2013

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Planta (2013) 238:599–614
DOI 10.1007/s00425-013-1926-9

EMERGING TECHNOLOGIES

Emerging technologies for non-invasive quantification of physiological oxygen transport in plants

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Received: 1 April 2013 / Accepted: 26 June 2013 / Published online: 12 July 2013

Abstract Oxygen plays a critical role in plant metabolism, stress response/signaling, and adaptation to environmental changes (Lambers and Colmer, Plant Soil 274:7–15, 2005; Pitzschke et al., Antioxid Redox Signal 8:1757–1764, 2006; Van Breusegem et al., Plant Sci 161:405–414, 2001). Reactive oxygen species (ROS), by-products of various metabolic pathways in which oxygen is a key molecule, are produced during adaptation responses to environmental stress. While much is known about plant adaptation to stress (e.g., detoxifying enzymes, antioxidant production), the link between ROS metabolism, O_2 transport, and stress response mechanisms is unknown. Thus, non-invasive technologies for measuring O_2 are critical for understanding the link between physiological O_2 transport and ROS signaling. New non-invasive technologies allow real-time measurement of O_2 at the single cell and even organelle levels. This review briefly summarizes currently available (i.e., mainstream) technologies for measuring O_2 and then introduces emerging technologies for measuring O_2. Advanced techniques that provide the ability to non-invasively (i.e., non-destructively) measure O_2 are highlighted. In the near future, these non-invasive sensors will facilitate novel experimentation that will allow plant physiologists to ask new hypothesis-driven research questions aimed at improving our understanding of physiological O_2 transport.

Keywords Oxygen · Reactive oxygen species · Sensor · Non-invasive

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Introduction

Oxygen plays a critical role in the physiology of photosynthetic organisms, including bioenergetics, metabolism, development, and stress response. Oxygen is involved in photosynthesis, respiration, and alternative oxidase activity. The metabolic rate of spatially distinct plant cells (and therefore O_2 flux and ROS production) is known to be affected by biotic stress (e.g., herbivory; Kerchev et al. 2012) and environmental stress (e.g., salt/nutrient stress, flooding; Bailey-Serres and Voesenek 2008). During aerobic metabolism, cells produce reactive oxygen species (ROS) as a by-product. Plants also produce ROS during adaptation to stress [e.g., abscisic acid (ABA)-mediated stress responses]. If stress conditions are prolonged, ROS levels surpass the capacity of detoxifying mechanisms within the cell, resulting in oxidative damage. While stress response pathways such as ABA-mediated mechanisms have been well characterized (e.g., water stress, inhibited

shoot growth, synthesis of storage proteins in seeds), the connection between ROS production, O_2 metabolism and stress response remain unknown. In part, this is because details of O_2 and ROS transport at the interface of cell(s) and the surrounding microenvironment remains nebulous. The link between ROS metabolism, O_2 transport, and stress response is under intense investigation by a number of labs (Foreman et al. 2003; Steffens et al. 2013).

In the last few decades, developments in nanomaterials and sensor technology have driven the fabrication of real-time, non-invasive sensors. While ROS sensors continue to suffer from issues of poor selectivity and slow response time, there are a number of new techniques for measuring O_2 near living cells/tissues. The availability of these sensors is an exciting opportunity for plant physiologists to probe the functional realm of cells and tissues in ways that were not previously possible. In the following sections, current and emerging technologies used to measure O_2 are described, followed by examples of sensor applications in plant physiology or life sciences research.

Current technologies for measuring oxygen

Probes

Most previous applications of O_2 sensors in plant physiology utilized a polarographic (Clark-type) metal electrode. Clark electrodes are easy to construct and usually contain a polarizable metal such as platinum connected to Ag/AgCl reference electrode immersed in electrolyte behind a Teflon membrane. Oxygen diffuses across the Teflon membrane and is reduced to the oxygen anion; the anion is then measured at the cathode as an electrical current. The sensor consumes the oxygen during measurement. Thus, the aqueous sample must be stirred at constant speed during measurement to obtain accurate readings. Due to diffusion-limited O_2 consumption at the sensor surface, Clark electrodes are highly susceptible to signal drift during measurement.

In the early 1990s, optical O_2 sensors were developed to eliminate inherent problems with Clark electrodes including stir sensitivity and drift (Krihak and Shahriari 1996; Kuhl and Jorgensen 1992; Lee and Okura 1997). These optical probes (also known as optrodes) contain an O_2-quenched luminophore that is immobilized at the tip of a fiber optic cable. The dye is interrogated using an excitation laser, and the emission is read by a photodetector. Once excited, luminescent dyes transfer energy to O_2 (Kuhl and Jorgensen 1992; Lee and Okura 1997), a process known as fluorescence quenching. During the quenching process, no O_2 is consumed (rather, energy is transferred between excited valence electrons and O_2). Quenching is described by the Stern–Volmer equation (Carraway et al. 1991) and is measured using either intensity-based techniques or lifetime-based techniques. Intensity-based techniques monitor emission amplitude at a fixed light wavelength. The quantum yield depends on the probability of a sufficient number of valence electrons populating the excited state to produce a measureable emission signal. This "intensity-based" approach is somewhat unreliable using standard photodetectors. To increase the probability of emission, excitation energy can be increased (e.g., the power supplied to the laser). The major drawback of this approach is increased photobleaching (i.e., permanent destruction of the dye).

Current optrodes resolve this problem by instead monitoring the lifetime of the excited luminophore, which is the time valence electrons remain in the excited state before emission. Lifetime-based measurement improves sensitivity and reduces photobleaching of the immobilized dye molecules, thereby increasing the longevity of the sensor (McEvoy et al. 2003; Wolfbeis 2004). The lifetime technique uses a frequency-modulated excitation signal (commonly a sine wave). Phase shifts in emission signal are measured at a fixed wavelength using a filter and photomultiplier tube. Figure 1 illustrates the behavior of the luminescent O_2-quenched dye platinum tetrakis pentafluorophenyl porphyrin (PtTPFPP) (McLamore et al. 2010a). The maximum phase shift (~60°) during calibration in phosphate buffered solution (PBS) occurs at an O_2 concentration of zero. According to the Stern–Volmer relationship, the measured phase shift asymptotically approaches a value of ~20° as O_2 concentration increases. The relationship between O_2 and phase shift is nonlinear over the range of 0–100 % O_2. However, from 0 to 21 % O_2 and also from 21 to 32 % O_2, the relationship is highly linear (usually $R^2 > 0.999$). Thus, for both commercial and custom optrodes, two-point

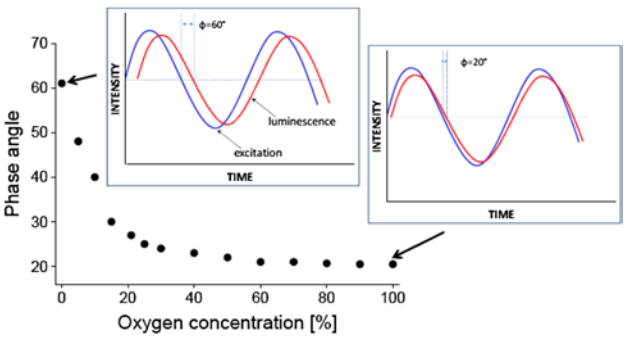

Fig. 1 Frequency-modulated excitation of an oxygen-quenched luminescent dye (platinum tetrakis pentafluorophenyl porphyrin). In this example from McLamore et al. (2010a, b, c), excitation at 470 nm is provided with an LED, and emission (luminescence) is monitored at 640 nm. Oxygen concentration is a function of phase shifts in measured excitation/emission signals (also known as phase angle, or ϕ)

calibration is carried out in deionized water (DI), nitrogen-purged DI, or O_2-saturated DI (McEvoy et al. 2003; Wolfbeis 2004; Chatni and Porterfield 2009; Chatni et al. 2009a, b; McLamore et al. 2010a).

Microprobes

Electrochemical (Clark) microsensors have been used to probe form–function relationships in various tissues and single cells (Dodds et al. 1999; Ober and Sharp 1996). These microelectrodes are commonly purchased or prepared using insulated Pt/Ir microelectrodes (Borisjuk et al. 2007; Land et al. 1999; Rolletschek et al. 2009). Disadvantages of Clark microelectrodes include stir sensitivity, drift, and "antenna noise". To alleviate these problems, optical microprobes (i.e., micro-optrodes) can be constructed based on the principles described in the previous section. A glass fiber optic cable is tapered using a CO_2 laser-based heat source to diameters between 5 and 10 μm (McLamore et al. 2010a). O_2 optrodes have many advantages over electrochemical microelectrodes, including improved sensitivity/selectivity, no O_2 consumption at sensor tip, faster response times (≈milliseconds), facile fabrication, and the ability to measure O_2 in liquid and/or air. This technology has recently been further enhanced by developing optrode systems that do not require a reference electrode (Chatni et al. 2009a). Even further, sensor performance can be improved, by addition of catalytic nanomaterials to the sensing membrane (Chatni et al. 2009b). Although these improvements have increased performance of microsensors, low signal-to-noise ratio often remains a challenge (see "Self-referencing flux sensors" for technological solutions to this problem).

Reagent-based techniques

Matsui and Tsuchiya (2006) developed a technique for measuring radial O_2 loss from roots by monitoring amperometric reduction of the oxygen-sensitive anthraquinone radical ion. This technique is an expansion of closed-chamber respiration studies using colorimetric monitoring of reducing agents such as titanium (III) citrate. Liquid media is pumped through a growth chamber and reductive current is measured using metal electrodes placed in the liquid.

Due to space limitations, an exhaustive review of the current techniques for measuring O_2 is not provided. Techniques not covered in detail here include pulse oximetry, near infrared spectroscopy, hyperspectral imaging, and F nuclear magnetic resonance imaging (FMRI). While a detailed review of these techniques is outside the scope of this manuscript, Ahmad and Kuppusamy (2010) described the use of these techniques (and others). Some of these technologies have been used for closed-chamber and open-chamber respirometry (Cloutier et al. 2009; Criddle et al. 1990; Gupta et al. 2009; Lamboursain et al. 2002; Verslues et al. 1998).

Applications of currently available technologies

Table 1 shows applications of probes and reagent-based techniques described above to study physiological O_2 transport in plants. Clark electrodes have been used in open- and closed-chamber respirometry for decades (Criddle et al. 1990; Lamboursain et al. 2002). Luminescent probes are beginning to be implemented in a number of facilities for continuous monitoring of O_2 using organometallic

Table 1 Summary of current technologies for measuring physiological oxygen transport

	Device	Organism	References
Respirometry	Anthraquinone reagent	*Typha latifolia* roots	Matsui and Tsuchiya (2006)
	Optical O_2 sensor	*Hordeum vulgare* root, *Brassica napus* seeds	Gupta et al. (2009)
	Clark electrode	*Rumex palustris* petiole; *Nicotiana tabacum* cv. *Hicks* callus and cell suspensions	Rijnders et al. (2000)
Micro sensor	Polarogaphic microelectrode	*Zea mays* primary root cells, *Eschscholzia californica;* stromatolites; *Glycine max, Eschscholzia californica, Ulothrix, cyanobacterial* (*Phormidium*)*/xanthophyte* (*Vaucheria*), *Ricinus communis*; *Ectocarpus siliculosus*, *Antithamnion plumula, Ricinus communis, Pisum sativum* L. 'Meteor' seeds*, Allium cepa* roots*, Eriophorum angustifolium, Spartina anglica*	Cloutier et al. (2009); Lamboursain et al. (2002); Verslues et al. (1998); Paterson et al. (2008); Armstrong et al. (2009); Dodds et al. (1999); Küpper et al. (2004); Shimamura et al. (2010); Shimamura et al. (2010); van Dongen et al. (2003)
	Luminescent microsensor	*Nicotiana tabacum* leaves, *Glycine max* (L.) Merr., *Pisum sativum*, *Brassica napus* seeds, *Arabidopsis thaliana*	Borisjuk and Rolletschek (2009); Borisov and Klimant (2007); Tyystjärvi et al. (1998); Vigeolas et al. (2003)

The sensors are organized based on use for either respirometry or microsensor studies of form–function relationship. Species name for application of the device is listed in the organism column

ruthenium dyes (Tyystjärvi et al. 1998), cyclometalated iridium complexes (Borisjuk and Rolletschek 2009), and platinum porphyrin dyes (Vigeolas et al. 2003) (Table 1). Most microsensor studies have measured O_2 concentration in living tissues after penetrating the surface with the sensor tip.

Clark microelectrodes have been used in the field for real-time measurement of biogeochemical profiles in photosynthetic mats (Kuhl and Jorgensen 1992; Revsbech and Jorgensen 1986). Sensors are either directly inserted into mats, or the mats are penetrated with a small drill and filled with agar prior to insertion of O_2 microsensors (Visscher et al. 1991). After 20–30 min, oxygen gradients reach steady-state equilibrium and spatial profiles are obtained using linear micro-actuators. This approach allowed researchers to determine the spatial location of key functional groups involved in biogeochemical cycling (Visscher et al. 1991; Visscher and Stolz 2005). Micro-optrodes have also been used to monitor O_2 in or near cells/tissues by impaling the specimen with a probe that has a 5–100 μm tip diameter (Table 1). Micro-optrodes were used to produce a large dataset regarding O_2 concentration in seeds (Borisjuk et al. 2007; Land et al. 1999), fruits, and roots (Rolletschek et al. 2009). This is a rapid, simple technique for characterizing physiological status of plants and photosynthetic organisms.

However, use of invasive microsensors to penetrate biological samples has many disadvantages. Use of this technique in roots, leaves, and shoots potentially causes significant experimental bias due to the limited detection range at the microsensor tip (range is limited to a sphere approximately 10–20 times the tip diameter) (Porterfield 2002). Due to this small "sample volume", collected data are potentially biased by local injury response (e.g., oxidative bursts) resulting from membrane damage. When using invasive probes, gas leakage makes it difficult to monitor O_2 over long periods of time (e.g., during diel cycling). In seeds, these invasive microsensor experiments are possible due to the O_2 barrier created by the testa, along with the relatively high metabolic demand of the developing seed (Bewley 1994; Rolletschek et al. 2009). However, great care must be taken to avoid tissue damage or fouling of the sensor tip. Mathematical methods have been developed for estimating O_2 in cells/tissues based on measurement of surface concentration, but these ad hoc estimations cannot account for temporally dynamic transport (Kochian et al. 1992). Thus, direct measurement of O_2 dynamics using non-invasive methods are preferred (these technologies are discussed in "Emerging technologies for measuring oxygen").

Matsui and Tsuchiya (2006) used the anthraquinone technique for monitoring radial oxygen loss in *Typha latifolia* L. roots and demonstrated improved performance over other reagent-based techniques. The anthraquinone method does not require the addition of phytotoxic salts and can provide rapid open-chamber estimations of root respiration. While this technique is fast, selectivity is low relative to optrodes.

Emerging technologies for measuring oxygen

Measuring the transport of O_2 using non-invasive (or minimally invasive) technologies provides an opportunity to study physiological phenomena in vivo for extended periods of time. Many of these emerging technologies provide increased sensitivity and selectivity compared to current and past technologies described in the previous section. If adopted, these emerging technologies will allow plant physiologists to carry out new experiments which were not possible a few decades ago (Fig. 2). For a comparison of various emerging technologies, see "Opportunities and challenges for emerging technologies".

Microrespirometry

Oxygen microassays permit rapid multiplexing analysis of cell suspensions (Alderman et al. 2004; Kratasyuk et al. 2001; Küpper et al. 2004; O'Riordan et al. 2000; Serrano et al. 2007). Kocincová et al. (2008) recently developed a fluorescent microassay for continuous measurement of H^+ and O_2. Excitation (X) is modulated sinusoidally with a 470 nm LED and emission (M) is continuously monitored

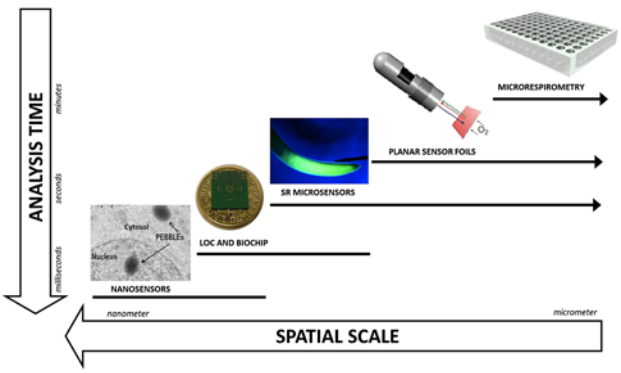

Fig. 2 Conceptual relationship between analysis time and spatial scale for O_2 sensors. *Horizontal lines* represent the approximate range of the spatial scale for each technology. *Horizontal lines with an arrow* indicate technology is applicable in the millimeter spatial scale (or higher). Microrespirometry, planar foils, and self-referencing sensors provide O_2 measurement at the micron scale with response times ranging from seconds to minutes. Nanosensors and biochip devices facilitate experimentation at the sub-micron scale with response times from milliseconds to seconds. Nanosensor image courtesy of Buck et al. (2004). Planar foil image courtesy of Tschiersch et al. (2012)

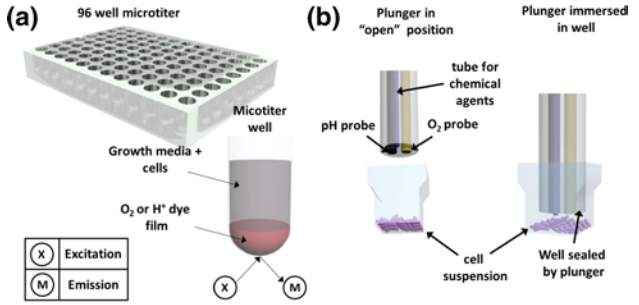

Fig. 3 **a** Schematic of microassay for simultaneous monitoring of O_2 and pH based on Kocincová et al. (2008). Excitation (X) is modulated sinusoidally with an LED and emission (M) is continuously monitored in the microtiter wells. **b** Conceptual diagram of microrespirometry assays using a plunger system to monitor changes in O_2 and H^+ in a sealed microtiter well (Brand and Nicholls 2011)

at 540 nm (Fig. 3a). Microrespirometry has recently been standardized for simultaneous O_2 and pH measurement by companies such as Seahorse Bioscience©, among others. Most commercial microrespirometry assays use a plunger system to seal the cell/tissue suspension (sample volumes are ~7 μL) while monitoring O_2 and H^+ over relatively short periods (5–10 min) (Brand and Nicholls 2011). Most instruments are capable of temperature control and autoinjection of pharmacological agents (e.g., potassium cyanide, dichlorophenyl-dimethylurea, salicyl hydroxamic acid). To date, studies have monitored O_2 concentration in microwells during inhibition/activation of respiration by addition of drugs known to affect oxidative phosphorylation.

In addition to direct monitoring of O_2, numerous microwell techniques have been developed for monitoring metabolites and small molecules associated with O_2 flux and redox homeostasis. These assays all monitor absorbance or fluorescence signals from exogenous reporter molecules. For example, Queval and Noctor (2007) developed a microtiter plate technique for monitoring antioxidants (ascorbate and glutathione) and pyridine nucleotides. For measuring ascorbate, homogenized tissues were treated with a cocktail containing ascorbate oxidase and dithiothreitol. Absence of absorbance at a wavelength of 265 nm (measured with a plate reader) was directly correlated to total ascorbate concentration. Similarly, glutathione was monitored by measuring the glutathione reductase-dependent reduction of 5-5′-dithiobis(2-nitro-benzoic acid) while monitoring absorbance at a wavelength of 425 nm. Pyridine nucleotide measurement was based on the reduction of dichlorophenolindophenol by phenazine methosulfate in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Decrease in A_{600} was correlated to NAD^+ and $NADP^+$ (acidified solution) or NADH and NADPH (basic solution) concentration.

Planar sensor foils

Tschiersch et al. (2011, 2012) described development and application of an optical device for measuring O_2 using planar sensor foils. The technique is based on ratiometric fluorescence quenching, which involves immobilization of two fluorescent dyes in a sensor foil (i.e., thin polymer film). One of the dyes is quenched by O_2, while the other is insensitive to O_2. The sensor foil is fixed to a transparent polyester support and positioned ca. 2 cm from the surface of a USB microscope. The apparatus is placed in contact with the biological sample (with a thin layer of water between the foil and the sample). Planar optrodes are used for non-invasive two-dimensional mapping of surface O_2 concentration.

Self-referencing flux sensors

McLamore et al. (2010a) developed a non-invasive technique for measuring physiological O_2 transport at the root–rhizosphere interface using a micro-optrode. This self-referencing (SR) microsensor technique converts traditional micro/nanosensors with low signal-to-noise ratio into dynamic flux sensors by filtering out signals not associated with biologically active transport. This is accomplished by oscillating a single sensor between two locations using computer-controlled stepper motors. The SR technique measures real-time changes in differential analyte concentration (ΔC) over a fixed excursion distance (ΔX) (Fig. 4).

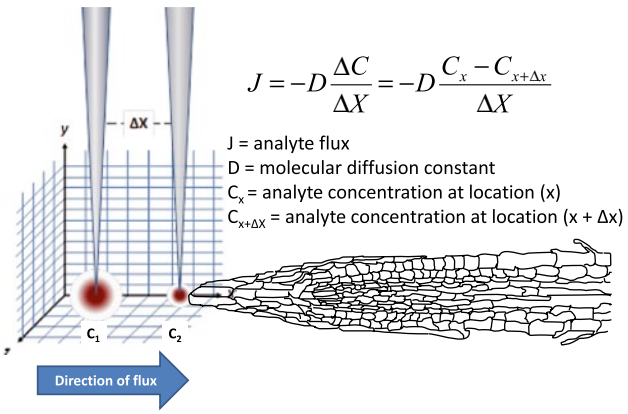

Fig. 4 Conceptual diagram showing the working mechanism for self-referencing microsensors. A single microsensor positioned within ≈1 μm of the surface of a root is oscillated in the direction of physiological O_2 transport (normal to the tangent plane of the tissue/cell surface). Differential concentration (ΔC) is measured without stirring the solution near the tissue surface. Since excursion distance (ΔX) is controlled by the linear stepper motors, flux is directly measured based on Fick's first law of diffusion. Three-dimensional stepper motors allow for surface morphology (e.g., root curvature) to be accounted for, providing real-time measurement of form–function relationships in a wide range of studies

Measurement and amplification of differential concentration signals significantly improve sensitivity, selectivity, and signal-to-noise ratio (Newman et al. 2012; Porterfield 2007; McLamore et al. 2010a, b; Shabala et al. 2012). SR micro-optrodes can be used in liquid or air for measuring O_2 flux under a wide range of experimental conditions (e.g., various lighting/heating, in presence of reagents), but cannot be used to monitor roots in soil.

Electron paramagnetic resonance (EPR) oximetry

EPR oximetry is a technique commonly used in medical applications for measuring O_2 levels in biological samples (an excellent review is given by Ahmad and Kuppusamy 2010). EPR is based on the absorption of electromagnetic radiation by unpaired electrons placed in a magnetic field. EPR oximetry is a variation of this technique that monitors energy transition states of exogenous "spin probes" near O_2. Soluble or particulate spin probes contain a paramagnetic material that changes EPR when near O_2. Spin probes are injected into the tissue where they are exposed to a magnetic field while monitoring radiofrequency energy.

EPR oximetry is a minimally invasive technique that can be used to provide three-dimensional mapping of oxygen distribution in a tissue environment for long periods of time (up to months in some cases). EPR oximetry is classified as "minimally invasive" due to the requirement of injecting spin probes that remain in the tissue during measurement.

Lab-on-chip and biochip

Lab-on-chip (LOC) and biochip technologies provide high sample throughput, remote data acquisition, multiplexing, and on-chip calibration (Ahn et al. 2004; Lee et al. 2008; Malins et al. 2000; Molter et al. 2009). Biochips are a subset of LOC that contain biological molecule(s) as an active component of the LOC. State of the art devices incorporate catalytic nanomaterials such as carbon nanotubes and graphene to improve sensitivity and response time (Claussen et al. 2009, 2011, 2012; ul Haque et al. 2007). LOC technologies typically employ microfluidics and microwell arrays to achieve improvements in sensitivity and selectivity. These devices can be mass produced in large volumes using rapid prototyping.

Figure 5 shows a conceptual design of a LOC device for measuring O_2 near individual cells/organelles. The substrate material for LOC can be fabricated from silicon, fused silica, plastic, or borosilicate glass. State-of-the-art devices for cell culture are capable of on-board cell culture (Kim et al. 2010). These devices typically include temperature control, analysis chambers/wells, and microfluidic channels to facilitate delivery/capture of growth media, cells/organelles, and test reagents. Precise control of

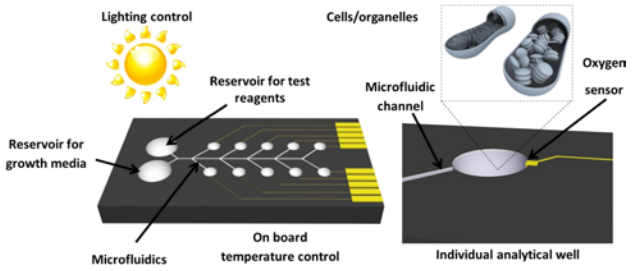

Fig. 5 Conceptual O_2 lab-on-chip device with on-board cell culture and temperature control. Microfluidic channels facilitate delivery/capture of growth media, cells/organelles, and test reagents. Analytical wells contain O_2 sensors in wells for measuring real-time O_2 changes

the microfluidic system utilizing micropumps and microvalves allows capture of a single isolated cell/organelle, or multiple cells based on the needs of the user. Optical or electrochemical sensors are fabricated within wells (up to 1,000 wells can be fabricated on a single 8 mm × 11 mm chip). For optical sensors in wells, a membrane containing an O_2-sensitive dye is commonly immobilized and interrogated with a microscope or camera fitted with optical filters. For electrochemical sensing, working and reference microelectrodes are fabricated on the device using photolithography, metal sputtering and etching. Current LOC developments are working toward disposable devices with potential field applications (Ahn et al. 2004).

Nanosensors

The accurate detection of cytosolic O_2 in single cells is now possible using sensors small enough to cross the cell wall through aquaporins or via endocytosis. These nanoscale sensors (i.e., sensors with at least one dimension smaller than 100 nm) have been developed in the last decade based on optical properties of noble metals at the nanometer scale (Xu et al. 2005). Examples include fluorescent indicator dyes immobilized on nanocomposites (Sud et al. 2005), quantum dots (Collier et al. 2011), magnetic nano-optrodes (Kuhl and Polerecky 2008), and biologically localized embedded nanoparticles (Buck et al. 2004).

For example, quantum dots (QDs) are semiconducting nanostructures (also called semiconductor nanocrystals) that are often employed as cellular probes. QDs are composed of nanoscale crystalline core structures in periodic groups II–VI (e.g., CdSe) or III–V (e.g., InP). QDs have many advantages over organic fluorescent probes, including reduced photobleaching, tunable (size-specific) emission wavelengths with distinct emission spectra, extreme emission brightness, and high spatial resolution (Alivisatos 2004; Chan et al. 2002). Due to the dependence of emission wavelength on QD size, multiple QDs may be imaged

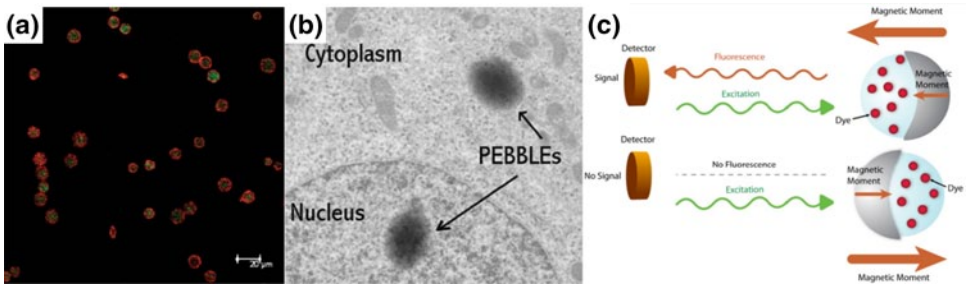

Fig. 6 Ratiometric quantum dots for measuring intracellular O_2 concentration. **a** In these confocal images, *green* represents emission of an O_2-quenched luminophore, while *red* represents emission by QDs (reference signal) (image courtesy of Collier et al. 2011). **b** Biologically localized embedded nanoparticles (PEBBLEs) for monitoring O_2 in organelles (image courtesy of Buck et al. 2004). **c** Magnetic-modulated optical nanoprobes (MagMOONs) for monitoring intracellular O_2 and thermal flux (image courtesy of Anker and Kopelman 2003)

simultaneously, providing accurate multiplexing capability that far exceeds traditional dye-based technology. The device by Collier et al. (2011) used multiple QDs for measuring intracellular O_2 concentration (see Fig. 5). This approach compares the emission from a QD coated with an O_2-sensitive dye ($\lambda \approx 650$ nm) to the emission from a reference QD (emission is in the near IR range; $\lambda \approx 800$ nm). At room temperature, the O_2-sensitive dye showed phosphorescence when excited in the Soret band (395 nm) or one of the two Q bands (508 or 541 nm) with very little spectral overlap on the reference QD (Fig. 6a).

Fercher et al. (2011) developed nanosensors by synthesizing nanoparticles using a cationic polymer (Eudragit RL-100) conjugated to an O_2-sensitive dye (PtPFPP). The nanosensors were capable of detecting physiological O_2 in a wide variety of media, including air-saturated phosphate buffer with various pH values (range 6.0–8.0) and ionic strength (50–300 mM). For intracellular monitoring, the net positive charge of the nanoparticles induces electrostatic attraction with negatively charged cell membranes, facilitating uptake across phospholipid bilayers. O_2 was measured in subcellular compartments of mammalian cells with high photostability and negligible cytotoxicity (described in detail in the Nanosensor applications section).

Applications of emerging technologies

Some of the emerging technologies listed here are being used in biomedical research applications, but have not yet been adopted for studies of plants or photosynthetic organisms. Translation of these technologies for monitoring O_2 in plant studies is now technologically feasible using currently available commercial equipment. In many cases, only small modifications are required for these tools to be available to plant physiologists (e.g., inclusion of a lighting system).

Microrespirometry applications

A variety of microrespirometry techniques have been applied in plant physiology research (Alderman et al. 2004; Kratasyuk et al. 2001; O'Riordan et al. 2000; Serrano et al. 2007). For example, Küpper et al. (2004) used luminescent probes to monitor O_2 near algae in a microwell. In addition to direct monitoring of O_2, microtiter plate assays such as the device by Queval and Noctor (2007) have been used to monitor small molecules involved in metabolism and redox homeostasis (pyridine nucleotides, glutathione and ascorbate). The device was used to monitor redox state in homogenized *Arabidopsis* leaves. Results correlated well with standard spectrometer and liquid chromatography analysis. While microassays can facilitate multi-analyte measurement, this technique has some disadvantages. Samples must be sealed in small chambers, often producing results that do not translate to in situ studies in the field. To extract meaningful data, many microassay techniques require external addition of reagents such as inhibitors or activators of photophosphorylation. With respect to assays requiring chemical pretreatment (Queval and Noctor 2007), these assays can be tedious and time consuming. In addition, most protocols require invasive/destructive sample preparation. Commercial plate readers have thermal control, but lighting control is a challenge for instruments that seal the individual microwells during measurement.

Planar sensor foils

Tschiersch et al. (2011, 2012) used planar optrodes for mapping millimeter resolution O_2 patterns in a variety of samples, including seedling roots of oilseed rape (*Brassica napus)*, *Cabomba caroliniana* leaves, pea seed/caryopsis (*Pisum sativum*), barley (*Hordeum vulgare*), potato stems (*Solanum tuberosum*) maize stems (*Zea mays*). O_2 spatial patterns associated with leaves from sycamore tree

(*Platanus occidentalis*), wandering Jew (*Tradescantia fluminensis*), hibiscus (*Hibiscus rosa-sinensis*) and maple tree (*Acer platanoides*) were monitored in the presence and absence of infection by powdery mildew (*Uncinula tulasnei*). This non-invasive technique provides a real-time estimate of respiration/photosynthesis in tissues or organs as determined by the size of the sensor foil (and adapter tubus). The approach is valuable for root growth in hydroponic solutions and monitoring shoots/leaves, but cannot be used for monitoring O_2 in soils (i.e., beneath the surface directly in contact with the foil).

Self-referencing microsensor applications

McLamore and Porterfield (2011) reviewed the use of non-invasive SR microsensors for measuring flux of molecules at the single cell and tissue level. SR polarographic microelectrodes have been used for studying O_2 flux in pollen tubes (Xu et al. 2006), protists such as *Spirogyra grevilleana* (Land et al. 1999; Porterfield et al. 1999; Porterfield and Smith 2000), olive leaves/roots (Mancuso et al. 2000), grape roots (Mancuso and Boselli 2002), and corn roots (Porterfield 2002; Shabala et al. 2006a). Oscillations in transmembrane O_2 flux have also been studied using this technique (Mancuso et al. 2000; McLamore et al. 2010a, b; Porterfield 2002; Shabala et al. 2006a). These ultradian oscillations are driven by ATP/ADP turnover and the dynamic timing of membrane pumps and channels. The oscillatory transport mechanism is critical for plant response(s) to changes in local environmental conditions, insect herbivory, and other stressors (Shabala et al. 2006b).

SR micro-optrodes were recently used to map two-dimensional O_2 flux along the surface of *Glycine max*, *Zea mays*, and *Phaseolus vulgaris* roots (McLamore et al. 2010b). In addition to studying O_2 transport due to oxidative phosphorylation and photosynthesis, the reversible data allow for studying dynamic systems such as cytochrome c electron flow and alternative oxidase electron shuttling in functioning roots of *G. max*, *P. vulgaris*, and *Z. mays* (McLamore et al. 2010a). Numerous reviews effectively highlight concerted efforts of various groups using SR microsensors to study pollen tube physiology (Holdaway-Clarke and Hepler 2003), ion transporters in roots (Newman 2001), and oscillatory transport at the root–rhizosphere interface (Shabala et al. 2006a). Wan et al (2011) provide a protocol for using SR micro-optrodes to study plant systems.

EPR oximetry applications

EPR oximetry has been used extensively for measuring O_2 concentration in single cells, small tissues, and organs in the medical field. These studies primarily focused on tumor physiology, cardiac tissues, wound healing, and basic organ studies (including liver, brain, kidney, and muscle). To date, there are a limited number of published EPR oximetry demonstrations for measuring O_2 in photosynthetic organisms. Ligeza et al. (1994) demonstrated the use of EPR to study O_2 concentration within bean leaves. A soluble cholestan spin probe was combined with isolated chloroplasts and injected into bean leaves (species not reported in manuscript). Yi et al. (2007) monitored O_2 evolution from isolated *Arabidopsis* thylakoid membranes using EPR oximetry.

Although there are only a few demonstrations of EPR oximetry in plant studies, a number of papers have shown the use of EPR for monitoring oxygen radicals near plant cells. The lifetime of O_2 radicals is in the order of 5–10 ns, preventing the direct use of EPR. Thus, spin-trap techniques are used to measure O_2 radicals with standard EPR. For example, Mojovic et al. (2005) demonstrated the use of EPR with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). DMPO was used to measure two oxygen radical species ($\cdot O_2^-$ and $\cdot$OH) produced by isolated plasma membranes from *Z. mays* L. (Mojovic). The use of various inhibitors together with DMPO was used to show that two independent sets of mechanisms are responsible for production of ($\cdot O_2^-$ and $\cdot$OH). In a similar study, Liszkay et al. (2003) used a different spin trap, ethanol/α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), for tracking $\cdot$OH in *Zea mays* (L.) coleoptiles and sunflower (*Helianthus annuus* L.) hypocotyls.

To date, most applications of EPR measured O_2 radicals near the surface of plant cells/tissues, but did not report measurements of O_2 concentration. This limitation is likely due to the inability of most spin probes to penetrate the plant cell wall, or artifacts caused by diffusion of O_2. For the latter, Presley et al. (2006) developed a protocol for correcting O_2 diffusion artifacts common to EPR oximetry. The technique allows users to differentiate from signals associated with O_2 consumption by cells in hypoxic, normoxic, or supersaturated conditions.

Lab-on-chip (LOC) applications

To date, most LOC applications have been used in medical research. For example, a glass LOC designed by Molter et al. (2009) used immobilized O_2 dyes in wells for measurement of O_2 uptake rate by isolated cells. The device is capable of measuring O_2 consumption rates with fmol min^{-1} resolution. Molter demonstrated the device using murine macrophage cells, human epithelial lung cancer cells, and human esophagus cells with on-chip temperature control. The transparent glass LOC could easily be integrated with a lighting system for analysis of plant cells.

Lee et al. (2008) developed fluorescent embedded microarrays for non-invasive O_2 sensing. This system monitors the cell culture environment in multiple culture wells at the same time without intervention. Malins et al. (2000) developed a dual-analyte LOC device based on the surface patterning of fluorescent dyes. LOC cell culture systems built upon previous microtiter plate platform (recall Fig. 3) by providing multiplexing capability and real-time monitoring of local O_2 at the single cell level (Molter et al. 2009; Oppegard et al. 2009). These devices are currently being used to develop 3D cell culture technologies (Huh et al. 2011).

Masi et al. (2009) developed a multielectrode array with 60 individual sensors for measuring bioelectric current at the surface of *Z. mays* (L.) root apex cells. While the device was not used to selectively measure O_2, the sensors could be easily modified to detect O_2 by coating with Teflon and platinum black as described by Porterfield (2007).

A recent thrust in LOC fabrication research has been to create inexpensive and easily manufactured devices, which is anticipated to expand the applications of LOC to widespread agricultural and environmental applications. Do et al. (2008) and Ahn et al. (2004) demonstrated the use of a disposable diagnostic LOC device for the measurement of O_2, glucose, and lactate in wells filled with human blood. This plastic LOC has a passive microfluidic manipulation system based on structurally programmable microfluidic technology. The optical device was able to detect gaseous and dissolved O_2 in cell cultures for flow rates ranging from 0.5 to 2 mL min^{-1}. The device uses on-chip pressurized air reservoirs as a source for fluid flow, which eliminated the need for complex microfluidic pumps.

A cellulose membrane microfluidic platform was developed by Hu et al. (2012) for electrochemical sensing of O_2. The low cost, paper-based electrochemical device was composed of ink-jet printed gold electrodes with ionic liquid (IL) as an electrolyte. Addition of IL and ferrocene to the backside of the cellulose membrane significantly improved sensitivity and response time. The planar electrode arrays are a promising inexpensive platform for detection of O_2 in a LOC platform and can be integrated with other sensors for multiplexing, if desired. The biocompatibility, low volatility of liquid solutions, and stability of gold nanoparticles make this device highly suitable for applications in plant cell system.

Nanosensor applications

Fercher et al. (2011) developed nanosensors using uncharged derivatives of Pt(II)-coproporphyrin I (PtCP), covalently linked to positively charged peptides. These probes were used to monitor subcellular O_2 localization in mammalian cells (i.e., PC12, HCT116, HepG2, HeLa, and SH-SY5Y cells). The study also monitored characteristic O_2 responses to stimulation with mitochondrial uncouplers and inhibitors. MitoXpress, a proprietary phosphorescent Pt–coproporphyrin dye conjugated to bovine serum albumin, is a commercial version of this intracellular O_2 nanosensor that has been used in a variety of mammalian cells (e.g., Jurkat, PC12, A549, HeLa, SH-SY5Y, and C2C12) (O'Riordan et al. 2002). MitoXpress can be monitored using standard fluorescent plate readers or a time-resolved fluorescent (TR-F) plate reader. MitoXpress is limited by relatively low cell-specific loading, and the need for transfection reagents (e.g., Endo-Porter) to activate endocytosis (Dmitriev et al. 2010; O'Riordan et al. 2007).

In a similar study, intracellular O_2 was directly monitored by tracking indicator dyes conjugated with QD (Cywinski et al. 2009). If not conjugated to an O_2 dye, QDs have a relatively low selectivity in living cells. Collier et al. (2011) improved sensitivity of QD by developing a ratiometric approach using two dyes (as previously described for the device by Tschiersch). Although this O_2 nanosensor has not yet been used in living cells, a number of improvements have been made in the last few years (Collier and McShane 2012).

Although new QD designs have excellent sensitivity, rapid response, and a low photobleaching rate, cytotoxicity has been a major deterrent against mainstream use (discussed in detail by Hardman (2006), Hoshino et al. (2011), and Tsoi et al. (2013), among others). To overcome the toxicity problem with QD, Buck et al. (2004) described the use of QD coated with biocompatible materials. These nanosensors are known as "probes encapsulated by biologically localized embedding" (or PEBBLEs) (Fig. 6b). PEBBLEs are nanoscale (20–600 nm in diameter) biocompatible probes that allow minimally intrusive sensing in subcellular environments. A fluorescent dye is immobilized on the surface of the QD, and the probe is functionalized with an optically transparent inert biocompatible matrix. PEBBLEs are then inserted into cells via gene gun delivery, pico-injection, liposomal delivery, or sequestration/endocytosis.

Anker and Kopelman (2003) expanded on the concept of PEBBLEs by developing magnetically "modulated optical nanoprobes" or MOONs. These minimally invasive nanosensors are designed for simultaneously monitoring of intracellular chemical and physical processes at the same time. MOONS are nanospheres that have a magnetic metal deposited on one half of the sphere via chemical vapor deposition. These magnetic nanospheres are then functionalized with an O_2-sensitive dye and delivered to intact cells using similar techniques as PEBBLEs. MOONs used to monitor chemical transport dynamics are called Mag-MOONs, while nanosensors functionalized with only magnetic thin films and no dye are called Brownian MOONs.

The Brownian MOONs monitor stochastic thermal motion at the cellular/subcellular level. Once oriented with an external magnetic field, the MOONS blink at a fixed frequency based on manipulation of the local magnetic field, where background fluorescence "shimmers" with no consistent pattern. This makes detection of emission by Mag MOONs and/or Brownian MOONs very simple to extract using commercial image acquisition systems. Using this frequency-modulated approach, MOONs increase signal-to-noise ratio by orders of magnitude over other nanosensors (Anker and Kopelman 2003).

Buck et al. (2004) compared the use of PEBBLEs to microsensor techniques and traditional confocal staining in neuroblastoma cells and found that the selectivity and sensitivity of the PEBBLEs was comparable. The response time and absolute detection limit for PEBBLEs and MOONs were significantly better than standard confocal staining. Anker and Kopelman (2003) used MagMOONs and Brownian MOONs in ivy epidermal leaves; MOONs also have been used to study endosome in macrophage (Behrend et al. 2004).

A final example for monitoring O_2 at the single cell level combines fluorescent nanosensor beads with a LOC device for 3D cell culture (Wang et al. 2013). The beads were composed of a silica core loaded with both an O_2-sensitive ruthenium dye and an O_2-insensitive reference dye (Nile blue); the entire surface was protected by a polydimethyl siloxane (PDMS) outer shell. The beads were immobilized within a collagen cell culture system on the chip, and O_2 was measured in wells during growth of human intestinal epithelium Caco-2 cells.

Few studies to date have directly used nanosensors to study O_2 transport at the cellular/subcellular level in plants. However, the core technology has been used to study cell expansion and stress response in plants (Kim et al. 2010; Kader and Lindberg 2010). In the next few decades, it is expected that many of these technologies will be used by laboratories working in plant biology. Table 2 below summarizes some emerging technologies for O_2 measurement.

Opportunities and challenges for emerging technologies

Some of the advantages and disadvantages for the emerging technologies reviewed above are discussed in this section (see Table 3). Commercial microrespirometry devices such as the device made by SeaHorse Bioscience© have provided a burst of excitement in cancer research. High throughput screening of mitochondrial activity in various cell lines currently allows researchers to connect genotype with phenotype through pharmacological studies (Brand and Nicholls 2011). The response time of the device for a single measurement (individual well) is in the order of 1–60 s, depending on the specific type of assay. The average lower limit of detection (LOD) for O_2 is approximately 1–5 μM. The major drawbacks of the device are that the chamber must be sealed during measurement. Mitochondrial activity may be affected by the temporal decrease in the partial pressure of O_2, which can induce hypoxia and bias data. Over a decade of instrument fine tuning and detailed studies on hysteric effects in mammalian cells, yeast and bacteria have reduced these problems significantly. A remaining drawback of this instrument is that analysis depends on addition of pharmacological agents.

Planar O_2 sensor foils allow the rate of O_2 production and/or consumption to be visualized in specific plant tissues at the sub-micrometer to millimeter scale. The technology is easy to use, and commercially available O_2-quenched dyes (such as platinum porphyrin) are robust and highly sensitive. Tschiersch et al. (2012) noted that although the technique is quantitative, the measured O_2 maps may not reflect the steady-state in vivo concentration. Rather, the two-dimensional maps are used to infer patterns of relatively high and low O_2 dynamics in living tissues. In addition, since the technique is confined to the surface of the sample, transport to/from underlying regions cannot be accounted for. Although no lower LOD was reported in Tschiersch et al. (2011, 2012), the ratiometric dyes used to fabricate the sensor foil have demonstrated detection of O_2 as low as 0.5 μM in other devices (Collier et al. 2011). Caution should be used when using sensor foils to conduct experiments for investigating hypoxia, as the lower LOD depends directly on the resolution of the USB camera used to acquire the fluorescent signal.

SR microsensors allow the measurement of both the magnitude and direction of O_2 transport in the unstirred layer. This direct measurement of physiological O_2 flux is critical for monitoring tissues such as leaves, where mitochondrial and chloroplast activity can occur in the same region of interest. SR has a response time (≈1 s) and a LOD of 1–2 μM, which allows rapid screening of physiological O_2 flux under hypoxic conditions, if desired. The technique is limited to the microscale domain, as the thickness of the boundary layer near most cells is larger than 3 μm (most boundary layers are 50–1,000 μm thick) (McLamore and Porterfield 2011). Another disadvantage is the requirement of specialty hardware/software for high precision analysis (e.g., vibration table, stepper motors, acquisition system with pre-programmed algorithms). One advantage of SR microsensors is the ability to combine the technique with other techniques (e.g., microscopy, nanosensors) for simultaneous measurement of boundary layer flux and intracellular activity in spatially distinct zones. This experimental approach represents an exciting opportunity to study transmembrane protein activity.

Table 2 Summary of emerging technologies for measuring physiological oxygen transport

	Device/approach	Organism	References
Microrespirometry	Optical microassay	*Schizosaccharomyces pombe*, *Spirulina platensis*, *Arabidopsis thaliana* seedlings	Kratasyuk et al. (2001); O'Riordan et al. (2000); Serrano et al. (2007)
	Multi-analyte microrespirometry (O_2 and H^+)	*Filamentous/thallous* algae, *Pseudomonas putida*, β cells, Hematopoietic stem cell, mouse podocyte, isolated mitochondria	Abe et al. (2010); Alderman et al. (2004); Brand and Nicholls (2011); Gurumurthy et al. (2010); Kocincová et al. (2008); Küpper et al. (2004)
Self-referencing microsensors	SR optrode/fluorescent optrode	*Europaea, Z. mays* roots, *Glycine max*, and *Phaseolus vulgaris*, mixed culture *phytoplankton* mats, *N. europaea* and *P. aeruginosa* biofilms, cancer tumor spheroids	McLamore et al. (2010a, b, c); McLamore and Porterfield (2011)
	SR microelectrode (Clark Style)	*Vitis rupestris ripera* and *vinifera Spirogyra grevilleana*, *Olea Europaea* leaves and roots), *monocytogenes, L. innocua, E. coli, L. lactis*, *L. bulgaricus, Lilium longiflorum* pollen tube, *Lycopersicon esculentum Mill* roots, *Pimephales promelas* eggs	Chatni and Porterfield (2009); Mancuso and Boselli (2002); Mancuso et al. (2000); Newman (2001); Porterfield (2002, 2007); Porterfield and Smith (2000); Sanchez et al. (2008); Shabala et al. (2006a); Shi et al. (2007); Xu et al. (2006)
EPR Oximetry	5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin probe	Isolated *Z. mays* (L.) plasma membranes	Mojovic et al. (2005)
	Isolated chloroplast mixed with cholestan spin probes	Bean leaf vascular O_2^a	Ligeza et al. (1994)
	Soluble spin probe	Isolated *Arabidopsis* thylakoid membranes	Yi et al. (2007)
	Ethanol/α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN) spin probe	*Zea mays* (L.) coleoptiles, *Helianthus annuus* (L.) hypocotyls	Liszkay et al. (2003)
Lab-on-Chip	60 multielectrode array (MEA)	*Zea mays* L. root apex cells	Masi et al. (2009)
	Microwell plate	Macrophage, epithelial lung cancer cell, human esophagus cell	Molter et al. (2009)
Nanosensor	Dye-conjugated nanosensors	Mammalian cells (e.g., *PC12*, *HCT116*, *HepG2*, *HeLa*, *SH-SY5Y*).	Dmitriev et al. (2010); O'Riordan et al. (2007); Fercher et al. (2011)
	QD	*Chlamydomonas* spp.	Cywinski et al. (2009); McLamore et al. (2010b) Lin et al. (2009); Collier et al. (2011)
	PEBBLE	Mouse oocyte, macrophage	Buck et al. (2004)
	MOONs	*Hedera rhombea* leaf, macrophage	Anker and Kopelman (2003); Behrend et al. (2004)
	QD + LOC	Human intestinal epithelial Caco-2 cells	Wang et al. (2013)
	Planar optrodes and nano-optrodes	*Dendrogyra cylindricus* tissues and photosynthetic mats	Dmitriev et al. (2010); Fercher et al. (2011); Kuhl and Polerecky (2008); O'Riordan et al. (2002)

Sensors are organized by use as either microrespirometry, self-referencing microsensors, EPR oximetry, lab-on-chip, or nanosensors. Species name for application of the device is listed in the organism column

[a] Species not reported

The advantages of EPR oximetry are that it is minimally invasive, and can accurately monitor O_2 concentration in a tissue environment. The technique is extremely rapid (response times are commonly in the nanosecond to microsecond range) and the LOD (1–5 μM) allows for use in a broad range of conditions. Spin probes can remain in tissues for up to months without losing oxygen sensitivity. The major disadvantages of EPR oximetry include heating of aqueous samples during measurement, low signal-to-noise ratio, low penetration depth, requirement of exogenous molecular probes, rapid bioreduction of probes, and motion artifacts (Ahmad and Kuppusamy 2010). Use of

Table 3 Summary of advantages and disadvantages for techniques covered in this review

Technique	Advantages	Disadvantages	LOD (μM)	Response time (s)	SNR
Clark electrodes	Simple to construct, wide acceptance, long shelf life	Consumes oxygen, stir sensitive, subject to signal drift, slow response time (diffusion limited)	≈1 to 5	≈1 to 60[a]	Med
Optrodes	Simple to construct, highly selective, can be used in air or water	Fragile glass core, relatively expensive, relatively expensive supporting hardware	≈0.7 to 1.4	≈1 to 3	High
Clark microelectrodes	Simple to construct, wide acceptance, long shelf life, inexpensive supporting hardware	Consumes oxygen, stir sensitive, subject to signal drift, slow response time (diffusion limited), low SNR, long response time[a]	≈1 to 2	1 to 25[a]	Low
Micro-optrodes	Simple to construct, highly selective, can be used in air or water	Fragile glass core, relatively expensive supporting hardware	≈0.5 to 1.2	≈1 to 2	High
Microrespirometry	High throughput, multiplexing capability	Sealed microwells can induce bias, exogenous reagents required, invasive/destructive sampling protocol, low SNR	≈2 to 5	≈7 to 10	Low
Planar sensor foils	Rapid, 2D mapping, facile sensor fabrication, high selectivity	Surface technique, may not directly translate to in vivo O_2 concentrations, performance limited by resolution of camera	NA[b]	≈20	Low
Self-referencing microsensors	Non-invasive, can monitor efflux or influx in real time, can be combined with other techniques (e.g., microscopy, LOC), high SNR	In vitro technique, limited to microscale domain, requires specialty hardware/software	≈1 to 2	≈1	High
EPR oximetry	Minimally invasive, reliable for months in a living tissue	In vitro technique, exogenous spin probes required, spin probes may not penetrate plant cell wall, low SNR	≈1.0 to 5.0[c]	$\approx 4 \times 10^{-8}$ [d]	Low
Lab-on-chip	High throughput, multiplexing possible, easily integrated with other hardware	Complex fabrication, limited to sub-millimeter domain, high cost, low SNR	≈0.5 to 1.0	≈1	Low
Quantum dots	Superior spatial resolution, can be conjugated with markers (e.g., antibodies) for improving localization, strong fluorescent signal	Toxicity after prolonged exposure, fate in tissues is unknown, difficult to penetrate plant cell wall	≈0.1 to 0.3	≈0.5 to 1.0	Med

If available, basic performance parameters are provided, including limit of detection (LOD), response time (t_{95}), and signal-to-noise ratio (SNR). LOD and response time values were calculated for at least four similar technologies from the published literature. For SNR, a qualitative ranking (low, medium, high) was assigned based on survey of at least 12 similar technologies in patents and published literature

[a] Value depends on stirring speed (i.e., local convection)

[b] Value not reported

[c] LOD and sensitivity varies widely among instruments

[d] Response time varies widely and can be up to 1 h

EPR oximetry for studying plant cells is particularly challenging, as there are few soluble or particulate EPR spin probes that are capable of crossing the plant cell wall.

The market and availability of LOC and biochip technologies is rapidly expanding. Integration of chip-based sensors with microfluidics allows researchers to design experiments suited to investigate a central hypothesis that might not be possible with commercial, "one size fits all" technologies. Biochips with integrated sensors are available for purchase from a number of companies, and microfluidic channels can be created using commercially available polydimethyl sulfoxide kits. LOC and biochips are capable of near real time (≈1 s response time) data collection under a wide range of conditions (as low as 0.5 μM O_2). The cost of biochip technologies is decreasing, and techniques such as mask-less photolithography, ink-jet printing, and screen-printing are allowing research labs without clean room access to develop microfluidic channels with feature sizes down to 15–20 μm. Use of disposable and biodegradable substrates further reduces device cost and prototyping time. A major advantage of LOC technology is the ease of integration with data-acquisition hardware, allowing

autonomous, compact systems to be developed. This allows LOC system to be utilized in field work, and other challenging environments such as outer space (ul Haque et al. 2007). The major disadvantages of LOC are the specialty equipment required for fabrication, relatively high cost, and limitation to the sub-millimeter domain.

Nanosensors are an exciting new technology with excellent sensitivity and spatial resolution far superior to other devices. However, many of these devices may require further investigation before the sensors are ready for mainstream use. Nanosensors are more biocompatible than direct use of indicator dyes, and some nanosensors can even be extracted from intact cells without inducing damage to cellular or subcellular structures. The advantage of this new technology is the ability to rapidly monitor O_2 in subcellular domains with remarkable spatial/temporal resolution; average response time and LOD are 0.5 s and 0.1 μM, respectively. To be used in cells, nanosensors must be surface functionalized for conjugation with proteins, organelles, or membranes. Uptake of nanoparticles across the cellulosic plant cell wall remains a considerable challenge, but developments in advanced functional materials are expected to make penetration across the plant cell wall possible in the coming years. The biggest opportunity is to couple high fidelity sensing with use of biocompatible materials that avoid cytotoxicity or activate adaptive/defense responses in cells. A major roadblock for use of metal-based nanosensors such as QD is the inherent toxicity of the Cd core after prolonged exposure. Lin et al. (2009) tested whether QDs would induce any toxicity by observing O_2 absorption and CO_2 depletion in single-celled green algae (*Chlamydomonas* sp.). In particular, this study focused on yellow fluorescent CdSe/ZnS QD. QD concentrations above 5 ppm decreased the rate of O_2 production to nearly zero, while CO_2 depletion was significantly reduced above 100 ppm of QD dosage. Thus, use of yellow fluorescent QD concentrations above 5 ppm should be avoided due to reduced metabolic activity. In general, these results are not deterrent to the use of QD, since the highly sensitive nanosensors can detect O_2 using QD concentrations as low as 0.5 ppm.

The cytotoxicity of QD and their subsequent effect on plant health has been investigated in a few plants, including tomatoes (Alimohammadi et al. 2011), but the mode of toxicity and fate of these particles remains elusive. Ongoing studies of the environmental and biological impacts of engineered nanoparticles are expected to provide insight into the interaction between nanoparticles and plants (Schmälzlin et al. 2005; Etxeberria et al. 2006; Hannah and Thompson 2008; Liu et al. 2009; Navarro et al. 2008; Zhu et al. 2008). In the mean time, development of new biocompatible materials and techniques for advanced sensor conjugation allow short-term use of nanosensors for probing living cells and tissues (Eggenberger et al. 2010; Martin-Ortigosa et al. 2012).

Exploratory biophysical models and bioinformatics are allowing us to improve our understanding of the processes used by photosynthetic cells for survival (Hardin 2000). However, we have only scratched the surface in terms of our understanding of the complex spatial and temporal dynamics of these networks involving O_2 and other small molecules (e.g., antioxidants, signaling molecules, redox mediators). In large part, this is due to a lack of tools for monitoring small molecule transport at the cellular and subcellular scale. While high fidelity ROS sensors are still under development, emerging technologies such as LOC devices and nanosensors are available for non-invasive monitoring of O_2 transport in real time. In the coming years, these tools will allow plant physiologists to conduct hypothesis-driven research that was not possible a decade ago.

Acknowledgments The authors also acknowledge the UF Excellence Award and the IFAS Early Career Award (CRIS No. 005062) for funding (McLamore). A special thanks to PIKL (Baltimore, MD) for help with graphic images.

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