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Optical Detection of Intracellular Quantities Using Nanoscale Technologies

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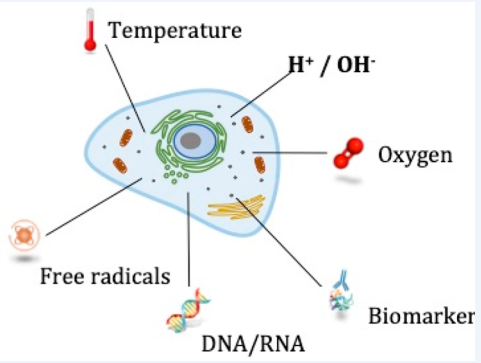
CONSPECTUS: Optical probes that can be used to measure certain quantities with subcellular resolution give us access to a new level of information at which physics, chemistry, life sciences, and medicine become strongly intertwined. The emergence of these new technologies is owed to great advances in the physical sciences. However, evaluating and improving these methods to new standards requires a joint effort with life sciences and clinical practice. In this Account, we give an overview of the probes that have been developed for measuring a few highly relevant parameters at the subcellular scale: temperature, pH, oxygen, free radicals, inorganic ions, genetic material, and biomarkers.



Luminescent probes are available in many varieties, which can be used for measuring temperature, pH, and oxygen. Since they are influenced by virtually any metabolic process in the healthy or diseased cell, these quantities are extremely useful to understand intracellular processes. Probes for them can roughly be divided into molecular dyes with a parameter dependent fluorescence or phosphorescence and nanoparticle platforms. Nanoparticle probes can provide enhanced photostability, measurement quality, and potential for multiple functionalities. Embedding into coatings can improve biocompatibility or prevent nonspecific interactions between the probe and the cellular environment. These qualities need to be matched however with good uptake properties, colloidal properties and eventually intracellular targeting to optimize their practical applicability. Inorganic ions constitute a broad class of compounds or elements, some of which play specific roles in signaling, while others are toxic. Their detection is often difficult due to the cross-talk with similar ions, as well as other parameters.

The detection of free radicals, DNA, and biomarkers at extremely low levels has significant potential for biomedical applications. Their presence is linked more directly to physiological and clinical manifestations. Since existing methods for free radical detection are generally poor in sensitivity and spatiotemporal resolution, new reliable methods that are generally applicable can contribute greatly to advancing this topic in biology. Optical methods that detect DNA or RNA and protein biomarkers exist for intracellular applications, but are mostly relevant for the development of rapid point-of-care sample testing.

To elucidate the inner workings of cells, focused multidisciplinary research is required to define the validity and limitations of a nanoparticle probe, in both physical and biological terms. Multifunctional platforms and those that are easily made compatible with conventional research equipment have an edge over other techniques in growing the body of research evidencing their versatility.

INTRODUCTION

In the last two decades, nanoscale technologies have given us access to information from biological systems with unprecedented resolution, specificity, and sensitivity. Engineered nanomaterials have provided many different ways in which physical and chemical parameters on the (sub)cellular scale can be transduced into an observable signal. Optimizing the relevance and utility of this wealth of information requires bridging the gaps between the physical sciences and life sciences, all the way to clinical practice. This Account provides an overview of some quantities that can be probed by nanoscale technologies on a subcellular level. We placed the emphasis on optical probes that can be used in cells and their (future) relevance in biology. These include temperature, pH, inorganic ions, levels of oxygen, free radicals, genetic material, and protein biomarkers.

Energy released as heat is associated with a manifold of biophysical and biochemical processes, which in turn are regulated by temperature. Similarly, finely controlled pH and oxygen levels are essential for cell homeostasis. Deviations from their equilibrium values can indicate various physiological

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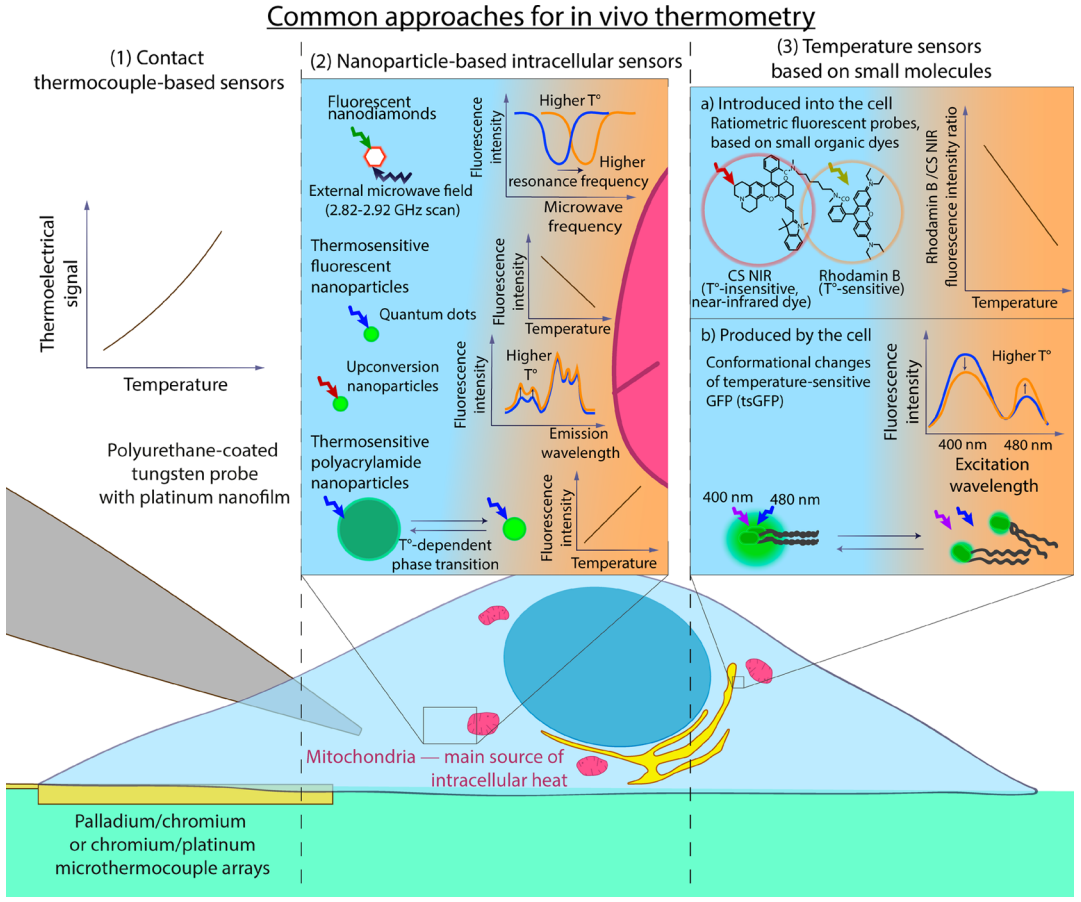

Figure 1. Approaches for intracellular thermometry in live cells. (1) Contact thermoelectrical sensors,[12,13] being the gold standard, usually lack spatial resolution and are invasive. (2) Approaches based on nanoparticles (such as FNDs[21] or quantum dots) allow for relatively noninvasive intracellular imaging. (3) Small thermosensing molecules can be internalized by the cells[17] or encoded in the cell DNA.[20]

and pathological processes. For instance, mechanisms of cancer and inflammation are linked to increased heat production[1] and acidic extracellular pH.[2] Complex mechanisms to maintain temperature, levels of acidity, and oxygen have developed over the course of evolution. Examples of such mechanisms under investigation include the role of brown fat in warm blooded animals and the effects of acidosis on disease onset and progression. To understand these processes, techniques for precise and reliable measurements of temperature, pH, and oxygen levels are in great demand for fundamental biomedical research.

Intracellular nanoscale probes can also contribute greatly to elucidating the roles of many inorganic ions in biology. Alkali and alkaline earth metals are common in most organisms and their functions include the regulation of osmotic values and membrane potentials. On the other hand, Cu^{2+}, Co^{2+}, and Mn^{2+} fulfill their role as cofactors in various processes in much lower quantities. Certain cations, such as mercury or lead, can be toxic and are undesirable in biological systems. Among anions, fluoride, chloride, and phosphate can be considered as ions with major biological relevance for which fluorescent probes have been reported. Detecting these ions at their relevant concentrations and locations makes for an interesting challenge, especially when it comes to achieving sensitivity and avoiding cross-talk with other quantities.[3]

Compounds that can be measured as an objective indicator for specific physiological or pathological processes are called biomarkers. They are mainly used for two purposes: (early) detection of diseases and monitoring of disease progression in response to therapy. Free radicals are molecules that contain at least one unpaired electron in an atomic orbital, which renders the molecule highly reactive and paramagnetic. They are associated with cell aging and degenerative diseases but also with physiological intracellular signaling and the neutralization of pathogens.[4] Deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), our genetic material, encode protein synthesis. Visualizing genetic material can be crucial for the diagnosis of genetic disorders or modifications[5] or detecting cancer[6] or the presence of certain viruses[7] or bacteria.[8] Methods that allow accurate localization and quantification of these compounds as well as known biomarkers can make significant impact in clinical practice.

■ NANOSENSOR PROPERTIES

The ideal probe for intracellular applications has to fulfill several requirements. It should be possible to read it out with high spatiotemporal resolution. It should be biocompatible and remain undisturbed by changes in parameters other than the quantity of interest.[9] Probes that transduce environmental changes into an optical signal are currently the most promising tools to meet these requirements. They have the advantage of spatial resolutions down to the diffraction limit and may be internalized noninvasively. Their spectral wavelengths, intensities, or luminescence lifetimes can depend on continuous changes in the physical environment (e.g., temperature or pH)

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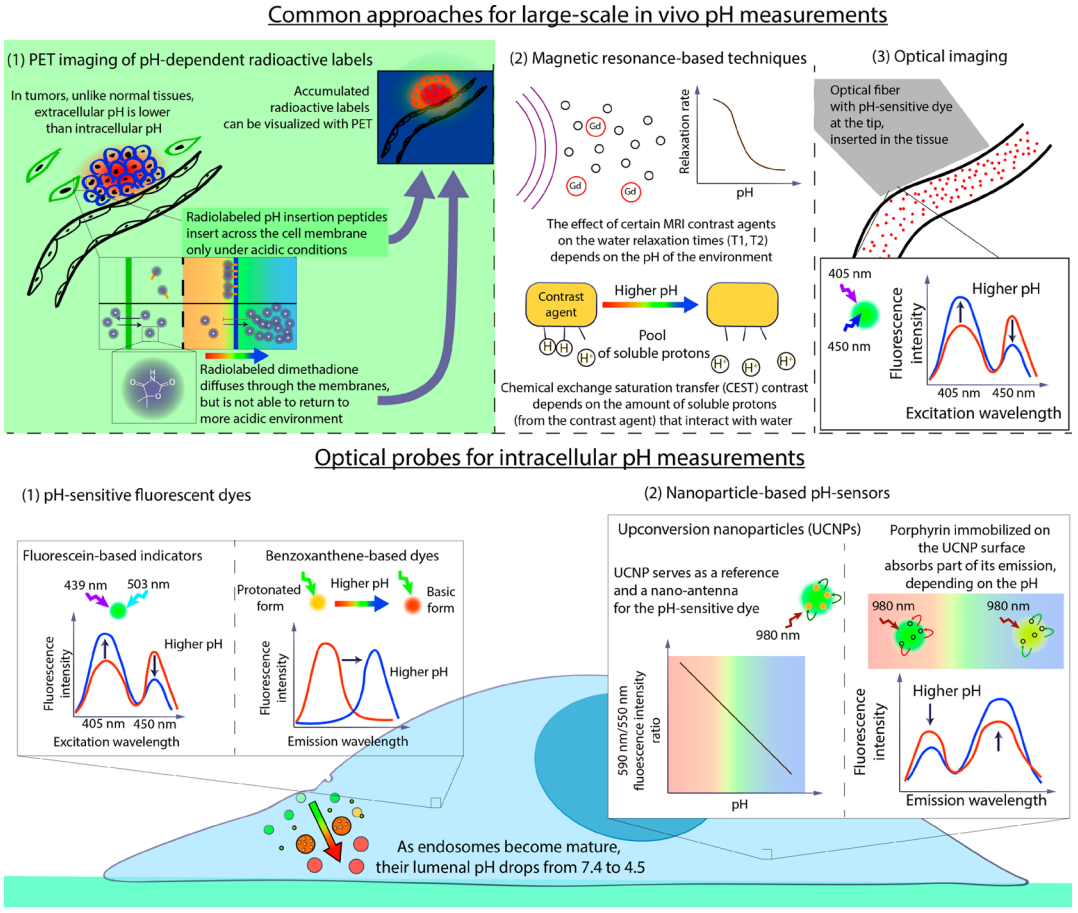

Figure 2. (top) Examples of macroscopic methods for pH measurement in cells.[2] (bottom) Intracellular luminescent pH probes.[26−28]

or on the occurrence of specific molecular events such as binding of compounds.

Advanced challenges for good nanoscale optical probes in biology are emission and excitation in the transparent biological window (in the near-infrared), specific organelle targeting, and internal calibration.

MEASUREMENT PRINCIPLES

In general, an optical probe for different quantities consists of a unit that changes its optical properties based on the environment. These optical properties have to be read out via one of several options. The most common is to measure fluorescence. The advantage is that rather high sensitivities can be achieved. Alternatively one can also measure color changes or absorption. This is simpler but usually also less sensitive. These readout techniques provide diffraction limited spatial resolution, but optical probes may also be designed to be compatible with super resolution techniques, resulting in a sub-diffraction-limit resolution.[10] Measuring changes in fluorescence lifetime offers an additional possibility to create contrast. Quantification is usually done by counting photons or comparing light intensities or a shift in wavelength.

PROBE UPTAKE

A crucial prerequisite for any measurements making use of intracellular probes is their uptake into cells. This varies dramatically depending on the cell type. Some cell types (for example, macrophages) readily ingest all kinds of particles that are provided since this is their purpose in biology. While many cells are almost as unrestrictive when it comes to particle uptake, others barely ingest any particles at all. There is an immense body of literature present from the gene transfection and the drug delivery field about how to bring particles inside cells. Also the field of optical labeling provides a large set of methods that can be applied for responsive probes too. For more information on this topic, we would like to refer to more specialized reviews.[11] As a rule of thumb smaller particles are taken up more easily than large ones. Due to the electronegative cell surface, electropositive particles are also preferred. Hydrophobic molecules also tend to enter more easily. But these are not universal rules.

TEMPERATURE

Local thermometry is a tool to improve fundamental understanding in cell biology. In addition, the technique becomes increasingly relevant to accompany new treatment modalities that apply local heating of pathological (cancerous) tissue.[12] Thermocouples provide a gold standard for thermometry in many applications and have also been developed on a sub-micrometer scale for subcellular measurements.[13,14] Compared to fluorescent probes, however, this approach is often invasive and limited in its spatial resolution. Extensive reviews on the different approaches for cellular thermometry have been published by Okabe et al.,[15] Wang et al.[16] and Bai and Gu,[9] and an overview of some reported methods is presented in Figure 1.

A ratiometric thermosensor consisting of two dyes, rhodamine B and CS NIR, was reported by Homma et al.[17] This

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thermosensor was targeted to mitochondria and, with only the rhodamine B part being temperature sensitive, provided ratiometric calibration in situ. Probes based on polyacrylamides, such as poly(*N*-isopropylacrylamide) (NIPAM), provide high sensitivity around their phase transitions, in which they undergo a structural change that leads to sharp increases in fluorescence intensity.[18] These probes could be made to penetrate the cell walls of yeast and enter mammalian cells and diffuse homogeneously through the cytosol.[19] Genetically encoded fluorescent proteins do not require internalization as they are endogenously expressed in specific organelles. A temperature sensitive probe based on the commonly used green fluorescent protein (tsGFP) was reported by Kiyonaka et al.[20] They achieved temperature sensitivity by introducing TlpA protein. This is a protein that can undergo conformational changes depending on the temperature.

Inorganic nanoparticles that have been used for biological nanoscale thermometry include quantum dots, upconversion nanoparticles, and fluorescent nanodiamonds (FNDs).[21] Their foremost advantage is their photostability, allowing for measurements over extended periods of time. The sensing capabilities of FNDs are linked to the magnetic states of the fluorescent nitrogen-vacancy (NV) center.[22] The electronic ground state of this atomic scale defect has distinct magnetic levels that can be brought into resonance with microwave radiation and induce a drop in the fluorescence. The frequency of this transition is sensitive to temperature changes and can thus be used for thermal sensing. What makes the NV center especially interesting is the fact that different readout modalities of its fluorescence are coupled to distinct physical parameters that can be relevant to biology, such as magnetic fields and spin fluctuations.[22] Due to their biocompatibility and high potential as a multimodal biological nanoprobe, FNDs have attracted much attention in research in recent years and will also be revisited throughout this Account.

pH

In contrast to temperature, pH levels may change in a more discontinuous fashion across membranes. Therefore, a relevant distinction between intracellular (pH_i) and extracellular (pH_e) can be made using targeted probes without sub-micrometer spatial resolutions. Changes in systemic pH levels can reflect altered pulmonary and renal functions[23] among others. At the cellular level, pH dependent processes include endocytosis, ion transport, and response to therapies.[24] For pH measurements in cells, magnetic resonance and optical probes[2,25] are mostly employed. On the subcellular scale, a wide variety of fluorescent nanoparticles and probes are available (see Figure 2). Recommended in-depth reviews on this topic are available by Schäferling,[24] Wencel et al.[26] and Han and Burgess.[27]

The most interesting probes fulfill a set of key properties: self-referencing (meaning that they contain an additional dye that is unperturbed by the pH, which can function as reference), high brightness, and good colloidal stability and uptake properties. Additionally, it is important that the probe can be read out with high accuracy in the weakly acidic range. Commonly used dyes for these purposes are 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), seminaphthofluoresceins (SNAFLs), and seminaphthorhodafluors (SNARFs). The former of these dyes has an absorption peak that shifts to longer wavelengths at a more alkaline pH. SNARFs and SNAFLs also show a shift in emission maxima between their protonated and deprotonated forms. Using the ratio of the excitation or emission of these dyes at their respective maxima in basic and acidic form gives a measure that is referenced internally.[27]

A notable class of optical probes are lanthanide doped upconversion nanoparticles (UCNPs). In the upconversion process, low energy (NIR) photons are used for excitation, while higher energy photons are emitted. As a consequence, the signal is free of autofluorescence. Measurement of pH has been demonstrated with resonant energy transfer from $NaYF_4:Yb^{3+},Er^{3+}$ crystals to pH dependent pHrodo Red[28] and polyglutamic porphyrin dendrimers.[29] Emission bands of $NaYF_4:Yb^{3+},Er^{3+}$ UCNPs have also been used for thermometry,[30] suggesting the potential for the development of multipurpose platforms. Another interesting alternative is pH sensitive fluorescent proteins. Such proteins have been made by Tantama et al.,[31] for instance. The authors were able to engineer red fluorescent protein into living neuro2A cells. These proteins then followed pH changes between 5 and 8 and were also able to measure different glucose levels, which result in a pH change.

INORGANIC IONS

Inorganic ions are indispensable in cell physiology. While colorimetric approaches are still used for ion detection,[32] most techniques for intracellular optical ion sensing are based on fluorescence. We will focus on the nanoparticle-based methods for cation detection. Application of NP-based sensors for inorganic anions has been limited, although probes for fluoride,[33] chloride,[34] hypochlorite,[35] and phosphate[36] have been reported.

An excellent review on the general types of fluorescent probes for transition metals has been published by Carter, Young, and Palmer five years ago.[37] An overview of nanoparticle-based fluorescent probes for intracellular imaging of metal ions has been published by Zhang et al.[38] Nanoparticles were originally employed as inert carriers, protecting the dye from the intracellular environment and vice versa.[39,40] They can also be used to improve the fluorescent properties of the fluorophore, for instance, with near-field fluorescence, when the fluorophore is affected by the local electromagnetic field of the metal NP.[41] Certain designs include selective ionophores, coloaded into the NPs with a pH-sensitive dye.[42] The cations of interest substitute protons within probes, and the pH change is reported by the dye.

Some classes of NPs can act as fluorescent reporters themselves. They usually carry molecules on their surface that selectively bind or react with specific ions, quenching[43] or enhancing[33,44] the signal. Some nanoparticles aggregate in the presence of certain ions, which results in altered fluorescence intensity.[45]

An elegant approach to metal ion sensing is based on DNAzymes, DNA molecules capable of performing enzymatic reactions in the presence of metal cofactors. DNAzymes can be fine-tuned to be extremely specific and selective for certain cations. The most common design features fluorescently labeled nucleotide sequences, bound to the inactive DNAzymes on the NP surface. The fluorescent signal is quenched by the NP or an additional quencher. Binding of the metal ion activates the DNAzyme, which cleaves the labeled sequence, releasing the fluorophore. Each NP can carry several different types of DNAzymes associated with different fluorophores, allowing simultaneous detection of multiple

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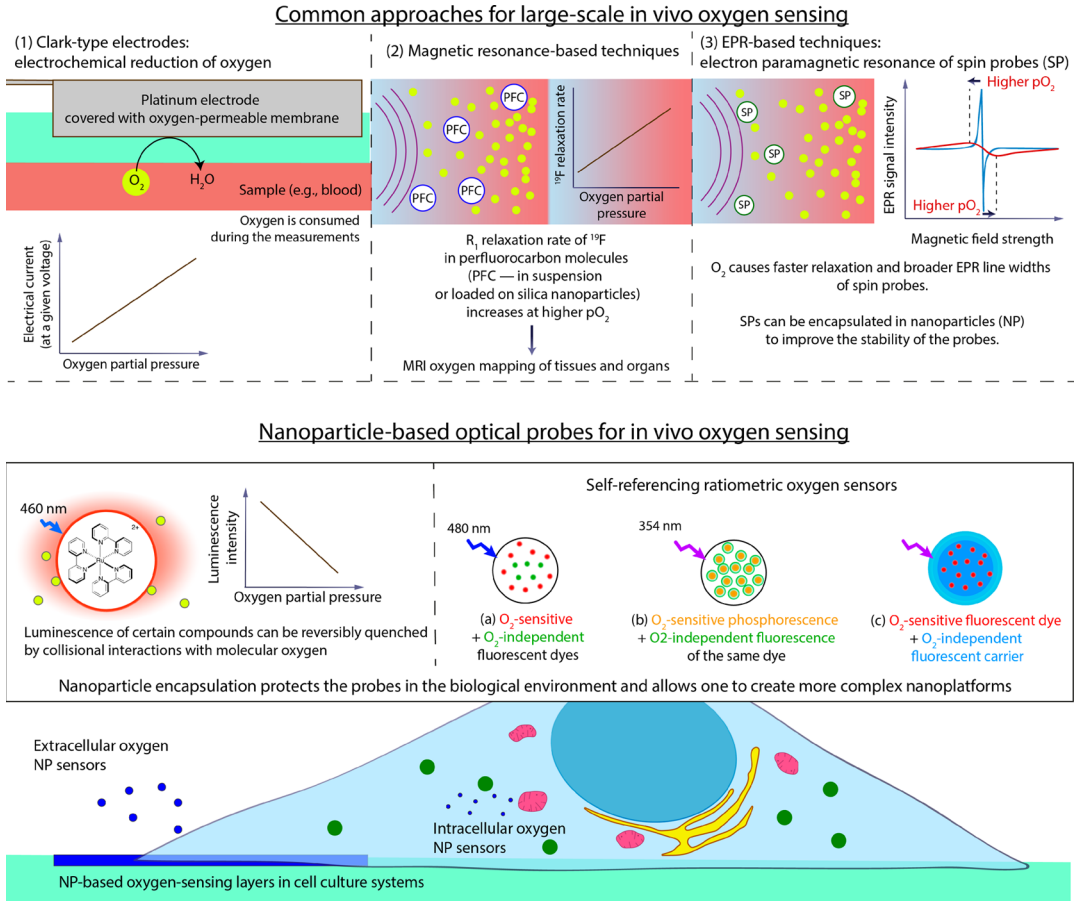

Figure 3. Examples of commonly used methods for macroscopic measurement of oxygen levels (upper) and nanoparticle-based luminescent probes (lower) for oxygen detection on the cellular level.[54−61]

different ions.[46] The drawback of this system is irreversible modification of the sensor. An additional complication is possible binding of extracellular ions by the DNAzymes, which can lead to premature cleavage of fluorescent labels. This problem has been addressed by photochemically caged probes, in which the DNAzyme only becomes active after irradiation with light.[47]

Another approach for optical ion sensing is based on highly specific and sensitive SERS probes. They do not suffer from photobleaching and can be illuminated with NIR light, which enhances the tissue penetration depth and decreases the phototoxicity.[48] With a proper SERS reporter, the same platform can be used for simultaneous detection of multiple ions of interest.[49] However, only a limited number of SERS-based platforms for ion detection in biological samples has been reported in the literature.

The field of nanoparticle-based optical sensors for inorganic ions still has potential for growth. Important problems of particle-based intracellular imaging of ions are highlighted in a review by Kantner et al.[3] It is necessary to take into account the potential effects of NPs on conformation of the sensing components.[50] Another drawback of many optical NP-based probes is the need for short-wavelength (350−405 nm) irradiation, which is scattered by biological samples and can be harmful for the cells.

OXYGEN

The approaches most often used for measuring oxygen levels for biomedical applications are amperometry, magnetic resonance imaging (MRI), electron paramagnetic resonance (EPR)-based approaches, and optical sensing (see Figure 3).

A recommended review on the topic of optical oxygen sensing has been published by Papkovsky and Dmitriev.[51] As phosphorescence of certain luminophores can be quenched by molecular oxygen, these compounds can be used for optical oxygen sensing. Reading out such probes is fully reversible, they do not consume oxygen and readout is fast.[52] At the same time, many of them are hydrophobic, which decreases their solubility in biological media and impedes the cellular uptake. These compounds can also be cytotoxic and may be degraded by cells. To solve these problems, the dye can be incorporated in nanoparticles.[53] Ratiometric measurements can be performed with oxygen-insensitive dyes loaded to the same nanoparticle.[53] There are two options to do this: nanoparticles exhibiting both oxygen-independent fluorescence and oxygen-sensitive phosphorescence[54] or intrinsically fluorescent nanocarriers, serving as internal reference.[55−57] Various designs of nanoparticle-based optical oxygen probes can be tailored for specific biomedical applications. They have been used to create oxygen-sensing layers in cell culture systems,[58,59] as free extracellular detectors present in the medium[60] or in 3D cell cultures[55,61] for intracellular measurements.[62] Apart from the proof-of-concept studies, nanoparticle-based oxygen-sensing systems have already been used to produce new biologically

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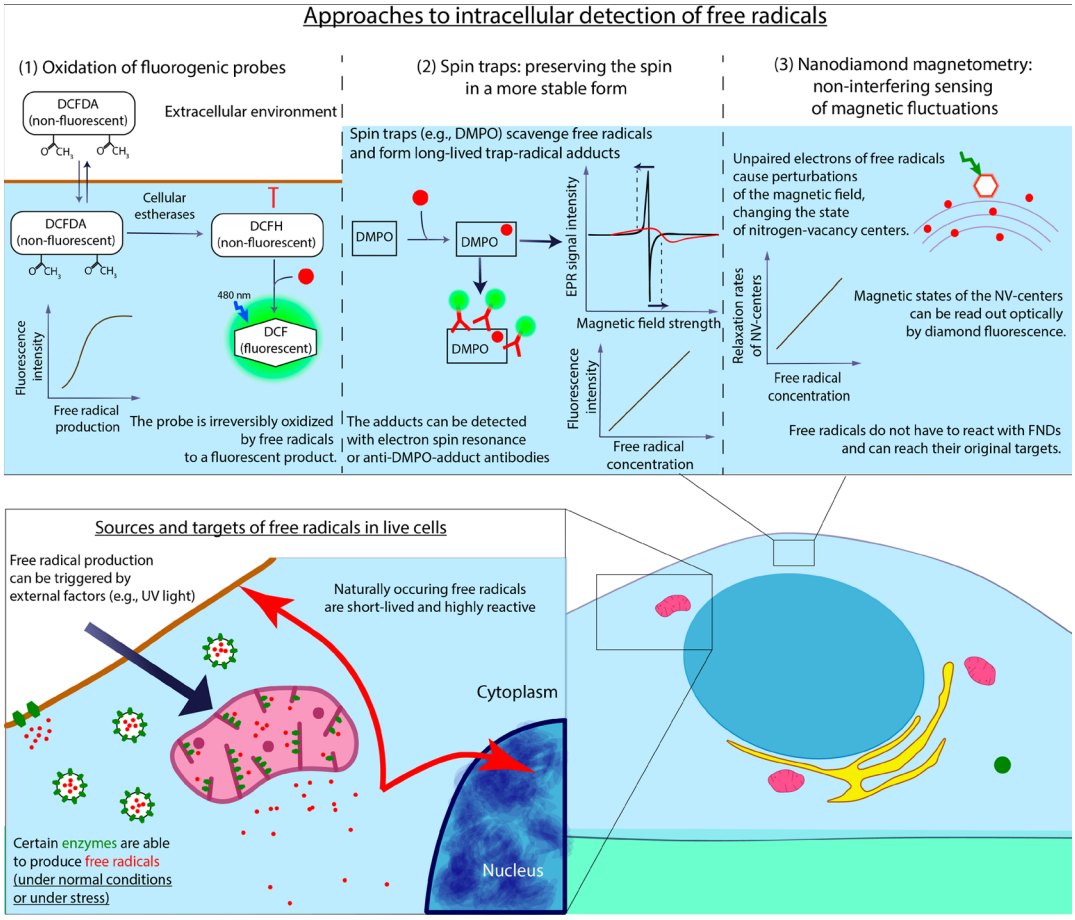

Figure 4. (top) Working mechanisms of free radical detection, based on fluorogenic dyes (e.g., DCFDA), DMPO spin traps, and a proposed method of diamond magnetometry. (bottom) External and internal factors leading to free radical production.

relevant data. Examples are concentrations and distribution of intracellular oxygen during the apoptotic events[62] or real-time imaging of oxygen consumption by neural cells, responding to a sensory stimulus.[63]

FREE RADICALS

Free radicals are produced during natural metabolism but can also be generated in the body by external sources. They are involved in intracellular signaling and pathogen neutralization; however, when the natural balance is disturbed, they also are associated with degenerative diseases and aging.

Due to their low baseline concentrations and short lifetimes, sensitive methods to detect free radicals in their biological environment are required. Possible methods are shown in Figure 4. The challenge lies in trapping the radicals in a reaction that is specific and yields a stable product for detection.

Electron spin resonance (ESR) is the gold standard for detecting free radicals, by which unpaired electrons can be detected directly. A major drawback however is its low sensitivity.

Using fluorescence based probes is another option for detecting free radicals. So far, the dye 2,7-dichlorofluorescin diacetate (DCFDA) is the most common probe. DCFDA can easily pass cell membranes, after which it is deacetylated by cellular esterase. The deacetylation process forms a non-fluorescent compound, $DCFH_2$, which is oxidized by reactive oxygen species (ROS) into 2,7-dichlorofluorescein (DCF). Several improvement points of DCFDA are its chemical stability and susceptibility to photobleaching.[64] Additionally, it is questionable whether the oxidation reaction is induced solely by cellular ROS, making it only a qualitative measure at best.

Although many alternatives for detecting free radicals are under investigation, none of them provide spatiotemporal resolution similar to luminescent probes for the parameters discussed in previous sections. A possible way to address the difficult challenge of visualizing free radicals in the intracellular environment is proposed by our own research group. The atomic sized nitrogen vacancy (NV) centers in fluorescent nanodiamonds (FNDs) have magnetic states that can be read out through their fluorescence and manipulated through optical and microwave excitation. External physical influences, especially magnetic fluctuations, are reflected in the relaxation and decoherence times of the NV's magnetic state. Since free radicals are paramagnetic due to their unpaired electron, they are expected to contribute mainly to the magnetic fluctuations in the intracellular environment.

As nanodiamonds have attracted much interest in recent years, ample evidence for their versatility is available in literature.[22] FNDs have been shown to be highly sensitive as spin probes, biocompatible with multiple cell types and organisms and suitable for different kinds of functionalization.

GENETIC MATERIAL

The most common approaches to visualizing DNA or RNA are explained in the following and summarized in Figure 5.

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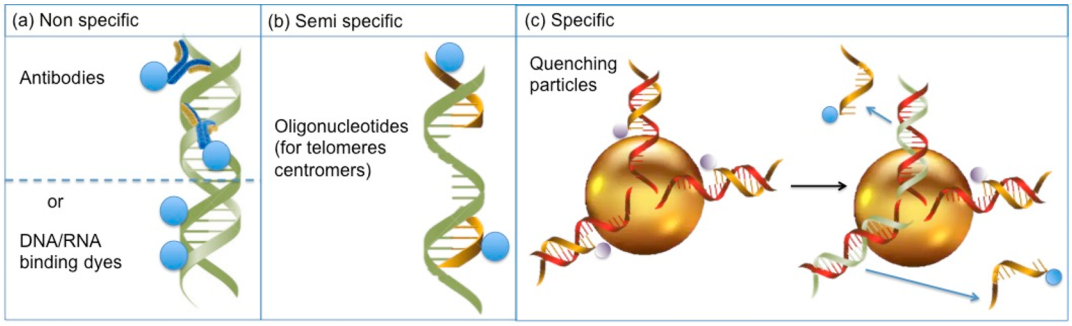

Figure 5. Most common strategies to visualize genetic material. (a) There are several dyes (blue) that bind directly to the target DNA (green). Additionally, there are antibodies that recognize DNA/RNA with a dye attached to it. (b) A semispecific approach makes use of small complementary sequences (yellow) with a dye attached to them. (c) Strands (red) are attached to a quenching particle (gold). Strands with a dye (yellow) are bound to these. Close to the particle, the dye is quenched (purple). When the target RNA (green) is present, the yellow strands are released, and the dye is active.

Traditionally, there are several molecular probes available that bind to DNA or RNA molecules but typically do not differentiate between sequences.[65,66] However, there are also an increasing number of probes that can differentiate between sequences.

DNA are double strands and RNA are single strands that consist of a specific sequence of 4 nucleic acid bases. Adenines present in one strand pair with thymines of a different strand, and guanines pair with cytosines. Thus, there is always a sequence that is complementary to a given strand of RNA or DNA. This principle can be used for analysis since one can offer a specific strand to which if the complementary strand is present, it will preferentially bind. This preferential binding is the basic principle of essentially all DNA/RNA probes.

In the fluorescent hybridization in situ (FISH) method, for instance, DNA is denatured, and small sequences with a fluorescent label attached to them can bind to the single strands.[67] This method works best with repetitive units with a high local concentration (for example, centromeres or telomeres, which are essential for cell division and senescence, respectively). An obvious disadvantage is that the DNA needs to be broken apart, and thus this is not suited for live cell imaging. There are also DNA specific antibodies or proteins available that bind somewhat specifically, but they usually also require fixing the samples.[68]

Another approach is to attach one strand that is complementary to the DNA or RNA of interest directly to a particle that is quenching. For this purpose, for example, gold particles,[69] carbon quantum dots,[70] carbon nanotubes,[71,72] graphene oxide,[73] or metal−organic frameworks[74] have been utilized. The other strand is a less good match to the first and has a quantum dot or dye attached to it. If the target RNA is present, the second strand and the quantum dot are replaced. The result is fluorescence from the quantum dot. This approach was used, for instance, by He et al. for cancer specific RNA,[69] where the authors used gold particles. Lin et al. used the same concept to detect mRNA (mRNA) in cancer cells.[75] However, instead of a gold particle they used an iron oxide particle for the same purpose. This has the advantage that iron oxide is visible in MRI and thus gives an opportunity for diagnostics.

Another feature that results from this complementarity and the ability to synthesize complementary strands (or pieces of strands): Several strands can be engineered into all kinds of shapes (also known as DNA origami). If there is another complementary strand present, it can interfere with this origami structure. If engineered smartly this interference can result in a change in measurable parameters (such as size or optical properties). This principle is also utilized in imaging probes.

However, using DNA or RNA probes also has several drawbacks. One issue that is especially crucial for in vivo studies is that the probes often contain DNA or RNA. This is problematic since free DNA is often associated with viruses and thus can be a red flag for the immune system.[76] As a result, the immune system often reacts to DNA or RNA (at least if this is not prevented). This means that often DNA or RNA containing probes do not reach their target location.[77] For the same reason, RNases or DNases, enzymes that degrade RNA or DNA, are omnipresent. Consequently, it is often necessary to work with RNase- and DNase-free media or to work under sterile conditions.[78] For a more detailed review on RNA and DNA imaging in cells, we would like to refer to the excellent review of ref 79.

BIOMARKERS

The term biomarker encompasses a wide variety of compounds the presence of which signifies a specific physiological or pathological process or condition. A biologically or clinically relevant role is therefore directly implied. The optical intracellular detection of biomolecules with ratiometric probes has been reviewed recently by Huang et al.[80] This broad category includes techniques based on fluorescence, photoacoustic measurements, resonant energy transfer, and surface enhanced Raman scattering (SERS). SERS is a well-established method for the ultrasensitive detection of biomolecules. The excitation of localized surface plasmons in metal core particles dramatically increases the Raman scattering intensity produced by molecules adsorbed to the surface, up to a factor 10,[10] resulting in single molecule sensitivity. Compared to luminescent probes, SERS probes encompass a large variety of nanoparticle platforms that can be used to detect a growing number of compounds with ratiometric calibration and multiplexed detection. An overview of intracellular SERS applications is given by Taylor et al.[81]

It should be noted that apart from intracellular applications for research purposes, the potential clinical impact of highly sensitive biomarker detection is tremendous. Detection methods of currently available biomarkers have their limitations including sensitivity and reliance on highly specific

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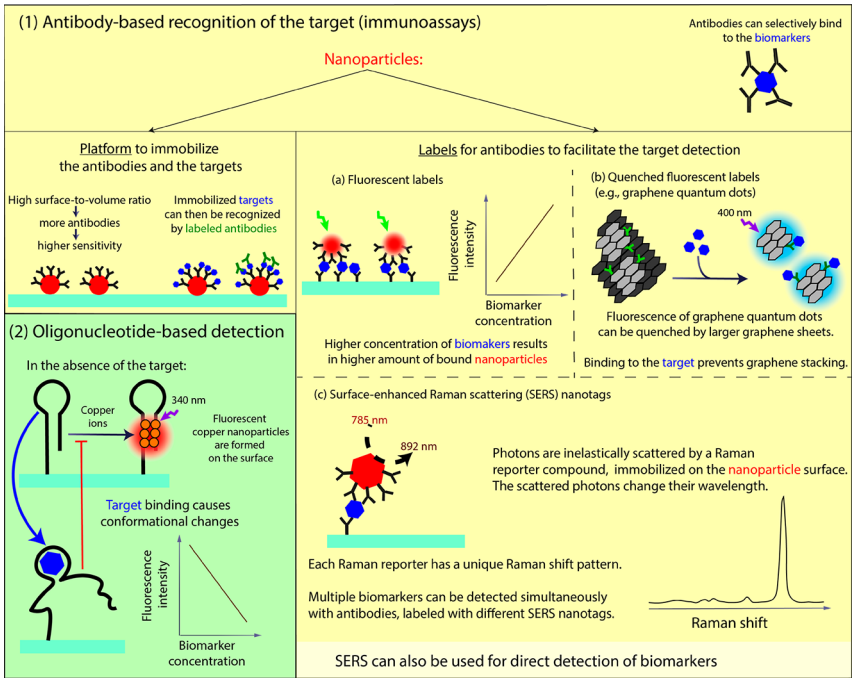

Figure 6. Overview of nanoparticle-enhanced detection of protein biomarkers.

reagents for detection, which require complex and expensive synthesis. Nanoparticles and nanostructured materials with large surface-to-volume ratios bring increased sensitivity. By increasing the surface to volume ratio, the biomarker interaction is amplified, allowing for an improved limit of detection. Additionally, the sensitivity can be increased by (fluorescence) signal amplification. In point-of-care testing, defined as medical testing near the treatment or consultation site, the speed of testing is of utmost importance.[82] Improving the speed and simplicity of biomarker detection, will be beneficial in these bedside tests. Another challenge is multiplex detection in order to reliably detect several biomarkers at the same time in complex samples, such as human serum or plasma.[82]

One of the important methods to detect biomarkers is antibodies. Their function relies on antibodies specifically reacting with corresponding antigens, with quantification generally achieved by measuring a specific activity of the label. The quantification is where nanomaterials can improve these probes, by providing quicker or more sensitive detection. Antibodies have been upgraded in several ways: greater flexibility by using nanoparticles with tunable fluorescence properties,[83] increased surface to volume ratio by applying a nanostructured platform,[84] and signal amplification by using bioconjugated nanoparticles.[84,85] A graphical overview of nanoparticle assisted detection methods of biomarkers through antibodies and oligonucleotides is provided in Figure 6.

Application of biomarkers can be found in innumerable medical fields, but oncology is one of the major medical fields that relies on the sensing of biomarkers. The detection of cancer-specific antigens by antibody probes also benefits from the properties of nanomaterials by either using them as nanoparticles or on nanostructured surfaces.[82,84,85] Next to cancer-specific antigens, nanomaterials are also used as labels for cancer and tumor biomarkers, for example, to detect tumor-specific receptors in cancers cells.[86] Furthermore, nanomaterials have been applied to detect biomarkers in several other diseases besides cancer. Among these, nanomaterials have been applied to improve detection of cardiac biomarkers,[87] to optimize detection of amyloid-β in Alzheimer's disease,[88] to recognize pathogenic bacteria (or their metabolites) in infections,[88] to determine viral infection stages,[89] and to identify chronic dry eye conditions.[90]

CONCLUSION

From a materials science perspective, many challenging steps have already been made toward intracellular imaging of physical and biochemical parameters. Molecular dyes to measure pH have already been widely applied. Improvement points for molecular luminophores include photobleaching and stability in the cellular environment. Polymeric nanoparticles can provide enhanced stability, uptake, and biocompatibility to (derivates of) molecular probes, as well as the possibility of combining multiple fluorophores in one particle. However, beyond the range of applications for which molecular dyes suffice, potential alternatives become exceedingly numerous. Contenders for setting a future standard in subcellular probing include quantum dots, nanocarbons and other inorganic particles. They come with improved optical properties, such as resistance to photobleaching and favorable excitation and emission wavelengths.

However, advanced probes also require advanced properties in the interaction with the cell in order to also guarantee the validity of their measurements. Nonbleaching probes that allow for longer experiments will also need to be evaluated for their uptake and trajectory through and excretion from the cell. Furthermore, the high spatial resolution provided by optical probes can only be used optimally in combination with effective targeting.

If, for example, the localization of an advanced luminescent probe depends on colocalization with a more toxic dye molecule, the practical value of its intrinsic biocompatibility as well as sensing properties are severely compromised. Ideally, biocompatibility and nontoxicity are well evidenced in various

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types of cells and microorganisms. Coating of probes (for instance with polymers) offers a possibility to prevent toxic effects on the cell or reduce nonspecific binding. However, also light itself can have an influence on cell biology. Thus, efforts have to be made to reduce light exposure or to use wavelengths with less impact.

Measuring protocols that can be incorporated in conventional fluorescence and scanning microscopes generally have an edge over those that require specialized light sources and filters for economic reasons. Platforms that do require modified or specialized equipment become interesting when they open up a wider range of applications. Examples that were highlighted in this Account are the well-established SERS method, diamond magnetometry, and upconversion nanoparticles. Applications in live cells require a vast amount of research on the biological effects of a nanoprobe to provide evidence for its measurement validity and its limits. Building this evidence requires focused, multidisciplinary research on each method, incorporating a range of biological model systems.

Another potential issue is cross-talk between different parameters. A change in ROS production might, for example, also cause a temperature change. Thus, it is of utmost importance to use proper controls and understand different influences on the signal.

Since the intracellular environment can be considered unexplored terrain for measurements in this range of resolution and sensitivity, their power lies in elucidating the links between the physical sciences and the life sciences. Multidisciplinary collaborations are therefore also essential to efficiently connect the solutions that are pushed by chemists and physicists to unresolved questions from life scientists and clinicians. Compared to the great advances that have been made in materials science, the applications of optical nanoprobes can be considered as only emerging in the life sciences. In order to make full use of their potential, it is important that the development of new optical probes is pushed beyond the proof-of-concept level. Nanomaterials have already proven themselves as useful tools for a wide range of biomedical applications, from fundamental research to diagnostics and treatment. There is no doubt that further combination of nanomaterials and life sciences will result in even more exciting scientific knowledge, practical solutions to long-standing problems, and new avenues for future research.

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