Intracellular probes for imaging oxygen concentration: how good are they?

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Intracellular probes for imaging oxygen concentration: how good are they?

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Keywords: 3D tissue models, FLIM, hypoxia, intracellular and cell-permeable probes, phosphorescence quenching, phosphorescent oxygen-sensitive probes

Abstract
In the last decade a number of cell-permeable phosphorescence based probes for imaging of (intra)cellular oxygen (icO2) have been described. These small molecule, supramolecular and nanoparticle structures, although allowing analysis of hypoxia, local gradients and fluctuations in O2, responses to stimulation and drug treatment at sub-cellular level with high spatial and temporal resolution, differ significantly in their operational performance and applicability to different cell and tissue models. Here we discuss and compare these probes with respect to their staining efficiency, brightness, photostability, toxicity, cell specificity, compatibility with different cell and tissue models, and analytical performance. Merits and limitations of particular probes are highlighted and strategies for development of new high-performance O2 imaging probes defined. Key application areas in hypoxia research, stem cells, cancer biology and tissue physiology are also discussed.

1. Introduction
Quantitative measurement and mapping of molecular oxygen (O2) using imaging techniques is required in many life science research and biomedical applications including cell biology, cancer metabolism, regenerative medicine and biomaterials science [1–4]. Among the different techniques described so far, the phosphorescence quenching method provides a direct, non-chemical, non-invasive measurement of O2, which can be tuned to the physiological O2 range (0–250 µM, equal to 0–21% O2 in atmosphere) and realized in different imaging modalities and formats (spatial, temporal, spectral, etc) [5–8]. General ease of use and compatibility across the available imaging platforms make this method superior over e.g. electrode based systems [9], instrumental techniques such as electron paramagnetic resonance (EPR), magnetic resonance imaging (MRI), pulsed oximetry, spectral imaging, or the use of indirect and non-quantitative hypoxia stains [1, 10–15].

Over the last decade, a novel class of phosphorescent probes have been developed which enable measurement of extra-, peri- and intracellular O2 (icO2) [7, 16]. While allowing the analysis and imaging of hypoxia, fluctuations in O2 availability, responses to stimulation and drug treatment at sub-cellular level with high spatial and temporal resolution, these probes differ in their chemical nature, spectral characteristics, sensitivity to O2 and general usability. For the different probes, marked differences exist in operational performance, compatibility with different cell and tissue models, imaging modalities and analytical tasks, which have not been analysed comprehensively. On the other hand, the diversity of experimental models of mammalian cells and tissue, which includes various in vitro, ex vivo and in vivo models, necessitates the O2 probe or sensor to match a significant number of analytical characteristics and practical requirements to perform adequately in complex physiological experiments. These requirements include loading method and efficiency, probe distribution in the specimen, intrinsic cytotoxicity and photo-toxicity, stability of calibration, sensitivity to environmental factors (pH, ionic strength, temperature, local distribution), chemical stability, safety and other parameters [1].

Cell-permeable (or self-loading) O2 probes usually contain an O2-sensitive indicator dye moiety, which is chemically modified or embedded in a supramolecular or nanoparticle (NP) structure, to acquire the ability to penetrate inside cells or tissue and remain there for periods of time sufficient for physiological and imaging experiments [5, 7]. For efficient intracellular accumulation, phosphor molecules can be modified with peripheral groups or substituents, such as positively charged groups or protein binding domains, which
facilitate probe interactions with plasma membrane and transport (specific or non-specific) into the cell. In such probes, the phosphor molecule remains exposed to the biological environment which can affect its characteristics via e.g. binding to proteins or different pH or viscosity in different cellular compartments.

In NP structures the phosphor can be shielded by a polymer matrix from unwanted molecular interactions and interference from the local environment, while still retaining its accessibility for O2 and sensing properties. Simple physical inclusion of indicator and reference dyes in NP O2 sensor structures broadens the range of imaging modes (phosphorescence lifetime-based imaging (PLIM) and ratiometric-referenced intensity measurements under one- or two-photon excitation), and increases probe brightness and photostability [5]. However, storage and operational stability of NP probes can be a serious issue [17].

So far, over 20 different icO2 probe structures have been reported (table 1) offering a difficult choice for new users. However, many probes do not provide the stated improvements, practical benefits and ability to work outside their developer’s labs [18] and only a few are available commercially. Insufficient characterization, testing and optimization of such probes, limited compatibility with key biological models or imaging modalities and practical validation are common, while the exchange of probes between different research groups, their direct comparison, proper technical support and troubleshooting are rare. Marginal improvements (‘re-inventing the wheel’), overstated performance, measurement artefacts represent the common issue.

We critically review the existing icO2 probes, highlighting their benefits for potential users and performance issues that have to be addressed. We also define key analytical and working specifications that need to be considered when selecting a suitable icO2 probe for particular biological models and applications, or when developing new probes. This review does not cover the large area of extracellular O2 probes, their imaging applications and specialized techniques. These are discussed extensively in recent reviews [1, 5, 7, 10].

2. Main practical considerations

To assess the suitability of a biological sample for the quantitative measurement of icO2, one should consider the loading efficiency, specific brightness of the probe, size and structural organization of the sample containing respiring cells/tissue, sensitivity of the available imaging system (microscope) and the format of the planned imaging experiments (time, frequency, speed). For brighter probes one can afford a lower loading efficiency/dose, or shorter loading time to achieve the minimally required optical signals, and also minimize the impact of the probe on cellular function (see section 2.2). In contrast, low phosphorescent signals and signal-to-noise ratio can prevent reliable and accurate determination of phosphorescence lifetimes and O2 concentrations [38–41].

2.1. Cell staining and brightness

Among key benchmarking parameters for icO2 probes are their efficiency and uniformity of cell staining, working concentrations and loading time, and achievable optical signals [7, 22, 39]. Unlike DNA constructs delivered by transfection [1], modern icO2 probes usually stain passively and uniformly all cells in the population by their simple addition to the media and incubation. However, cell specificity and staining patterns can vary significantly. Not many icO2 probes have been evaluated with representative panels of different cell types. Some probes were only demonstrated with easy-to-load phagocytic cells which effectively ingest particles and solutes from surrounding media [42–44]. Also staining of heterogeneous tissue samples containing different cell types or multi-cellular aggregates can be complex, or limited mainly to the surface layer (spheroids) or one particular cell type. Loading should also occur in a reasonably short period of time, typically 0.5–24 h.

Specific brightness is determined by the photophysical characteristics of a phosphorescent indicator. The long-decay fluorescent complexes of Ru(II) and Ir(III) and phosphorescent metalloporphyrins such as PdTPCPP and PtBP have been used (see table 1), however, bright, photostable, easily available and inexpensive PdTFPP is currently the most popular dye [1, 5]. At the same time, the molecular organization of the probe and how the O2 sensitive, cell-targeting and other moieties are assembled together is also important.

Small molecule probes produced by the functional modification of a phosphorescent dye possess small size and fast cell loading, occurring in as little as a few minutes [19, 20, 23]. However, these ‘probes’ also have disadvantages: hydrophobic core, possible self-aggregation, uncontrolled interaction with cellular components (proteins, lipids) which can alter their photophysical characteristics and O2 calibration. The phosphorescent moiety is not shielded and quenching interferences can potentially occur. Although flexible with chemical modifications, the fine-tuning of such probes to improve cell permeability and staining efficiency is not so straightforward, due to rather limited knowledge of structure-activity relationships for phosphorescent dyes [20].

Supramolecular probes comprise covalent conjugates of phosphorescent dyes with delivery vectors of biological nature, such as cell-penetrating peptides or saccharide moieties [7]. These have been shown to be more efficient than modified phosphorescent dyes [45]. Initially, the synthesis of such bioconjugates was limited mainly to moderately hydrophobic phosphorescent dyes, such as PtCP and [Ru(bpy)2(pic)]2+ [19, 22] but more recently it was extended to the hydrophobic PtTFPP dye via click-modification of pentafluorophenyl groups [25]. Therefore, a Pt-Glc probe based on PtTFPP
indicator dye shows good in-depth staining of animal tissue and performance in O2 imaging. Nonetheless, the specific brightness of the small molecule and supramolecular icO2 probes containing only one phosphorescent moiety per structure are moderate while the doses needed for efficient cell loading are high (1–20 µM).

Certain types of NPs, produced from cationic and amphiphilic polymers, show efficient intracellular

<table>
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<th>Table 1. Composition and photophysical characteristics of existing cell-permeable O2 probes.</th>
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<tr>
<td>[Ru(bpy)2(pic)]2+ conjugate with R8 peptide [19]</td>
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<tr>
<td>PtTFPP in polystyrene and dodecyltrimethoxysilane hybrid NP (PLL-NP). Size 77 nm [37]</td>
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</table>

ND—data not reported.

a Emission lifetimes for unquenched state (0% O2) are presented.
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Each NP contains multiple dye molecules. On the other hand, their large size can compromise cell penetration and loading efficiency. Nonetheless, current NP probes are among the brightest (table 1). In some cases, a minimal degree of staining provided by the NP structure can be more efficient than highly efficient loading with small molecule conjugate [25]. When selecting a suitable NP icO2 probe, the balance between cell loading efficiency and specific brightness, stable retention of dye molecules inside the NPs without any leakage, stability of sensor NP during operation, storage and leaching, and their spectral properties should be considered.

Cellular uptake of such NPs is thought to be mediated by electrostatic interactions with negatively charged plasma membranes and specific transport mechanisms, e.g. clathrin-mediated endocytosis [46]. Staining is not very cell-specific and usually slower (12–48 h) than for small molecule probes, and is not possible for some cell types. More recently, anionic NP O2 probes based on PMMA-MA also showed efficient staining of various cells and 3D tissue models with comparable performance in imaging experiments [34]. They also internalize via energy-dependent endocytosis mechanisms.

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Table 2. Characterization experiments performed with selected icO2 probes.

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<td>None observed</td>
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<td>Stable, no leaching</td>
<td>ND</td>
<td>ND</td>
<td>Stable, no leaching</td>
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<td>Ratiometric, lifetime, two-photon</td>
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ND, data not reported; ECA, extracellular acidification; PDT, photodynamic therapy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.
more and high throughput equipment such as a time-resolved fluorescence reader (cell populations) and fluorescence activated cell sorter (FACS) (individual cells) [20, 21, 29]. Thus, plate readers can be used for rapid optimization and comparison of different probes for their suitability for icO2 imaging, compatibility with different cell lines, working concentrations, time and conditions of cell loading [39]. For example, such analysis of phosphorescence intensity signals under similar staining conditions shows better performance of NanO2 probe than PEPP0 with respect to HepG2 cells [39].

2.2. Toxicity
For many icO2 probes, their technical description and demonstration were mainly focused on their photophysical and O2 sensing properties, rather than their general usability. In practice some of the reported structures (especially NP probes) show pronounced toxicity, cell specificity, complex staining patterns, high doses and/or slow accumulation in cells. First generation icO2 probes used invasive loading methods such as microinjection, gene gun, electroporation, or facilitated delivery with additional transfection reagents [28, 38, 51, 52]. These approaches are associated with a pronounced impact on cell viability, low and uneven cell loading and stochastic distribution in cells. They were subsequently superseded with probes capable of efficient passive self-loading.

The absence of intrinsic toxicity for an icO2 probe and minimal impact on cellular function are extremely important, particularly when studying cell metabolism and physiology [53]. O2 sensing or targeted delivery moiety (e.g. peptide, polymer components) can interact with endogenous molecules, receptors and nutrient transporters, affect endo- and exocytosis and normal cell function. For NP and macromolecular probes the size of <100 nm is considered optimal for cell penetration [54], and staining concentration should be kept below 1–10 µM (probes) or 50–500 µg ml⁻¹ (NPs). Unfortunately, rather crude and non-specific methods of toxicity assessment are used with many probes (cell viability or MTT assays—see table 2). These results are not very trustworthy and can easily miss sub-lethal but significant effects on the cell. Also toxic effects can evolve slowly (e.g. changes in cell proliferation or gene expression) [47, 55]. For proper toxicological assessment of icO2 probes, more sensitive and specific tests are required, such as the ATP, extracellular acidification (ECA) and oxygen consumption rate (OCR) assays which detect minor changes in cell bioenergetics, mitochondrial function and metabolism [56]. It is worth noting that icO2 probes themselves can inform on drug-induced toxicity and changes in mitochondrial function, for example by measuring oxygen consumption, self-deoxygenation or local O2 gradients in cultured cells [37, 50].

Photogeneration of singlet oxygen upon illumination is another mechanism of tissue damage by O2 probes [1, 5]. This may be less critical for cell-impermeable O2 probes administered systemically in animal vasculature, due to the short lifetime of singlet oxygen and remote location of its critical targets (cell membranes, DNA) [57, 58]. On the other hand, for intracellular probes their location inside the cell and the presence of nearby targets are critical; thus, cytosolic and lysosomal localization are preferred, while nuclear, plasma membrane and mitochondrial localization is less desirable. Bright probes with high cell permeability allow minimization of unwanted effects on cellular function by low doses of the probe and low illumination intensity.

2.3. Cell-specificity and intracellular distribution
Many NP and small molecule probes enter the cell by endocytosis pathway [46, 59] (table 2). However, some cell-penetrating peptides (protein transduction domains) structures utilize direct translocation (ATP- and temperature-independent mechanisms) and can facilitate transport to such intracellular organelles as mitochondria [20, 24]. However, upon conjugation with phosphorescent moiety transport and targeting properties can be altered significantly (e.g. hydrophilicity, charge, binding activity) and are hard to predict. Studies of PtCP conjugates with Arg-rich peptides helped to produce O2 probes with whole cell (cytosolic) distribution, whereas similar conjugates of PtTFPP showed aggregation and poor O2 sensing performance.

Cationic NPs based on RL100 hydrogel stain many different cell lines, but are inefficient with neural cells [34, 47]. Mixed endocytosis mechanism of cell entry shown by many NP probes allows efficient staining of different cell types [29, 33, 34]. However, based on the available data (tables 1 and 2) [7], their staining efficiency and sub-cellular distribution in different cell types are still difficult to predict and regulate. When entering the cell by endocytosis, the probe usually remains in recycling endosomes and lysosomes. In order to exit endosomes and reach mitochondria, endoplasmic reticulum or cytosol, the probe has to experience ‘endosomal escape’ [59], a process which is not so easy to regulate externally. In the case of direct translocation (well-studied for peptide-based structures or delivery by electroporation) [21, 24, 28, 60], the probe is not trapped in endosomes and can diffuse into the cytoplasm and other compartments. This uptake pathway can help overcome the problems of cell-specificity (no requirement of specific interaction with cell membrane) and sub-cellular targeting.

Most of the probes currently used in O2 imaging show preferential endosomal localization (co-localize with lysosomal stains such as LysoTracker Green). Lysosomal localization also protects the cell from photo-damage, as damaged lysosomes and their content can be repaired or replaced. One NP probe shows weak co-localization with mitochondria [37]. The PtCP-based probe PEPP0, which shows mitochondrial localization, is poorly suited for high-resolution
imaging experiments due to low photostability [21]. So far, only a few studies have attempted sub-cellular targeting of icO₂ probes [27, 61]. This necessitates detailed studies of structure-activity relationships and mechanisms of intracellular transport and localization for phosphorescent icO₂ probes. Also probes should be tested with various cells (table 2), particularly those which proved difficult to stain.

On the other hand, the small dimensions of eukaryotic cells (~10 μm), large diffusion distances for O₂ molecules and easy movement across membranes (unlike other metabolites or ions), make sub-cellular O₂ gradients, produced by the network of respiring mitochondria acting as O₂ sink, very flat or non-existent [62, 63]. As a result, icO₂ concentration can be measured with high (sub-cellular) spatial resolution with many existing probe structures, particularly those located in endosomes which are abundant in most cells [1, 50].

2.4. Detection (imaging) modes

Depending on the particular biological model, available icO₂ probe structure(s) and live cell imaging platform, researchers can choose the preferred imaging mode for their study. The important factors to keep in mind are:

(a) the physical size and structural organization of the biological model and associated limitations for in-depth penetration of the icO₂ probe, as well as the excitation and emission of light. These factors dictate the preferred spectral characteristics for the probe, excitation light source and detector; [64–66]

(b) the available read-out modes for the measurement of phosphorescent signals and O₂ quantification. These can be conventional intensity measurements, ratiometric intensity or PLIM (sometimes called as ‘microsecond fluorescence lifetime imaging microscopy (FLIM)’) [7, 16].

Conventional biological models are 2D cultures of adherent cells where the icO₂ probe can be detected on standard widefield (LED-based) or laser-scanning confocal microscopes. By nature, phosphorescent O₂ probes usually emit in the red and near-infrared range (600–800 nm), and are excitable with UV (380–405 nm)—intense bands—or visible light (500–640 nm) (see table 1). Shorter excitation wavelengths (<450 nm) can be destructive to the cell, and the 550–640 nm range is preferred. On the other hand, probes with near infrared emission (>700 nm) require specialized red-sensitive detectors while most of conventional confocal scanners normally operate at wavelength <700 nm [67].

For larger and physiologically more relevant 3D tissue models, such as cells grown in porous scaffolds, multi-cellular aggregates (spheroids) or explants of live animal tissue, typical dimensions/thicknesses range from 50–100 μm to about 1 mm [65, 68]. Here, one-photon confocal microscopy providing penetration depths in tissue of up to 200–300 μm can access only surface parts of the sample. For better quality and deeper imaging, two-photon excitation microscopy should be used which provides a penetration depth up to 700–1000 μm. Current small molecule O₂ probes, although staining 3D tissue models well [25, 69], are inefficient under two-photon excitation [25], while NP probes containing two-photon antennae (such as polyfluorene in MM2 probe) have low staining depths [17, 34]. New two-photon phosphors [70] and improved NP probes [34] can aid to tackle this problem.

The preferred and more accurate mode for O₂ quantification by means of icO₂ probes is phosphorescence lifetime-based imaging, which provides stable, one-off calibration. However, it requires special hardware and software to support time-gated or time-correlated single photon counting (TCSPC) detection, which is often absent on standard live cell imaging systems. One the other hand, in live cell imaging FLIM is quickly gaining popularity, a number of systems supporting PLIM mode, e.g. based on TCSPC [40], are now available commercially. Pt-porphyrin based icO₂ probes having phosphorescence lifetime values 20–70 μs and convenient spectral characteristics (table 1) measured in PLIM mode are the most efficient in terms of their overall performance of O₂ imaging. Eu-based probes are also attractive and due to shorter lifetimes (few μs) they can provide faster image acquisition rates [71]. However their weak quenching can result in poor sensing performance at low O₂.

Ratiometric-intensity-based detection is a more accessible method for O₂ imaging, as it can be implemented on standard live fluorescence imaging platforms [5, 16]. This mode requires the presence of an additional oxygen-insensitive reference signal in the probe - usually a fluorescent dye of comparable brightness and spectrally distinct (e.g. blue or green). It is anticipated that signal ratio for the probe depends only on the O₂ concentration, but in reality it is also affected by background signals (higher autofluorescence/ blanks at shorter wavelengths) and attenuation of each signal by the tissue (changes with depth). So far, only a few studies describe the quantitative ratiometric-intensity imaging of icO₂ in complex biological specimens and compare it with PLIM [34, 35, 47].

Intensity measurements at a single wavelength is the last and least preferred option for icO₂ imaging. In this case, signals produced by the probe are influenced by many parameters including geometrical alignment, excitation intensity, photobleaching, image acquisition settings. In this mode, it is rather difficult (but not impossible) to obtain stable O₂ calibration and perform accurate quantification of O₂. At the same time, this simple mode is useful for tracing the relative changes in O₂ concentration and oxygenation state of respiring samples during physiological experiments and pharmacological treatments [29, 72, 73].

To foster wider use of tissue O₂ imaging, several ‘multi-modal’ probes have been developed, including MM2 and PA2 (tables 1 and 2) [17, 34]. They combine in one NP reference and two-photon antenna
(polychloro) and O₂-sensitive reporter (PtTFPP), which makes them compatible with all the above listed imaging modalities. Supramolecular O₂ probes with FRET-antenna were also described, but evaluated only briefly [23, 74].

2.5. O₂ calibration and quantification

Apart from the above considerations, the calibration of an icO₂ probe is another critical issue. The ease of this procedure is determined by the features of the probe, read-out mode (ratiometric intensity or lifetime) and possible matrix effects originating from the sample being imaged. Instrumental settings are also very important and can often lead to measurement artefacts. Ideally, the probe itself should provide stable and reproducible calibration, unaffected by cell loading and time and adequate for the experimental conditions used. Calibration experiments are normally performed at the stage of evaluation and optimization of the imaging method, prior to applying it to complex physiological studies.

On the other hand, a number of factors such as dye leaching (dye doped NPs), sensitivity to pH, ionic strength effects, protein content or probe internalization, can influence probe calibration (table 2) [17, 27, 33], but these are rarely examined. Solution and in-cell calibrations can differ significantly, especially for small molecules but also for shielded dendrimeric structures [20–22, 75]. NP such as NanO2 and PA2 show low dependence from the environment since the reporter of probe lifetime and O₂ calibration on the concentration was reported for some probes [33], which is probably due to self-quenching. Such effects can seriously affect the precision of O₂ measurement.

To avoid this, the calibration conditions should resemble as close as possible the experimental conditions used in physiological studies. In-situ calibration using cells loaded with the probe and treated to block their respiration and avoid the formation of local O₂ gradients (e.g. with antimycin A) are preferred. Such samples are then measured at several different O₂ levels (atmospheric or dissolved set in the incubation chamber), and the resulting average signals (phorescence lifetime, intensity or ratio) are processed to work out the calibration function.

Similar to solid-state O₂ sensors [5, 8], many icO₂ probes display a non-linear Stern–Volmer relationship (table 1), due to the complex and heterogeneous environment of indicator dye. In this case, mathematical or physical models (e.g. the two-site model [76]) should be used for fitting calibration data points. Different types of icO₂ probes based on Pt-porphyrin dyes usually have a similar sensitivity to O₂ and can operate over the whole physiological range (0–200 µM or 0–21%) [7]. The range of lifetime changes 2–5 fold (\(\tau_o/\tau_{21%}\)) close to optimal. Ratiometric intensity response can vary, but is usually kept around 1.5–3 fold (\(R_{0%}/R_{21%}\)) (table 1).

3. Practical applications: biological models and physiological studies

The high application potential of icO₂ probes has been demonstrated in a number of physiological studies with different 2D and 3D cell models (table 3). Tissue hypoxia and its effects on cell physiology, metabolism, bioenergetics and signalling are the main areas of research. Even in routine studies, cultured cells must be maintained under controlled and physiologically relevant O₂ levels (usually below 5% or 50 µM O₂), unlike the widely used ambient conditions (21% O₂) which in fact are hyperoxic. It has been demonstrated that extracellular, local intracellular and pericellular O₂ levels can be very different in cultures of actively respiring cells, even 2D monolayers [50, 77], and if cell oxygenation is not precisely controlled, different experiments can produce artifactual results due to the adaptive responses of cells to hypoxia. Thus, adaptation of neural-like PC12 and SH-SY5Y cells to hypoxia (3% O₂) drastically changed their balance of energy production pathways and activity of HIF-2α signalling pathway [48]. Many drugs and pharmacological agents can affect cell respiration and thus modulate cell oxygenation or tissue hypoxia [78–80]. Also the therapeutic action of drugs (metabolic effectors, differentiation agents), which are influenced by hypoxia, can be misinterpreted [81, 82]. icO₂ probes can help to devise and elaborate such effects.

Sub-cellular O₂ gradients, particularly near the mitochondria, were also attempted to measure with icO₂ probes, but studies conducted so far are not conclusive and require further verification (table 3). On the other hand, pronounced O₂ gradients in live tissue do exist, both laterally and in-depth, and can be mapped by the imaging method in 2D and 3D and over time [65]. In such studies, icO₂ probes are advantageous over extracellular, as they can be applied locally (rather than systemically) and used over long periods of time after once-off application (table 3).

Several NP and small molecule based icO₂ probes were used with cultured tumour spheroids and neurospheres [25, 34, 47]. A standard method of imaging neurosphere oxygenation and assessment of drug action and toxicity was described [47], with examples of different staining conditions and multi-parametric imaging with other fluorescent probes, both live cell and immunostaining of fixed tissue. Thus, 3D analysis of spheroid oxygenation reports on its heterogeneity, viability or functional properties of different cells within. The information provided by O₂ imaging can be used to optimize culturing conditions (atmospheric hypoxia, perfusion) and design better and more relevant physiological tissue models [47]. On the other hand, PEBBLE probe and liposomal reagents with brain tissue [83] and Oxyphor G2 probe (designed for vasculature imaging) with tumour spheroid model [69] were seen to be problematic for O₂ imaging in 3D cell models.
Cultured organotypic tissue slices are also widely used in physiology and imaging studies. Here, sample thickness, heterogeneity and presence of multiple different cell types, their different metabolic and proliferation states (quiescent or actively dividing or respiring cells which form hypoxic niches), contribution of extracellular matrix, complex toxicity effects, gentle nature and relatively short lifetime in culture are the main challenges which make their analysis difficult. Dedicated icO2 probes for such samples are highly needed.

Direct comparison the phosphorescence lifetime-based O2 imaging method with conventional hypoxia stains (antibody-based and redox probes) proved its superiority and indirect and semi-quantitative nature of these alternative methods [25, 87]. Tissue O2 imaging becomes a powerful tool when used in combination with other probes for pH, Ca2+ and ROS and reactive nitrogen species (RNS). Development of advanced FLIM-PLIM platforms for high-resolution imaging of live cell and tissue samples is anticipated [40, 90], which will allow more detailed studies of intra- and extracellular...

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ROS, reactive oxygen species; GFP, green fluorescent protein; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form.
O₂ gradients, viability and other important parameters determining the physiology of normal and diseased tissue in vivo [34].

4. Conclusions and future trends

Having analysed the available icO₂ probes, significant differences in their performance characteristics and applicability with different tissue models can be seen. This knowledge can be used for rational selection of the most appropriate icO₂ probe for particular cell/tissue model or physiological study, and for designing new high-performance probes.

The main selection criteria are cell/tissue staining efficiency, toxicity, cell specificity, intracellular distribution, in-depth penetration into tissue, spectral characteristics, compatibility with advanced imaging modalities, such as PLIM under two-photon or near infrared excitation and emission, and capabilities of multiplexing with conventional fluorophores.

In the coming years, we anticipate a broad adoption of icO₂ probes by researchers in different disciplines, and their active use in complex physiological studies with primary cells, 3D cell and tissue models, and in vivo applications. Large application niche and limitations of the existing probe structures will also drive development of novel probe structures with improved brightness, sub-cellular targeting and general usability [91].

O₂ imaging will also benefit from rapid development of imaging techniques and optoelectronic components, including multiplexed FLIM-PLIM, super-resolution imaging systems, light sheet microscopy and in vivo imaging [92–95]. We also believe that many emerging icO₂ probes and imaging technologies so far demonstrated as a proof-of-concept [18] will progress towards quantitative and multiplexed systems and be available for ordinary users. With regards to biological models and applications, high-resolution imaging of tissue O₂, probing of hypoxic niches and local O₂ gradients are very exciting and relatively unexplored areas for phosphorescent icO₂ probes and imaging method.

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