Chapter 6

# General Discussion

Fluorescent nanodiamonds (FNDs), with Nitrogen-Vacancy (NV) centers doped in the crystal lattice, are attractive for many biological applications. In this dissertation, the projects were performed to study the interplay between cells and FNDs and providing insights into the activity of FNDs in the cells. Additionally, it explores the potential quantum applications of FNDs in biological fields. In this chapter, we will discuss the findings of each project and implications for the future.

**6.1 Cellular uptake and fate of FNDs**

Endosomal escape is crucial for efficient intracellular delivery and subcellular targeting. Quantitative fluorescence analysis is the most popular method to estimate the endosomal escape efficiency[1]. However, the precision of the result is usually constrained by the quenching and bleaching issues of fluorescent dyes[2]. In contrast, the colour centers of nanodiamonds reside beneath the surface, being isolated from the surrounding environment. As a result, FNDs become ideal candidate for data analysis. In **Chapter 2**, we evaluated endosomal escape efficiency using FNDs at the single-cell level in two different cell types. Adequate cellular uptake of FNDs is the prerequisite for quantitative analysis. The internalization of FNDs was calculated following the previous report[3]. Compared to HeLa cells, we found that human umbilical vein endothelial cells (HUVECs) ingest more FNDs. Besides, the number of FNDs per cell is significantly unevenly distributed between cells. The distribution of FND in cells shows a cucurbit shape which means that most cells contain a few particles while several cells have more. This indicates that the cell diversity, even in the same cell type, is usually underestimated.

Next, we assessed the endosomal escape of FNDs with the calcein assay. Calcein is a cell membrane impermeable dye and is internalized into cells only via endocytosis. It shows green fluorescence in the intracellular vesicles and diffuses into the cytoplasm after endosomal escape. If there is no colocalization between green and red signals, it means

that FNDs escaped from the endosomes successfully. As expected, we found a large variation in the results of single cells while whole image analysis represents an average result of multiple cells. Nevertheless, we observed remarkable endosomal escape of FNDs in HUVECs after another 4h of incubation while we did not observe the same in HeLa cells. One potential reason is that HUVECs are primary endothelial cells and HeLa cells are cancer cells.

While FNDs are generally easily internalized by cells, this is not the case for HeLa cells. HeLa cells require longer incubation time and higher concentrations of FNDs to achieve sufficient uptake[4]. To tackle this problem, in **Chapter 3**, we modified FNDs with pHrodo Green Dextran. The pH-sensitive coating not only significantly enhances the cellular uptake of FNDs but also indicates the subcellular location by the changes in fluorescence intensity. We observed a 5-fold increase in the cellular uptake of FND compared to bare particles. After the cells were exposed to the nanodiamonds, the endocytic process was clearly revealed by the 1h live cell imaging. pHrodo Green Dextran is a pH-sensitive dye and emits higher green fluorescence with decreasing pH. With this property, we further investigated the interactions between cells and FNDs at various time points. FNDs were translocated from early endosomes to late endosomes and lysosomes with time. In the end, most of FNDs were excreted by the cells.

Although the surface modification brings many superior properties, the coated FNDs lost their quantum sensing abilities. We were not able to find characteristic peaks with optically detected magnetic resonance (ODMR). The coated FNDs retained the red fluorescence while losing the capability of quantum sensing. We suspected that the positively charged pH-sensitive dye caused the charge conversion in FNDs and turned $NV^-$ centers into $NV^0$. We also did some follow-up experiments to explore it. We coated FNDs with dextran or dextran-$NH_2$ to mimic the coating. While dextran coated FNDs preserved

the quantum properties, the bonus of highly efficient cellular uptake also disappeared.

**6.2 Biocompatibility of FNDs**

The biocompatibility of FNDs is widely acknowledged among various cell types. Sperm cells are distinguished from other cells by their unique structure and functions. In **Chapter 4**, we systematically investigated the biocompatibility of FNDs on boar sperm, considering variations in size, concentration, and maturity stages. We conduct experiments from different aspects such as metabolic activity, cell membrane integrity and redox response. The results showed that the interaction between FNDs and sperm cells is not consistent across all conditions. Specifically, FNDs with larger sizes and lower concentrations exhibit less stress on the sperm cells. 1 μg/mL 70nm and 120nm FNDs are safe to act as bio labels in sperm cells.

**6.3 Diamond relaxometry**

Due to the remarkable quantum properties at room temperature, FNDs have emerged as exceptionally promising material in the sensing field. In **Chapter 5**, we applied diamond relaxometry to detect the free radical dynamics in primary bronchial epithelial cells isolated from either chronic obstructive pulmonary disease (COPD) patients or healthy donors. The primary cells were exposed to different concentrations of cigarette smoke extract (CSE) for 20 minutes. We found that, with 15% CSE treatment, COPD donors exhibited strong reactivity to the stimulation, leading to increased production of free radicals compared to healthy donors. If the concentration of CSE increased to 35%, cells from both groups were overstressed and showed low T1 values. Therefore, no statistical differences were found. It is worth noting that the diamond relaxometry was performed on the single cell level and not impacted by the complex chemicals in the smoke extract. Besides, FNDs were neither consumed nor reacted with surrounding molecules during the measurement. In combination with tiny inserts during the cell culture, we obtained real-time dynamics of

free radicals in a few cells with nanoscale spatial resolution.

During the measurement, we observed notabe individual differences. Primary cells may have a different baseline of free radicals or different responses even though they're from the same health status. Variation in individuals is very common in clinical studies[5]. In our study, we don't have any further information regarding each donor. The only criterium for the selection is whether he/she has COPD. The presence of unknown information, such as age, gender, and underlying health status, introduced considerable uncertainty into the results. Additionally, the inherent variability among individual cells further contributed to the overall uncertainty. Increasing the sample size may reduce the error.

**6.4 Further perspective**

With diamond relaxometry, the surrounding magnetic noise (e.g. free radicals) can be converted to optical readout. The sensitivity and spatial resolution allow measurements down to the nano scale. This emerging technology brings new opportunities alongside some challenges.

One direction is to use our technique to gain a deeper understanding of the radical involvement of physiological mechanisms or disease development. Conventional methods typically require a large number of cells, involve destructive procedures and long time reaction. Even though something happened in the early phase, due to the limitations mentioned above, we poorly understand them until the accumulated phenomena is pronounced. Diamond relaxometry is sensitive and powerful but often lacks comparable supporting data. If our measurements are coupled with follow up tests (such as single cell analysis), in combination with location and time details, they will provide a great chance to shed some light on free radical generation.

Another direction is to apply our method to more complex models. 2D cell models are simple and widely used. But the communication among various cells and the tissue

architecture are often underestimated. Our technique is compatible with basic organisms to sophisticated animal models. In the meantime, diamond relaxometry is an optical based system and therefore shares all the advantages and limitations of light microscopy. The bio autofluorescence, penetration depth, how to keep the sample still and alive will be the potential bottlenecks.

**References**


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