engineering, or experiments under hypoxic environment. Examples of such tools and measurement set-ups shown in Fig. 2 include: (1) a hypoxia chamber (macro-system) which can be set at different pO₂ levels; (2) solid-sate O₂ sensors placed inside the chamber at different locations; (3) a handheld optical scanner which interrogates with the sensors (from outside or inside the chamber) and reads current O₂ concentration; (4) a tissue culture flask (mini-system) with built-in sensor spots also measurable with the
Conclusions and future prospects

Overall, a fairly broad variety of different $O_2$ sensing probes, measurement formats and applications have been described and tested, and each of them possesses merits and limitations. To ensure proper selection and use for a particular biological model or measurement task, it is necessary to demonstrate their analytical performance under relevant experimental conditions, determine structure–function relationships and work out detailed operational protocols that can be easily adopted by ordinary (non-skilled) users. Thus far, only some of these probes and techniques (see Table 2) have been shown to provide quantitative and reproducible measurement of $O_2$ with stable and accurate calibrations. Many others have stuck at the stage of proof of concept, with incomplete optimisation, unsatisfactory working specifications and analytical performance, and semi-quantitative or qualitative data output (relative changes in $O_2$ concentration or use under ‘clean’ conditions). This limits their adoption by the broad community of biomedical researchers. Another practical issue is the low level of understanding by the end-user of the basic principles underlying each particular $O_2$-sensing technique and defining the scope of its applicability. This often leads to negative results in first experiments and dissatisfaction with the method which is then difficult to overcome. To make a way towards wider practical use, these probes and techniques, which exist in many variations outlined above, require substantial development and improvement, comprehensive validation in biological experiments and demonstration in cutting-edge physiological studies.

On the application side, initial bioenergetic, metabolic, cell biology and toxicological studies using $O_2$-sensing techniques have been applied to rather simple, macroscopic samples, such as mitochondrial preparations [118], suspension cell lines [57] and perfused respiring tissue [24]. Nowadays, the focus is shifting towards more complex models and real-life systems: adherent differentiated cells [83–86], ex vivo and in vivo systems, such as intact respiring brain, muscle, tumour tissue and vasculature [90, 91]. More recently, detailed mapping and reconstruction of $O_2$ gradients on a micro-scale and in 3-D have been demonstrated in tissue and even in individual cells [26, 81], which require thorough verification. New ‘super-resolution’ imaging platforms are now emerging [119]; however, their usability with the long-decay $O_2$ probes has still to be demonstrated.

Measurement of local $O_2$ concentrations and gradients in tissues and within the cell is important for research areas such as cancer metabolism, neuroscience, effects of hypoxia on cell physiology, biomedical devices and wound healing. For macro-objects, such as tissue slices, organs or whole organisms, $O_2$ is also a valuable parameter, for example in radio- and chemotherapy, assisted reproductive technology and organ transplantation [120]. The demand for non-invasive $O_2$ measurement systems spans well beyond the above mentioned areas and models. It is also
highly relevant for biotechnology, environmental monitoring, food, and chemical and industrial process control.

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