Intracellular Relaxometry, Challenges, and Future Directions

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1. INTRODUCTION

Beyond their use in jewelry, diamonds are used as abrasives and drilling materials for their hardness. Protected within an inert crystal lattice, specific defects are present or can be engineered. These so-called color centers do not bleach or blink and are infinitely photostable. Hundreds of color centers are described in diamonds. The nitrogen vacancy (NV) defect, the most studied type, consists of a nitrogen atom with a vacancy next to it. NV centers exhibit bright fluorescence from 550 to 800 nm. Nanodiamonds (NDs) with these color centers are attractive for long-term fluorescent imaging as well as super-resolution microscopy. NV centers usually exist in neutral NV0 and negatively charged NV− forms. The NV− is used in quantum-sensing applications to detect external electromagnetic fields on the basis of the optical readout. The fluorescence intensity is dependent on its magnetic environment. Since fluorescence detection is very sensitive, this method even allows one to record the signals of single electrons or a few nuclei.

NV centers allow one to investigate magnetic nanostructures, domain walls in magnetic structures, paramagnetic ions in solution, spin-labeled molecules, or proteins with metallic parts. They can also be used to increase the sensitivity of biosensors. Since their magnetic resonance is very temperature sensitive, NV centers can be used to detect temperature changes in the milli-Kelvin range with nanoscale resolution. Recently, NV centers have been used for measurements in cells. Davis et al. imaged spin-labeled slices of fixed cells, while McGuinness et al. measured the orientation of a particle within a cell. Later, even measurements of metabolic activity have been achieved in live yeast cells, immune cells, and sperm cells during the particle’s transport inside the cell, or during viral infection. The first relaxometry measurements in primary cells from donors have been demonstrated. Here, we discuss the current limitations of this method and the future direction this exciting field might take.

2. THE RELAXOMETRY PRINCIPLE

The NV center has three electronic states available to its six electrons: a triplet ground state, a triplet excited state, and a singlet metastable state. Each of the triplet states, in turn, has three spin sublevels: the degenerate \( m_S = \pm 1 \) sublevels and the \( m_S = 0 \) sublevel. By default, the electrons are in a thermal equilibrium between the \( m_S = \pm 1 \) and the \( m_S = 0 \) sublevels of the ground state. In a relaxometry experiment, the NV centers are first pumped into the bright \( m_S = 0 \) state and then left to relax into the (darker) natural stochastic combination of \( m_S = 0 \) and \( m_S = \pm 1 \). The relaxation happens faster in the presence of external magnetic noise from unpaired electrons of free radicals or spin labels (see Figure 1(2)). The current state of
the NV center can be read out by its fluorescence intensity. This approach allows one to perform the sensing, using only the optical means. It can be implemented in a confocal microscope-like setup if one can make the excitation laser pulse.

3. CURRENT LIMITATIONS AND FUTURE PROSPECTS
A summary of future prospects that we expect is given in Figure 2.

3.1. Other Defects or Materials. So far, only NV centers have been used for quantum sensing in living cells. Currently, one of the biggest challenges of using NV centers is reproducibility from one particle to the next. One way to achieve this goal is to use large ensembles of NV centers. However, since it is a necessity that the NV centers are close to the diamond surface and thus to the sample, the number of NV centers that can be used is limited. At the same time, there are a few color centers that have been proven useful in physics. However, in biology, they need to operate at room temperature and be stable in NDs in a high enough concentration. The latter problem might be resolved in the future. Due to the autofluorescence of biosamples, it is preferable to have emission in the near-infrared region, the so-called biological window. Besides defects in diamonds, also entirely different materials, might emerge in the future, potentially hosting color centers. Interesting candidates are other wide bandgap semiconductors like silicon carbide or gallium arsenide. Also, molecular systems might prove to be valuable.

3.2. ND Internalization into Cells. Fortunately, NDs are exceptionally biocompatible in cells, in animal testing, and even in humans. Many cells can easily take up nanoparticles via different pathways. By functionalizing the surface of NDs, it is also possible to change the uptake pathway and efficiency. The uptake is usually impeded, if the cell is small (bacterial cells) or has a pronounced cell wall (bacterial, yeast, plant cells). Certain mammalian cells (e.g., neurons or some epithelial cells) also do not internalize nanoparticles easily. The uptake can be forced by more invasive procedures such as electroporation, microinjection, and chemical transformation. While these approaches generally increase the uptake rates, they can harm the cells. Moreover, the intracellular fate of NDs can differ, depending on the exact internalization procedure.

3.3. Controlling the Intracellular Location. The full potential of the technique can only be used if we know or even control where the measurement takes place in the cell. (6) In order to collect an optical signal from a diamond, one needs optical access. This is a serious challenge for thick, pigmented, or highly refractive samples. The application that is shown is a measurement in a skin tissue or on the intact skin of a patient.
generally encapsulated in intracellular vesicles: endosomes or phagosomes. These vesicles eventually fuse with lysosomes, and the majority of NDs ultimately exit the vesicles through endosomal escape, generally not entering the nucleus or other organelles. The timing and efficiency of the endosomal escape depends on the cell type and shape of the NDs, among other factors. "Prickly" NDs have a higher chance of escaping the endosomes. Another approach to increase endosomal escape is to functionalize the ND surface with cationic polymers causing rupture of the vesicles and releasing the cargo into the cytoplasm. Lastly, the vesicular compartment can be bypassed using nonendocytic uptake protocols (electroporation, microinjections, chemical transformation). In certain cases, one might want retention of the NDs in the vesicular compartment. More rounded as well as larger ND particles can increase the endosomal retention.

A number of approaches have been used to achieve targeting of the NDs with intracellular localization sequences or antibodies. One challenge is to preserve the functionality of the targeting moiety, as it is exposed to the complex mixture of proteins and salts, changing pH of the endolysosomes, and intracellular enzymes.

A different approach is to deduct the properties of the ND’s environment from the way the particles moved during the experiment. It is possible to combine single-particle tracking and trajectory analysis with $T_1$ measurements to get a map of $T_1$ values as the ND moves through the cell. This approach requires a complicated analysis of the trajectories, as NDs move in complex patterns through a highly nonuniform intracellular environment.

### 3.4. More Complex Samples

While measurements in isolated living cells have been successfully performed, there are other interesting, more complex samples. While in cultured cells optical access is easily achieved, this is challenging in thick tissues or large organisms. In ex vivo studies, individual cells can be extracted from a tissue sample and cultured. However, many processes can only be studied when cells are in their biological context. These include biodistribution, clearance by the body, or certain processes in disease progression. A further challenge is to know where the diamond particle is within a complex sample (e.g., in the cell of which type) and to provide the biological context for the measurements. To study some interesting phenomena, one might need to measure deep within the body. One solution is to provide optical access via an optical fiber with NDs or macroscopic diamonds attached.
Relaxometry is a versatile technique with distinct advantages including nanoscale resolution, real-time measurements, and sensitivity for free radicals.

4. CONCLUSIONS

Relaxometry is a versatile technique with distinct advantages including nanoscale resolution, real-time measurements, and sensitivity for free radicals. The method is also all-optical and thus relatively straightforward to implement. In the future, we anticipate new directions in the field, including an extension to more complex samples. We expect that in the future relaxometry data will be further correlated with other biological information as well as data from other single-cell techniques.

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Notes
The authors declare no competing financial interest.

REFERENCES

(1) Gruber, A.; Dräbenstedt, A.; Tietz, C.; Fleury, L.; Wrachtrup, J.; von Borczyskowski, C. Scanning Confocal Optical Microscopy and Magnetic Resonance on Single Defect Centers. Science 1997, 276 (5321), 2012–2014.
(2) Kurtsiefer, C.; Mayer, S.; Zarda, P.; Weinfurter, H. Stable Solid-State Source of Single Photons. Phys. Rev. Lett. 2000, 85 (2), 290–293.
(3) Aharonovich, I.; Castelletto, S.; Simpson, D. A.; Su, C.-H.; Greenleaf, A. D.; Prawer, S. Diamond-Based Single-Photon Emitters. Rep. Prog. Phys. 2011, 74 (7), 076501.
(4) Raman Nair, S.; Rogers, L. J.; Vidal, X.; Roberts, R. P.; Abe, H.; Ohshima, T.; Yatsui, T.; Greenleaf, A. D.; Jeske, J.; Volz, T. Amplification by Stimulated Emission of Nitrogen-Vacancy Centres in a Diamond-Loaded Fibre Cavity. Nanophotonics 2020, 9 (15), 4505–4518.
(5) Hemelaar, S. R.; de Boer, P.; Chipaux, M.; Zuidema, W.; Hamoeh, T.; Martinez, F. P.; Nagl, A.; Hoogenboom, J. P.; Giepmans, B. N. G.; Schirhagl, R. Nanodiamonds as Multi-Labeling Labels for Microscopy. Sci. Rep. 2017, 7 (1), 720.
(6) Vicidomini, G.; Moneron, G.; Han, K. Y.; Westphal, V.; Ta, H.; Reuss, M.; Engelhardt, J.; Eggeling, C.; Hell, S. W. Sharp-Low-Power STED Nanoscopy by Time Gating. Nat. Methods 2011, 8 (7), 571–573.
(7) Karaveli, S.; Gaathon, O.; Wolcott, A.; Sakakibara, R.; Shemesh, O. A.; Peterka, D. S.; Boyden, E. S.; Owen, J. S.; Yuste, R.; Englund, D. Modulation of Nitrogen Vacancy Charge State and Fluorescence in Nanodiamonds Using Electrochemical Potential. Proc. Natl. Acad. Sci. U. S. A. 2016, 113 (15), 3938–3943.
(8) Grinolds, M. S.; Hong, S.; Maletinsky, P.; Luan, L.; Lukin, M. D.; Walworth, R. L.; Yacoby, A. Nanoscale Magnetic Imaging of a Single Electron Spin under Ambient Conditions. Nat. Phys. 2013, 9 (4), 215–219.
(9) Mamin, H. J.; Kim, M.; Sherwood, M. H.; Rettnier, C. T.; Ohno, K.; Awichalom, D. D.; Rugar, D. Nanoscale Nuclear Magnetic Resonance with a Nitrogen-Vacancy Spin Sensor. Science 2013, 339 (6119), 557–560.
(10) Cuija, K. S.; Boss, J. M.; Herb, K.; Zopes, J.; Degen, C. L. Tracking the Precession of Single Nuclear Spins by Weak Measurements. Nature 2019, 571 (7764), 230–233.
(11) Juraschek, D. M.; Meier, Q. N.; Trassin, M.; Trolier-McKinstry, S. E.; Degen, C. L.; Spaldin, N. A. Dynamical Magnetic Field Accompanying the Motion of Ferroelectric Domain Walls. Phys. Rev. Lett. 2019, 123 (12), 127601.
(12) Perona Martínez, F.; Nusantara, A. C.; Chipaux, M.; Padamati, S. K.; Schirhagl, R. Nanodiamond Relaxometry-Based Detection of Free-Radical Species When Produced in Chemical Reactions in Biologically Relevant Conditions. ACS Sens. 2020, 5 (12), 3862–3869.
(13) Ermakova, A.; Pramanik, G.; Cai, J.-M.; Alagara-Siller, G.; Kaiser, U.; Weil, T.; Tzeng, Y.-K.; Chang, H. C.; McGuinness, L. P.; Plenio, M. B.; Nayanov, B.; Jelezkov, F. Detection of a Few Metallo-Protein Molecules Using Color Centers in Nanodiamonds. Nano Lett. 2013, 13 (7), 3305–3309.
Biocompatible Fluorescent Nanodiamonds. Sci. Rep. 2015, 4 (1), 5004.

(46) Zhang, Y.; Sharmin, R.; Sigaeva, A.; Klijn, C. W. M.; Mzyk, A.; Schirhagl, R. Not All Cells Are Created Equal — Endosomal Escape in Fluorescent Nanodiamonds in Different Cells. Nanoscale 2021, 13 (31), 13294—13300.

(47) Pang, Y. T.; Ge, Z.; Zhang, B.; Xiu, P.; Li, Q.; Wang, Y. Pore Formation Induced by Nanoparticles Binding to a Lipid Membrane. Nanoscale 2020, 12 (14), 7902—7913.

(48) Chu, Z.; Zhang, S.; Zhang, B.; Zhang, C.; Fang, C.-Y.; Rehor, I.; Cigler, P.; Chang, H.-C.; Lin, G.; Liu, R.; Li, Q. Unambiguous Observation of Shape Effects on Cellular Fate of Nanoparticles. Sci. Rep. 2015, 4, 4495.

(49) Zhang, X.-Q.; Chen, M.; Lam, R.; Xu, X.; Osawa, E.; Ho, D. Polymer-Functionalized Nanodiamond Platforms as Vehicles for Gene Delivery. ACS Nano 2009, 3 (9), 2609—2616.

(50) Hazira, S.; Mohan, N.; Loe-Mie, Y.; Lepagnol-Bestel, A.-M.; Massou, S.; Adam, M.-P.; Le, X. L.; Viard, J.; Plancon, C.; Daudin, R.; Koebel, P.; Dorard, E.; Rose, C.; Hsieh, F.-J.; Wu, C.-C.; Potier, B.; Herault, Y.; Sala, C.; Corwin, A.; Allingquant, B.; Chang, H.-C.; Treussart, F.; Simonneau, M. Fluorescent Nanodiamond Tracking Reveals Intraneuronal Transport Abnormalities Induced by Brain-Disease-Related Genetic Risk Factors. Nat. Nanotechnol. 2017, 12 (4), 322—328.

(51) Bray, K.; Cheung, L.; Hessain, K. R.; Aharonovich, I.; Valenzuela, S. M.; Shimoni, O. Versatile Multicolor Nanodiamond Probes for Intracellular Imaging and Targeted Labeling. J. Mater. Chem. B 2018, 6 (19), 3078—3084.

(52) Chan, M. S.; Liu, L. S.; Leung, H. M.; Lo, P. K. Cancer-Cell-Specific Mitochondria-Targeted Drug Delivery by Dual-Ligand-Functionalized Nanodiamonds Circumvent Drug Resistance. ACS Appl. Mater. Interfaces 2017, 9 (13), 11780—11789.

(53) Leung, H. M.; Chan, M. S.; Liu, L. S.; Wong, S. W.; Lo, T. W.; Lau, C.-H.; Tin, C.; Lo, P. K. Dual-Function, Cationic, Peptide-Coated Nanodiamond Systems: Facilitating Nuclear-Targeting Delivery for Enhanced Gene Therapy Applications. ACS Sustainable Chem. Eng. 2018, 6 (8), 9671—9681.

(54) Lake, M. P.; Bouchard, L.-S. Targeted Nanodiamonds for Identification of Subcellular Protein Assemblies in Mammalian Cells. PLoS One 2017, 12 (6), e0179295.

(55) Sharmin, R.; Hamoh, T.; Sigaeva, A.; Mzyk, A.; Damle, V. G.; Morita, A.; Vedelaa, T.; Schirhagl, R. Fluorescent Nanodiamonds for Detecting Free-Radical Generation in Real Time during Shear Stress in Human Umbilical Vein Endothelial Cells. ACS Sens. 2021, 6 (12), 4349—4359.

(56) Fedotov, I. V.; Doronina-Amitonova, L. V.; Voronin, A. A.; Levchenko, A. O.; Zibrov, S. A.; Sidorov-Biryukov, D. A.; Fedotov, A. B.; Velichansky, V. L.; Zheltikov, A. M. Electron Spin Manipulation and Readout through an Optical Fiber. Sci. Rep 2015, 4 (1), 5362.

(57) Rendler, T.; Neburkova, J.; Zemek, O.; Kotek, J.; Zappe, A.; Chu, Z.; Cigler, P.; Wrachtrup, J. Optical Imaging of Localized Chemical Events Using Programmable Diamond Quantum Nanosensors. Nat. Commun. 2017, 8 (1), 14701.

(58) Li, C.; Soleymani, R.; Rohandel, M.; Cappellaro, P. SARS-CoV-2 Quantum Sensor Based on Nitrogen-Vacancy Centers in Diamond. Nano Lett. 2022, 22 (1), 43—49.

(59) Holzgrafe, J.; Gu, Q.; Beittner, J.; Kara, D. M.; Knowles, H. S.; Atätüre, M. Nanoscale NMR Spectroscopy Using Nanodiamond Quantum Sensors. Phys. Rev. Applied 2020, 13 (4), 044004.

(60) Sangtawesin, S.; Dwyer, B. L.; Srinivasan, S.; Allred, J. J.; Rodgers, L. V. H.; De Greve, K.; Stacey, A.; Donschuk, N.; O’Donnell, K. M.; Hu, D.; Evans, D. A.; Jaye, C.; Fischer, D. A.; Markham, M. L.; Twitchen, D. J.; Park, H.; Lukin, M. D.; de Leon, N. P. Origins of Diamond Surface Noise Probed by Correlating Single-Spin Measurements with Surface Spectroscopy. Phys. Rev. X 2019, 9 (3), 031052.

(61) Aslam, N.; Přender, M.; Neumann, P.; Reuter, R.; Zappe, A.; Fávaro de Oliveira, F.; Denissenko, A.; Sumiya, H.; Onoda, S.; Isoya, J.; Wrachtrup, J. Nanoscale Nuclear Magnetic Resonance with Chemical Resolution. Science 2017, 357 (6346), 67—71.