Beyond the sparkle, other properties of diamond have gained increasing attention in the past few decades among chemists and physicists. Color centers—impurities formed by one or a few foreign atoms or vacancies in the diamond lattice—are one reason for this. While pure diamond is transparent, the presence of color centers causes changes in coloration. Color centers introduce additional electronic states in the wide band gap of diamond, giving rise to transitions that absorb and emit light in the visible spectrum. To this date, hundreds of different color centers have been described, and the number is steadily increasing. Since they are protected within the diamond lattice, their physical and optical properties are unprecedentedly stable. Many different techniques based on diamond color centers have been developed, which allow...
the visualization, localization, and tracking of (bio)molecules or the observation of chemical reactions. These methods can be roughly subdivided into two categories.

The first is the use of color centers as stable photon emitters in nanodiamonds for labeling in microscopy (discussed in the section on Labeling). Nanodiamonds with fluorescence spanning the optical spectrum have been fabricated successfully. The biggest strength of diamond color centers here is that they can be used for (long-term) labeling and tracking. Especially important is the visibility of diamond color centers with many different imaging modalities and thus the possibilities for correlative microscopy. The second way to utilize diamond color centers for chemical analysis and localization of molecules is to use their unique properties for quantum sensing. Especially the so-called NV center is increasingly popular, due to the remarkable experimental successes achieved with it. The NV center transduces information on its surroundings into changes in its fluorescence intensity. Apart from detecting these defects offer magnetic resonance measurements with unprecedented sensitivity. Apart from detecting magnetic signals for chemical analysis, color centers are also influenced by other quantities. Thus, they can be used to detect temperature, electric fields, strain, or pressure, which are not discussed further here.

The aim of this Review is to look at how diamond defects can be used in chemical and biochemical analysis. We first discuss different diamond materials and their surface and defect properties and then discuss applications. We differentiate between applications which make use of the unique photostability of the defects (discussed in the section on Labeling) and applications relying on the quantum properties (discussed in NV Center-Based Sensing and Magnetometry). For more specific reviews on diamond applications that do not rely on color centers (not covered here), or diamond surface chemistry as well as emerging applications in biology, we refer readers to more specific literature, as cited. For work on chemical analysis with diamond based on electrical properties rather than color centers, we refer readers to another review.

### DIAMOND MATERIALS

There are several different types of diamond materials that can host color centers. While natural diamonds can also host color centers, there is no good control over the exact chemical composition. Additionally, the cost of natural diamonds is another reason why synthetic diamonds are preferred. We will first discuss bulk synthesis and then describe nanoscale materials. Diamond synthesis is a large field in itself, and we will only briefly introduce basic concepts here. For a more in-depth discussion, we refer the reader to refs 21 and 22.

**Bulk Synthesis.** Since the 1950s, synthetic diamond can be obtained using the high-pressure high-temperature (HPHT) synthesis method, in which a hydraulic press reproduces the conditions of natural diamond formation.

Diamonds produced by this method typically contain relatively high concentrations of nitrogen (from atmospheric nitrogen that is present during the process) and some nitrogen vacancy centers. These diamonds have a yellow hue due to the inclusion of nitrogen and are the cheapest form of bulk diamond.

In the 1980s, chemical vapor deposition (CVD) diamond was developed. The main advantage of this method is the greater control of material purity. Especially for applications which rely on quantum effects (discussed in NV Center-Based Sensing and Magnetometry), this is critical. High-purity CVD diamonds typically contain very low numbers of color centers. These can be introduced by ion implantation or delta doping (growing a layer that contains foreign atoms, such as nitrogen). Especially worth mentioning are the possibilities to grow isotopically pure diamonds containing almost exclusively $^{12}$C and to include color centers along a preferred crystallographic direction. Both are very relevant for quantum-based applications. Isotopic purity is desired when the nuclear spins of $^{13}$C contribute to magnetic noise that obscures other signals in magnetic sensing purposes.

**Nanodiamond Synthesis.** For nanodiamonds there is an even greater variety of synthesis methods available. The shape, size, and surface chemistry of fluorescent nanodiamonds (FNDs) depend on the FNDs manufacture method.

Historically the oldest and still most frequently used nanodiamonds are produced by detonation synthesis. They are obtained by controlled detonation of graphite mixed with explosives (e.g., hexogen/RDX) or the detonation of high-energy explosives (TNT/hexogen/RDX). These particles are spherical and about 5 nm in diameter. Due to their small size, they only host a very low numbers of color centers. Since their small size is very attractive for application, there have been several attempts made to increase the color center yield in detonation nanodiamonds (DNDs). However, so far these processes result in only one detectable color center in every 10,000 particles. For quantum applications, these color centers in DNDs are typically of insufficient stability. The stability of color centers in DNDs of typically small diameter (~5 nm) is compromised by their proximity to the surface and quality of the crystal lattice.

The nanodiamonds obtained by grinding of micro-sized particles synthesized in a static HPHT process have been shown to be more suitable for biolabeling based on color centers than particles produced by detonation. The main reason is that HPHT nanodiamonds are larger and can thus host more color centers. A 100 nm FND can contain up to around 1000 NV centers after irradiation treatment (with 3 MeV electrons at a fluence of $5 \times 10^{19}$ e/cm$^2$ in this case).

The HPHT FNDs are available with the smallest average size of 10–20 nm and have a broad size distribution. This polydispersity can be improved for instance by centrifugation. Larger particles sediment faster and thus end up in the pellet, while smaller particles remain in the supernatant. This way particles down to 4 nm can be separated from mixtures with a broad size distribution. However, only the FNDs larger than 30 nm have enough NV centers to emit sufficient signal for tracking biomolecules with setups commonly used in the biological laboratories. The most frequently used FNDs are below 100 nm. In comparison to the spherical DNDs, the HPHT FNDs have irregular shapes with sharp edges.

The nanodiamond shape correlates with the number and type of crystallographic planes of various energetic levels and preferential abundance of different functional moieties on the surface. Inhomogeneity of surface chemistry hampers control of particles’ behavior in solution as well as efficient biolabeling.

Another less popular but potentially interesting method that might allow chemically controlled production of NV centers is to use diamondoid-like or adamantane seeds, which already have nitrogen or other well-defined atoms in place and grow nanodiamonds around these. This has the striking
advantage that there is control over the nanoscale environment. So far, however, the yields that have been achieved are impractically low.

Great possibilities might also emerge from a method shown by Trusheim et al.44 The authors microfabricated nano-diamonds out of a high-purity bulk material. This technique also has advantages in terms of control over the purity of the starting material as well as the shape of the final particles. But as for the previous technique yield so far is problematic.

Crane et al. recently showed an interesting approach for nanodiamond synthesis.45 They used high-pressure, high-temperature conditions but made use of a doped aerogel as precursor. The doping of the aerogel allows control over the types of color centers the particles contain. In their pioneering work, the authors showed the creation of diamonds containing silicon vacancy centers.

The Influence of Particle Size. One of the most critical parameters for nanodiamonds is their size. While the size matters in most applications, it does so for different reasons. For quantum sensing applications discussed in NV Center-Based Sensing and Magnetometry, it is crucial that the analyte that should be measured is as close as possible to the color centers to achieve the best possible sensitivity. An exception here is temperature sensing, where the distance to the surface is less problematic.

For biological applications there are restrictions in terms of size too. For labeling applications (discussed in the section on Labeling) the particle needs to be as small as possible (and as bright as possible). Molecules that should be labeled might be impaired in their function if a large label is attached. This is of course less of a problem if the structures that are labeled are also large (as for instance entire cells).

Large labels do not diffuse through cells or other biological matter as freely and thus might not even reach their target. Furthermore, there are several barriers in biology which cannot be overcome by particles above a certain size. Examples for this are the blood brain barrier, placenta barrier, clearance from the body via the kidneys (= renal clearance, up to around 15 nm46) in organisms. On the cell level there are size restrictions for uptake into cells (there are huge differences between different cells types and in some instances even between individual cells; some cells ingest up to micron-sized particles47) or delivery into the nucleus (in humans the nuclear pores are 5.2 nm;48 larger particles might enter during division). However, this issue is much more complicated than that. A pore might hinder much smaller particles from entering than its actual diameter (for instance if the charge of the pore structure and the particle repel each other). The great selectivity of biological barriers is evident when observing how well small molecules are differentiated. Thus, crossing biological barriers is always also a matter of chemistry.

Color Centers. Of the hundreds of different defects that are known, we will here discuss the ones that are most interesting for chemical and biochemical analysis. A more specialized review article on color center creation is available from Smith et al.49 Figure 1 summarizes what foreign elements have been used already (blue) and what has been at least investigated theoretically (in yellow). The elements forming the most interesting color centers are marked in red. To form a stable color center, the foreign element has to fulfill a few criteria: it has to be in a certain size range that is similar to carbon, so it can be incorporated into the lattice during growth without causing too much strain. Additionally, the foreign atom has to be able to form stable bonds to the surrounding diamond lattice. For implantation there is also a restriction on the size: large elements cause damage in the diamond, which leads to graphite formation, making them unsuited for the creation of diamond color centers.

For labeling applications, it is desirable to have particles that are as bright and as small as possible. Sadly, these two

![Periodic table of diamond color centers](image)

**Figure 1.** Periodic table of color centers in diamond. The table shows the most important elements which have been found related to color centers. The most important color centers that have been used with the most promising properties are shown in red. Elements in other color centers are shown in blue. Color centers that have been studied theoretically are depicted in yellow. Representative references can be found for the respective color centers: H50, Li51, N52, O53, F53, Mg53, Si53, P54, S54, Ca53, Sc55, Ti56, Cr57, Mn55, Fe55, Co55, Ni58, Cu55, Zn55, Ge59, Sn59, Xe60, Pb59, and Er51.
properties are inversely correlated, and thus one is usually traded off for the other. It is of course also interesting to have access to labels of different colors. At this point, color centers in all kinds of spectral regions have been found. Compared to organic dyes, color centers generally have quite broad excitation and emission bands but differ greatly between color centers. So if a limited number of different particles are commercially available (hosting NV or NVN centers) from Adamas Nanotechnology or FND Biotechnology.

**NV Centers.** By far the most prominent diamond color center is the so-called nitrogen vacancy center. Thus, most examples described in this paper use the NV center. This defect consists of a nitrogen atom, which replaces a carbon atom in the diamond lattice and an adjacent empty lattice site. The negatively charged NV$^-$ defects are responsible for the far-red fluorescence emission. The NV$^-$ center has a zero-phonon line at 637 nm and a broad band centered at 700 nm wavelength when excited by green-yellow light. This defect occurs naturally (HPHT diamonds for instance usually contain nitrogen and some NV centers) but can also be fabricated. There are several methods available for this. In diamond material that already contains nitrogen, vacancies can be created by irradiation with all kinds of high energy particles including irradiation with electrons or different ions. Afterward, the diamonds are annealed at high temperature. During this process the vacancies become mobile and combine with the nitrogen atoms to form NV centers. Nitrogen can be introduced either during diamond growth or afterward. For introducing nitrogen during growth, delta doping is most commonly used. This is a method where first pure diamond is deposited and then a layer that contains nitrogen, vacancies can be created by irradiation with high energy particles including irradiation with electrons or different ions. Afterward, the diamonds are annealed at high temperature. During this process the vacancies become mobile and combine with the nitrogen atoms to form NV centers. Nitrogen can be introduced either during diamond growth or afterward. For introducing nitrogen during growth, delta doping is most commonly used. This is a method where first pure diamond is deposited and then a layer that contains nitrogen. Nitrogen can be introduced either during diamond growth or afterward. For introducing nitrogen during growth, delta doping is most commonly used. This is a method where first pure diamond is deposited and then a layer that contains nitrogen. By adjusting the implantation energy, one can vary the depth (higher energies result in deeper NV centers). Implanting at specific spots or through masks allows control of the lateral position. The greatest precision has been achieved by implanting through an AFM tip with a small hole which can be placed where NV centers are desired.

There are also several other methods that aim to obtain NV centers with a preferential alignment along one direction. This is interesting for sensing applications with ensembles, which require alignment of the NV axis with an external field (see NV Center-Based Sensing and Magnetometry for discussion on magnetic sensing).

**Other Color Centers.** The impressive properties of NV centers and their drawbacks for certain applications (most notably the hope that it might be possible to pack other color centers more densely or into smaller particles, low brightness, or emission in a broad and undesired wavelength range) have spurred a search for ideal color centers.

**Other Nitrogen-Based Color Centers.** Apart from NV$^-$ centers there are several other nitrogen-based color centers which are potentially useful. Dei Cas et al. systematically investigated the creation of these different color centers. As a starting material they used HPHT diamonds which were milled to reduce the size and irradiated to form vacancies. They then varied the subsequent thermal annealing step systematically. They found that depending on the time and annealing temperature it is possible to tune which defects are formed. Basically, the higher the temperature and the longer the annealing time the more defects with multiple nitrogen atoms they observed. A summary of their findings is shown in ref 2. They explain these differences by the increased mobility of N at higher temperatures.

**Silicon Vacancy Centers.** The most prominent color center for quantum sensing apart from the NV center is the silicon vacancy (SiV) center. The two most important ways to create SiV centers are implantation or diamond growth in the presence of silicon substrates. Compared to the NV center, the SiV center emits a much higher proportion (about 70%) of light in the zero-phonon line
and thus yields emission in a very narrow wavelength range. This can be advantageous for labeling applications discussed in the section on Labeling. While it is less bright than the NV, the fact that the emission is in a narrow band. Thus, by discarding all signals that are not within this spectral range most background can be removed. Additionally, this is advantageous for quantum applications where photons need to be identical so they can be entangled (not discussed here; for further information, see ref 70). For biological applications the SiV has the advantage that its emission is at 738 nm in a wavelength range where there is less background fluorescence from cells. Another advantage of the SiV seems to be that SiV centers can be stable in much smaller nanodiamonds. Vlasov et al. showed stable emission from single SiV centers in particles down to just 400 atoms (or 1.1 nm), while the stability of NV centers is already compromised at around 5 nm, where particles show blinking. The largest disadvantage of the SiV compared to the NV center is that it only has the desired quantum sensing properties at low temperature. Another disadvantage is that silicon vacancy centers are less bright than NV centers.

**Color Centers with Other Foreign Atoms.** The germanium vacancy (GeV), the tin vacancy (SnV), and the lead vacancy (PbV) have properties similar to those of the SiV. These centers have so far been produced via ion implantation. Also these color centers emit a relatively large proportion through the zero phonon line. However, due to the larger size of the foreign atom the ground state splitting is further apart which might offer an advantage for quantum sensing applications. For a more detailed comparison between these color centers we would like to refer to an excellent article by Bradac et al. A further drawback of all color centers apart from the NV is that their limited availability (and the requirement for low temperature for quantum sensing), these color centers have not been used widely in sensing yet.

### SURFACE CHEMISTRY

Starting from the materials described above, there are several reasons to alter the surface chemistry, which are summarized in Figure 2 and discussed in more detail in the following section. The most common “untreated” surface is oxygen terminated. This termination results from a treatment with oxidizing acids, which is usually done as a last step in diamond fabrication. This starting material contains a mixture of various chemical groups. The different ways to alter the surface chemistry shown in Figure 2 are similar for nanodiamonds and bulk diamonds. However, the individual procedures and approaches are not always transferable between nanoparticles and bulk materials. Depending on the diamond material, different aspects have to be considered. In nanodiamonds, aggregation always plays an important role. In bulk diamonds on the other hand reactivity is typically much lower, and thus often harsher conditions have to be used.

The surface chemistry of (nano)diamonds can also be influenced by the synthesis method and the purification treatments. Several examples for such differences have been reported in the literature. For instance, graphitic impurities on the surface greatly alter the surface properties, but these impurities can be removed by cleaning. Also, residues from the milling material can influence the diamond properties.

**The Effect of Surface Chemistry on Fluorescent Properties.** There are two ways in which the surface chemistry can influence the fluorescent properties of (nano)diamonds. Understanding and controlling this is essential for the applications discussed in the following sections on Labeling, FRET with Diamonds, and NV Center-Based Sensing and Magnetometry. The first way in which the surface has an influence is of moleculesfunctionalities on the surface fluorescence (or quench fluorescence). In DNDs, fluorescence mostly originates from non-diamond surface-localized defects, including defects in the sp³ shell of nanodiamond and impurities, although color centers have also been observed in these particles. There are several factors such as size of the nanodiamond, graphitization on the surface, and surface moieties of the nanodiamond host which can affect the surface states. The recombination of donoracceptor pairs can determine the surface role in nanodiamond luminescence. Delocalized p-electrons in aromatic hydrocarbons can create conditions for the strong absorption and re-emission of photons.

Another way in which the surface can influence fluorescence properties is by interacting with the color centers. Here the best-known process is the charge conversion in negatively charged NV centers that are converted into neutral NV. This happens if molecules on the surface compete with the color centers for the electrons. This process has an influence on applications that rely on fluorescent brightness and has an even greater effect on quantum sensing properties. While both NV and NV are fluorescent, NV centers are slightly brighter.

**The Effect of Surface Chemistry on Sensing Performance.** Surface chemistry also has a dramatic effect on quantum sensing-based applications (discussed in NV Center-Based Sensing and Magnetometry). The basic principle in quantum sensing is that you prepare the NV centers in a defined state and measure for how long the NV center is able to retain this state. Depending on the exact pulsing sequence that is used, these times are called T1 time, T2 time, or T2*. (further details are discussed in NV Center-Based Sensing and Magnetometry). Especially for magnetic sensing, the surface has a profound effect and is widely seen as the most important bottleneck to improve performance. In these applications, color centers (especially the NV center) are used to transduce physical parameters, such as magnetic field fluctuations from spins external to the diamond, into an optically measurable signal. This signal decreases quickly with increasing r (where r is the distance between the NV center in diamond and the sensing target beyond the diamond surface). Therefore, shallow NV centers are required for sensing applications.

However, shallow NV centers are impacted by the surface in two ways. The first is charge conversion to NV which was mentioned before. This is detrimental for sensing since only NV has optical and spin properties required for sensing. Another detrimental effect that the surface can have is to cause a strong signal itself. This is the case if there are dangling bonds (or free electron spins) located close to the color centers. Such dangling bonds cause paramagnetic noise and in the worst case render the color center useless. Thus, it is of utmost importance for sensing performance to optimize the surface chemistry and minimize dangling bonds.

**Effect of Oxygen Surface Treatment.** In the following section we will review the most promising surface modifications and the effect they had on the sensing performance in detail.

Kim et al. reported the effect of oxygen plasma and thermal oxidation on optical properties on NV centers. They
observed a 3-fold increase in $T_2$ (a direct measure for sensing performance) upon oxidation of surface at 550 °C. Oxygen plasma exposure clearly damages near-surface NV centers and appears to create extra vacancies at the surface. Mild oxidation at 550 °C improves $T_2$ and substantially decreases background fluorescence. Higher temperatures such as 580 and 600 °C resulted in further decline in $T_2$ for most NV centers, attributed to oxidative etching. Apart from improving the surface chemistry, oxidation provides fine control over the depth of shallow NV centers.

An increase in charge stability of NV$^-$ centers has been reported by Yamano et al. by oxygen post-treatment of acid-cleaned diamond. The potential reason for the instability of NV$^-$ centers upon acid treatment is the high probability that not all of the C−H bonds are oxidized by the acid treatment. Usually, a H-termination causes a strong upward band bending owing to negatively charged surface adsorbates being attracted by C−δ−H$^+$ δ dipoles. If the surface oxidation is not complete and residual C−H bonds remain on the diamond surface, upward band bending can occur depending on C−H density. This could cause the charge state instability of shallow NV centers. However, additional oxidation treatments, such as UV/ozone exposure and oxygen annealing, could oxidize the surface effectively. This can contribute to the stabilization of NV$^-$ centers near the surface. Ozone treatment alone is another promising technique, which was also reported to eliminate the non-diamond carbon and leads to a uniform surface.

Effect of Nitrogen Surface Treatment. As with virtually all diamond surface modification techniques, the resulting surface is covered with a variety of different groups rather than homogeneously modified. In this case the resulting surface is partially covered with nitrogen, with N−H, NH$_2$, and N=N groups present on surface. Shallow NV$^-$ were stabilized similar to oxygen-treated surfaces. The sensing performance of nitrogen-terminated diamond was discussed in detail by Kawai et al. Nitrogen-terminated diamond formed by nitrogen radical beam exposure stabilized shallow NV$^-$, and the spin properties were comparable to those of oxygen-terminated diamond. In their article the author claimed that $T_2$ increased 2-fold compared to that of bare diamond.

Effect of Fluorine Surface Treatment. Osterkamp et al. reported the creation of shallow NV centers less than 3 nm below the surface. They proposed to activate (dark) NV$^+$ centers that are very close to the surface of diamond. After fluorinating the surface, they observed a 4-fold increase in NV$^-$ formation yield.

Shanley et al. reported the increase in formation of NV centers by the usage of NF$_3$ and compared it with H-terminated diamonds. They observed a substantial increase in the ratio of shallow NV$^-$ to NV$^0$ centers, and due to the high electronegativity of fluorine it stabilizes the charge.

One theory proposed by the authors for the increased amount of NV$^-$ centers is that H-terminated diamond exhibits upward bending of bands in the presence of H$_2$O due to its acidic nature. In contrast, the large electronegativity of fluorine inverts the polarity of the surface dipole, causing the downward bending of bands. Fluorination of H-terminated diamond, therefore, switches the charge state of near-surface NV$^-$

Figure 3. Overview of surface modifications of the diamond surface to prevent aggregation and protein corona formation as well as target cell components. The diagram depicts two types of surface modifications that can be applied on nanodiamonds. The first layer of modifications prevents aggregation and the formation of a protein corona (blue circumference). In some articles this layer has been used to attach a second layer of modifications for targeting (d)−(f) (orange circumference). Some of the surface modification strategies for preventing aggregation and the formation of a protein corona includes (a) carboxylation or hydroxylation, (b) silk fibroin, (c) lipid coating, (d) serum albumin, (e) antibodies or peptides, and (f) polymer coating. Modified surfaces of FNDs can be linked to ligands (L) such as antibodies and streptavidin/biotin complex to target specific molecules.
centers. Hence, this increases the number of NV\textsuperscript{−} centers formed or increases the stabilization of charge.

Jelezko and co-workers further investigated the effect on shallow NV\textsuperscript{−} centers with \ensuremath{SE_{l}} as the fluorinating agent. X-ray photoelectron spectroscopy measurements showed the presence of only fluorine atoms on the surface, in contrast to previous studies, indicating very good surface coverage. \ensuremath{T_{2}} measurements of shallow NV\textsuperscript{−} centers (less than 5 nm), revealed a rather short average coherence time of about \ensuremath{T_{2} = 4 \pm 1 \mu s}, typical for implanted NV. This value could be explained by a strong influence from paramagnetic defects (e.g., dangling bonds) on the diamond surface.

**Effect of Silicon Surface Treatment.** Kawai et al. recently prepared silicon-terminated diamond by using silicon beam deposition at high temperature.\textsuperscript{95} The silicon-terminated (001) diamond surface exhibited a (3×1) low-energy electron diffraction pattern, and its negative electron affinity (NEA) was estimated to be \textasciitilde−0.86 eV using angle-resolved photoemission spectroscopy and near-edge X-ray absorption fine structure (NEXAFS) analysis. They observed surface upper band-bending when depositing MoO\textsubscript{3} on a Si-terminated surface, similar to hydrogen termination. They observed a decrease in the number of shallow NV\textsuperscript{−} centers (<3 nm) compared to the starting material, which was O-terminated. In addition, there was photoblinking in the shallow NV centers in the presence of silicon.

**Effects of More Complex Coatings.** Similar to surface groups, chemically or physically attached macromolecules can affect the photoluminescence of (nano)diamond or their quantum sensing performance.\textsuperscript{96} In general, the same principles of stabilization that have been introduced in the previous sections apply here. However, since this is more difficult to understand, such effects are less studied in more complex coatings. These coatings are usually applied to achieve a biological function (discussed in Effects of Surface Chemistry on Biological Functions and further in the section on Labeling). In addition, there are also coatings in use which serve a function for sensing. These are discussed in NV Center-Based Sensing and Magnetometry.

**Effects of Surface Chemistry on Biological Functions.** Apart from influencing the color centers, surface chemistry can also be used to alter how particles interact with a biological environment. Below we discuss different nanoparticles which achieve a certain goal. However, often the different strategies achieve multiple goals (see Figure 3). Biocompatibility is generally considered very good and also discussed in more detail elsewhere.\textsuperscript{18,19}

**Preventing Aggregation.** The aggregation of nanoparticles depends on their size, shape, and surface chemistry. It has been shown that below 100 nm particles are more prone to aggregation, unless a very specific surface treatments are made. On top of that, in a biological environment, particles do not appear as bare particles. Nonspecific protein adsorption (protein corona formation) occurs. This can lead to FND aggregation in cell culture media or body fluids and a decrease in detection sensitivity to optomagnetic signals.\textsuperscript{97} So far, a few coating strategies that rely on either noncovalent or covalent interaction have been successfully applied to obtain colloidal stable FNDs. Generally, introducing charges on particles improves colloidal stability because charged particles repel each other. Harvey et al. made use of this principle when they coated nanodiamonds with 1,3,4-dihydroxyphenylalanine (L-DOPA).\textsuperscript{98} This coating renders the surface electro-negative. Furthermore, the coating provides chemical groups (amines, carboxylic acid, alcohols, and conjugated Michael acceptors) on the surface, which allow further functionalization. Using charged peptides or proteins is useful for this purpose as well.\textsuperscript{99}

Silica shells are also useful for avoiding aggregation (and fulfill other biological functions).\textsuperscript{100} The most interesting property of this technique is probably that the resulting particles are spherical and very uniform. Additionally, also this method allows further functionalization. A potential drawback is that the shells are typically a few nanometers in size and thus add distance between the color centers and the spins in the environment which impairs sensing performance.

**Preventing/Reducing the Formation of a Protein Corona.** Common cell media as well as body fluids contain a large variety of proteins. When nanodiamonds (or any nanoparticle) come in contact with these media, the surface is quickly covered with all kinds of proteins. This is to some extent unavoidable or might even be beneficial (certain proteins facilitate uptake or improve colloidal stability). On the other hand, corona formation is often seen as problematic if there is poor control over which proteins and how many of them attach.

Machova et al. observed that protein interactions with nanodiamonds depend more on their surface chemistry rather than size.\textsuperscript{101} The surface termination of particles leads to formation of a different protein corona, which might have an impact on cytotoxicity. A precise understanding of how the corona forms and evolves, and its influence on cytotoxicity, is lacking. Garcia-Bennett et al. postulated stronger aggregation of the protein corona on the surfaces of negatively charged nanodiamonds.\textsuperscript{100} The negatively charged nanodiamonds showed a decrease in ROS generation and better compatibility with cells when incubated with a pre-formed protein corona.

So far, the several strategies have been developed to prevent formation or regulate the composition of the corona (see Figure 3). It has been demonstrated that treatment of FNDs with peptides and proteins such as a serum albumin\textsuperscript{101} or silk fibroin\textsuperscript{102} prevents aggregation in physiological solutions. Nevertheless, these proteins do not fully prevent nonspecific binding.

The noncovalent conjugation approach makes use of interactions of lipids with the FNDs surface.\textsuperscript{103} The lipid layer enables better protection against undesired binding of proteins; however, it is not suitable for a long-term preservation due to its high susceptibility to fluctuations in biological environments. Such fluctuations include, for instance, pH change or molecular crowding. The greatest advantage of described noncovalent functionalization methods is the simplicity. Additionally, it has been reported that some molecules retain their function better if they are attached noncovalently. This approach should find application whenever it is not crucial to target FNDs into specific cellular location. Otherwise, the desired labeling precision and stability could be achieved by covalent modification of FNDs surface with antifouling films such as polyethylene glycol (PEG)\textsuperscript{104} or tetraethylene glycol (TEG) functionalized with zwitterionic head groups\textsuperscript{97} or hyperbranched polyglycerol (HPG).\textsuperscript{105} These polymers could be easily linked with antibodies or another ligand molecule for highly specific recognition of receptors. Zou et al. fabricated a polyglycerol-based coating which outperformed PEG in preventing the formation of a protein corona.\textsuperscript{106} Their coating was so efficient that it prevented particle uptake by macrophages. This is of great
interest for in vivo applications, where this behavior would help the particles avoid the immune system and thus make them more likely to reach their target. Torelli et al. in addition used polyglycerol to avoid corona formation.\textsuperscript{107} The authors also demonstrated that polyglycerol shells can be further functionalized. In their case they attached Vascular Endothelial Growth Factor. This molecule is recognized by receptors which indicate blood vessel formation in tumors and is thus an important biomarker in cancer research.

\textit{Surface Chemistry for Targeting.} The FNDs have been covalently linked with antibodies or short peptides to target the cell membrane or cytoplasmic molecules as well as organelles such as the nucleus or mitochondria.\textsuperscript{108} Here it has to be noted, that the nucleus as well as mitochondria are enclosed with a double membrane which hinders entry of nanodiamonds. Thus, nuclear or mitochondria targeting means targeting of the nuclear or mitochondrial surface. The only exception where it might be possible to enter the nucleus is during cell division.

Different strategies are summarized in Figure 7 below. Hsieh et al. treated the integrin α5 receptors with azide-modified antibody to conjugate them with alkyn−HPG FNDs directly on the cell membrane.\textsuperscript{99} Zou et al. introduced a triphenylphosphonium (TPP) mitochondrial targeting moiety in low and high densities on the surface of nanodiamond functionalized with polyglycerol (PG), used to prevent protein corona formation.\textsuperscript{109} Despite presence of the PG, the TPP attracted proteins to form a corona layer. A small amount of proteins on the surface of nanodiamonds with low density of TPP did not affect its mitochondrial targeting ability. Contrarily, the targeting was affected by higher amounts of proteins on particles with high density of TPP moieties. Biotin−streptavidin interactions are, after antibodies and peptides, the second most commonly used approach for tagging FNDs. These and other studies show various modification strategies that avoid protein corona formation and aggregation of FNDs.\textsuperscript{110} However, it is still an open question how this chemistry influences nanodiamond uptake, intercellular trafficking, and finally the biosensing performance. Another concern is whether or not the functionality of the attached molecules is retained after attachment to a nanodiamond particle. This might differ greatly depending on where and how the molecules are attached.

\textit{Altering Uptake by Cells.} While there are many cell types which spontaneously ingest nanodiamonds in high quantities, there are also many cells which do not take up particles in the desired amounts or do not show uptake at all. For these types of cells different strategies have been developed to increase uptake. One of these strategies is to use positively charged coatings. These coatings are effective because they reduce electrostatic repulsion between electronegative (from the surface termination) diamond surfaces and the electronegative cell membrane. Han et al., for instance, achieved this goal by attaching cationic human serum albumin to the surface of fluorescent nanodiamonds.\textsuperscript{112} Zheng et al. made use of a positively charged peptide.\textsuperscript{75} As a result, the authors observe increased uptake into HeLa as well as colon cancer cells. Such charged coatings also offer the advantage that they improve colloidal stability.

Another strategy to increase uptake is to coat particles with molecules that are recognized by receptors in cells. Zhang et al. applied this concept when they used a coating which contains folic acid.\textsuperscript{113} This molecule is recognized by cancer cells and thus promotes uptake by these cells.

\textit{Embedding Diamonds into Complex Materials.} Apart from tailoring the surface of (nano)diamonds or binding molecules to the surface, it is also possible to construct more complex structures which contain nanodiamonds. This has recently been achieved by Guarino et al., who incorporated nanodiamonds in a poly-caprolactone matrix, which can be 3D printed.\textsuperscript{114} The authors produced a scaffold material that is useful for growing cells for instance for tissue regeneration. They could show that it is still possible to perform fluorescence detection from particles in these scaffolds. Khalid et al. incorporated FNDs into electrospun silk fibroin membranes.\textsuperscript{102} They demonstrated that they could sense temperature with these nanodiamonds. They tested the biological properties of their material in a murine model where they promoted wound healing. The presented approach (which might potentially be extended for other sensing applications) is promising for wound monitoring.

Additionally, nanodiamonds have been used as a supplement for materials which are used in tissue regeneration. Fox et al. showed that nanodiamonds embedded in poly[(L-lactide)-co-(ε-caprolactone)] scaffolds can be beneficial in bone tissue engineering.\textsuperscript{115} The authors could show that adding nanodiamonds to their polymer improved cells growth on their implanted scaffolds.

\textit{Diamond Hybrid Materials for Sensing.} Diamonds can also be combined with other materials to achieve chemical or biochemical sensing. Here we discuss how different coating materials, which were responsive to an external quantity, can work together with diamond color centers.

Recently, Petrakova et al. developed NV center-based pH sensors.\textsuperscript{116} The authors coated nanodiamonds with a cationic polymer, poly(allylamine) or poly(diallyldimethylammonium chloride). These polymers change their electrical properties with pH. As a result, the charge states of the NV centers are shifted. When the NV$^-$ state switches to NV$^0$, there is a change in fluorescence. By monitoring the fluorescence spectra, the authors could monitor molecular binding events in proximity to the nanodiamond surface.

Recently, Rendler et al. developed alternative diamond particles, which are sensitive to pH or the redox status.\textsuperscript{117} They made use of the magnetic properties of NV centers and used nanodiamonds that were coated with poly[(2-hydroxypropyl)methacrylamide] (HPMA)-based co-polymer chains. These polymer chains were loaded with gadolinium. If the shell is close to the diamonds the NV centers thus have low $T_1$. When the pH is lowered, the polymer chains are cleaved from the particles, which results in an increase in $T_1$.

Barton et al. developed a hybrid material based on nanodiamonds coupled with a spin label. When the spin label encounters a radical it is converted into a stable radical. This radical can in turn be detected via the quantum sensing properties of the color centers within the diamond.\textsuperscript{118} The material made by Zhang et al. also makes use of the fact that NV centers work distance dependently.\textsuperscript{119} They used a hydrogel which swells in response to external factors. When the hydrogel swells, spins on the surface move farther away and thus the signal reduces. In their pioneering work, the authors used temperature as stimulus but in principle this concept could also used with different polymers, which are responsive to other stimuli.
Yet another hybrid material was developed by Almotiri et al., who used fluorescence from NV centers to detect creatinine, a metabolic waste product in the body. Its detection in blood and urine serves as crucial parameter to assess kidney health. The nanodiamonds they used were embedded in a molecularly imprinted polymer for creatinine. Such imprinted polymers are selectively binding certain molecules (in this case creatinine). If the creatinine is incorporated in the polymer, it alters the nanoparticle—molecule surface charge interaction and thus shifts the NV−/NV0 ratio.120

Characterization of Diamonds. Due to the reasons given above, the nature of the diamond core as well as its outer shells must be well characterized because it strongly affects the surface reactivity and sensing ability of diamonds. To investigate the composition and surface chemistry of diamonds after purification or functionalization, several methods have been applied. Table 1 gives an overview of the techniques which have been used and the information that can be obtained with them.

Table 1. Overview of Different Characterization Techniques That Have Been Used for Diamonds and the Type of Information They Reveal

<table>
<thead>
<tr>
<th>characterization method</th>
<th>information</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTIR</td>
<td>functional groups on the surface carbon—hydrogen (C−Hx, O−H, C−O, C−O−C, C=C, C−N, or N−H)</td>
<td>121</td>
</tr>
<tr>
<td>Raman spectroscopy</td>
<td>C−H surface bonds, composition and structure for bulk carbon phases (1332 cm−1 for diamond, D and G bands at 1410 and 1590 cm−1 for amorphous and graphitic carbon)</td>
<td>122</td>
</tr>
<tr>
<td>XPS</td>
<td>element composition, surface termination, sp2 and sp3 hybridization</td>
<td>123</td>
</tr>
<tr>
<td>AES</td>
<td>sp2 and sp3 hybridization (more sensitive than XPS)</td>
<td>124</td>
</tr>
<tr>
<td>HR-TEM</td>
<td>structure of the outer shells at the atomic scale</td>
<td>125</td>
</tr>
<tr>
<td>HREELS</td>
<td>surface chemistry (hydrogen concentration of hydrogenated diamonds, reference on microcrystalline material)</td>
<td>126</td>
</tr>
<tr>
<td>Boehm titration</td>
<td>nature of oxygen-containing groups</td>
<td>127</td>
</tr>
<tr>
<td>NMR</td>
<td>surface functionalization, ozone-treated, hydroxylated, fluoride-functionalized NDs</td>
<td>128</td>
</tr>
<tr>
<td>TDMS</td>
<td>thermal stability, amount of adsorbed molecules (reference from detonation NDs)</td>
<td>129</td>
</tr>
<tr>
<td>TGA</td>
<td>quantitative weight analysis of outer shells of NDs</td>
<td>130</td>
</tr>
<tr>
<td>DLS/Zeta</td>
<td>hydrodynamic size/surface charge of NDs</td>
<td>47</td>
</tr>
</tbody>
</table>

“Abbreviations: FTIR, Fourier transform infrared spectroscopy; XPS, X-ray photoelectron spectroscopy; AES, Auger electron spectroscopy; HR-TEM, high-resolution transmission electron microscopy; HREELS, high-resolution electron energy loss spectroscopy; NMR, nuclear magnetic resonance; TDMS, thermal desorption mass spectrometry; TGA, thermogravimetric analysis; DLS, dynamic light scattering; ND, nanodiamond.

LABELING

The most intriguing chemical processes occur in living organisms. Many of them stay mysterious as there are a limited number of non-invasive visualization methods and related probes available. Even fewer techniques provide the ability to monitor biochemical reactions with high spatiotemporal resolution in real time. By far the most commonly used methods are based on optical imaging. Reineck et al.131 compared properties of various biolabels. Among these probes organic fluorophores are widely used by researchers due to their small size (below 1 nm), high fluorescence quantum yield, and variety of types that cover the spectrum from visible light to the near-infrared. A major limitation of using organic probes is that they bleach during the exposition time. Moreover, it is important for probes to be water-soluble and to emit in the near-IR region of the spectrum. Emission in the near IR range is favorable due to the low autofluorescence of biological matter within this range. Semiconductor quantum dots, gold nanoclusters, carbon nanoparticles, and nanodiamonds are promising alternatives for organic biolabels. Quantum dots are more photostable than organic dyes; however, they are larger and composed of toxic elements (e.g., Cd, Se, Te). Gold nanoclusters and carbon dots exhibit intermediate levels of photostability and brightness when compared with other biolabels. Nanodiamonds, which will be discussed in the following sections, offer a good compromise between sustained fluorescence, brightness, and biocompatibility.

FNDs Tailored for Multiple Microscopy Techniques. Apart from their photostability and biocompatibility, nanodiamonds have one more distinct advantage. They are easily detectable in many different microscopy techniques; for example, diamond color centers are easy to visualize in fluorescence microscopy or confocal microscopy.

To improve and push the limits of spatial resolution, super-resolution techniques can be applied (Figure 4a–c). The first super-resolution technique, which was applied to color centers is stimulated emission depletion (STED) (Figure 4b).132 This technique allowed localization of NC centers with a resolution of down to 6 nm. Chen et al. applied a technique called deterministic emitter switch microscopy (DESM)133 (Figure 4c). They were able to localize nanodiamonds with down to 12 nm resolution and claim that their technique is faster than STED. However, so far the required equipment is very specialized, and the measurements remain challenging.

Apart from optical techniques (which can visualize color centers) nanodiamonds are also easily imaged in electron microscopy (Figure 4d–f). Certain color centers (for instance, SiV or NV134,135) can be seen in cathodoluminescence (this means that they emit light when they are irradiated with an electron beam). Interesting for correlative microscopy is that they remain fluorescent even after sample preparation for electron microscopy, while conventional dyes usually are destroyed during the typical staining procedures (Figure 4e,f).3 Paramagnetic defects in diamond themselves are also visible in conventional magnetic resonance imaging (MRI) in T1 mode. T2 contrast can be achieved via metallic impurities in the diamond material.136 However, unless they are coated with conventional contrast agents, they are not particularly well visible due to their small size and comparably low defect concentration.137 Manus et al. conjugated gadolinium(III) (Gd(III)) to the surface of NDs for improving MR imaging. NDs enhance T1 and T2 contrast, while Gd(III) is typically effective for T1 positive contrast. Their work demonstrates that ND–Gd(III) aggregates can be used as cell tracking reagents for MR imaging for both of the modalities.138 This approach was later applied for tracking of cancer cells labeled with ND-Gd(III) aggregates in a tumor environment in a mouse (Figure 4g,h).139 The authors could show that labeled tumoral tissue has shortened relaxation times (T1 contrast) compared to non-labeled tumoral tissue and surrounding muscle (Figure 4h).

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On the other hand, the quantum properties of nitrogen-vacancy center in nanodiamonds have been used to increase the contrast in fluorescence for imaging purposes. Irigashi et al. developed real-time in vivo imaging with reduced background of nanodiamond aggregates inside a mouse (Figure 4i) based on a selective imaging protocol with microwave (MW) irradiation (further explained in the section on More Complex In Vivo Labeling).140

**Figure 4.** FNDs tailored for multiple microscopy techniques. Overview of imaging techniques based on fluorescent nanodiamonds. Super-resolution microscopy techniques based on fluorescent nanodiamonds can be applied. (a) Schematic figure of unresolved and resolved FNDs. (b) STED microscopy on FNDs shows a shrinking of a confocal spot of 223 nm diameter down to 8 nm.142 (c) Deterministic emitter switch microscopy (DESM) allows resolving NV centers with a resolution down to 12 nm.133 Nanodiamonds can be visible under different microscopy modalities allowing correlative microscopy. (d) Schematic figure of multicellular environment tagged with FNDs. FND immunolabeling of a membrane receptor on HT29 cells imaged on confocal microscope (e) as well as with an electron microscope in cathodoluminescence, secondary electron, and backscattered electron mode (f).141 In vivo imaging based on paramagnetic defects in diamond. (g) Schematic figure of FNDs in in vivo imaging. (h) Magnetic resonance image of a mouse with a tumor labeled with nanodiamond functionalized with Gd (left side) and unlabeled tumor (right side) of the image. The saturation−recovery plots show a shortening in relaxation time in the labeled tumor compared to unlabeled tumor and surrounding muscle.140 (i) Real-time, background-free in vivo imaging of nanodiamond aggregates inside a mouse. Fluorescence images of the mouse obtained without (upper image) and with (lower image) selective imaging protocol (SIP) described by Irigashi et al.140 Time courses of fluorescence intensity for the nanodiamond aggregate (FND, red line) and autofluorescence (blue line). FND shows rapid modulation as a consequence of selective imaging protocol with microwave radiation turned on and off every 10 ms.140 Reproduced images: (1) Reprinted with permission from ref 132. Copyright 2009 Nature Publishing Group. (2) Reprinted with permission from ref 133. Copyright 2013 American Chemical Society. (3) Reprinted with permission from ref 4. Copyright 2017 Nature Publishing Group. (4) Reprinted with permission from ref 139. Copyright 2016 American Chemical Society. (5) Reprinted with permission from ref 140. Copyright 2012 American Chemical Society.**

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**Tracking of Molecules and Biochemical Processes.**

The human body is a complex, highly dynamic, and molecularly crowded environment.141 Further understanding of this fascinating system requires a real-time visualization of biochemicals at the cellular level. The FNDs with their unique optical and magnetic properties are promising labels for long-term tracking of the molecules inside and on the surface of living cells. Additionally, combining localization with the sensing capability, which is discussed in FRET with Diamonds and NV Center-Based Sensing and Magnetometry, would allow chemical reactions to be monitored. In the next sections we follow the FND particles step by step on their way through the cells.

**Nanodiamonds in Contact with the Cell Membrane.** An insight into the dynamics of cell membrane proteins is an important first step for revealing cellular responses to environmental stimuli.

Hsieh et al. labeled integrin α5, which is a key cell migration receptor, with alkyne-HPGFNDs.142 The movement of these membrane proteins was tracked in HeLa cells for up to 2 h by fluorescence imaging. It has been found that transport of integrin α5 on the filopodia of migrating cells is a time-dependent process. The alkyne-HPGFNDs of different sizes
(100 and 50 nm) have been also applied to track integrin β1 on human fibroblasts and HeLa cells for more than 10 h.

Undeniably, this shows that FNDs are excellent probes to conduct fluorescence imaging of the membrane molecules with an arbitrarily long time without blinking, photobleaching, or cytotoxic effect. However, further improvement of the FNDs-based probes is required to ensure monovalent labeling of the targeted membrane proteins to improve temporal and spatial resolution of fluorescence analysis.

**Nanodiamonds inside Cells.** In order to tag and track molecules inside the living cell, first FNDs need to pass through the membrane. The cellular uptake of FNDs occurs in a natural way due to endocytosis. The nanodiamond probes could be also introduced into the cells via injection or electroporation or with support of chemical treatment. The latter methods affect cell viability. However, unlike endocytosis, these do not require an engineered escape strategy to deliver FNDs into the cytosol or to organelles. The forced internalization can prove to be extremely useful for cells that do not easily take up particles.

Injection and nanowire-assisted delivery of FNDs into single cell are still underrepresented protocols as they are time-consuming and require a high precision. On the other hand, these methods allow control of the concentration of nanoparticles delivered based on injection pressure and time. Injection procedures with FNDs have so far only been applied to multicellular systems, such as *Caenorhabditis elegans*, or exceptionally large cells, such as human embryonic fibroblasts. While the injection of FNDs is associated with breaking the physical continuity of the membrane of one cell at a time, electroporation works for many cells simultaneously. Electroporation involves high-voltage electric pulses that create pores on the cell membrane. Tseng et al. applied electroporation to introduce FNDs (30 nm) coated with BSA and α-lactoalbumin into HeLa cells. The authors found that the FNDs were uniformly distributed inside the cytosol and did not form undesired aggregates. However, the electroporation protocol resulted in a significantly lower number of FNDs inside the cell compared to internalization via endocytosis. Moreover, only 40% of cells stayed alive after the procedure. Similarly, our group has applied electroporation to introduce FNDs (70 nm) into yeast cells. Also, in this case the number of live cells decreased significantly. Therefore, one could assume that an electroporation should be a method of choice if the cell viability is not crucial for the experiment.

Alternatively, we adapted chemical transformation protocols to overcome the thick cell wall and membrane permeability obstacle in the yeast cells. Among various tested reagents, we found that the chemical transformation mix (PEG4000 and 1 M lithium acetate) in combination with DMSO was the least invasive to induce fluorescent nanodiamond uptake. We showed that Triton treatment removed an undesired excess of FNDs from the yeast surface. This is essential for tracking analysis because it ensures selection of particles that are inside the cell. Our group has also developed a protocol for internalization of lipid coated FNDs into yeast spheroplasts. Liposomes cationic polymers or dendrimers also support uptake of FNDs by mammalian cells. It has been shown that shear stress and other types of mechanical stimulation may increase the FNDs uptake by cells. Nevertheless, these techniques are associated with transport of particles within endosomes.

Generally, FNDs are readily internalized into different type of cells via energy-dependent, clathrin-mediated and receptor-mediated endocytosis. Once inside the cells, the particles may stay trapped in the endosomes or be released. Like for many other nanomaterials, efficient delivery to the intracellular environment plays a key role in extending FND’s intended functions of precise tracking, biolabeling or any other organelle-specific research. To the best of our knowledge, there is a lack of systematic studies on endosomal escape of FNDs and dependence on the cell type and lifecycle stage. The time range from internalization until release of FNDs as well as if there is release or not varies greatly per cell type.

Recent studies showed that the shape and surface modification of FNDs can influence cellular uptake and endosomal escape. FNDs have been recognized in several studies as a safe and effective material which can escape the endosomes. Chu et al. compared the internalization behaviors of round and fluorescent nanodiamonds with sharp edges and found that the latter can easily rupture the endosome membranes and move to the cytoplasm. Pang et al. further revealed the mechanism of pore formation caused by the sharp edges. They further pointed out that being both sharp (R ≤ 4–5 nm) and large enough (L ≥ 20 nm) are requirements for FNDs to escape the endosomes. For better cellular internalization, surface modification has been widely used to prevent the aggregation of FNDs and improve the colloidal stability under physiological salt conditions. At the same time, the functionalization strategies may have an impact on the sorting of FNDs into transporting vesicles of different fate. It is worth noting that some coated FNDs can be trapped in endosomes or lysosomes, while some of them will escape.

Nowadays, it is still a big challenge to clearly understand the endosomal escape of FNDs due to the limitation of detecting and quantifying methods. There is no consensus yet on how to evaluate this critical step in the intracellular delivery process. Fluorescent endosome markers sometimes are not as informative as expected. Acidotropic dyes (such as Lysotracker Green), for instance, can label only 70% of intracellular vesicles, which might influence the results. Differences among cells and variety among size, shape, and surface chemistry of diamonds or their surface coatings add more uncertainty to this topic.

The internalization pathway defines the destination of FNDs. Upon entering the cell via endocytosis, the vesicles containing FNDs undergo rounds of fusions and fissions to form early endosomes. At this stage endosomes are initially sorted and their fate is defined. Ultimately, FNDs that do not escape from the vesicle can be recycled back to the plasma membrane, sent to the trans-Golgi network or to the lysosome for degradation. Prabhakar et al. reported two populations of FNDs in human breast adenocarcinoma cells. The group of aggregated particles (size up to 2 μm) was confined to the endosomes perinuclear area, whereas the dispersed nanodiamonds were found in lysosomes near the plasma membrane. Moreover, other authors have shown that during the proliferation process, FNDs were first localized near the nucleus and then equally redistributed among the two daughter cells. The fate of FNDs introduced into the cells by methods overcoming vesicle transport remains unknown.

Further studies are also necessary to unravel the possible mechanisms that govern FNDs-loaded vesicle trafficking occurring in the cytosol. It is known that the actin...
microfilaments and microtubules play a role in the transport of endosomes.\(^{160}\) It has been suggested that actin is involved in a short-range movement of small vesicles.\(^{161}\) The microtubules together with kinesin and dynein motors may be responsible for long-range transport of larger objects. It is difficult to confirm these observations based on a single particle tracking of organic molecules due to their photobleaching. For the standard probes, the trajectory length and the spatial resolution are limited to a few seconds and tens of nanometers, respectively. The FNDs seem to be much better labels for 3D tracking of vesicles and investigate mechanisms of their intercellular movement due to a high spatiotemporal resolution and contrast from the surrounding environment. The NV\(^{-}\) centers in FNDs have a fluorescence lifetime which is about 5 times longer than the autofluorescence of cell. Haziza et al.\(^{3}\) investigated endosomal trafficking with FNDs in synaptic terminals and found differences between healthy and diseased state of neurons. Hui et al. showed that surface functionalization has an influence on the diffusion rate of the nanoparticles in cytoplasm of the HeLa cells.\(^{162}\) 40 nm lipid-coated FNDs were moving 1 order of magnitude faster than the bare probes. Our group has made a similar comparison in the yeast cells.\(^{163}\) We found no significant difference in intercellular movement between lipid-coated and bare FNDs. Also nanodiamonds containing SiVs have already proven to be useful for tracking in cells.\(^{164}\) As mentioned earlier, these have the advantage that they emit in a narrow wavelength range in the near-infrared. Liu et al. incorporated their nanodiamonds into polypeptide chains to improve colloidal stability and then tracked their movement inside HeLa cells. Merson et al. used SiV centers for labeling in neural precursor cells.\(^{165}\) As expected, they observed that the nanodiamonds with SiVs were similarly biocompatible as FNDs with NV centers. The narrow emission wavelength allows efficient removal of background or differentiating the emission from other labels that are present in the sample. However, SiV centers are less bright than NV centers. In addition, the fabrication process is less established, and so far, there are no

Figure 5. (a) Overview of FNDs’ pathways within the cell. Images (1)–(4) show different approaches for artificial internalization of FNDs, while (5) represents internalization of FNDs through endocytic pathways. FNDs can remain in endosomes and follow the endocytic pathway to lysosome and the Golgi complex, escape to the cytosol, or be excreted. If FNDs are functionalized it is possible to direct them to different subcellular compartments such as nuclei and mitochondria. (b) Overview of FNDs destination throughout cellular processes, such as cell—cell communication (14), proliferation (15), migration (16), and trafficking inside cells (17). Representative literature can be found for each image: (1)\(^{11,142,143}\)(2) and (3)\(^{144}\)(4)\(^{145}\)(5)\(^{146}\)(6)\(^{147}\)(7)\(^{148}\)(8)\(^{149}\)(9)\(^{150}\)(10)\(^{151}\)(11)\(^{152}\)(12)\(^{153}\)(13)\(^{154}\)(14)\(^{155}\)(15)\(^{156}\)(16)\(^{157}\)(17)\(^{158}\).
nanoparticles that contain high concentrations of SiV centers. While this might be beneficial for some of the quantum sensing applications presented in NV Center-Based Sensing and Magnetometry, this is not desirable in labeling applications where high brightness is needed.

Even if the given examples and other excellent studies demonstrate that the first steps in 3D tracking with FNDs have been made with great success, this field still needs further development. It is necessary to control FNDs’ aggregation on the cell membrane and understand how aggregate size influences the tracking outcomes and FNDs’ destination. Better understanding of FNDs trafficking via endosomes requires co-localization with other biomarkers which still seems to be a challenge on the labeling and image processing levels. Another challenge is to understand what the recorded trajectories mean. So far only limited information has been extracted from FNDs tracking. Data from other nanoparticles give reasons for optimism that more information can be extracted from trajectories. Recently, Lin et al. applied nanodiamonds to find slow-proliferating/quiescent cancer stem cells in spheroids. This proves that FNDs may also contribute to imaging of 3D structures and therefore support further development of organ-on-chip technology.

Exocytosis of Diamond Nanoparticles. FND potentially could become long-term cell tracking markers both in vitro and in vivo. The exocytosis of FND was investigated in co-culture which could become long-term cell tracking markers both in vitro and further development of organ-on-chip technology. Contrast agent instead of widely used gadolinium nanoparticles, advantage over other

More Complex In Vivo Labeling. Besides the previously discussed intracellular labeling, FNDs can be used as probes to track the fate of therapeutics or cells in the organs of living organisms. Imaging in a tissue is challenging due to its high autofluorescence. The sub-nanosecond pulsed laser excitation and time-resolved detection of signal give the NV centers an importance for the future development of gene therapies. Lake et al. successfully targeted and visualized nucleoporin (Nup98) with FNDs. Nucleoporin plays a crucial role in regulating the transport of molecules to and from the nucleus. Morita et al. demonstrated targeting to the nuclear pore complex in yeast cells. There are also several articles on drug delivery, which demonstrate targeting but since there are no color centers involved in this process they are not discussed here in detail. The same challenge has to be addressed in order to visualize molecules or processes at the surface and inside mitochondria. Mkandawire et al. are so far the only researchers who have successfully labeled the “powerhouse” of cells using FNDs conjugated with antibodies. One could say that labeling of the cytosol content would be a much easier task than tagging specific cell compartments. However, targeting molecules in this molecularly crowded and highly dynamic space still remains a challenge. There are only a few studies that reported successful use of FNDs for that purpose. Morales-Zavala et al. functionalized FNDs with a bifunctional peptide that allowed sensitive in vitro detection of amyloid fibrils and aggregates in the cytoplasm of cells from Alzheimer’s disease mice brains. FNDs are also promising for protein and drug delivery into the cytosol. They stabilize and protect the molecules they carry, as well as enabling in situ visualization of their intracellular route. Su et al. introduced FNDs complexed with luciferase into the cytoplasm of human mesenchymal stem cells. Luciferase is an oxidative enzyme that produces bioluminescence. Apart from that it is worth mentioning research focused on targeting tissue and extracellular molecules. Fu et al. shed a new light on regulation of matrix metalloproteinase-2 (MMP-2) activity. The authors developed a type of glioma-specific FND complex with chlorotoxin-like peptide from scorpion venom to inhibit the cell invasiveness by blocking MMP-2 activity. Thus, they show simultaneous tracking and drug delivery. More Complex In Vivo Labeling. Besides the previously discussed intracellular labeling, FNDs can be used as probes to track the fate of therapeutics or cells in the organs of living organisms. Imaging in a tissue is challenging due to its high autofluorescence. The sub-nanosecond pulsed laser excitation and time-resolved detection of signal give the NV centers an advantage over other fluorescent probes used in vivo and ex vivo. FNDs also have a high potential to be applied as an MRI contrast agent instead of widely used gadolinium nanoparticles, which need surface modification to be permeable through the cell membrane and has confirmed cytotoxicity. First attempts to target and visualize biomolecules with FNDs in multicellular systems have used C. elegans, which is a model organism in developmental and cell biology. This small nematode is built up out of complex tissues and is optically transparent, which provides relatively simple fluorescence signal detection from NV centers. Kuo et al. used time-gating-based imaging to track movement of nanodiamonds conjugated with yolk lipoprotein complex toward an oocyte, over 6 h after injection into the distal gonad of C. elegans. Jones et al. used differential magnetic imaging to detect nanodiamonds within the gut of this model organism. FNDs have also been employed to investigate cytoplasmic movement in zebrafish embryos and another model organism, the fruit fly Drosophila melanogaster. Moreover, FNDs have been applied for in situ animal studies. Moscariello et al. showed that fluorescent nanodiamonds are able to cross the blood–brain barrier in healthy mice. The given examples provide
promising evidence for future nanotheranostic applications of FNDs in neurological diseases. Nanodiamonds with color centers have also captured the interest of researchers developing strategies in stem cell-based therapy for the treatment of human diseases. It has been shown that FNDs could provide information about the distribution of grafted cells and pharmacokinetics of drugs in various animal models. It has been shown that fluorescent nanodiamond labeling did not affect stem cell proliferation and differentiation. Su et al. 177 applied magnetic modulation for a tracking of FND-labeled mesenchymal stem cells in miniature pigs. Other researchers combined FNDs with fluorescence-activated cell sorting, fluorescence lifetime imaging microscopy, and immunostaining to identify and track engrafted lung stem/progenitor cells in mice. 185,186 The given examples and others summarized in Table 2 provide clear evidence that FNDs are appealing for pre-clinical and clinical trials. So far, FNDs have found application in clinical dentistry for root canal treatment. There are also already several studies which have demonstrated biocompatibility in vivo. 187–191 However, it is necessary to also keep long-term effects of nanodiamonds and their clearance pathways in mind. 192 This is especially important when new and less explored hybrid materials or surface-modified particles are considered.

While in vivo imaging has already been achieved several times, there are still challenges that need to be overcome. Generally, the smaller the particles the less bright they are. This poses a challenge for in vivo imaging, where background fluorescence is a severe problem. Using bright, large particles just partly solves this issue. Particles that are hundreds of nanometers in size do not penetrate biological material well. Also, if the labeling structures are very large, they deteriorate the spatial resolution. Furthermore, fluorescence imaging is generally strongly limited in terms of penetration depths into tissues. Despite emission in the near-infrared (the most transparent wavelength region for tissues) penetration of (green) excitation light probably is expected to be below 2 cm from the surface. So far, the only potential solution might be endoscopic systems where a fiber is inserted which provides optical access to deeper tissues.

There are already a few strategies that have been developed to improve background fluorescence. A possible solution is to first bleach the background fluorescence from tissues and only then image diamond particles. While background fluorescence usually disappears after illumination, the signal from color centers remains. However, this technique is obviously less practical for living samples. Igarashi et al. developed an elegant way to remove background, which makes use of the quantum properties of color center. In this approach it is utilized that there is a difference in brightness between spin states of the NV center (Figure 4i). 194 The spin polarization of color centers can be achieved by optical pumping with a green laser. Then the NVs are exposed to microwave radiation of 2.87 GHz (resonant with the energy gap between the NVs are exposed to microwave radiation of 2.87 GHz) to trigger decrease of the fluorescence intensity. Only the fluorescent spots that respond to the pulsing are attributed to diamond. The subtraction of the data between images acquired with or without microwaves results in high-resolution images of FNDs. A similar imaging strategy, which also exploits the spin properties of nanodiamond color centers, relies on the application of modulated magnetic field instead of microwaves. The magnetic field is used to create a mixture of |m_s = 0) and |m_s = ±1) sublevels in order to trigger a change in a fluorescence intensity of polarized spins. 198

Table 2. Summary of In Vitro and In Vivo Tracking Research

<table>
<thead>
<tr>
<th>FND size (nm)</th>
<th>coating</th>
<th>cell/organism</th>
<th>method</th>
<th>results</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 none</td>
<td>HeLa</td>
<td>epifluorescence, 3D SPT</td>
<td>tracking for 200 s, diffusion coefficient of D = 3.1 × 10^{-3} μm^2 s^{-1}</td>
<td>193</td>
<td></td>
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<tr>
<td>35 PEG–folic acid</td>
<td>HeLa</td>
<td>epifluorescence, 3D SPT</td>
<td>tracking for 370 s, diffusion coefficient of D = 2.0 × 10^{-3} μm^2 s^{-1}</td>
<td>113</td>
<td></td>
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<td>50 none</td>
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<td>proof-of-concept experiment for in vivo tracking of FNDs in animal blood vessel</td>
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## FRET WITH DIAMONDS

Förster resonance energy transfer (FRET), is a tool to determine distances between (parts of) molecules very accurately. It is based on a nonradiative dipole–dipole interaction between two molecules. It has become an efficient
and important tool for studying biological interactions. When two molecules are close to each other, one molecule as donor transfers its energy to the other receptor molecules. Therefore, the fluorescence intensity and lifetime of the donor decrease. Fluorescent nanodiamonds containing NV$^-$ centers as fluorophores are leading candidates for FRET donors due to their excellent photostability, high biocompatibility, and long fluorescence lifetime.

Several different receptors have been combined with FND donors in FRET. Mohan et al. first reported that sub-20-nm FNDs are useful as far-red fluorescent donors with a FRET efficiency around 7%. Tisler et al. observed a highly efficient energy transfer (86%) between a single NV center and a single quencher. These findings pave the way toward FRET-based scanning techniques using NV donors. Alghannam et al. proposed the creation of shallow layers of NV centers in bulk diamond. By using the FRET technology, they successfully proved that most of NV centers are within 3.6 nm from the diamond’s surface. Börsch et al. developed FND-based FRET technology to better understand the details and mechanism of the rotary motions and elastic energy storage of a single biological nanomotor F0F1-ATP synthase.

There are still some problems to be solved before FNDs will be widely applied in FRET. The size is a vital parameter to characterize. It will determine the mobility and movement range of FNDs (inside a cell) and the potential of alerting the properties of target molecules or environment. Besides, the smallest detectable distance is also restricted by the depth of NV centers to the surface of nanodiamonds. So far, the large-scale production of size-controlled smaller and brighter FNDs remains challenging.

### NV$^-$ CENTER-BASED SENSING AND MAGNETOMETRY

Color centers allow sensing in many ways. While the previous sections discussed methods which rely solely on detecting the fluorescence of color centers, we will here review techniques and applications which rely on their spin properties. Among known color centers in diamond, the negatively charged nitrogen vacancy center (NV$^-$) is currently the most widely used and successful system for sensing purposes. In this section we will first discuss its spin properties. Then we will introduce optically detected magnetic resonance (ODMR) and relaxometry-based sensing of (electron) spins. The last part of this section treats nanoscale nuclear magnetic resonance based on color centers. Although they are very similar in principle, nuclear spin sensing is more challenging, since they cause smaller signals than electron spins. In the literature, the acronyms ESR (electron spin resonance) and EPR (electron paramagnetic resonance) are sometimes used to refer to the detection of the NV$^-$'s own electron spin, as well as the detection of external electron spins via the NV$^-$ center’s fluorescence. In our discussion, we will use the term EPR for the detection of external electron spins using the NV$^-$ center as a probe. Probing the electron resonance of the NV$^-$'s own electron can also transduce physical information, we will refer to this technique as ODMR (optically detected magnetic resonance) and discuss its merits later on in this section.

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**Figure 6.** (a) Atomic structure of the NV$^-$ center. (b) Energy level diagram of the NV$^-$ center indicating fluorescent and non-fluorescent transitions. A typical ODMR spectrum at zero magnetic field and non-zero magnetic field is displayed in the inset. (c) The axis of the defect is aligned along the [111] or an equivalent crystallographic direction. (d) Detailed diagram of the NV$^-$ electronic ground state indicates the zero-field splitting $D$, Zeeman coupling, and hyperfine interactions.
Finally, we will discuss hybrid materials where diamonds are coated with responsive materials, which increase the signal from the environment.

**Introduction of Magnetic Properties of NV Centers.** The key features of the NV− center are its optically detectable and controllable spin states, which have long coherence times at room temperature. Its structure is depicted in Figure 6a, while Figure 6c shows how NV centers can be positioned with respect to the diamond lattice. The first of these properties let us distinguish between the \( \text{m}_\text{s} = 0 \) state, which yields bright fluorescence, and the \( \text{m}_\text{s} = \pm 1 \) state, which is relatively dark due to an alternative, non-fluorescent decay route. On top of that, the “dark” decay route preferably repopulates the bright \( \text{m}_\text{s} = 0 \) state, allowing for optical spin polarization as well. By applying (pulsed) microwave radiation resonant with the \( \text{l}_\text{m} = 0 \) \( \rightarrow \text{l}_\text{m} = \pm 1 \) transition, the NV− spin states can be controlled further with high fidelity. These properties are the basis of various sensing modalities, ranging from qualitative imaging to sensitive quantitative detection of chemicals. The NV− structure and energy level diagram are depicted in Figure 6.

Magnetic resonance techniques, including electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) spectroscopy, are among the most versatile techniques for chemical analysis. They provide non-invasive and non-destructive options for this purpose. However, typical instrumentation requires large samples with macroscopic numbers of spins in order to provide sufficient signal strength.  

Detecting magnetic resonances with an atomically sized probe with optical readout is highly promising for improving the sensitivity and resolution of these methods.

Although there is an active ongoing search for color centers in semiconductors, this search is mostly motivated by the poor efficiency at which the NV− center generates spin-entangled photons, which are desirable for quantum network research.

**Optically Detected Magnetic Resonance.** As mentioned earlier, ODMR refers to a technique where the magnetic resonances of the NV center itself are read out (in contrast to applications where the NV center is used to read out the magnetic resonance of an external spin in a sample which is discussed in Nuclear Magnetic Resonance Spectroscopy). Figure 6b shows the outcome of a typical ODMR measurement in the top-right inset. At zero field (black curve), there is just one dip in fluorescence which corresponds to the difference between \( \text{m}_\text{s} = 0 \) and \( \text{m}_\text{s} = \pm 1 \), since at zero field \( +1 \) and \( -1 \) are equal in energy. If there is a magnetic field \( \text{B} \) applied, the two transitions are not equal anymore due to the Zeeman effect. This is shown in the orange curve in Figure 6, as well as the detailed energy level structure. The difference between two dips is proportional to the magnetic field.

Although the ODMR sequence measures the magnetic resonance of the NV center it can reveal information on the sample because there are several properties which influence the ODMR signal. Among these properties are magnetic fields, temperature, or the spatial orientation.

The most obvious application of ODMR is sensing of magnetic properties. This has been done for several different magnetic nanoparticles. Le Sage et al. used ODMR measurements to map the magnetic fields around magnetotactic bacteria that can produce and store magnetic nanoparticles.  

To this end, they used a bulk diamond with a dense ensemble of NV− centers and measured the magnetic fields produced by the magnetic particles in living bacteria.

Another important application of ODMR is temperature sensing. While this technique is not used for chemical sensing yet, researchers aim to detect temperature changes caused by chemical reactions in the future.

Another property that can be utilized is the fact that NV− centers detect the projection of the \( \text{B} \)-field vector along the NV axis. As a result, the ODMR contrast is highest if the NV center is aligned with the field. If the NV center or magnet is turned with respect to each other the contrast decreases. Thus, it is possible to determine for instance the rotation of a nanodiamonds containing NV centers. McGuinness et al. presented the proof-of-principle experiments where researchers applied either a magnetic field or microwaves to track the orientation of 50 nm FNDs in HeLa cells. This was a significant milestone, which opened the door to the orientation tracking of FNDs based on fluorescence detection and control of spins polarization state inside living cells.

**Electron Spin Detection.** \( T_1 \) Relaxometry for Spin Sensing. Relaxometry is a relatively simple sensing scheme that can be performed without the assistance of microwave radiation, which is an important advantage when studying aqueous samples (almost all the biological samples). It measures the rate at which the initially spin-polarized NV− center decays into its equilibrium population of magnetic states. The inverse of this relaxation rate is the longitudinal relaxation time \( T_1 \). Relaxometry is sensitive to magnetic noise, which can come from diffusion or flipping of any non-zero spin. Since electron spins have high magnetic moments compared to nuclei, the signal is dominated by electron spins if they are present. To perform a relaxometry (or \( T_1 \)) experiment, only a pulsed laser is essential. This laser is used to pump the NV centers to the (bright) ground state. After a varying dark time, another laser pulse is applied to probe whether the NV center is still there and to repolarize the spin. In the presence of fluctuating magnetic fields, redistribution of the spin population to equilibrium occurs faster. The relaxation time gives a quantitative measure that can be related to the conditions of the environment, such as the amount of spins present in the surrounding of the NV center. Figure 7 displays a schematic of the principles of \( T_1 \)-based sensing and spectroscopy.

Tetienne et al. demonstrated relaxometry-based sensing of gadolinium. They used nanodiamonds of different sizes to detect gadolinium (a standard spin label) in solution. In their early work they demonstrated that relaxometry is suitable to quantify spin labels. Ziem et al. also detected gadolinium as well as manganese and ferritin (a protein with an iron core). However, they used a different sensing scheme. In their work a microfluidic device was used which was microfabricated into a bulk diamond. This scheme is more demanding to fabricate but has the advantage that color centers in bulk diamond have longer relaxation and coherence times. Ermakova et al. also demonstrated detection of ferritin with relaxometry in the same year. Recently, this method was applied even within cells. In their study Wang et al. used again a different setup. They investigated dead cells that were embedded in a resin (as used routinely for electron microscopy). These cells were attached to a tuning fork, which was scanned over the diamond surface. In their work they were able to visualize ferritin within these cells.
show that they can differentiate between dendritic cells from human donors which were differently aggressive.

**EPR Spectroscopy.** $T_1$ relaxometry can also be performed in a spectroscopic mode, in which the relaxation rates are recorded at different magnetic field strengths.\(^{219}\) When the external magnetic field is increased, the frequency of the $l m_s = 0$ transition of the NV center’s electron decreases, until it comes into resonance with the $l m_s = -1/2$ transition of an external electron of the sample at 512 G (see Figure 7). In this situation, both electronic systems are able to exchange energy rapidly, leading to a steep reduction of the relaxation time $T_1$. Using this method, the spectral density of spin distributions may be reconstructed.

Finding the resonance condition with an external magnetic field may also be used to optimize contrast in $T_1$ imaging and may be extended to meeting the condition for cross-relaxation with nuclear spins.

As featured in Figure 7, NV$^-$-based EPR spectroscopy results in the detection of the electron spin through cross-relaxation, which occurs at external magnetic fields around 512 G, due to the electron g-factor of 2.\(^{220}\) Obtaining chemically relevant information requires that the technique is able to resolve small differences in similar g values. Fortman et al. reported NV-EPR using pulsed double electron–electron resonance (DEER) at high magnetic fields to resolve the EPR spectrum of nitrogen impurities (P1 centers) in the diamond.\(^{221}\) Since such an experiment has yet to be demonstrated with spins external to the diamond, this poses an open challenge in the field.

**Nuclear Magnetic Resonance Spectroscopy.** Nuclear magnetic resonance has revolutionized chemistry and medicine in the 20th century. Extending the utility of this technology to the nanoscale is of great interest for the study of microscopic samples, which cannot be analyzed using conventional coil-based equipment. Using the nitrogen vacancy center in diamond as a spin sensor presents a promising pathway toward achieving this goal.

Unlike macroscopic NMR, the scope of the field that develops the detection methods for nuclear spins and their quantum state has large overlap with the development of quantum information processing techniques. In this section of our Review, we will discuss the progress in NV$^-$-based detection of nuclear species viewed in the perspective of its future value for chemical analysis. We will discuss the principles, challenges pertaining to future applications, progress in the field, and an outlook on mitigating current roadblocks.

**Principles: Detecting Nuclear Moments with Multi-pulse Sequence.** Magnetometry using the NV center relies on the detection of the optical contrast between its electronic spin states, indicated as $l m_s = 0$ (bright) and $l m_s = \pm 1$ (dark). The presence of nuclear magnetic fields is typically probed through their influence on the decoherence of the superposition $|0\rangle + i|1\rangle$. The evolution of the state’s phase factor is dependent on external magnetic influences. With fast and powerful microwave pulses, the electronic spin state can be brought into the superposition state and rotated along the Bloch sphere according to designed sequences (dynamical decoupling sequences). Each pulse of the dynamical decoupling sequences induces a rotation over $\pi$ of the state vector. As a result, the phase accumulated over time by magnetic field components before the $\pi$-pulse is negated after

Figure 7. EPR using NV centers in diamond. (a) A probe NV center interacts with the surrounding spins on the surface and external to the diamond. An external magnetic field can be applied to tune the Zeeman splitting of the NV$^-$ electronic spin and surrounding single electronic spins to the resonance condition, facilitating cross-relaxation and reducing the longitudinal relaxation time. (b) Measurement of longitudinal relaxation time $T_1$. (c) Longitudinal relaxation time as a function of external B field. (d) Transition frequency for the NV center (blue) and a single electron (orange) as a function of the external magnetic field.

Apart from chemical sensing, relaxometry is also useful to characterize magnetic properties of materials.\(^{212,213}\) For instance, Schmid-Lorch et al. used this method to characterize iron oxide nanoparticles.\(^{214}\) In their article they attached the nanoparticles on the tip of an atomic force microscope probe. Then they recorded $T_1$ while they moved the tip over an NV center in a bulk diamond.

It has also been shown that relaxometry measurements are influenced by electrical conductivity of a material nearby.\(^{215}\) In their proof of principle study Ariyaratne et al., who used a scanning magnetometry device showed that they can read out changes in conductivity from a nearby metal structure.

Barton et al. used relaxometry to detect the chemical redox state as well as radicals.\(^{118}\) They used a sensitive coating with spin labels. These compounds are converted into stable radicals when they react with radicals in the sample. As a result, the $T_1$ decreases.

Recently, relaxometry was applied to measure free radical generation in aging cells.\(^{216}\) This is the first time that NV centers were used to detect metabolic activity in living cells with nanoscale resolution. The authors used nanodiamonds containing ensembles of NV centers, which were introduced into living cells. They were able to clearly differentiate between genetic mutants. Additionally, relaxometry enabled them to follow the aging process and to assess the effects of anti-aging drugs or chemical stress factors. The same concept has later been used to measure free radical generation in mitochondria of macrophages (immune cells).\(^{217}\) The authors were able to direct nanodiamonds to the mitochondrial surface by coating them with antibodies. Additionally, they demonstrated relaxometry from single isolated mitochondria for the first time. Recently, also the first measurements in primary cells from donors have been conducted.\(^{218,219}\) Nie et al. were able to...
the pulse. Only when the external field components change sign at the same rate as the pulse repetition, will they drive the electron spin away from its initial state. The resulting phase can be extracted by projecting the state back onto \( |0\rangle \) or \( |1\rangle \) with a \( \pi/2 \) rotation and observing the fluorescence contrast. Thus, when the pulse repetition matches \( 2\omega_0 \) (double Larmor frequency), a signal is observed, which shifts proportionally to a weak external bias field. The manipulations and evolution of the Bloch state vector are illustrated in Figure 8.

Dynamical decoupling sequences thus serve a two-fold purpose: they protect the initial state from decoherence and serve as a band-pass filter that selects a specific frequency to which the NV becomes sensitive. The method described above can be considered the canonical method used to measure the NMR fingerprints of nuclei on the nanoscale.

\( T_1 \)-Based NMR. Alternatively, \( T_1 \)-based NMR can be performed without the necessity of microwave pulsing sequences. In this method, nuclear spins can be detected through mutual suppression of the longitudinal relaxation time \( T_1 \) of the NV electronic spin, provided that the Zeeman splitting is increased to approach the ground-state level anticrossing (GSLAC). This method was demonstrated by Wood et al. for the detection of protons in immersion oil.228 Although this method is promising for cases in which microwave manipulation is difficult or undesirable, its spectral resolution is limited by the NV dephasing time \( T_1^* \) rather than \( T_2 \). This makes further improvement a matter of surface engineering and may impose a limit on optimal performance.

Developments. The current progress in NV-based nanoscale NMR has evolved from early demonstrations of highly sensitive spin detection with NV centers, in which decoherence and dephasing measurements record the presence of spins external to the diamond.229–231 Building upon these results and methods, actual measurement of the nuclear gyromagnetic ratios of external H atoms were first demonstrated in 2013.232,222

The possibility of spectrally discriminating different nuclei in a widefield setup was shown by DeVience et al.228 Widefield setups provide the advantage of a larger sensing volume and field of view, without the need to select an optimal NV center. This comes at the cost of signal-to-noise ratio, which suffers from the fluorescent signal emitted by NV centers not aligned with the bias field (and thus not contributing to the signal), as well as magnetic field inhomogeneities over the surface. Despite these compromises, it was shown that sufficient sensitivity could be achieved to spectrally identify \(^1\)H, \(^19\)F, and \(^31\)P nuclei.

Improving the detection sensitivity in NV NMR is limited by the photon detection efficiency, readout fidelity, and coherence time. The first two determine the number of sequence repetitions required in order to distinguish between the bright and dark spin states. The latter limits the meaningful interaction time between the sensor and the sample. In short, it is key to extract information as efficiently as possible from the sample within the ultimate time limit imposed by the NV’s coherence time.

Using NV ensembles can also be a method for enhancing the signal and sensitivity, as was shown in ref 232. Nanoscale grating was etched into the diamond in order to maximize the contact between the sample and the NV centers. In this configuration, minimum detectable concentrations were as low as \((6–11) \times 10^{-24} \) \(^{19}\)F spins per liter.

Lovchinsky et al.224 demonstrated a method for improving the sensitivity of NMR detection of single proteins by making use of an ancillary nuclear spin belonging to the \(^{15}\)N nucleus of the NV center probe.233 By coupling the ancillary nuclear spin to the NV’s electron spin, the information contained in the electronic spin state after applying the multipulse sequence can be stored in the neighboring nuclear spin as longer lasting memory. In doing so, the state can be read out repetitively, without the information being lost by repolarization of the NV electron spin after exciting the fluorescent transition. This method allows for a sharp reduction in sequence repetitions needed to properly distinguish the \( m_n = 0 \) and \( m_n = 1 \) states.

**Nanodiamond NMR.** A requirement for performing dynamical decoupling NMR measurements is the alignment of an external B field with the symmetry axis of the NV center. Furthermore, high crystal quality and surface cleanliness are necessary to extend the coherence times of the NV center as much as possible. Neither of these can be easily achieved when using NV centers in nanodiamonds, since their material purity and surface controllability are inferior to

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**Figure 8.** Bloch sphere representation of (NV electron) spin evolution and manipulations and different NMR setup configurations demonstrated in the literature. (a) Preparation of superposition with \((\pi/2)\) pulse. (b) Phase accumulation during free precession. (c, d) \( \pi \) and \( \pi_x \) rotations redirecting the phase accumulation toward the initial state. (e) Proton/multispecies NMR detection with single NV.8,222,223 (f) Single molecule/protein NMR detection.224 (g) Wide-field/NV ensemble NMR.225,226 (h) Nanodiamond NMR.227
those of bulk diamonds. Regardless of the difficulties, nanodiamonds are of great interest because of their ability to reach otherwise inaccessible places such as a cell’s interior and to be traced with high spatial resolution. The possibility of performing NMR spectroscopy with an NV center in nanodiamond was demonstrated by Holgrafe et al. 227 By immobilizing the nanodiamonds in a perfluoroalkoxy polymer (PFPE) coating and using a magnet with two rotational degrees of freedom, the external field could be aligned with a randomly oriented NV center. The hydrogen signal in the NMR measurement was attributed to the nanodiamond surface, as the analyte should be free of hydrogen atoms. This way, the H signal was used as a calibration for estimating the particle shape, which is highly variable, but important for inferring analyte properties from the NMR signal.

The constraining conditions of this demonstration show that applications like intracellular NMR are not in reach for the time being. However, several valuable steps in coherent spin control in nanodiamond have been taken in recent years that were deemed unfeasible in the past. These include the development of low-power dynamical decoupling sequences 234 and coherent control of nuclear spins in nanodiamond. 235

Sensors Based on \( T_2 \) for Unlimited Spectral Resolution.

The power of NMR hinges on its spectral resolution, allowing us to not only derive chemical composition of a sample, but also structural information from, e.g., chemical shifts and \( J \)-coupling. The spectral resolution of NV-based NMR is limited by the time during which the state of the quantum sensor evolves coherently under influence of the sample’s magnetic field. With the dynamical decoupling methods described before, the spectral resolution is ultimately limited by the NV’s coherence time \( T_2 \). 236 Being in the order of milliseconds, spectra recorded this way will not have line widths below the order of kHz, being insufficient for resolving chemical shifts and finer spectral information. In the past 5 years, the field has advanced from the basic measurement method described before to develop several new techniques that surpass this limitation.

One of these techniques is the concatenation of subsequent dynamical decoupling measurements by synchronizing these measurements with an external clock. 237, 238 Instead of averaging multiple measurements that are all limited in spectral resolution by \( T_2 \), synchronization with the external clock combines subsequent “stroboscopic” measurements to reconstruct the signal as if recorded over the full sampling duration. With this method, only the external clock’s stability and available sampling time impose an intrinsic limit on the achievable frequency resolution.

A correlation spectroscopy method can also be used to circumvent the NV’s intrinsic coherence time limit. 239 In this method, two consecutive decoupling sequences record the nuclear spin states, separated by a waiting time \( t \). During this waiting time, the nuclear spins undergo free precession, which correlates the two measurements, and a signal equivalent to the nuclear free induction decay can be recorded. Instead of the coherence time, it is now the longitudinal relaxation time \( T_1 \) of the sensor that limits the sampling time (and therefore the spectral resolution). However, the spectral resolution can be improved further by making use of long-lived nuclear spin states as memory. 240 This method was applied as basis for probing molecular dynamics 241 and to perform two- and three-dimensional localization of nuclear spins inside a diamond crystal. 237, 242, 243

Most notably within the scope of this Review are the works by Aslam et al. 244 and Glenn et al. 245 Although these methods have not been integrated with NV NMR yet, improvement of state-of-the-art NV NMR protocols with nuclear hyperpolarization has been demonstrated by Bucher et al. 246 Polarization from TEMPOL radicals was transferred to proton samples through Overhauser dynamic nuclear polarization, while the spectrum was recorded using the CASR protocol mentioned before. This way, the signal magnitude was enhanced by 2 orders of magnitude.

### CONCLUSION

Diamond color centers have emerged as powerful tools in chemical and biochemical analysis. Among the most remarkable properties are the excellent biocompatibility and unique stability. These are ideal properties for biolabels. Especially when long-term labeling is required or it is desired to see the labels with many different methods, nanodiamonds outperform conventional probes. However, despite their promising properties, their use in biology is so far limited. For many applications conventional labels are good enough, and thus there is little drive to change. For other applications their relatively large size, large size distributions, irregular shapes of particles, and their surface chemistry and relatively low brightness are still major challenges. Additionally, tuning and precisely controlling the surface chemistry is crucial.

The possibility to perform quantum sensing with color centers is unique and potentially very useful. NV centers allow extremely sensitive measurements with nanoscale spatial resolution. Unlike other systems they work at room temperature and under ambient conditions. With this system it is possible to detect spin labels or paramagnetic species as free radicals. By using responsive coatings, it is now possible to achieve specificity for chemicals which would otherwise not be accessible (as for instance H⁺ in pH sensing). Making use of the versatile tools chemists have developed, it is likely that this area will expand substantially in the near future.

The first groundwork has been laid to use quantum sensing to detect chemical reactions within living cells or even entire organisms. However, the presented applications are all in their infancy. Many of the articles that have been discussed here have a proof-of-principle character, and the full potential of the technology still has to unfold in various applications. Since many of the presented techniques offer unique datasets, validation is often tricky. For quantum sensing applications heterogeneity of particles/defects is even more severe than in biolabeling and thus poses a major challenge for material scientists to optimize diamond materials.

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The authors declare no competing financial interest.

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Claudia Reyes-San-Martin completed her bachelor’s degree in medical technology with a major in morphophysiology and cytodiagnostics at the University of Chile. She is currently a Ph.D. candidate in Prof. Romana Schirhagl’s group at the University Medical Center of Groningen, where she studies the use of fluorescent nanodiamonds for biomedical applications.

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