Is a quantum biosensing revolution approaching? 
Review on biocompatible ODMR techniques

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Understanding the human brain remains one of the most significant challenges of the 21st century. As theoretical studies continue to improve the description of the complex mechanisms that regulate biological processes, in parallel numerous experiments are conducted to enrich or verify these theoretical predictions and with the aim of extrapolating more accurate models. In the field of magnetometers for biological application, among the various sensors proposed for this purpose, NV centers have emerged as a promising solution due to their perfect biocompatibility and the possibility of being positioned in close proximity and even inside the cell, allowing a nanometric spatial resolution. There are still many difficulties that must be overcome in order to obtain both spatial resolution and sensitivity capable of revealing the very weak biological electromagnetic fields generated by neurons (or other cells). However, over the last few years, significant improvements have been achieved in this direction, thanks to the use of innovative techniques, which allow us to hope for an early application of these sensors for the measurement of fields such as the one generated by cardiac tissue, if not, in perspective, for the nerve fibers fields. In this review, we will analyze the new results regarding the application of NV centers and we will discuss the main challenges that currently prevent these quantum sensors from reaching their full potential.

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Introduction

Electromagnetic field sensing is a highly important field in current scientific research, pushing towards the creation or improvement of sensors. There are many electromagnetic field sensors that have emerged over these years. The goal of the research is aimed at the implementation of devices capable to reveal less and less intense fields with an increased spatial resolution. In particular, these sensors will find application in biology, where high sensitivity sensing coupled to high resolution is of the utmost relevance. Furthermore, research is aimed at the use of such devices not only to gain insight on the processes, but also to monitor them (once understood), thus trying to reveal anomalies and seek a cure for them. For example, monitoring neuronal fields would allow not only the investigation of brain currents during cognitive processes in order to improve neurological diagnostic systems, but also to identify the early stages of neurodegenerative disease, like Parkinson’s, Alzheimers’s disease and other forms of dementia.[12]

In the biological field, the study of processes would be enriched if sensors were also available to detect changes in temperature. Most processes involve a local increase in temperature, especially diseases, such as cancer. Among the various devices that have emerged over the years, promising sensors for the detection of biological fields are the color centers in diamonds. The color centers are impurities in the crystalline matrix that, when stimulated, emit fluorescence. In particular, the nitrogen-vacancy (NV) complex is by far the most promising. The spin energy levels of the NV center are sensitive not only to electromagnetic fields, but also to temperature variations. This dependence becomes even more attractive thanks to the possibility to optical initialization and spin readout by means of the Optically Detected Magnetic Resonance (ODMR) technique. These exceptional properties makes the NV complex a very promising candidate as a sensor for biological application.

The paper is structured as follows: section 1 gives a brief description of the theory of quantum sensing, section 2 analyzes the magnetic field generated by mammalian neuronal cells and cardiac tissue, section 3 deals with experiments aimed at the detection of cells fields and finally in section 4 the experimental techniques, used to enhance the sensitivity of NV centers to be used as biosensors, are highlighted.

1 The theory of quantum sensing with NV\(^{-}\) centers

The nitrogen-vacancy (NV) defect is a natural complex of impurities in diamond crystalline matrix. This complex is composed of a substitution nitrogen atom and a vacancy-type defect, located in adjacent reticular sites.[2] This system has a pyramidal symmetry (C\(_{3v}\)) and it has, as axis of symmetry, the line that connect the nitrogen atom with the vacancy (see Fig.1b). Compared to the tetrahedral structure of the diamond, there are 4 possible orientations of this defect, each with equal probability in conditions of conventional syntheses. Moreover, there are two charged states in which it is possible to find the nitrogen-vacancy defects and they are distinguished by the number of electrons involved. The 3 carbon atoms surrounding the vacancy contribute to sharing 1 electron each to the complex, while nitrogen contributes with 2. If, in total, in the system only these 5 electrons are present, the center is electrically neutral and it is referred to as NV\(^{0}\), with total electronic spin S = 1/2. Alternatively, the defect can trap 1 additional electron from the surrounding lattice, creating the NV\(^{-}\) center. In this case the electrons developed an electronic quantum spin number S = 1, with spin component along symmetry axis \(\{|m_s = 0 >, |m_s = +1 >, |m_s = -1 >\}\). The most promising configuration for quantum sensing exploits the spin property of the NV\(^{-}\) complex. These sp\(^{3}\) orbitals linearly combine to form 4 molecular orbitals: the lowest energy state of the ground configuration that is the orbital singlet, spin triplet state \(^{3}A_2\) and the electronic excited states that are orbital doublet, spin triplet \(^{3}E\), and spin singlet orbital singlet \(^{1}E\), and \(^{1}A_1\).

As can be seen from the simplified image (Fig.1b) of the NV\(^{-}\) energy level structure, by irradiating the complex with a 532 nm pump laser, the electronic state is excited in a non-resonant way and then relaxes to the fundamental state with subsequent emission at room temperature between 637 nm (zero phonon line) and 800 nm (phonon sideband). While optical excitation from the \(|m_s = 0 >\) state is spin preserving, excitation from \(|m_s = \pm 1 >\) has a finite branching ratio into the metastable singlet \(^{1}E\), with a lifetime of 300 ns. This singlet state relaxes into \(|m_s = 0 >\) through non-radiative processes and weak infrared emission peaking at 1042 nm. This leads to a drop in fluorescence output up to 30% for a single NV\(^{-}\), or 1-2% for a large NV\(^{-}\) ensemble, compared to the situation when the system is initialized in \(|m_s = 0 >\), allowing optical read out of the spin state.

As we focus on sensing, from now on we will refer to NV\(^{-}\) as NV for simplicity.
FIG. 1: a) Diamond crystalline structure with nitrogen-vacancy defect; b) NV\(^-\) radiative state transitions that occur during laser pumping. Radiative optical transition \( ^3E \rightarrow ^3A_2 \) with 637 nm zero phonon line (ZPL), and non optical transition \(^1E \rightarrow ^1A_1\) with 1042 nm ZPL. Non radiative intersystem crossing (ISC) transitions subsist between \( ^3E \rightarrow ^1A_1 \) and between \(^1E \rightarrow ^3A_2\).

1.1 NV ground electronic state

The Hamiltonian of \(^3A_2\), the ground spin state of the NV system, can be written in the following form\(^[14,15]\):

\[
\hat{H}_{gs} = \frac{\hat{S} \hat{D} \hat{S} + \hat{S} \hat{A} \hat{I}}{\hbar} + \hat{I} \hat{Q} \hat{I}
\]

where \(\hat{S} = (\hat{S}_x, \hat{S}_y, \hat{S}_z)\) and \(\hat{I} = (\hat{I}_x, \hat{I}_y, \hat{I}_z)\) are the dimensionless electron and nitrogen nuclear spin operators, respectively. The first term represents the fine structure splitting due to the electronic spin-spin interaction, that it couples with the fine structure tensor \(\hat{D}\). The second term is generated by the hyperfine interaction between NV electrons and the nitrogen nucleus (I=1 for a \(^{14}\)N nucleus, while I=1/2 for a \(^{15}\)N nucleus), with the hyperfine tensor \(\hat{A}\). Finally, the third term represents the nuclear electric quadrupole interaction, with the electric quadrupole tensor \(\hat{Q}\). It should be noted that, in this notation, the component \(z\) coincides with the NV axes of symmetry. Due to the symmetry of the NV center, \(\hat{D}, \hat{A},\) and \(\hat{Q}\) are diagonal in the NV coordinate system\(^[20]\) and, in terms of the natural spin-triplet basis \(\{|m_s = 0>, |m_s = +1>, |m_s = -1>\}\), the Hamiltonian can be written as:

\[
\hat{H}_{gs} = \frac{D_{gs}}{\hbar} \left( \hat{S}_z^2 - \frac{\hat{S}_z^2}{3} \right) + \frac{A_{gs,14N}}{\hbar} \hat{S}_z \hat{I}_z + \frac{A_{gs,15N}}{\hbar} \left( \hat{S}_y \hat{I}_x + \hat{S}_x \hat{I}_y \right) + \frac{Q_{gs}}{\hbar} \left( \hat{I}_z^2 - \frac{\hat{I}_z^2}{3} \right)
\]

where \(D_{gs} \approx 2.87\) GHz is the zero field splitting, \(Q_{gs}\) is the nuclear electric quadrupole parameter, \(A_{gs,\parallel}\) and \(A_{gs,\perp}\) are the axial and non-axial magnetic hyperfine parameters\(^[20]\). The parameters values are shown in the table\(^[1]\).

| Hyperfine parameters          | Value          |
|-------------------------------|----------------|
| Zero field splitting          | \(D_{gs} \approx 2.87\) GHz |
| Axial hyperfine term          | \(A_{gs,14N} \approx -2.14\) MHz |
|                              | \(A_{gs,15N} \approx 3.03\) MHz |
| Transverse hyperfine term     | \(A_{gs,14N} \approx -2.70\) MHz |
|                              | \(A_{gs,15N} \approx 3.65\) MHz |
| Nuclear electric quadrupole   | \(Q_{gs} \approx -5\) MHz |

TABLE I: Hyperfine parameters for the NV defect determined at room temperature.
1.2 The Optically Detected Magnetic Resonance technique

One of the characteristics that renders the use of the NV center as a sensor attractive and convenient is the possibility to discriminate the spin components of the electronic state. This is allowed by the different coupling of the \(|m_s = 0 >\) state with a metastable level, compared to the \(|m_s = \pm 1 >\) state and results in a variation of the photoluminescence (PL) of the defect under laser non resonant excitation. Optically Detected Magnetic Resonance (ODMR) consists in the application of a microwave field (MW) on the sample, simultaneously with its exposure to a non-resonant laser at a higher frequency with respect to the resonant frequency corresponding to the energy gap between the ground and the \(3E\) level (e.g. 532 nm) of the NV (see Fig.2a). Considering an ensemble on NV centers, when the frequency of the MW reaches the ground state resonance \(D_{gs}\) of the NVs, with a certain probability (depending on the MW power), those NV centers will be initialized in the states \(|m_s = \pm 1 >\) rather than \(|m_s = 0 >\). As said, this corresponds to a reduction in photoluminescence of the NV centers, at it can be seen, e.g., in Fig.2b where a typical ODMR spectrum is reported with the expected fluorescence dip at the zero field splitting \(D_{gs}\).

![Diagram of NV radiative state transitions and fluorescence collection](image)

**FIG. 2:** a) NV radiative state transitions that occur during laser pumping and microwave (MW) excitation. The coupling of the state \(|m_s = \pm 1 >\) with the metastable level generates a statistically lower fluorescent emission than when the electronic state was initialized in \(|m_s = 0 >\). b) Fluorescence collected by the NV center, in a certain interval of time, when the MW frequency varies. Dip in correspondence of the zero field splitting \(D_{gs}\). (resonance frequency of the undisturbed NV center, at room temperature).

The coupling terms of NV center with the electric, magnetic fields and local temperature variations will be analyzed in the remainder of this section.

1.3 Magnetic field sensing

A static magnetic field produces the well-known Zeeman effect, that it is described by the following expression:

\[
\hat{V}_{gs} = \frac{\mu_B g_{gs}^//}{\hbar} \hat{S}_z B_z + \frac{\mu_B g_{gs}^\perp}{\hbar} (\hat{S}_x B_x + \hat{S}_y B_y) + \frac{\mu_N g_N}{\hbar} \hat{I} B
\]

(3)

where \(\mu_B\) is the Bohr magneton, \(\mu_N\) is the nuclear magneton, \(g_{gs}^//\) and \(g_{gs}^\perp\) are the components of the ground state electronic g-factor tensor and \(g_N\) is the isotropic nuclear g-factor. In the presence of relatively weak magnetic fields, it’s possible to approximate the almost diagonal g-factor tensor in a diagonal form, with constant \(g_e = 2.003\ [1]\). As can be seen in Tab.\[\text{II}\], the interaction of the magnetic field with the nucleus is 2000 times smaller and, consequently, may be neglected. \[\text{II}\]. The presence of external fields eliminates the energy degeneracy of the levels \(|m_s = \pm 1 >\), which split with an amplitude given by \(\gamma_e B_z\), where \(\gamma_e = \frac{\mu_B g_e}{\hbar}\) (see Fig.3).

If, instead of a single NV center, an ensemble of NV centers is considered, up to eight magnetic resonance dips can be observed, due to the four possible orientation of the NV axis in the diamond’s crystalline matrix (see Fig.4). The angle between each different pair is 109.4°. For certain directions of the magnetic field, some resonances can be degenerate. A NV-based magnetometer can be realized, for example, by applying a bias field along the NV axis, removing the degeneracy, so that changes in the magnetic field projection along this axis affect the resonance
FIG. 3: NV ground-state $^3A_2$ scheme. Above: a) $^{14}$N hyperfine states and b) $^{15}$N hyperfine states. Below: schematic ODMR spectra. The spectra are shown considering Zeeman splitting and hyperfine splitting.

frequencies almost linearly. Another option is to use all four NV alignments; although the eight ODMR frequencies have more complicated dependence on $\mathbf{B}$, this option yields information about the direction of magnetic field.13 The use of NV center as a magnetic field sensor firstly was proposed in14 and demonstrated with single39 and NV ensembles7 in 2008.

FIG. 4: ODMR spectra a) in the absence of a magnetic field and b) in the presence of an external bias magnetic field. The magnetic field lifts the degeneracy of the $|ms = \pm 1\rangle$ states and results in two separate dips in the ODMR spectrum. c) An example ODMR spectrum (excited at 532 nm) with a magnetic field in an arbitrary direction for an ensemble NV centers in diamond. Each of the four NV alignments has a different magnetic field projection along its quantization axis, leading to eight ODMR peaks (two for each NV alignment). For each dip a coupling with the nuclear spin of the N14 atom generates additional three hyperfine level.18
1.4 Electric field sensing

The Hamiltonian describing the interaction with the electric field was derived from molecular orbit theory by Doherty et al.\textsuperscript{15} and it can be written in the following form:

\[
\frac{\hat{V}_{gs}}{\hbar} = d_{gs}^\parallel (E_x + F_x) [\hat{S}_y^2 - \frac{\hat{S}_z^2}{3}] + d_{gs}^\perp (E_x + F_x)(\hat{S}_y^2 - \hat{S}_z^2) + d_{gs}^d (E_y + F_y)(\hat{S}_z\hat{S}_y + \hat{S}_y\hat{S}_z)
\]

where \(d_{gs}^\parallel\) and \(d_{gs}^\perp\) are respectively the axial and non-axial Stark shift components of the permanent electric dipole moment \(d_{gs}^d\) in the ground triplet state\textsuperscript{20}. \(\hat{E}\) is the electric field and \(\vec{F}\) is the mechanical strain.

According to Eq. 4 the effect of the electric field \(\hat{E}\) plays the same role as mechanical strain \(\hat{H}\textsuperscript{20,21}\). The strain depends on the diamond material: in single-crystal samples, the mechanical strain field is substantially negligible; while, in polycrystalline ones, a relatively high strain field is induced by the growth conditions, leading to a splitting of the spin state \(|m_s = ±1\rangle >\) even in absence of external magnetic fields.

The frequency shift caused by the electric field is much smaller than the shift produced by the presence of a magnetic field (see Tab. I). For this reason, in order to reliably measure this second order effect caused by the Stark shift, it is necessary to decouple it from the Zeeman shift.

To summarize, the fine structure Hamiltonian of the NV ground state, describing the energy levels of the electronic spin states due to the spin (\(\hat{S}\)) interaction with the static magnetic (\(\vec{B}\)), electric (\(\hat{\vec{E}}\)), and strain (\(\vec{F}\)) fields, can be written in terms of the natural spin-triplet basis \(|m_s = ±1\rangle >, |m_s = 0\rangle >\) in the following matrix form:

\[
\hat{H}_{gs} = \begin{pmatrix}
0 & -\mu B g_e B_x \frac{B_x - i B_y}{\sqrt{2}} & -\mu B g_e B_x \frac{B_x + i B_y}{\sqrt{2}} \\
-\mu B g_e B_y \frac{B_x + i B_y}{\sqrt{2}} & hD + \mu B g_e B_y B_z & -h d_{gs}^d (P_y - i P_y) \\
-\mu B g_e B_x \frac{B_x - i B_y}{\sqrt{2}} & -h d_{gs}^d (P_y + i P_y) & hD - \mu B g_e B_z
\end{pmatrix}
\]

where it’s possible to observe that the natural-spin basis vectors are eigenstates of the Hamiltonian only in the presence of both the magnetic and electric field aligned with the NV axis. In this condition, \(D = D_{gs} + d_{gs}^d P_{z}\) describes the frequency shift of the resonance lines resulting from the zero-field splitting and from the Stark effect associated with the component of the vector \(\vec{P} = \hat{E} + \vec{F}\). Otherwise external fields not aligned to NV symmetry axis produce a non-diagonal matrix, and therefore energy levels of undefined spin. In particular, the presence of additional transverse strain and electric-field components \(P^\perp\) modifies the ground-state structure.

The Hamiltonian assumes a quasidiagonal form considering a new spin basis \(|0\rangle >, |+\rangle >, |−\rangle >, obtained by a field-dependent mixing of the \(|m_s = +1\rangle >\) and \(|m_s = −1\rangle >\) spin states according to the following unitary operator:

\[
\hat{U} = \begin{pmatrix}
1 & 0 & 0 \\
0 & e^{i \frac{\pi}{2} \sin(\frac{\theta}{2})} & e^{-i \frac{\pi}{2} \sin(\frac{\theta}{2})} \\
0 & e^{i \frac{\pi}{2} \cos(\frac{\theta}{2})} & -e^{-i \frac{\pi}{2} \sin(\frac{\theta}{2})}
\end{pmatrix}
\]

where \(\tan(\phi) = P_x / P_y\) and \(\tan(\theta) = (d_{gs}^d P^\perp) / (\mu B g_e B_z)\) are the field-dependent phases defining the spin state mixing. The Hamiltonian takes the following form in the \(|0\rangle >, |+\rangle >, |−\rangle >\) basis:

\[
\hat{H}_{gs} = \hat{U} \hat{H}_{gs} \hat{U}^\dagger = \begin{pmatrix}
0 & c_1 B g_e B^\perp & c_2 B g_e B^\perp \\
c_1 B g_e B^\perp & hD + W & 0 \\
c_2 B g_e B^\perp & 0 & hD - W
\end{pmatrix}
\]

with

\[
W = \sqrt{(hd_{gs}^d P^\perp)^2 + (\mu B g_e B_z)^2}
\]

The constants \(c_1\) and \(c_2\) represent the phase of the matrix elements and \(B^\perp\) is the transverse component of the magnetic field with respect to the NV axis. If \(B^\perp \approx 0\), the non-diagonal terms can be neglected and the Hamiltonian can be regarded as diagonal in the basis \(|0\rangle >, |+\rangle >, |−\rangle >\), with energy difference between the \(|0\rangle >\) and the \(|±\rangle >\) states is given by \(hD ± W\), corresponding to ODMR resonances are separated by a 2\(W/h\) frequency splitting (depending on the strengths of the magnetic, electric, and strain fields, as well as their orientations with respect to the axes of the NV center). Since the states \(|±\rangle >\) are a coherent superposition of the states \(|m_s = ±1\rangle >\), we underline that the ODMR resonance is observed also in this case as a reduction in the fluorescence emission at the new MW resonance frequencies.
1.5 Temperature sensing

Another interesting feature of the NV complex is the dependence of its spin levels on changes in temperature.\textsuperscript{22} Indeed, the microscopic origin of $D_{gs}$, the zero field splitting (ZFS) parameter, is due to spin-spin interactions in the NV’s orbital structures, and the value depends on the lattice length, which is strongly correlated to the local temperature. When the local temperature increases the diamond lattice spacing of the NV center increases as well, lowering the spin-spin interaction and reducing the ZFS parameter $D_{gs}$. Under ambient conditions $D_{gs} \approx 2.87 \text{ GHz}$ and the temperature dependence is $dD/dT \approx -74 \text{ kHz/K}$.\textsuperscript{22} In general, the ZFS parameter shows a non-linear dependence, and its value increases with the temperature decreases.\textsuperscript{23}

To realize a NV-based temperature sensor, the most obvious solution is exploiting the $D_{gs}$ temperature dependence.

| Property                | Coupling coefficient          |
|-------------------------|-------------------------------|
| Magnetic field          | $\gamma_e = \frac{e\mu_B}{h} \approx 28 \text{ GHz/T}$ |
|                         | $\gamma_N = \frac{\mu_N g_N}{h} \approx 15 \text{ MHz/T}$ |
| Electric field          | $d_{/gs} \approx 3.5 \text{ mHz/(Vm}^{-1})$ |
|                         | $d_{\perp,gs} \approx 0.17 \text{ Hz/(Vm}^{-1})$ |
| Temperature             | $\partial D_{gs}/\partial T \approx -74 \text{ kHz/K}$ |

TABLE II: Coupling coefficient of the NV center with the external fields.

This require that no external fields is present ($\vec{B}, \vec{E}, \vec{F} = 0$), i.e. $|m_s = \pm 1>$ is degenerate. In this case, an increase in temperature leads to a decrease in the resonance frequency, associated with a shift of the degenerate levels $|m_s = \pm 1>$ towards the level $|m_s = 0>$. We note that this does not seem to be the optimal solution, since, even in the absence of applied fields, the sample may have an internal strain and may be affected by the Earth’s magnetic field. Unless it is possible to find a diamond sample with negligible $\vec{F}$ and to design an experimental set up able to reasonably compensate for the external magnetic field (e.g. Helmholtz coils), the dips would not be perfectly overlapped because of the non perfect degeneracy of $|m_s = \pm 1>$, thus showing a larger full-width-at-half-maximum (FWHM) and therefore a lower resolution.

A better solution is to apply an external magnetic field in order to significantly separate the spin levels. However in this configuration, a single dip can shift for a temperature variation, but also for a variation of magnetic field. To decouple the two contributions it is enough to monitor both $|m_s = +1>$ and $|m_s = -1>$ spin states at the same time, using simultaneous driving of the microwaves in ODMR technique.\textsuperscript{24} As it can be seen in the Figure\textsuperscript{5} by simultaneously monitoring the initial dips (red curves), it is in principle possible to understand if there are variations in the magnetic field (the dips move in opposite directions) or in temperature (the dips move in the same direction).

FIG. 5: Example of magnetic and thermal shifts of the spin resonance, in ODMR spectra. Dips with equal colors correspond to paired resonances. The colors represent the timeline of the dips. The initial dips is red, then green and finally blue.

A recent technique consists in the application of an intermediate transverse magnetic field $B_\perp$.\textsuperscript{25} Similarly to the case just discussed, the application of $\vec{B}$ removes the degeneration of $|m_s = \pm 1>$ and therefore improves FWHM. The intensity and the transverse direction of that field, on the other hand, create a quantum superposition of states which is insensitive to magnetic fields but sensitive to temperature. This quantum superposition has a small expectation value of the spin along any direction, this implies the degeneration of the hyperfine structure between the levels.
\[ |m_I = \pm 1 > \text{ (except for the quadrupole contribution } Q_{gs}, \text{ which separates } |m_I = 0 > \text{ from } |m_I = \pm 1 >\). The figure shows the corresponding scheme of the spin energy levels (only the }^{14}\text{N isotope is considered as it is the most common). In this situation, the ODMR spectrum has two dips (instead of 6), providing a substantial improvement in the signal-to-noise ratio. This particular orientation of the magnetic field ensures the protection of the measurements from the noise of other possible magnetic field. In fact, the NV spin is non-sensitive to the magnetic field fluctuation, because the contribution of the magnetic component enters only in the second order in the Hamiltonian.

![FIG. 6: NV ground-state }^{3}A_2 \text{ scheme, in presence of intense transverse magnetic field } B_{\perp}.](image)

2 Bio-sensing

Before describing the experiments focusing on the NV-based sensor it is necessary to specify the type of biological specimens we intend to analyze, the expected magnitude of the electromagnetic field produced by these specimens and the principal parameters such as sensitivity and spatial/temporal resolution, required from the NV-based sensors. This section, after reviewing some of the devices typically used for biosensing, analyzes in detail neuronal and cardiac cells. Higher sensitivity and resolution of electromagnetic fields is considered necessary by researchers who want to expand the understanding of the fundamental processes regulating the interaction of these cells.

2.1 From the conventional electrophysiological techniques to NV sensors

Electrophysiology deals with the study of electrical phenomena associated with physiological ones. In electrophysiology is possible to detect the membrane potential using two different techniques in vitro: patch clamp and MEA (Micro Electrode array). The patch clamp technique is a measure that involves the use of an electrode immersed in a glass capillary with a physiological solution, which allows the measurement of changes in membrane potential in response to a current flowing through specific ion channels. The relative measure corresponds to a typical physiological response of the cell, such as the action potential. This technique has the great advantage of being able to perform an intracellular measurement on a single cell. However, it is a rather invasive technique, as it destroys the cell membrane through the recording electrode, so once the measurement is complete it is no longer possible to repeat it on the same cell, it is a complex technique and requires many measures to have a consistent statistic. Otherwise, the multielectrode technique (MEA) involves measuring the membrane potential variations on multiple cells simultaneously. The MEA is a device consisting of an array of electrodes, which can vary in shape, chemical composition and number, immersed in a typically glassy (insulating) double layer. Typically, the electrodes are made of titanium or indium tin oxide (ITO), and have a diameter that can vary between 10 and 30 µm. Unlike the patch clamp technique, the MEA is a rather versatile and non-invasive technique, allowing to repeat the same measurement several times. For example, in the field of neuroscience the MEA allows to study the development of a neuronal network over time. The disadvantage is that it does not allow studying the single cell with the typical sensibility of the patch clamp. The greatest goal in biophysics would be to enclose in a single technique the peculiarities of the two techniques mentioned above. For this reason, the need of high sensitivity biosensing, non-invasive and iterative detection for biological applications has prompted the study and realization of different devices.

In the following we describe and compare the principal ones.

As for the detection of weak magnetic fields, in addition to the NV-based sensors, the set of emerging devices comprise the superconducting quantum interference device (SQUID) sensors and chip-scale atomic magnetometers (CSAMs). Until now the measurement of very weak magnetic fields was the domain of SQUIDs sensors. These sensors have reached sensitivity levels of (0.9–1.4) fT/Hz^{1/2} with a pick-up coil area of the order of 1 cm^2. However,
SQUIDs require cryogenic cooling, which, in addition to implying significant cost and maintenance complexity, requires positioning the sensor a few centimeters from the sample. An alternative is offered by the CSMAs, that are based on microfabricated alkali vapor cells integrated with small optical components such as diode lasers and fiber optics. These devices have reached sensitivities below 5 fT/Hz$^{1/2}$ at sensor volume 8 mm$^3$. However, despite the exceptional sensitivity, the minimum working distance between sensor and magnetic source for CSAM or SQUIDS remains at least few mm, that makes them unsuitable for monitoring individual cell signals or small tissues, being the amplitude of the magnetic field decreasing quadratically with the distance.

As for the detection of electric fields, the sensors emerging in the last few decades are single-electron transistors (SETs), that are a promising candidate for achieving higher detection sensitivity due to the Coulomb oscillations. However, only in recent years emerged on the existence of a SET-based biosensor, probably because of their difficulty of the room-temperature operation.

Finally, in recent years there has been a growing interest in the use of temperature sensors capable of operating on a nanometric scale. It has been known for some time that local temperature variations at the intracellular level play a fundamental role in cellular activities related to body temperature homeostasis and energy balance. Particular attention is paid to the possibility of measuring local temperature variations of cell organelles (i.e. nucleus, mitochondria, etc.) or ion channels. For example, different simulation models showed a hypothetical variation in temperature at the level of the ion channels, due to the flow of the ions from the inside to the outside of the plasma membrane, during the genesis of the action potential. Due to the difficulty of this local measurement, no one has ever measured this thermal variation. Interestingly, Guatteo et al. using patch clamp techniques observed that there is a change in the firing frequency when temperature change. Currently fluorescence probes are powerful method used to study intracellular temperature variation thanks its high spatio-temporal resolution. The probes typically used for this measurement are organic or inorganic fluorescent probes, such as fluorescent proteins, organic dyes, quantum dots (QDs) and many others. Organic probes are biocompatible probes, rather stable and very easy to chemically target. But there are different problems related the use of these probes: these are often autofluorescent and to avoid the phenomenon it is necessary to add specific quenchers; they cannot be used for a long time, in fact these sensors suffer from photobleaching and unstable photoluminescence. In the best case scenario, the probe degradation consists of fluorescence suppression, in the worst case scenario it releases an electron that binds to nearby molecules making them toxic. These probes are organic and by their nature they are also subject to even weak pH variations, for this reason it is fundamental a strict control of the cell environment. The inorganic probes such as quantum dots (QDs) have the advantage of being stable in fluorescence, have a high sensitivity to temperature variations, the nanometric size allows obtaining a spatial resolution useful for cellular measurements. Although the size of these sensors would allow spatial resolution limited by the diffraction limit only, their chemical composition is found to be non-biocompatible in most of the cases. Other temperature sensors are based on up converting nanoparticles (UCNPs): nanoscale particles (diameter 1-100 nm) that exhibit photon upconversion, i.e. when stimulated by incident photons they are able to emit fluorescence of shorter wavelength. They are usually composed of rare-earth based lanthanide or actinide-doped transition metals. Their core-shell structure allows sensor compatibility, however, sensitivity is not high. Extremely interesting devices able to realize all these measurements (magnetic, electrical and temperature sensing) eventually at the same time, are one based on the NV. The advantages of these sensors are manifold: they have stable photoluminescence in the visible and near-infrared range, their chemical composition ensures resistance to photobleaching and delineates them as an inert and therefore biocompatible material so cell/neurons can be grown directly on its surface or nanodiamonds can be injected inside them, allowing for bub-cellular spatial resolutions and it is a non-invasive technique. Finally, they can operate at room temperature and, in more detail, their dynamical range of temperature sensing extends further 500 K for both bulk and nanoscale diamonds.

### 2.2 NV center as sensor for neuronal signals

In the last decade, neuroscience has attracted great interest beyond the scientific community. Because of the increase in life expectation, cases of neurodegenerative diseases such as Parkinson’s, Alzheimer’s, Huntington’s disease and many others are constantly growing. Currently, these diseases are incurable, even symptoms mitigation is difficult because of late diagnosis when most of the neurons involved have been irreparably damaged. This reason strongly prompts develop to new increasingly precise and sensitive techniques, allowing a deeper understanding of neuronal circuits ranging from functioning of the single neuron to the behavior of the entire synaptic network. Neurons are the functional units of the nervous system. They communicate via electrical signals, known as action potentials.

The action potential (AP) consists in the variation in time of the membrane potential $V_m$, where $V_m = \Phi_{in} - \Phi_{out}$ is the electrical potential difference between the inside and the outside of the cell membrane. The AP rapidly rises
and falls, creating a few ms long voltage pulse, with the characteristic shape shown in Fig.7. The AP impulse is caused by several ionic species (among which the main ones are Na\(^+\), K\(^+\)), which cross the neuronal membrane in specific conditions.

![Diagram of a neuron](image)

**FIG. 7:** a) Single neuron. In the upper box a zoom of the neuronal membrane is reported, where the ionic current and the field generated by it are schematized. In the lower one the axial current and the relative field are schematized. b) Neuronal action potential (AP) typical shape. In rest conditions \(V_m\) is regulated to -70 mV. If, thanks to the initial stimulus, \(V_m\) exceeds the threshold, membrane depolarization begins. In this phase the Na\(^+\) channels open, allowing sodium to enter in the neuronal cell, bringing \(V_m\) to about 35 mV. Then begins the repolarization phase in which, once the Na\(^+\) channels are closed, the K\(^+\) channels open, letting out the potassium, until \(V_m\) becomes \(\approx -93\) mV (hyperpolarization). Finally, the sodium-potassium pump restores the initial conditions. During the depolarization, the influx of positive charges produces local internal and external longitudinal currents. They are responsible for the PA propagation in the axon adjacent area. The propagation directionality is guaranteed by the refractory period: although the local currents arrive from both the previous and the next segment cell, in the first one the PA will still be in the hyperpolarization phase and it will not be able to trigger a new cycle.

As already said, the two techniques developed in electrophysiology to study the physiological and synaptic mechanisms in a neuronal network are the patch clamp and the MEA. In the last decade scientists have tried to study more and more specifically the path of the electrical signal from the cell body (or soma) to the whole dendritic tree. In other words, the goal would be to create a device that allows to scan the neuron point by point from the soma to the axon and the dendrites, following and characterizing the electrophysiological variations of the electrical signal during its propagation. The technology closer to this ambitious goal is the one of the CMOS-MEA, that allows having a much higher density of electrodes with respect to the traditional MEA technology. Numerous studies have managed to scan the path of the electrical signal in a neuronal network at the level of the single neuron.\(^{55-58}\)

Bakkum and colleagues\(^{58}\) recently have developed a high electrode density CMOS-MEA device capable of stimulating a specific area and simultaneously scanning the signal along some points from the soma to the axon. Clearly, this technique is much more sensitive than MEA, but given the stochasticity of the cell’s placement in space, it requires cells to be marked in order to follow their path. Recently several groups have correlated this technology to the technique of optogenetics. They tagged the genes of interest and activated them following an optical stimulation and simultaneously followed the signal thanks to the integration of the CMOS-MEA.\(^{59,60}\)

However, these techniques do not allow following the entire dynamics of the action potential, but to have a scan of a region depending on the position of the electrodes with respect to the neuron with its axon and its dendritic body.

NV sensors may therefore have a huge impact on these applications: nanodiamonds can be positioned on the neurons membrane or the cell can be plated on a bulk diamond.\(^{61}\) Indeed, diamonds are bio compatible and color centers in diamonds have an exceptional spatial resolution, one can imagine that these properties can be exploited for reconstructing the AP dynamics. Furthermore, the possibility of positioning them adjacent to the cell membrane has the advantage of experiencing strong magnetic fields. However, since neuronal magnetic fields are extremely weak, their detection appears to be challenging even for NV-based sensors, at least for mammalian cells, while measurements have been performed on giant neurons of invertebrates.\(^{62}\)

To predict the electromagnetic fields intensity created by the AP, and therefore to understand what sensitivity of the NV sensors is needed to sense it, it is necessary to model how the AP develops and propagates.
Hodgkin-Huxley model\textsuperscript{[42,43,44,45]} allows estimating the ionic current flowing through the neuron membrane (when the ion channels are open). For the human neuron, the total estimated ionic current, sum of the single channels contribution $I_{\text{ion}}$ is:

$$I_\perp = \sum I_{\text{ion}} \simeq 2 \text{ pA/\mu m}^2 \quad \Delta T \simeq 1 \text{ ms}$$

Each $I_{\text{ion}}$ generates a magnetic field (see Fig.\textsuperscript{[7]}), which can be estimated by means of the Biot-Savart law:

$$\vec{F}_C, \vec{B}_{\text{ion}} \cdot \vec{l} = \mu_0 I_{\text{ion}}.$$  However, the resulting amplitude of these fields depends on the channels density, which largely varies depending on the axon area being considered. Furthermore, we note that the $\vec{B}_{\text{ion}}$ fields, sum of the contributions of the field produced by the various channels, is typically vanishingly small on average, because of the different fields directions. The condition in which the channels are locally in a cluster may represent a very significant situation.\textsuperscript{[45,46]} Assuming a current of 100 pA/\mu m\textsuperscript{2} and considering that the NV sensor can be positioned at an average distance of few nanometers from it (as can be easily achieved by targeting the channel with functionalized NDs, a magnetic field of about 0.1 – 5 nT (or even higher) can eventually be obtained. Nevertheless knowledge in this regard is still insufficient and therefore this hypothesis deserves further investigation.

In reality, the current flowing through the membrane is not the only charge flow: there are also longitudinal currents, which flow along the neuron axis $I_{\text{axial}}$. These currents propagating, both internally and externally with respect to the neuron membrane, are responsible for the propagation of the AP pulse. They also generate a magnetic field, around the neuron (see Fig.\textsuperscript{[7]}). Both the axial current and the corresponding magnetic field have been estimate.\textsuperscript{[21,23,29]} In particular, Ref.\textsuperscript{[23]} goes beyond the simplification of the Hodgkin-Huxley model, introducing the spatial and temporal progression of the AP along the various neuronal compartments, into which they have divided the axon. The theoretical prediction is a maximum field $B_{\text{axial}} \simeq 3$ pT on the external membrane near the Ranvier node and a field $B_{\text{axial}} \simeq 2.3$ pT on the myelin sheath external surface in those regions where the axon is wrapped by it. The maximum magnetic field was also calculated by Isakovic et al. in Ref.\textsuperscript{[13]} for the nerve composed of 100 axons, obtaining only $B_{\text{axial}} \simeq 6$ pT. This is due to the cancellation of the magnetic field component, caused by different axons within the same nerve, bringing opposite directional currents. This estimated magnetic fields, in reality, are the same fields detected by magnetoencephalography (MEG). The reason why MEG detects fields of $10^{-15}$ T is due to the distance from the source.\textsuperscript{[20]}

Using these values, a NV sensor positioned on the neuron surface or a few micrometers from it, should have a temporal resolution of about 0.1 ms (in order to be able to trace the time variation), and spatial resolution of about 10 \mu m\textsuperscript{3} (which would allows a good reconstruction of the AP propagation, being the axon length ranging from 0.1 \mu m to 1 m). Thus, the NV sensor should have a minimum sensitivity of $^{[41]}

$$\eta = \delta B_{\text{min}} \sqrt{\Delta T} \simeq 3 \text{ pT} \sqrt{0.1 \text{ ms}} \simeq 30 \frac{\text{pT}}{\sqrt{\text{Hz}}}$$ \text{ (6)}$$

The NV sensor optimal sensitivity is in principle limited by the quantum projection noise. This fundamental sensitivity limit for spin-based magnetometers is given by $^{[42]}

$$\eta_q = \frac{1}{\gamma_e \sqrt{nT_2}} \text{ (7)}$$

Where $\gamma_e$ is the magnetic coupling coefficient (Tab.\textsuperscript{[4]}). $n$ represents the number of NV centers and $T_2^*$ their characteristic dephasing time. It is important to underline that the number of NV centers $n$ refers to the sensing volume. As mentioned, for the single PA detection the sensing volume should be around 10 \mu m\textsuperscript{3}, the size of the cell. In the Ref.\textsuperscript{[29]}, the estimation of the parameters $n \simeq 3 \cdot 10^6$ cm\textsuperscript{-3} and $T_2^* \simeq 450$ ns determines a spin projection noise value of $\eta_q \simeq 30 \frac{\text{pT}}{\sqrt{\text{Hz}}}$ for the sensing volume of 10 \mu m\textsuperscript{3} (the experimental sensitivity reached is instead $\eta_q \simeq 15 \frac{\text{pT}}{\sqrt{\text{Hz}}}$ for the sensing volume of 5 \cdot 10^6 \mu m\textsuperscript{3}). This value is still 1000 times larger than the sensitivity required for the detection of a single AP. However, how it will be discussed in section 4, it is possible to work on both the above mentioned parameters to improve the result.

Once the biomagnetic field $\vec{B}(x,t)$ has been measured, to trace the unknown source it is necessary to carry out the inverse problem. In general, its solution is not unique, due to the existence of the so-called “magnetically silent” current sources (i.e. the ones producing magnetic fields that almost cancel each others) and due to the fact that the magnetic field can be influenced by the electric field.\textsuperscript{[51,52]} However, in the single axon case, it can be uniquely
resolved. On the contrary, in the biological tissue case and in the 3D structures case, that cannot be traced back to standard models (such as a spherically symmetrical conductor or a horizontally layered medium), the solution is not unique. In some cases this is resolved by the knowledge of the electric field on the conductor surface.

In conclusion, albeit the neuronal field generated by biocurrents is very weak, it is potentially in the range of NV ODMR techniques. Moreover, other mammalian cells are eventually generating more substantial biocurrents.

### 2.3 NV center as sensor for cardiac signals

The human (and animal) heart generates the body’s most intense electromagnetic field. In particular, by comparing measurements performed externally to the human body, the electric field generated by the heart, measured through the electrocardiogram (ECG) is about 60 times stronger than that of the brain, recorded by an electroencephalogram (EEG). In addition, the heart magnetic field detected by the magnetocardiogram (MCG) is about 5000 times higher than the neuronal magnetic field detected by magnetoencephalography (MEG): 0.05 nT (heart) vs 1 fT (neuron). Thus, ODMR based on NV sensors can also find very significant applications in studying cardiac cells and tissues. To achieve a first qualitative estimation of entity of the magnetic field in this case, one can start from a very simplified model: the spherical heart. Although this model is not physiologically accurate, it allows to extrapolate analytical solutions.

In the reference, another assumption concerns the origin of the currents. There are two currents sources in the heart: the first consists of intracellular currents, the second is given by the anisotropy of the tissue. Regarding the first current contribution, the authors consider a spherical shell of cardiac tissue, which covers a blood cavity and is surrounded by an external bath of unlimited electrical conduction. The heart fibers propagate in the $z$ direction and a variation of the membrane potential $V_m$ is assumed following the activation of the action potential (AP), started at $\theta = 90^\circ$ (Fig. 8a). In this work the electric field is evaluated using the bidomain model and considering a situation of quasi-stationarity (although $V_m$ depends on time due to the action potential propagation, it is assumed that, given a certain $V_m(t_0)$, one can derive current and magnetic field in a quasistatic way).

Thus, the electric potential is obtained, using the continuity equations and the boundary conditions, the current density distribution is obtained using Ohm’s law and finally the magnetic field using Biot-Savart’s law. Considering the anisotropic electrical conductance data, the $V_m$ values and typical heart dimensions, it turns out that the magnetic field is stronger near the internal and external surfaces while it is weaker in the heart wall. The peak value of the magnetic field is around 14 nT (Fig. 8b).

At the heart center, instead, the magnetic field reduces to $B = 2 \text{nT}$. This is due to the fact that intracellular and extracellular currents are in opposite directions with almost the same magnitudes in the depths of the tissue and, therefore, tend to cancel each other their relative magnetic field. Considering a planar cardiac tissue sample, the spherical shell method is no longer valid. In this last case it has been
found that the magnetic field reaches a peak value $B = 1\ nT$.

The heart AP is about $\Delta T = 1\ ms$ long, as in the neuronal case. Considering a human heart, a NV sensor positioned on the heart surface should be sensitive to magnetic field $B = 14\ nT$, with a temporal resolution of about 0.1 ms (in order to be able to trace the time variation $\Delta T$), and a spatial resolution of about $10\ \mu m^3$ (which would allow a good reconstruction of the PA propagation also in the heart case, being the heart radius of about 40 mm). That is, it should have a minimum sensitivity:

$$\eta = \delta B_{\min} \sqrt{\Delta T} \simeq 14nT\sqrt{0.1\ ms} \simeq 14\ \sqrt[p]{Hz}$$

(8)

This value represents an excellent intermediate step for the application of actual biosensing technologies using NV, to arrive at the detection on neuronal signals.

3 Methods and bio-applications

To exploit NV-centers for biosensing, it is necessary to set up an optical microscope coupled to an ODMR apparatus: a microwave antenna positioned near the diamond sample designed for a microwave source operating in the range of 2-4 GHz, as described in section 1.2. The next sections are devoted to the presentation of several biosensing experiments exploiting NV centers in diamonds. Some of them are proof-of-principle tests on a cell culture (in vitro cells), others are experiments carried on living organisms (in vivo cells).

3.1 Bulk diamond applications

As discussed in the previous section, the electromagnetic fields produced by the living cells (as neurons, chromaffine, heart cells), even in mammals, are typically extremely weak. For this reason, several "proof-of-principle" experiments addressed measurements of fields produced by cells with peculiar electromagnetic properties. Among the most suitable ones are magnetotactic bacteria (MTB), containing magnetite ($Fe_3O_4$) or ferrite ($Fe_2O_3$) bacteria magnetic particles (BMP). The nanometer size of the BMPs is small to generate a single magnetic domain, but sufficient to create a permanent magnetic moment $\mu_{\text{BMP}}$. This produces a cell magnetic moment $\mu_{\text{MTB}} = \sum \mu_{\text{BMP}}$, given by the sum of the BMP individual dipoles, which is exploited by the MTB to orient itself with respect to the earth’s magnetic field.

Among the various uses in the biomedical field, Sage et al. used *Magneto凭ilum magneticum* AMB-1 for biomagnetic imaging. The bacteria used in this work create magnetic nanoparticles with cubo-octahedral morphology and an average diameter of 50 nm. The experiment was performed both with bacteria dried on the surface of diamond chip implanted with NV centers, as well as with bacteria stored in phosphate-buffered saline (PBS) and laid on the chip surface (in vitro experiment).

The diamond sensor used to perform this experiment is a high-purity single-crystal diamond chip, with a 10 nm layer thickness of NV centers. The estimated surface density of nitrogen-vacancy centers is $3 \cdot 10^{17} \ cm^{-3}$ in the case of experiments on bacteria in the liquid medium and $10^{18} \ cm^{-3}$ for dry bacteria.

In the case of dry bacteria the objective was to demonstrate the possibility of measuring of their static magnetic field, exploiting ODMR measurements at different bias magnetic field orientations ($B_{\text{bias}} = 3.7\ mT$).

In the case of live bacteria in the liquid medium, it was shown that it is possible to evaluate the magnetic field generated by the bacteria dipole $\mu_{\text{MTB}}$ along the [111] crystallographic axis of the diamond, when also the bias magnetic field is oriented along this axis. Furthermore, cell viability was assessed immediately after magnetic imaging (lasting 4 minutes), using a standard fluorescence-based "live-dead" assay obtaining a viability of about 44%. Cells mortality was attributed to the laser heating, since preliminary tests showed that 1 hour exposure to microwaves did not cause substantial cells mortality. Cells vitality was however partially preserved thanks to the strategy used to decouple laser light from the biological sample. Indeed in this set-up the laser impinges on diamond at an angle greater than the critical angle for the diamond–water interface, resulting in its total internal reflection within the diamond.

A wide field optical microscope was used for both MTB samples, with a field of view of $100 \times 30 \ \mu m^2$ of the sample surface and a resolution of 400 nm.

A sCMOS camera was sufficient to image the single magnetic nanoparticles inside the MTB. Their magnetic field is of the order of mT. Thanks to these measurements, the total magnetic moment $\mu_{\text{MTB}}$ was determined by numerically fitting the modeled field distribution to the measured ones, with a mean value of $5 \cdot 10^{-17} \ m^3A$.

The magnetic field estimated from the ODMR measurements was compared with a scanning electron microscope...
Various experiments were therefore conducted to evaluate the cell viability, e.g. in HeLa cells. The two measurements were in excellent agreement and their values were compatible with the data reported in [50, 51]. This highlights the potential of NV centers, able to perform sub-cellular magnetic field measurements at room temperature, allowing real-time imaging of magnetic dipole creation, single MTBs chain dynamics [88] and magnetic particles formation in various organisms [92, 93].

Barry et al. [10] studied individual neurons of marine worms (Myxicola infundibulum) and squids (Loligo pealei). The marine worm has a long axon [49], which stretches over its entire length (tens of mm and diameter of about 5 mm). The giant squid neuron (about 0.5 m long) did not extend over the entire length and is isolated following specific protocols [95]. An initial proof-of-principle test is performed on isolated neurons for both species. The action potential AP is stimulated by means of a current pulse, received by an electrode directly in contact with the neuron. The pulse is generated by a current of about 10 mA, has a duration of about 1 ms and is repeated with a frequency of 0.4 Hz for the worm and 100 Hz for the squid.

The AP generation and its propagation is verified by micro-electrodes, (Fig.9A). From this axonal AP intracellular time trace, it can be modeled [50, 52, 53] the shape of the associated magnetic field (Fig.9B). This is compared with the experimentally measured magnetic field, performed with the NV-based sensor in contact with the excited single neuron. Time traces are shown in Fig.9C and 9D respectively for the worm and the squid neuron. The measurements was performed with ODMR technique at bias magnetic field $B_{bias} = 0.7$ mT, oriented along two diamond axes and perpendicular to the axon axis (being the magnetic field generated by the AP pulse perpendicular to this last one). In Ref. [10] Barry et al. carried on also a measurement on a living worm. The worm was directly fixed on the diamond and the distance between the neuron and the active NV layer was about 1.2 mm (see Fig.10A). The magnetic field generated by the propagation of the AP pulse measured by ODMR technique is shown in figure 10B. It is smaller than the one measured in the excised neuron, but its value is compatible with the increasing sensor distance. The diamond sensor, exploited an electronic grade (N < 5 ppb) single crystal chip, with a NV center layer of 13 μm. This layer has a NV centers density of $d = 3 \times 10^{17}$ cm$^{-3}$ and a characteristic dephasing time $T_2^* = 450$ ns. The sensing volume is $V = 5 \times 10^{-6}$ cm$^3$, consequently the number of potentially stimulated centers is $n = 15 \cdot 10^{11}$.

Referring to the Eq. 7 the fundamental sensitivity limit becomes: $\eta \approx 10 \, fT/Hz^{1/2}$, while the sensitivity reached experimentally is $\eta \approx 15 \, pT/Hz^{1/2}$, allowing, anyway, a reliable measure of the magnetic fields generated by these animal species (of the order of nT) but it is still not sufficient for reveal those of human neurons (of the order of pT).

### 3.2 Nanodiamonds

The techniques for the creation of NV centers in diamond are well established also for nanodiamonds (NDs). The minimum dimensions reached by nanodiamonds-based sensors exploits colloidal suspensions of single diamond particles of diameter 4-5 nm, but on average the nanodiamonds typically used in experiment have a size of 50-100 nm. The nanometer size makes nanodiamonds-based sensor of extreme interest for bio-sensing application, furthermore they are potentially usable in vivo experiments. Nonetheless, they have also important drawbacks such as e.g. the increased sensitivity of NV spins to environmental noise. Indeed, while in a bulk diamond the coherence time $T_2^*$ is mainly influenced by the electronic impurities and nuclear spins in the surrounding, for nanodiamonds the coherence time is further reduced by to the surface spin noise. This should be accounted for in the estimation of the sensitivity limit (Eq. 7). Despite this limitation, nanodiamonds have attracted interest also as a non-toxic alternative to quantum dots for biomedical imaging, then as magnetic sensors and finally as drug transporters (thanks to the discovery of the possibility to functionalize the diamonds surface in various ways, exploiting the covalents carbon bonds). The great interest and the exceptional range of applications of NDs is pushing improvement in fabrication technologies already able to provide very pure nanodiamonds with controlled surface chemistry at a low cost [99, 100].

#### 3.2.1 Biocompatibility and functionalization studies

To understand the perspective in bio-medical application, deep investigation of NDs biocompatibility is required. More specifically, it is important to understand how they diffuse in tissues and their long-term biological effect. While bulk diamonds are non-toxic and inert, NDs interaction with cells should be carefully investigated [101, 102]. There is a huge variety of nanodiamond specimens because of their different possible dimensions and to the various way in which their surface can be functionalized. Each of them could potentially interact in a different way with the cell sample, therefore, it is fundamental to check their non-toxicity before their real use. Various experiments were therefore conducted to evaluate the cell viability, e.g. in HeLa cells [103, 104, 105] (a cell line...
FIG. 9: Measured AP voltage and magnetic field from excised single neurons, taken from the reference\textsuperscript{[49]}. A) Measured time trace of intracellular axonal AP voltage $\Phi_{in}^{meas}(t)$ for giant axon from \textit{M. infundibulum} (worm). B) Calculated time trace of AP magnetic field $B(t)$ for \textit{M. infundibulum} extracted from data in A. C) Measured time trace of AP magnetic field $B(t)$ for \textit{M. infundibulum} giant axon with $N_{avg} = 600$. D) Measured time trace of AP magnetic field $B(t)$ for \textit{L. pealeii} (squid) giant axon with $N_{avg} = 375$. Gray box indicates magnetic artifact from stimulation current.

FIG. 10: Single-neuron AP magnetic sensing exterior to live intact organism, taken from the reference\textsuperscript{[49]}. A) Overhead view of intact living specimen of \textit{M. infundibulum} (worm) on top of NV diamond sensor. In configuration shown, animal is stimulated from posterior end by suction electrode, APs propagate toward worm’s anterior end, and bipolar electrodes confirm AP stimulation and propagation. (Scale bar 20 mm). B) Measured time trace of AP magnetic field $B(t)$ from live intact specimen of \textit{M. infundibulum} for $N_{avg} = 1,650$ events.

deriving from tumoral human cells), in human neurons\textsuperscript{[106,107]}, in human trachea\textsuperscript{[108]}, in the translucent \textit{Caenorhabditis elegans} worm\textsuperscript{[109]} and intravenous infusion\textsuperscript{[110]}. Briefly, it is found that the nanodiamonds of size between 50 and 100 nm are correctly incorporated by the cells, without damaging them. In particular in Guarina et al\textsuperscript{[guarina2018nanodiamonds]} an ODMR detection scheme with NV centers in nanodiamonds internalized in hippocampal neurons was performed in suitable conditions (3 mW of excitation power, -20 dBm of continuous-wave MW power), demonstrating that tested cells were not affected by the implementation of the measurement protocol, in their spontaneous firing (bursts synchronization was preserved, as well as the amplitude of spontaneous inhibitory and excitatory events), even if some alteration both at the single-cell level and in neuronal networks was observed in neuronal firing, which was principally attributed to the effects of nanoparticles aggregation. The aim of the work was to assess the feasibility of in \textit{vitro} imaging and targetable drug delivery via nanodiamonds, but the same argument holds for sensing applications. Furthermore, if properly functionalized, the nanodiamonds can anchor themselves to the surface of the cell sample in the desired areas.

3.2.2 Nanodiamonds applications

Once the biocompatibility of nanodiamonds is assessed, it is necessary to understand to which extent the sensing techniques developed for sensor based on NV in bulk diamond can be extended to nanodiamonds based sensors, functionalized and incorporated in the cells of interest.

A proof-of-principle demonstration of quantum control techniques to map the intracellular temperature of a neuronal network was performed by Simpson et al\textsuperscript{[109]}. The NDs were dispersed in cell media in concentration of 6 $\mu$g/mL, sonicated for few minutes, and then applied to the primary cultures during a routine change of cell media. The 170 nm diameter NDs contained approximately 500 NV centers each.
Using ODMR techniques in combination with standard wide-field microscopy with a field of view of 80 × 80 μm² was possible to observe NV resonance frequency in only 6 seconds. Specifically, in Ref. the ODMR signal presents two fluorescence dips (see section 1.4). This effect is negligible in bulk diamonds while nanodiamonds crystal lattice suffers strong deformation inducing line splitting. In that paper the two dips, spaced by few MHz, were modeled as a single one with higher spectral broadening. By interpolating the ODMR graph with a Lorentzian function, it was estimated the mean crystal field splitting $D_{gs} = (2868.59 ± 0.17) \text{MHz}$.

To demonstrate the NV thermo-sensor performance in biological measurement, the temperature of the neuronal solution was reduced by 1.9 °C. Repeating the ODMR analysis for a total acquisition time of 12 s, a resonance frequency shift was observed. The respective temperature variation was estimated using the temperature coupling coefficient $dD/dT \approx −74 \text{kHz}/K$ (see section 1.5). The distribution reported a mean temperature change of $−1.36 ± 0.08)\degree C$, consistent with the reduction in environmental temperature.

We underline that NDs allowing to create maps of the spatial distribution of the temperature inside the cells allowing to have new insight of the biological system. There are many biological processes whose knowledge would be enriched by using this kind of measurements. For example, it could be verified if in neurological disorders, such as epilepsy, the temperature changes is responsible for the increase of neuronal impulses, or even if temperature increases are really generated following the opening of the ion channel.

Another biological application reported by Ermakova et al. using nanodiamonds with NV centers as thermo-sensors, optically-induced thermal gradients for thermogenetic neural modulation. This thermal gradient is generated at the transient receptor potential channels (TRP channels): a group of ion channels that are normally found on the plasma membrane of numerous types of animal cells. A particular specialized form of these ion channels appears to be highly sensitive to temperature changes. Some species of snakes can use TRP channels to detect the thermal build-up caused by infrared IR radiation emitted by nearby prey, allowing them to estimate the direction and distance of the IR source.

To experimentally recreate this local temperature change and therefore study the TRPs response, Ermakova et al. used IR laser. This method, respect to conventional techniques as environmental heating or TRPs chemical agonists allows cellular spatial resolution and ultrahigh temporal resolution. The precise temperature control is performed by means of rapid IR laser pulses and varying the laser intensity, whose actual thermal impact is monitored by the nitrogen-vacancy complex. This quantum probe (whose dimension is of about 300 nm) is integrated on the tip of an optical fiber, together with a microwave antenna. The optical fiber is positioned near the cell irradiated by the IR laser, allowing a measurement of its temperature by the ODMR technique.

Specifically, in this experiment, it was initially evaluated if thermal stimulation via IR laser of two TRP channels of the snake. The TRP channels considered were the Crotalus atrox TRPA1 (caTRPA1) and the Elaphe obsoleta lindheimeri TRPA1 (elTRPA1). Fluorescent proteins (caTRPA1-IRES-EGFP) had been added to the channels, allowing to monitor the calcium channels opening and closing. Thanks to the IR laser pulses it was possible to slowly increase the cell temperature, to monitor it with the NV sensor and then to obtain the threshold temperature, inducing opening of the calcium channels. The threshold temperatures were found to be $T_0 = (27.8 ± 0.6)\degree C$ for caTRPA1 (see fig.11) and $T_0 = (38.5 ± 0.7)\degree C$ for elTRPA1 (see fig.11).

Once estimated the threshold temperature $T_0$, Ermakova et al. experimented the technique on other animal species. The cells used were mouse neurons and zebrafish larvae, whose thermogenetic activation is induced by TRPA1 channels causing responses.

In the case of caTRPA1 channels, the in vivo neurons were maintained at a temperature of 27 °C lower than the threshold temperature obtained before for this channel; in the case of elTRPA1-expressing, neurons were kept at basal temperatures of 35.5 °C.

As expected, they found that the thermal increase induced by the IR laser activates the TRP channels triggering the generation of the neuronal AP, measured through conventional electrophysiological techniques. When to measurements on live samples is considered, the sample can no longer be kept at the desired temperature, therefore it is necessary to choose the TRP channel suitable for body temperature of the animal species analyzed. As for the zebrafish neurons, whose body temperature is found to be 26 °C, the elTRPA1 channels may be suitable. As for the mammalian brain, the perfect TRP candidate has yet to be found. For example, the mouse body temperature is too close to the threshold temperature of elTRPA1 and it may be desensitized.

The results of the application of this technique in living zebrafish showed that it is possible to thermogenetically activate neurons using the IR laser. In particular, the technique demonstrated a spatial resolution of 60 μm (fiber size in which the IR laser was focused on the sample), allowing one or few neurons to be stimulated. As for the IR laser intensity, Emarkova et al. observed that 30 mW laser power induced the escape behavior exhibition of 93% of the larvae. NV-based temperature sensors allow careful monitoring of the temperature reached by the cells with high spatial resolution and temperature sensitivity up to 0.1 °C. To preserve cellular integrity and to avoid cell ablation is essential to heat-up the tissues by a few degrees only and for a time interval not exceeding a few
FIG. 11: Activation of snake TRPA1 in cells expressing TRPA1-IRES-EGFP using femtosecond IR laser pulses, taken from the reference.\textsuperscript{111}

a) R-GECO1.1 fluorescence (black line) reflects \(Ca^{2+}\) dynamics in the cytoplasm with the 20 mW laser beam turned on at \(t=30\) s and off at \(t=60\) s.

b,c) With the temperature of HEK293 cells expressing snake TRPA1 increased in a stepwise fashion using properly adjusted IR laser radiation, the activation thresholds of caTRPA1 b) and eolTRPA1 c) were determined.

d) A similar heating of control cells does not induce \(Ca^{2+}\) elevation. The black line is the fluorescence response. The red line is the temperature in the medium.

### 4 Techniques for improving ODMR sensitivity

In this section we discuss some technological solutions to improve the sensitivity of NV-based sensor as well as the precautions to be taken when it is used as a bio-sensor.

Considering Eq.\textsuperscript{7}, where the expression of the ultimate sensitivity limit reachable is reported, it is obvious that the number \(n\) of NV centers and their decoherence time \(T_2^*\) play a key role. To increase \(n\), while maintaining the same spatial resolution, it is necessary to have diamonds with an increased NV centers density. This can be achieved by enhancing the number of nitrogen implanted in the diamond and improving the N-to-NV conversion efficiency, minimizing the concentration of residual paramagnetic substitutional nitrogen\textsuperscript{17}. In parallel, to increase \(T_2^*\), it is also recommended the production of ultra-pure diamonds, with reduced unwanted electronic impurities (e.g. the P1 centers) and nuclear spins impurities (e.g. the paramagnetic \(^{13}\text{C}\) isotopes, whose natural abundance is about 1.1 \%).\textsuperscript{119,120,121} It is important to note that the NV density increase will necessary worsen the decoherence time of the NVs themselves, because of their mutual interaction. Consequently, an optimal trade-offs between these parameters must be sought.

In addition to the NV-density and diamond sample engineering, the sensitivity can be improved by implementing specific experimental techniques, that are based on laser and microwave pulses of particular duration, synchronized appropriately.\textsuperscript{122,123}

For example if an unknown electromagnetic field, responsible for the ODMR resonance frequency shift, is constant or slowly varying, it is possible adopt the experimental \textit{pulsed} ODMR protocols\textsuperscript{125} or the \textit{Ramsey} method\textsuperscript{14}, instead of the continuous wave (\textit{CW}) ODMR\textsuperscript{124}. The \textit{CW} ODMR is the simplest and most widely employed magnetometry method with NV-based sensors, wherein the microwave driving and the optical polarization and readout occur simultaneously. Although this technique is easy to be implemented, the relative ODMR spectrum dips are affected by the broadening induced by the continuous exposure of the laser beam and microwave field on the sample. With \textit{pulsed} ODMR techniques this broadening effect is substantially suppressed, allowing to obtain a narrower FWHM of the ODMR spectrum dips and therefore to improve the sensitivity measurement. This protocol, in fact, uses temporally separated optical laser initializations, \(\pi\) microwave control pulses, and laser readout pulses. The \(\pi\) pulses, whose name derives from the representation of the process on the Bloch sphere, is an oscillating microwave field that brings the electronic state from the state \(|m_s = 0 >\) to \(|m_s = \pm 1 >\). \textit{Ramsey} ODMR spectroscopy, on the other hand, consists on in the application of two \(\pi/2\) pulses, separated by a time \(\tau\). Also the \(\pi/2\) pulse is an oscillating microwave field that brings the electronic state from the state \(|m_s = 0 >\) to a balanced superposition of \(|m_s = +1 >\) and \(|m_s = -1 >\). By varying the time \(\tau\), the so-called ”\textit{Ramsey fringes}” are obtained, from which it is possible to extrapolate a measure of the fields amplitude. Also this technique allows sensitivity improvement with respect to the \textit{CW}: the decoupling from the MW and laser power offering the possibility to increase the MW power to improve the contrast, without degrading the FWHM.
In the case of time-varying electromagnetic fields, there are other even more complex microwave pulse sequences, capable of decoupling the measurement from surrounding spin environment. In this way the decoherence time of the NV centers increases and consequently it becomes possible to interrogate the quantum system for longer times, improving the measurement statistic and therefore the sensitivity. One of these experimental protocols is the Hahn Echo sequence, which refocuses the dephasing NVs spin, applying an additional $\pi$ pulse in the middle of Ramsey sequence. The characteristic time of the spin coherence decay, measured with this protocol, is called $T_2$ and it is typically one or two orders of magnitude longer than $T_2^*$. Even more complex dynamic decoupling sequences, which apply multiple refocusing $\pi$ pulses further improving the decoherence time $T_2$ have been devised. Among these, the most famous are the Carr-Purcell-Meiboom-Gill (CPMG) and the XY8 sequences, which differ in the rotation axes (around which the spin rotates): the first method applies the pulses along the same axis, while the second chooses a different one for each $\pi$ pulses. It is useful to underline that, although these techniques allow to extended the coherence time of the NV centers, they cannot go beyond the spin-lattice relaxation time $T_1$, that for an NV spin ensemble in bulk diamond is about 3 ms.

The figure briefly summarizes the above mentioned pulse sequences.

![Diagram of NV measurement protocols](image.png)

**FIG. 12:** NV measurement protocols, taken from the reference. Schematic of timing and duration of laser pulses, microwave pulses, and readout sequences relative to the field being sensed for common NV diamond protocols.

It is useful to underline that the sensitivity formula in Eq.7 describes an idealized measurement with a perfect readout mechanism. On the contrary, typically the readout mechanism add noise in the measurement, that can be described introducing, in the previous equation, the spin-readout fidelity factor $\mathcal{F}$.

$$\eta = \frac{1}{\gamma_e \sqrt{nT_2^*}} \frac{1}{\mathcal{F}}$$

Keeping the usual optical-readout, but improving the photon collection is expected to increase $\mathcal{F}$ (see for different methods to improve photon collection). Ancilla-assisted repetitive readout, which is based on mapping the NV spin state to the nuclear spin state also improves $\mathcal{F}$.

When the ultimate goal is bio-sensing, some constraints rise limiting the implementation of the above described pulse sequences. One constraint is the frequency bandwidth. In fact, the dynamic decoupling techniques mentioned above are capable of measuring time-varying external field only if this time variation is of the order of the time interval separating the $\pi$ pulses. Furthermore, in order to control the system quantum state, the time between these pulses cannot exceed the coherence of the NV center. Consequently, the frequency of the signal to be measured must be of the order of the coherence time of the NV centers. In the biological case, the electromagnetic fields pulse lasts about 1 ms. This value is very far from $T_2$, marking a boundary for the use of these techniques in biological applications. Another constraint is associated to the optical laser power. The higher the laser power the better the sensitivity in measurements with ensembles, since it increases the percentage of excited centers and consequently the fluorescence signal. However precautions must be taken to avoid cells and proteins damaging. An efficient solution can be to direct the laser beam towards the diamond sample at an angle allowing total reflection (Brewster angle). In this way only the fluorescence emitted by the NV centers travels through the cells, placed on the other diamond surface. In this case, however, precise control over sensing volume would be lost, deteriorating spatial resolution.
In a standard configuration, where the laser impinges perpendicularly on the sample, it is necessary to limit the optical power reaching the cells to few mW. In this regard, Fig. 13 shows a sensitivity curve versus the laser optical power, obtained by adopting the technique described in Moreva et al.\textsuperscript{25}. As anticipated in the introduction, the application of a transverse bias magnetic field $B_{\text{bias}} \simeq 3\, mT$, allows to improve the sensitivity of a the NV center based thermo-sensor with respect to other standard techniques in CW regime. In Ref.\textsuperscript{25}, the temperature sensitivity reached is $\eta \simeq 4.8\, mK/Hz^{1/2}$ in a sensing volume of $1\, \mu m^3$, obtained at a power level (80 mW) that can present biocompatibility problems. However, the sensitivity obtained is even beyond the one required to monitor biological mechanisms, usually requiring sensitivities of the order of $1 \, ^\circ C$. Thus, Fig. 13 shows that it is possible to perform the temperature measurement with a lower laser power, finding an ideal compromise between the temperature sensitivity and laser intensity impinging on the cell sample. Indeed, with a power of a few mW it is already possible to discriminate biological processes with a sensitivity of the tenth of a degree.

![Graph showing temperature sensitivity versus the excitation power at 532 nm.](image)

**FIG. 13:** Temperature sensitivity versus the excitation power at 532 nm, with reference to the technique described in \textsuperscript{25}. The inset shows the inverse of the thermal sensitivity versus the excitation laser power.

**Conclusion**

Sensors based on the NV centers in artificial diamonds are one of the emerging quantum technologies as a technology of huge potential interest in biological applications, thanks to both their practicality and their technical performances. In fact, the ability to initialize and read out optically the spin state at room temperature, makes the use of this quantum sensor convenient and powerful even for biological applications. Furthermore, the levels of sensitivity and spatial resolution achieved are extremely high, which in principle allows potential application towards the detection of very weak electromagnetic fields as the one generated by mammalian, and potentially human, cells. Even if an eventual use of NV sensors for the detection of biological electric fields is more problematic due to its weak coupling constant, regarding the magnetic field sensing and especially temperature measurements astonishing results have already been achieved. Indeed, the thermal gradients generated by biological phenomena can be reliably observed thanks to the actual sensitivity of the NV-based sensors. This is of utmost importance because localized intracellular temperature gradients may affect neuronal functionality (including vesicular dynamics and neurotransmitter release) or may provide indirect measurement of mitochondrial activity. Regarding the detection of bio-magnetic fields, the NV-based sensors have already good results with peculiar biological cells, presenting either an intrinsic magnetic field (magnetotactic bacteria) or a generated magnetic filed in axon of squids or long worms, much larger than the one generated in the human ones. The improvement of these devices suggests the possibility of exploiting NV-base sensors also for the detection of weaker but more fascinating biological magnetic fields. In particular, an estimate of the cardiac magnetic field that is generated on the heart surface was here reported. This value is in the range of present measurement exploiting the NV center properties, together with optimized diamond sample engineering and the adoption of pulsed measurement protocols in order to improve the diamond coherence time. Another estimate reported in this paper concerns the analysis of the magnetic field associated to human neuron activity. The weakness of these fields requires further improvements of this detection technique, in particular for the single action potential while measurement of clustered channels is probably a target in the range of present technology. However the considerable
interest in the neuronal field detection for its perspectives as diagnostic and therapeutic tools for neurodegenerative diseases and aging effects, together with the recent years progress of these techniques (partially covered by this review), is expected to boost the technological developments and eventually the market success of quantum assisted biosensing based on NV centers.

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