University of Groningen

Diamond Nanosensors for Age and Stress Related Changes in Cells

Morita, Aryan

DOI:
[10.33612/diss.136220127](https://doi.org/10.33612/diss.136220127)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Morita, A. (2020). *Diamond Nanosensors for Age and Stress Related Changes in Cells*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.136220127>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Diamond Nanosensors for Age and Stress Related Changes in Cells

Aryan Morita

Diamond Nanosensors for Age and Stress Related Changes in Cells

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Tuesday 3 November 2020 at 9.00 hours

by

Aryan Morita

born on 23 August 1987
in Yogyakarta, Indonesia

Thesis artworks: Aryan Morita

Supported by LPDP Scholarship from Ministry of Finance Indonesia

Supervisor

Prof. R. Schirhagl

Co-supervisor

dr. A.I. Mzyk

Assessment Committee

Prof. C.F. Calkhoven

Prof. T. Cordes

Prof. A. Hermann

Dedicated to my beloved family and Andri

Paranymphs

Linyan Nie

Isti Hidayati

Contents

Chapter 1	General introduction	9
Chapter 2	Cell uptake of lipid-coated diamond	21
Chapter 3	The fate of lipid-coated and uncoated fluorescent nano diamonds during cell division in yeasts	49
Chapter 4	Targeting nanodiamonds to the nucleus in yeast cells	87
Chapter 5	Quantum monitoring the metabolism of individual yeast mutant strain cells when aged, stressed or treated with antioxidant	113
Chapter 6	General discussion	159
	Summary and samenvatting	165
	Acknowledgement	172
	Curriculum vitae	175
	List of publications	176

1. General introduction

Chapter 1

1.1 Free radicals, oxidative stress, and ageing

Free radicals, chemical substrates with at least one unpaired electron, strongly contribute to physiological and pathological processes¹. Free radicals are by-products of the normal cellular metabolism that mostly takes place in mitochondria. A schematic representation of the cell's metabolism in mitochondria is presented in figure 1.

Figure 1. Mitochondrial metabolism. Mitochondria as powerhouse of the cells generate energy in form of ATP by using oxygen (O_2) and transport electrons (a). Mitochondria also have a system to eliminate waste byproducts with the help of enzymes including superoxide dismutase (SOD) (b). This enzyme converts free radicals to less reactive molecules like oxygen and hydrogen peroxide (H_2O_2). (Reprint from Spinelli and Haigis, 2018)²

Accumulation of free radicals during cell metabolism or an inadequate clearance system will lead to mitochondrial dysfunction³. Mitochondrial dysfunction has been proposed as driving force behind the aging process⁴ and oxidative stress in cells⁵. Oxidative stress itself plays a major role in development of chronic and degenerative diseases in the human body.

1.2 Free radicals and antioxidants

Cells have mechanisms to neutralize the effect of free radicals by producing antioxidants. Mechanisms of antioxidants to destroy free radicals have been classified in two ways. First, free radicals are converted into less reactive molecules by donating or removing the electron. The second mechanism is prevention of free radical formation by scavenging free radicals or stabilizing transition metal radicals like copper and iron⁶. Although cells can produce endogenous antioxidants like glutathione, exogenous antioxidants also play an important role in the cell's defense against free radicals. Some of these antioxidants are promising anti-ageing drug candidates.

1.3 Detection methods for free radicals

Detection methods for free radicals have been developed for many years. Since these substrates occur in very small concentrations and have short lifetimes, finding the best method is not easy. One way is via detecting the expression of certain genes which encode enzymes involved in coping with stress indicating molecules (e.g superoxide dismutase or catalase)⁷. These gene expressions are then investigated using quantitative polymerase chain reactions (qPCR). Instead of detecting expressed genes one can also analyze the enzymes, which these genes encode for (most commonly by western blotting). The major advantage of these methods is that these enzymes are specific for certain radicals and one can

differentiate between them. However, the enzymes have to be known in advance. Furthermore, spatial and temporal information on location and time of generating free radicals are lost. Another indirect method that can be used is the lipid peroxidation assay⁸ or DNA damage profiles⁹. Both detect damage caused by free radicals, reactive oxygen species (ROS) or other damaging sources. These methods suffer from low specificity and do not reveal spatial information.

In contrast imaging approaches provide spatially and temporally resolved data. The two most common, non-destructive 3D imaging techniques for biomedical applications are fluorescence imaging and magnetic resonance imaging. Figure 2 shows some approaches for detecting free radicals.

Figure 2. Approaches for detecting free radicals. (1) Using fluorescence based dyes and (2) spin trap for measuring free radicals production. (Reprint from Sigaeva et al, 2019)¹⁰

Fluorescence imaging allows direct detection of free radicals. Additionally, it is relatively easy and very sensitive. The assays are based on an organic dye (e.g. H₂DCFDA, luminol), which fluoresces when reacting with ROS.

However, there are two problems with this approach. First, often the ROS are consumed in the process and thus distinct time points and no real time curves are obtained. Furthermore, these dyes generally are non-specific and react with a wide range of different reactive molecules (as reactive oxygen species or reactive nitrogen species). Additionally, organic dyes, which are used in these techniques suffer from photo-bleaching and thus can only be used for short term studies. Quantum dots can be very photo-stable but are usually at least to some extent toxic¹¹. Additionally, one does not obtain chemical information on the environment.

Magnetic resonance imaging (MRI) and electron spin resonance spectroscopy (ESR) are the gold standard in many different scientific disciplines. Especially valuable is the ability to non-destructively reveal 3D information, which is element specific. Thus it allows for functional contrast. However, especially when high spatial resolution is required or only limited amounts of sample (as the interior of a single cell) are available the methods approach their limits. In conventional MRI, approximately 10^{12} - 10^{18} nuclei (or a factor of 1000 less electron spins) are needed to generate an observable signal. This limits the resolution to about $3 \mu\text{m}^3$ at its best^{12,13}.

1.4 Diamond magnetometry as a tool for detecting free radicals

Diamond magnetometry combines the advantages from fluorescence imaging (easy and sensitive) with specificity from magnetic resonance methods. The technique is based on a defect called nitrogen vacancy (NV) center in diamond crystals that can convert magnetic noise to an optical signal. The basic principle of diamond magnetometry is shown in figure 3.

Figure 3. The basic principle of diamond magnetometry: (a) NV center in diamond¹⁴. (b) Energy diagram of an NV-center. (c) Optically detected resonance of an NV-center at different fields^{15,16}.

After excitation with a green laser the NV-center emits red photons. If the electron is in the $m_s = \pm 1$ state there is also an alternative way to the ground state over a dark state. As a result less red photons are emitted and a decreased fluorescence is observed. If a microwave at the resonance frequency is applied that equals the difference between the two states (2.88 GHz at zero field) the spins flip into the $m_s = \pm 1$ state. This effect can be observed as a drop in fluorescence. In presence of a magnetic field the $m_s = \pm 1$ states are not equal in energy any more resulting in two resonance lines.

Their distance is proportional to the field, which thus can be determined. The exact position of the resonance peaks reveals the presence of near-by (within some tens of nm distance from the defect) radicals. That this fluorescence can be read out with a confocal microscope constitutes an advantage since photons can be read out more sensitively. This new technique has already been successfully used to measure magnetic vortices¹⁷, record the magnetic signature of magnetosomes in living magnetotactic bacteria¹⁸, and quantify biomarker expression from cells with nanoparticle labels¹⁹.

1.5 Thesis objectives

The aim of this thesis is to apply diamond magnetometry to measure free radicals in cells during the ageing process and when they are triggered by oxidative stress. To achieve this goal, in **chapter 2**, we established a diamond uptake protocol as initial step. In **chapter 3**, we followed the particles movement during cell division. To be able to control the location of diamond particles, in **chapter 4** we modified the particles by using a specific antibody for targeting specific organelles. In **chapter 5**, we performed the first diamond magnetometry measurement in living cells. More specifically we detect free radicals formation during oxidative stress responses in two different cell stages (young and aged cells). Additionally, we were able to clearly differentiate between knock out mutants with an impaired metabolism. Finally in **chapter 6**, we discussed about the importance and relevance of this thesis.

References:

1. Halliwell, B. & Gutteridge, J. M. C. Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol.* **186**, 1–85 (1990).
2. Spinelli, J. B. & Haigis, M. C. The multifaceted contributions of mitochondria to cellular metabolism. *Nat. Cell Biol.* **20**, 745–754 (2018).
3. Lane, R. K., Hilsabeck, T. & Rea, S. L. The role of mitochondrial dysfunction in age-related diseases. *Biochim. Biophys. Acta - Bioenerg.* **1847**, 1387–1400 (2015).
4. Harman, D. The Free Radical Theory of Aging. *Antioxid. Redox Signal.* **5**, 557–561 (2003).
5. Valko, M. *et al.* Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39**, 44–84 (2007).
6. Young, I. & Woodside, J. Antioxidants in health and disease. *J. Clin. Pathology.* **56**, 176–186 (2001).
7. Digniseppi, J. & Fridovich, I. The Toxicology of Molecular Oxygen. *Crit. Rev. Toxicol.* **12**, 315–342 (1984).
8. Gaschler, M. M. & Stockwell, B. R. Lipid peroxidation in cell death. *Biochem. Biophys. Res. Commun.* **482**, 419–425 (2017).
9. Cadet, J. & Richard Wagner, J. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harb. Perspect. Biol.* **5**, 1–18 (2013).
10. Sigaeva, A. *et al.* Optical Detection of Intracellular Quantities Using Nanoscale Technologies. *Acc. Chem. Res.* **52**, 1739–1749 (2019).
11. Soenen, S. J. *et al.* The cytotoxic effects of polymer-coated quantum dots and restrictions for live cell applications. *Biomaterials* **33**, 4882–4888 (2012).
12. Ciobanu, L., Seeber, D. A. & Pennington, C. H. 3D MR microscopy with resolution 3.7 μm by 3.3 μm by 3.3 μm . *J. Magn. Reson.* **158**, 178–182 (2002).
13. Glover, P. & Mansfield, P. Limits to magnetic resonance microscopy. *Rep. Prog. Phys.* **65**, 1489–1511 (2002).

14. Schirhagl, R. *et al.* Nitrogen-Vacancy Centers in Diamond: Nanoscale Sensors for Physics and Biology. *Annu. Rev. Phys. Chem.* **65**, 83–105 (2014).
15. Balasubramanian, G. *et al.* Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature*. **455**, 648–652 (2008).
16. Nagl, A., Hemelaar, S. R. & Schirhagl, R. Improving surface and defect center chemistry of fluorescent nanodiamonds for imaging purposes-a review. *Anal. Bioanal. Chem.* **407**, 7521–7536 (2015).
17. Rondin, L. *et al.* Stray-field imaging of magnetic vortices with a single diamond spin. *Nat. Commun.* **4**, 1–5 (2013).
18. Le Sage, D. *et al.* Optical magnetic imaging of living cells. *Nature*. **496**, 486–489 (2013).
19. Glenn, D. R. *et al.* Single-cell magnetic imaging using a quantum diamond microscope. *Nat. Methods*. **12**, 736–738 (2015).

Chapter 2

2. Cell uptake of lipid-coated diamond

Aryan Morita^{1,2}, Felipe Perona Martinez¹, Mayeul Chipaux¹, Nicholas Jamot¹, Simon Hemelaar¹, Kiran van der Laan¹, Romana Schirhag¹

1. Department Biomedical Engineering, University of Groningen, The Netherlands
2. Department Dental Biomedical Sciences, Universitas Gadjah Mada, Indonesia

Published in *Particle and Particle Systems Characterization* 36:1900116, 1-8 (2019)

Abstract

Fluorescent nanodiamonds (FNDs) can be used as nanoscale magnetic resonance sensors and stable optical labels. As a first step for using FNDs as nanosensors inside cells, they have to be ingested. Several techniques that improve particle uptake have been used. A simple approach based on commercially available liposomes is used to improve uptake. Uptake into colon cancer cells (HT-29 cells) is demonstrated. Additionally, it is shown for the first time that one can facilitate diamond uptake into yeast cells by removing the cell wall and creating a so-called spheroplast. Finally, the characteristics of FNDs coated with lipids and their behavior inside the cells are evaluated.

2.1 Introduction

Fluorescent nanodiamonds (FNDs) are a promising material for various biomedical applications including drug delivery, therapeutics, and imaging^{1,2}. FNDs have superior physical and chemical properties including hardness and Young's modulus, high thermal conductivity and electrical resistivity, chemical stability and resistance to harsh environments, and good biocompatibility^{1,3}. Nitrogen vacancy (NV) centers in FNDs, atomic-sized defects with stable fluorescence, possess outstanding optical and magnetic properties⁴⁻⁶. The fluorescence is perfectly stable, which qualifies FNDs as interesting biolabels. Additionally, when irradiated with microwaves, the NV fluorescence changes based on its magnetic surrounding. As a result, it can be used as a magnetic resonance sensor that can be read out optically. To utilize these sensors in cells, they first have to enter the cell. Although some cells spontaneously ingest FNDs⁷⁻¹², most cells do not ingest FNDs by themselves.

Introducing FNDs to the intracellular environment has been investigated by a variety of methods in a range of cell types. Methods that can be used include surface functionalization, pinocytosis, picoinjection, and gene gun bombardment¹³. Different coatings have been applied to achieve diamond uptake, such as recombinant polypeptide¹⁴, silica^{15,16}, polymers, and nucleic acids¹⁷. However, the above mentioned methods require complex synthesis and are either rather harsh to the cells or might disturb their natural behavior.

It has also been demonstrated that diamonds can be incorporated in lipid membranes¹⁸, which changes their crystallization properties. In combination with FND, several different lipid coatings have been investigated too. Hsieh et al. used a somewhat more complex protocol that allows elegant functionalization¹⁹. Vavra et al. also used a somewhat more complex approach based on silica-coated nanodiamonds which allows the introduction of spin labels into the coating²⁰.

Sotoma et al. used a different lipid composition which is based on a cross linked diacetylene containing coating²¹. This approach is different since the cross-linked coating likely is more stable and remains on the diamond and it is unlikely that it supports membrane fusion. Hui et al. used yet another composition of lipids which leads to particles with rather different properties²². Their particles become very hydrophobic and are soluble in organic solvents.

Here, we use a simple alternative lipid coating for cell uptake of FNDs for the first time. The advantage of such a coating is the simple coating process, and the possibility to tune the lipid composition^{23–25}. Most interestingly, liposomes might be able to fuse with the cell membrane and thus offer a way to circumvent the natural endocytosis or speed up uptake. The same process has already been demonstrated for other nanoparticles that were encapsulated with lipids^{26,27}. This is advantageous for all applications where the desired location is not the endosome. Since endosomes usually fuse with lysosomes, the particle is subjected to a harsh environment. From what we know so far, this likely does not affect the diamond particle itself. Furthermore, a large body of knowledge is available from the gene transfection or drug delivery fields on how to achieve targeting with lipid coatings. This knowledge can in a similar way also be applied to FNDs. Such a coating is also relatively biocompatible and biodegradable²⁸.

We have demonstrated the uptake of FNDs with lipid coating into cells and we have characterized the lipid coating used in this study (see Figure 1). We have also investigated the lipid-coated FNDs (FND-lip) behavior inside HT-29 cells, a colon adenocarcinoma cell line, and *Saccharomyces cerevisiae* that has a thick cell wall. Neither of these cells spontaneously ingest diamond particles. Finally, we showed the suitability of FND-lip for optical labeling and quantum sensing applications.

Figure 1. Schematic representation of diamond uptake into yeast. 1) The cell wall is removed. The remaining yeast cell, which is only covered by the cell membrane, is called spheroplast. 2) Nanodiamonds containing fluorescent defects are coated with lipids (FND-lip). 3) Finally, FND-lip particles are added to the spheroplasts. When their membranes fuse, diamond particles are released into the cytosol of the yeast cell.

2.2 Results and discussions

2.2.1 Characterization of FND-lip

Characteristics of FND-lip have been investigated using dynamic light scattering (DLS), cryo TEM (transmission electron microscopy), and optically detected magnetic resonance (ODMR). The DLS results (Figure 2) show an increase in particle size of the FND-lip compared to FNDs but the difference is not significant ($P > 0.05$).

Figure 2. Size determined by dynamic light scattering of FND-lip and FNDs. The measurement was performed at 25 °C.

Both FND-lip and FNDs are colloidally stable in water ($PdI < 1$). Zeta potential measurement showed that the 70 nm FNDs were electronegative (-15.73 ± 0.89 mV). After adding liposome, the particles became electropositive (35.67 ± 2.64 mV). We used cryo TEM to confirm our results because DLS is not ideal for aggregates due to sedimentation and because diamond particles are nonspherical. Figure 3 shows the results of these measurements. We compare FNDs (a) with FND-lip (b). In the inset of Figure 3b, lipids are visible both free and as a coating. The liposomes form a tight layer around the diamond particles. The thickness of the lipid layer on diamond particles was 4.8 ± 1.2 nm. Performing optically detected magnetic resonance measurements on FNDs and FND-lip (Figure 4d) did not reveal any significant differences.

Figure 3. TEM images of FNDs a) and FND-lip b). The insets show a zoom-in at representative areas. Diamonds are shown in blue (false color) and liposome (free and as a coating are indicated with the red arrows).

2.2.2 Cell uptake behavior

We used two different cell types to investigate cell uptake behavior, FND-lip and FNDs. To investigate the uptake into cells, we performed confocal imaging after uptake. Confocal images of HT-29 cells are shown in Figure 4.

Figure 4. a) Uptake of FND-lip into HT-29 cells (the image is $100 \times 100 \mu\text{m}^2$). b,c) are zooms into representative areas. Typical ODMR spectra taken from NV centers inside FND-lip in such HT-29 cells are depicted in d). Diamonds can be identified by their typical double peak pattern. We did not see any significant difference between ODMR spectra between coated and uncoated particles. e) shows uptake of bare FNDs into HT-29 cells (the image is $50 \times 50 \mu\text{m}^2$). f) is again a zoom in. Blue arrows point to diamonds that are clearly inside the cells.

The insets are included to give the reader a better view of the particle size and confirm that some of the particles are indeed within the cells. The images in Figure 4 are part of a z-stack. While large amounts of diamonds are present when using FND-lip (Figure 4a–c), considerably less uptake is observed with FND only (Figure 4e–f). To identify diamond particles and to show that it is still possible to do quantum measurements inside the cells, we performed ODMR measurements (see Figure 4d). The particles could indeed be identified as diamonds and coating did not alter the characteristic ODMR peaks from NV centers.

As a next step, we quantified the uptake into different cell types. Here, it is interesting to differentiate between objects and particles. All adjacent bright

pixels are attributed to an object, which means that an object can be either a single particle or an aggregate. The number of particles is calculated by dividing the total number of bright pixels in the red channel by the number of bright pixels for single particles.

Figure 5 shows that cells with FND-lip contain higher numbers of particles but less objects than FNDs. It indicates that FND-lip has a tendency to create aggregates. Compared to previous experiments by Hemelaar et al.⁸, the number of FND-lip and FNDs that can be internalized by yeast cells is lower than for spheroplasts. A possible reason is that once the cell wall is removed also naked FND can enter easily. Permeability changes also affect time that is required by particles to enter the cells which can be seen from comparing with Hemelaar et al.⁸.

Although we were not able to directly prove membrane fusion (see Figure 8), using liposome as a coating agent for FNDs helped the particles entering cells. It has been shown that liposome coating changed FNDs zeta potential from electronegative to electropositive. When FND-lip are taken up by the cells, they bind to the negatively charged plasma membrane. A previous study showed that cells ingested positively charged particles better than negatively charged ones²⁹.

As is known from the gene transfection field, spheroplasts have the disadvantage that they are more fragile than native cells³⁰. This is likely also the reason why we see some reduced viability after the uptake protocol. Although spheroplasts cannot proliferate by budding, they still have abilities of normal yeast cells. Spheroplasts can also regenerate their cell wall and revert to normal reproducing cells when they are inoculated in solid medium supplemented with osmostabilizer³¹.

Figure 5. Quantification of FND-lip and FNDs ingested by yeast spheroplast cells and HT-29 EpCAM-GFP cells. In a,b) control group, yeast cells without any treatment for their cell wall were mixed with FND-lip and FNDs. For all situations, 100 cells were selected randomly and analyzed. In c,d), quantification of particle uptake for FND-lip and FNDs has been done in HT-29 EpCAM-GFP by selecting ten cell clusters (these cells grow in clusters not as individual cells). Data from all groups were analyzed by a homemade Fiji program.

To test if the cells were still viable after diamond uptake, we performed MTT assays. The results are shown in Figure 6 for HT-29 cells (a) and as well for yeast spheroplasts in comparison to regular yeast cells (b).

Cells incubated with FND-lip survived better than FNDs for all the cell types. While FND-lip group has better survival rate, there is no significant difference between FND-lip and FNDs groups (P value > 0.05 with 95% confidence interval). The FND-lip group has the highest percentage of cell survival compared to the other groups. The experiments indicate that adding liposomes have no harmful effect to cells. This is expected because liposomes are

also natural cell membrane components. The relatively low viability compared to literature values with diamonds alone here most likely comes from uptake protocol. Especially for yeast cells, lower viability is expected since they undergo an uptake protocol where the cell wall is removed or partially removed. So, the “toxicity” does not come from the diamond themselves but from the method we use to achieve uptake.

Figure 6. MTT assays for a) HT-29 cell and b) yeast cells/spheroplasts. 100% is the value that is obtained for a control with untreated cells. For all cell types, liposomes alone are favorable while FNDs slightly decrease the viability. A positive control with H₂O₂ shows decreased viability as expected.

2.3 Conclusion

Coating with liposomes offers a way to deliver FNDs into HT-29 cells and yeast cells, which do not ingest particles spontaneously. Through the creation of yeast spheroplasts, this method can even be extended to cells with a thick cell wall as yeast. This spheroplasting method could also be applied to use any of the other lipid-based coatings in literature for yeast cells. Since liposomes change the zeta potential of FNDs and since they might also fuse directly with the cell membrane, higher amounts of particles can be ingested. This could be

especially useful in combination with coatings which prevent aggregation or which lead to targeting.

2.4 Experimental section

The uptake behavior was evaluated with two different cell types: a colon cancer cell line called HT-29 and a *S. cerevisiae* BY4741 strain expressing HxT6-GFP (green fluorescent protein). For HT-29, the cells were simply mixed with FND-lip. Since yeast cells have a thick cell wall, it is necessary to remove the cell wall first and then fuse with the liposomes. A schematic representation of uptake process into yeast is shown in Figure 1.

Preparation of FND-coated liposomes: FNDs used in this study were purchased from Adamas Nanotechnologies, Inc. (NC, USA; ND-NV-70, >300 NV centers/diamond particle) and have a diameter of 70 nm with concentration 1 mg mL⁻¹ in deionized H₂O. They were oxygen terminated by acid cleaning from the vendor. Liposome kit (Sigma cat no. L4395) that contained 63 μmol L-α-phosphatidylcholine and 9 μmol cholesterol was mixed with deionized H₂O. Size of liposomes could be decreased using a sonicator. To prepare FND-lip, 2 μg mL⁻¹ of FND solution was added into liposomes and was mixed by vortexing for 30 s.

This liposome composition was chosen to contain a mixture of two phosphatidylcholines (PCs). The first were phosphatidylcholine (PC from egg source) head groups leading to liposomes with neutral zeta potential which were heavily hydrated³². The second had positively charged head groups. It was shown for ingesting other particles with this coating that positive charges from choline head in liposomes interacted with negative charges in cell membranes. Phospholipid bilayers of PCs were relatively soft membranes because of their unsaturated alkyl chains. During hydration process, flabby network-like

structures formed and gave them an unstable structure. The structure stabilized through relaxation of membrane tension and appropriate amount of cholesterol³³.

This was promoted by alkyl chains in PCs group³⁴. If there is endocytosis, the presence of liposome can provoke destabilization of endosomal membrane³⁵. Unsaturated alkyl chains also decrease transition temperature (melting temperature) of phospholipid bilayer membrane in cells and induce membrane fluidity, behavior, permeability, membrane fusion, lateral pressure, and flip-flop dynamic³⁶.

Characterization of FND-lip—Optically detected magnetic resonance (ODMR)

measurement: To test if the FND-lip particles are still useful for quantum measurements, ODMR measurements were performed. For the measurements, customized equipment similar to what is commonly used in the field and as described previously was used⁵. In short, the equipment was a homebuilt confocal microscope with built in microwaves and sensitive detection with avalanche photodiodes.

The sample suspension was dropped onto a microscope cover slide and evaporated in room temperature. The instrument was set to -12 dBm of microwave power, 1 mW of laser power, and 100 repetitions.

Characterization of FND-lip—Dynamic light scattering (DLS) measurement:

The DLS measurement was used to determine the particle size and its changes after applying the coating. It was performed using Zetasizer nano system to determine diameter of particles. Samples were measured in triplicate and mixed in between to prevent sedimentation. DLS is not an ideal method to measure diamond aggregates due to sedimentation and the nonspherical shape of

diamonds³⁷ is also not ideal for DLS. However, DLS is a fast method and averages over large particle numbers; therefore, it was still used here to get an impression on the particle sizes.

Characterization of FND-lip—Zeta Potential Measurement: The sample of 5 $\mu\text{g mL}^{-1}$ FND-lip was diluted in sterile deionized water. The measurements were performed in triplicate and 5 $\mu\text{g mL}^{-1}$ FND70 (FNDs with a hydrodynamic diameter of 70 nm) was used as control. All the measurements were performed at 25°C.

Characterization of FND-lip—Cryo TEM: To compensate the drawbacks of DLS, the results were also confirmed by using cryo TEM for complementary results. Samples were transferred to holey carbon-coated copper grids (Quantifoil 3.5/1) and frozen by rapid injection into liquid ethane (Vitrobot, FEI) and examined with FEI Tecnai T20 electron microscope operating at 200 keV. Images were taken under low dose conditions with a slow scan charged coupled device camera.

Cell uptake behavior—cell culture of HT-29 cells: Cell culturing was done using standard conditions for this cell type. For this study, two different HT-29 cells were used. First, HT-29 EpCAM-GFP cell line that overexpresses the epithelial cell adhesion molecules fused to green fluorescent protein was used for particle uptake analysis. Second, HT-29 non-GFP cell line that has been used for evaluating lipid membrane interaction was used. The cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (penicillin/streptomycin), and 1% glutamax. They were cultured in T-75 flask containing 10 mL of DMEM and grown in humidified incubator under an atmosphere of 95% air and 5% CO₂ at 37°C.

Culture medium was replaced every 48 h. When the cells reached 75–80% confluency, the medium was aspirated and the cell monolayer was treated with 3 mL of 0.25% trypsin EDTA and incubated for 5 min at 37°C. After that, cells were visualized using a microscope to ensure complete detachment and resuspended in DMEM complete medium.

Cell uptake behavior—FND-lip uptake in HT-29 EpCAM-GFP cells: The cells with 60% confluency were put in a glass bottom cell dish with four compartments with 300 μL DMEM complete medium for each compartment. 5 $\mu\text{g mL}^{-1}$ FND-lip and FNDs were added per compartment and incubated for 2 h. To preserve the cells for later measurements they were fixed. To this end, all media were aspirated and 3.7% paraformaldehyde (PFA) solutions were added in each compartment. After incubation for 15 min at room temperature, the solution was replaced by 1% of PFA.

Cell uptake behavior—FND-lip uptake in yeast spheroplast: *S. cerevisiae* BY4741 strain expressing HxT6-GFP was used since this strain is regularly used as a model organism to study aging on a molecular level. The HxT6-GFP mutant is a strain, which has GFP labels in the cell membrane. This allows to determine the cell borders via fluorescence. The yeasts were grown in synthetic dextrose (SD) complete medium supplemented with 5% D-glucose at 30°C with constantly shaking at 200 rpm. Creating yeast spheroplasts were done by following a modification procedure by Karas et al³⁸. The procedure is described in short in the following. After reaching an OD₆₀₀ (optical density at 600 nm) of 2.5–3, the cells were centrifuged at 2500 $\times g$ for 5 min at 10 °C and then the supernatant was removed. The cells were resuspended in 20 mL 1 M D-sorbitol and vortexed, and then centrifuged at 2500 $\times g$ for 5 min at 10°C and the supernatant was removed.

The pellet was resuspended thoroughly in 20 mL SPEM (containing 1 M D-sorbitol, 10×10^{-3} M EDTA pH 8, and 10×10^{-3} M sodium phosphate) buffer solution followed by vortexing. Then, zymolyase-20T (Amsbio, UK) and β -mercaptoethanol (Sigma, Netherlands) as an activator were added. The mixture was incubated for 30 min at 30°C while constantly shaking at 75 rpm. To stop the spheroplasting process, 20 mL of 1 M D-sorbitol was added and the mixture was centrifuged at $1000 \times g$ for 5 min at 10°C. After that the supernatant was removed. The pellet was incubated in 2 mL STC (contains 1 M D-sorbitol, 10×10^{-3} M Tris-HCl, 10×10^{-3} M CaCl_2 , and 2.5×10^{-3} M MgCl_2) buffer solution at room temperature for 20 min. 200 μL yeast spheroplast suspension was combined with 50 μL of 5 $\mu\text{g mL}^{-1}$ FND-lip solution and 50 μL of 5 $\mu\text{g mL}^{-1}$ FNDs as control group. Then, the samples were mixed by gently flicking the tube and incubated at room temperature for 5 min. This was a lot shorter than a few hours, which is typically used for endocytosis. Yeast spheroplasts were fixed using 1% PFA in buffer PBS for microscopy imaging. As a negative control, yeast cells without any treatment of the cell wall were incubated with FND-lip and FNDs with the same concentration.

Cell uptake behavior— Confirming spheroplast formation via secondary electron microscopy: First, yeast cells and yeast spheroplasts were embedded in epon and osmium stained as described before³⁹. In short, yeast cells and yeast spheroplasts were fixed with 1% glutaraldehyde and 4% PFA in 0.1 M cacodylic acid and resuspended in 1% of low melt agarose and then cut into $\approx 1 \text{ mm}^3$ sections. All samples were post fixed in 1% osmium tetroxide/2.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 2 h and then dehydrated through an increasing graded ethanol series (30%, 50%, and 70%) for 10 min per concentration and three times 100% ethanol for 20 min. The samples were left

overnight in 1:1 ethanol and epon mixture at room temperature and replaced with pure epon and then incubated for 3 h at room temperature. After that, they were placed at 200 mbar vacuum for 10 min to remove air bubbles and then put at 58°C over the weekend.

After embedding, semi thin sections of around 300 nm were prepared with a glass knife. Use of more expensive diamond knives was avoided to prevent potential damage of the knife by diamonds in the material. The sections were transferred to commercially available indium tin oxide (ITO)-coated glass plates. SEM measurements were performed on a Hitachi SU5000 using 2.5 keV and 29.6 μs dwell time and backscattering electron detection. The results in Figure 7 show a typical yeast cell as well as a spheroplast, after removal of the cell wall.

Figure 7. Yeast cells a) compared to yeast spheroplasts. b) Yeast cells are surrounded by a thick cell wall, which is removed to create a yeast spheroplast.

Cell uptake behavior— Evaluation of cell membrane interaction with FND-Lip:

Interaction between cell membrane and liposomes was investigated. The main purpose of this experiment was to evaluate if the lipid coating remains with the diamonds and to evaluate if the liposomes are able to promote membrane fusion. This experiment was performed with HT-29 non-GFP cells. Liposome was labeled with 0.5% N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (NBD-PE) (Thermo Fisher, Netherlands) in

chloroform. After chloroform was evaporated, liposome-NBD PE was diluted in sterile deionized water and incubated with the cells for 20 min at 37°C with 5% CO₂. Interaction was analyzed by using Zeiss LSM 780 confocal laser microscope (Zeiss, Germany) at 463/536 nm. Figure 8 shows the interaction between liposome-NBD PE (in green) and cells.

Figure 8. Interaction between liposome-NBD PE with HT-29 non-GFP cells. Green represents liposome-NBD PE particles. While some of the liposomes remain in the cell membrane, part of the liposomes entered the cell. a) shows the sample with FND-lip and b) shows a sample with liposome only. While we cannot directly proof membrane fusion, we clearly observed uptake and we can see that the lipid coating remains with the diamond particles.

Cell uptake behavior—Evaluation of endosomal pathway of FND-Lip: To further investigate the uptake mechanism, testing was performed for colocalization with endosomes. This was done in HT-29 non-GFP cells. First, cells with FND-lip were fixed with 4% PFA in phosphate buffered saline (PBS) pH 7.4

for 10 min at room temperature followed by cell permeabilization with PBS containing 0.5% Triton X-100 (Sigma, Netherlands) for 10 min.

Cells were incubated with 1% bovine serum albumin in PBS (PBSA) for 30 min to block unspecific antibody. Early endosomal antibody 1 (EEA1) (Thermo fisher, Netherlands) was used as primary antibody and diluted in 0.1% PBSA (1:200). It was incubated with the cells for 1 h at room temperature and followed by washing step with 1% PBSA.

After incubation with primary antibody, cells were incubated in secondary antibody donkey anti rabbit FITC (Thermo Fisher, Netherlands) in 0.2% PBSA (1:100) for 45 min at room temperature followed by a washing step with 1% PBSA. All samples were wrapped with tin foil to avoid light exposure. Evaluation was performed with a confocal laser scanning microscope at 463/536 nm and colocalization analysis Coloc2 plugin in Fiji software. Figure 9 shows endosomes (green) and FND-lip (red) in HT 29 non-GFP cells. It is clear from the analysis that diamonds do not colocalize with early endosomes.

To quantify this finding, the Pearson correlation (r) was calculated as defined by Manders et al⁴⁰. A value of 0.12 ± 0.61 was received which means FND-lip has little correlation with EEA1.

Figure 9. Colocalization: Early endosomes are shown in green and FNDs in red after incubation with FND-lip for 2 h at 37 °C. It can be seen qualitatively that diamond particles do not colocalize with the endosomes.

Cell uptake behavior—Characterization of FND-Lip uptake and particles

analysis: To quantify FND uptake, 4 μL fixed yeast spheroplast cells were put between a poly-L-lysine-coated glass slide and a cover glass, and imaged by using Zeiss LSM 780 confocal laser scanning microscope (Zeiss, Germany). HT-29 EpCAM-GFP cells that were fixed in 1% PFA were observed by using confocal laser scanning microscope. FND particles were imaged at 561/650 nm and GFP was imaged at 488/525 nm. The number of particles that were ingested by cells was determined by a homemade Fiji program⁶. In short, 100 yeast and spheroplast cells and 10 clusters of HT-29 EpCAM-GFP were selected randomly and an algorithm was applied to find area of the well in 3D. The volume close to the membrane was automatically subtracted to avoid false positive results from particles on the surface.

Cytotoxicity assay—MTT assay for HT-29 cells: The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide] assay was performed for assessing metabolic activity and cytotoxicity in HT-29 cells. The cells were seeded at a density of 1.5×10^4 cells per well in 96-well microliter tissue culture plates and placed in a 5% CO_2 humidified incubator until 60% confluency. DMEM complete medium was removed and $5 \mu\text{g mL}^{-1}$ FND-lip, $5 \mu\text{g mL}^{-1}$ FND, or $5 \mu\text{g mL}^{-1}$ liposome in culture medium were added. Pure medium and cytotoxic hydrogen peroxide (H_2O_2) served as negative and positive controls. All samples were incubated for 2 h. Following the treatments, medium was removed and 100 μL MTT solution (5 mg mL^{-1} MTT in sterile PBS) was added to each well followed by incubation for 4 h. Subsequently, the MTT solution was removed and 200 μL isopropanol was added to each well, to dissolve formazan crystals, followed by incubation for 10 min at 37°C . Optical densities were read with microplate reader

Fluostar Optima (BMG Labtech, Ortenberg, Germany) at 540 nm. Cell viability rate was calculated as percentage of MTT absorption as follows.

$$\% \text{ survival} = (\text{mean experimental absorbance} \div \text{mean control absorbance}) \times 100\%$$

Cytotoxicity assay—MTT assay for *S. Cerevisiae* cells: Cells were grown in SD medium to early exponential or stationary phase and washed with sterile water. Cell density was adjusted to an OD_{600} of 2. 1 mL of cell suspension was mixed with $5 \mu\text{g mL}^{-1}$ FND-lip, $5 \mu\text{g mL}^{-1}$ FND, or $5 \mu\text{g mL}^{-1}$ liposome in SD medium and incubated for 2 h in an incubator at 37°C . The MTT assay was done by following Gerlier and Thomasset's (1986) protocol⁴¹. The only adaptation made was using lower speed of centrifugation ($1000 \times g$) for the spheroplasts, compared to the speed used for yeast cells with a cell wall.

References:

1. Mochalin, V. N. *et al.* The properties and applications of nanodiamonds. *Nat. Nanotechnol.* **7**, 11–23 (2011).
2. Laan, K. J. van der. *et al.* Nanodiamonds for In Vivo Applications. *Small.* **1703838**, 1–17 (2018).
3. Chipaux, M. *et al.* Nanodiamonds and Their Applications in Cells. *Small.* **1704263**, 1–25 (2018).
4. Balasubramanian, G. *et al.* Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature.* **455**, 648–652 (2008).
5. Dolde, F. *et al.* Electric-field sensing using single diamond spins. *Lett. Nat. Phys.* **7**, 459–463 (2011).
6. Bradac, C. *et al.* Observation and control of blinking nitrogen-vacancy centres in discrete nanodiamonds. *Nat. Nanotechnol.* **5**, 345–349 (2010).
7. Hemelaar, S. R. *et al.* The interaction of fluorescent nanodiamond probes with cellular media. *Microchim. Acta.* **184**, 1001–1009 (2017).
8. Hemelaar, S. R. *et al.* Generally Applicable Transformation Protocols for Fluorescent Nanodiamond Internalization into Cells. *Sci. Rep.* **7**, 5862 (2017).
9. Kucsko, G. *et al.* Nanometre-scale thermometry in a living cell. *Nature.* **500**, 54–58 (2013).
10. McGuinness, L. P. *et al.* Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells. *Nat. Nanotechnol.* **6**, 358–363 (2011).
11. Perevedentseva, E. *et al.* Nanodiamond internalization in cells and the cell uptake mechanism. *J. Nanoparticle Res.* **15**, (2013).
12. Chu, Z. *et al.* Unambiguous observation of shape effects on cellular fate of nanoparticles. *Sci. Rep.* **4**, 4495 (2015).
13. Webster, A. *et al.* The delivery of PEBBLE nanosensors to measure the intracellular environment . The delivery of PEBBLE nanosensors to measure the intracellular environment. *Biochem. Soc. Trans.* **35**, 538–543 (2007).
14. Zheng, T. *et al.* Recombinant Protein Polymers for Colloidal Stabilization and Improvement of Cellular Uptake of Diamond Nanosensors. *Anal. Chem.* **89**, 12812–12820 (2017).
15. Guerrero-Martínez, A., Pérez-Juste, J. & Liz-Marzán, L. M. Recent progress on silica coating of nanoparticles and related nanomaterials. *Adv. Mater.* **22**, 1182–1195 (2010).
16. Weng, M. F. *et al.* Cellular uptake and phototoxicity of surface-modified fluorescent nanodiamonds. *Diam. Relat. Mater.* **22**, 96–104 (2012).
17. Petrakova, V. *et al.* Imaging of transfection and intracellular release of intact, non-labeled DNA using fluorescent nanodiamonds. *Nanoscale.* 12002–12012 (2016).
18. Hughes, Z. E. & Walsh, T. R. Elucidating the mechanisms of nanodiamond-promoted structural disruption of crystallised lipid. *Soft Matter.* **12**, 8338–8347 (2016).
19. Haziza, S. *et al.* Fluorescent nanodiamond tracking reveals intraneuronal transport abnormalities induced by brain-disease-related genetic risk factors. *Nat. Nanotechnol.* **12**, 322–328 (2016).
20. Hsieh, F. J. *et al.* Correlative Light-Electron Microscopy of Lipid-Encapsulated Fluorescent Nanodiamonds for Nanometric Localization of Cell Surface Antigens. *Anal. Chem.* **90**, 1566–1571 (2018).
21. Vavra, J. *et al.* Supported Lipid Bilayers on Fluorescent Nanodiamonds: A Structurally Defined and Versatile Coating for Bioapplications. *Adv. Funct. Mater.* **28**, 1–9 (2018).
22. Sotoma, S., Hsieh, F. J., Chen, Y. W., Tsai, P. C. & Chang, H. C. Highly stable lipid-encapsulation of fluorescent nanodiamonds for bioimaging applications. *Chem. Commun.* **54**, 1000–1003 (2018).
23. Hui, Y. Y. *et al.* Two-photon fluorescence correlation spectroscopy of lipid-encapsulated fluorescent nanodiamonds in living cells. *Opt. Express.* **18**, 5896 (2010).
24. Johnston, M. J. W. *et al.* Characterization of the drug retention and pharmacokinetic properties of liposomal nanoparticles containing dihydrosphingomyelin. *Biochim. Biophys. Acta - Biomembr.* **1768**, 1121–1127 (2007).
25. Fanciullino, R. & Ciccolini, J. Liposome-encapsulated anticancer drugs: still waiting for the magic bullet? *Curr. Med. Chem.* **16**, 4361–4373 (2009).

26. Akbarzadeh, A. *et al.* Liposome : classification , preparation , and applications. *Nanoscale Res. Lett.* **8**, 1 (2013).
27. Lira, R. B. *et al.* Studies on intracellular delivery of carboxyl-coated CdTe quantum dots mediated by fusogenic liposomes. *J. Mater. Chem. B*, **1**, 4297–4305 (2013).
28. Allen, T. M. & Cullis, P. R. Liposomal drug delivery systems: From concept to clinical applications. *Adv. Drug Deliv. Rev.* **65**, 36–48 (2013).
29. Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomedicine*. **7**, 5577–5591 (2012).
30. Kawai, S., Hashimoto, W. & Murata, K. Transformation of *Saccharomyces cerevisiae* and other fungi: methods and possible underlying mechanism. *Bioeng. Bugs*. **1**, 395–403 (2010).
31. Svoboda, A. Regeneration of yeast. *Exp. Cell Res.* **44**, 640–642 (1966).
32. Liu, J. Interfacing zwitterionic liposomes with inorganic nanomaterials: Surface forces, membrane integrity, and applications. *Langmuir*. **32**, 4393–4404 (2016).
33. Nomura, S. I. M. *et al.* Changes in the morphology of cell-size liposomes in the presence of cholesterol: Formation of neuron-like tubes and liposome networks. *Biochim. Biophys. Acta - Biomembr.* **1669**, 164–169 (2005).
34. Manconi, M. *et al.* Intracellular distribution of fluorescent probes delivered by vesicles of different lipidic composition. *Colloids Surfaces B Biointerfaces*. **57**, 143–151 (2007).
35. Torchilin, V. P. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* **4**, 145–160 (2005).
36. Ibarguren, M., López, D. J. & Escibá, P. V. The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health. *Biochim. Biophys. Acta - Biomembr.* **1838**, 1518–1528 (2014).
37. Ong, S. Y., *et al.* Shape and crystallographic orientation of nanodiamonds for quantum sensing. *Phys. Chem. Chem. Phys.* **19**, 10748–10752 (2017).
38. Karas, B. J. *et al.* Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat. Protoc.* **9**, 743–750 (2014).
39. Kuipers, J., de Boer, P. & Giepmans, B. N. G. Scanning EM of non-heavy metal stained biosamples: Large-field of view, high contrast and highly efficient immunolabeling. *Exp. Cell Res.* **337**, 202–207 (2015).
40. Manders, E. M. M., Verbeek, F. J. & Aten, J. A. Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* **169**, 375–382 (1993).
41. Gerlier, D. & Thomasset, N. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods*. **94**, 57–63 (1986).

Supporting information

Figure S1. Interaction between liposome-NBD PE with HT-29 non GFP cells. Green represents liposome-NBD PE particles. While some of the liposomes remain in the cell membrane, part of the liposomes entered the cell. All images show the sample with FND-lip (as Fig 3 of the main manuscript)

3. The fate of lipid-coated and uncoated fluorescent nanodiamonds during cell division in yeasts

Aryan Morita^{1,2,†}, Thamir Hamoh^{1,†}, Felipe P. Perona Martinez¹, Mayeul Chipaux¹, Alina Sigaeva¹, Charles Mignon¹, Kiran J. van der Laan¹, Axel Hochstetter³, Romana Schirhagl¹

1. Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
2. Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
3. Division of Biomedical Engineering, School of Engineering, University of Glasgow, Glasgow G12 8LT, UK

Published in *Nanomaterials* 10(3); 1-15 (2020)

Chapter 3

Abstract

*Fluorescent nanodiamonds are frequently used as biolabels. They have also recently been established for magnetic resonance and temperature sensing at the nanoscale level. To properly use them in cell biology, we first have to understand their intracellular fate. Here, we investigated, for the first time, what happens to diamond particles during and after cell division in yeast (*Saccharomyces cerevisiae*) cells. More concretely, our goal was to answer the question of whether nanodiamonds remain in the mother cells or end up in the daughter cells. Yeast cells are widely used as a model organism in ageing and biotechnology research, and they are particularly interesting because their asymmetric cell division leads to morphologically different mother and daughter cells. Although yeast cells have a mechanism to prevent potentially harmful substances from entering the daughter cells, we found an increased number of diamond particles in daughter cells. Additionally, we found substantial excretion of particles, which has not been reported for mammalian cells. We also investigated what types of movement diamond particles undergo in the cells. Finally, we also compared bare nanodiamonds with lipid-coated diamonds, and there were no significant differences in respect to either movement or intracellular fate.*

Keywords: fluorescent nanodiamonds; cell division; yeast

3.1 Introduction

The fluorescent nanodiamonds (FNDs) are promising long-term biolabels due to their unprecedented photostability¹⁻³. They can host fluorescent defects such as the nitrogen vacancy (NV) center. These centers can be excited with a green laser (532 nm) and emit red fluorescence (a broad peak above 600 nm). NV centers occur naturally in nanodiamonds from high pressure high temperature (HPHT) synthesis, but their numbers can be increased by irradiation in several different ways. These increase the number of color centers and thus their fluorescence intensity⁴. Possibilities include irradiation with silicon ions⁵, helium ions⁶, or electrons^{7,8}. For biological applications, the excellent biocompatibility of fluorescent nanodiamonds is also crucial⁹. In several previous studies, FNDs are introduced in mammalian cells and have shown no negative effects¹⁰⁻¹². From these studies, it is known that mammalian cells passively take up FNDs in different ways depending on the cell type and exact conditions. The most reported uptake path is endocytosis. When nanodiamonds are endocytosed they are engulfed in an endosome and eventually escape from it¹³. Besides, little is known so far about the behavior of these FNDs after uptake or about what happens to them during cell division. Although most studies are limited to short times where division does not occur, there is a small number of articles on the behavior of FNDs during cell division in mammalian cells^{14,15}.

In this study, yeast cells were used as model organism. We have shown before that FNDs can be brought inside these cells¹⁶. For yeast cells, the uptake mechanisms of nanoparticles are unknown. As they are covered with a thick cell wall, the uptake is also a lot more artificial than in mammalian cells and there is probably no natural mechanism that the uptake is comparable to. To achieve uptake, two protocols have been established: One option is to permeabilize the cell wall, which allows the diamond particles to enter. It has also been shown in

a previous work that the cells could proliferate after being treated with FNDs^{16,17}. Another option, which we used here, is to remove the cell wall entirely. This method allows the diamonds to enter and regrow the cell wall¹⁸. Here, we investigate for the first time what happens during and after cell division in yeast. This is especially interesting for yeast cells, because the division is asymmetric. Asymmetric division can manifest itself in different ways, for instance, different cell content. In yeast cells, division results in differently sized mother and daughter cells. Before cell division, a diffusion barrier keeps molecules (in this case, FNDs) in the membrane of the mother and prevents them from entering the membrane of daughter cell. In the FNDs, other particles or molecules only leak into the daughter cells if they detach from the membrane in the mother cells or if the diffusion barrier becomes permeable¹⁹. This mechanism is in place to protect daughter cells from harmful substances like aging factors²⁰.

Compared to other organisms, yeast cells have several advantages for this kind of research. They are a relevant model to study the aging process, and they are widely used in biosynthesis and in food industry^{21–23}. They are undemanding in cultivation and allow for easy genetic and molecular modifications²¹.

Cell division is a very important step in the aging process of yeast cells. When investigating the transfer of FNDs during cell division, in principle, there are four possible outcomes after cell division: 1. FNDs could remain (preferentially) with the mother, for example, because they are regarded as harmful by the cell (see Figure 1a). 2. FNDs might (preferentially) move into the daughter cells (see Figure 1b). 3. FNDs might be excreted (see Figure 1c). 4. FNDs might end up randomly distributed between both mother and daughter cells (see Figure 1d).

Figure 1. In asymmetric cell division, a yeast cell produces a smaller daughter cell. Except for the size, the daughter cells are similar to the mother cells. Both of them have nuclei (red), a vacuole (green), and other organelles (gray). When fluorescent nanodiamonds (FNDs) (purple) are inside the cells during division, they can (a) stay in the mother cell, (b) move to the daughter cell preferentially, (c) being excreted by the cells, or (d) being equally distributed.

The goal of this article is to determine which one of these possibilities is the case for nanodiamonds. To answer this question, we used FNDs in yeast cells using the spheroplasting process^{18,24} (i.e., removing the cell wall) and we followed them during cell division. We investigated here which of the four possible outcomes occur and in what frequency.

3.2 Materials and methods

3.2.1 Diamond starting material

Bare particles. Throughout this article we used fluorescent diamonds with a hydrodynamic diameter of 70 nm (FND70) from Adamas Nanotechnology (Raleigh, NC, USA). They have a relatively broad size distribution and irregular shape²⁵. According to the vendor, these particles are irradiated with an electron beam at 3 MeV to 5×10^{19} e/cm² fluence followed by high temperature annealing above 600°C under vacuum for 2 h²⁶. The NV content was measured by the manufacturer by electron paramagnetic resonance to be approximately 2–2.5

ppm. This means each particle hosts approximately 300 nitrogen vacancy centers. We measured their fluorescence spectrum (see supplementary Figure S3) on a Thermo Fisher Varioskan microplate reader, with excitation wavelength at 532 nm, and analyzed it as shown by Fu et al.²⁷. We found that the particles contain almost exclusively NV⁻ centers, which is also in line with what others found for similar particles²⁸. With our homebuilt confocal microscope we can detect ~1,000,000 counts per second for a single particle. This was determined in previous works where we spread particles evenly on a surface (confirmed by SEM) and measured the counts²⁵. As they undergo a cleaning process in oxidizing acid, their surface is oxygen terminated and electronegative with zeta potential of -16 ± 1 mV.

Coated particles. For facilitating FNDs uptake in yeast cells, a liposome kit (Sigma, Zwijndrecht, The Netherlands) has been used as coating material. This kit contains 63 μmol L- α -phosphatidylcholine and 18 μmol stearylamine. After the coating process, the zeta potential value of FNDs becomes electropositive (36 ± 3 mV)¹⁸. To prepare FNDs coated with lipids (FND-lip), 2 $\mu\text{g mL}^{-1}$ of FND solution was added into liposomes and was mixed by vortexing for 30 s.

Particles characterization. Characterization of diamond and lipid-coated diamond particles (FND-lip) has been performed in a previous study¹⁸. There we characterized several properties for these particles. The findings are summarized here shortly. No significant differences in size between FND-lip and FNDs were observed. There we found that both FND-lip and FNDs are colloiddally stable in water ($\text{PdI} < 1$). To further characterize the particles, we performed an analysis of the zeta potential. To this end, 4 $\mu\text{g mL}^{-1}$ liposome-coated FNDs were diluted in sterile deionized water and 1 mL of the solution was injected into the cuvette and

4 $\mu\text{g mL}^{-1}$ FND70 was used as control. The measurements were performed with a Malvern Zetasizer Nanosystem (Malvern, Cambridge, UK). All the measurements were set in 25°C. Each measurement takes ~2 min. The zeta potential measurement showed that the 70 nm FNDs were electronegative (-15.73 ± 0.89 mV). After adding liposome, the particles became electropositive (35.67 ± 2.64 mV)¹⁸. Cryo TEM (recorded with Tecnai, Oregon, USA) revealed that the thickness of the lipid layer on diamond particles was 4.8 ± 1.2 nm¹⁸. It is also apparent from this previous study that the FNDs are actually coated by lipids. Performing optically detected magnetic resonance measurements (as routinely used in the field) on FNDs and FND-lip did not reveal any significant differences¹⁸. Although FNDs generally are known to have excellent biocompatibility^{9,29}, a very small decrease in metabolic activity has been reported for yeast cells and FND-lip¹⁸.

3.2.2 Fluorescence Nanodiamond Particles Uptake

Saccharomyces cerevisiae BY4741 and HxT6-GFP strains were used as model organisms. According to the *Saccharomyces* genome database, the wild type strain BY4741 was used as a parent strain for an international systematic *S. cerevisiae* gene disruption project. Thus, it was chosen here for its broad use. These wild type cells were used for tracking intracellular movement of FNDs. The HxT6-GFP strain was used for quantifying FNDs. This modified strain expresses Hexose transporter 6 (glucose transporter) with green fluorescent protein (GFP) in the cell membrane, thus allowing imaging of the cell boundaries. Both cells were grown in synthetic dextrose (SD, Formedium, Norfolk, UK) complete medium until midlog phase ($\text{OD}_{600} = 1.05$). The spheroplasting protocol was modified from Karas et al.²⁴ and was performed to get the FNDs inside cells. The adaptation from the original protocol was that after spheroplasting they put the

spheroplast on specific medium and we did not do that. In the spheroplast protocol, the cell wall is removed entirely from the yeast cells to create spheroplasts. To obtain these spheroplasts, the cells were washed with sterile demineralized water and centrifuged for 5 min at $2500 \times g$ at 10°C . The supernatant was discarded, and 20 mL of 1 M D-sorbitol was added to the cells. The cells were again centrifuged for 5 min at $2500 \times g$ at 10°C . After discarding the supernatant, 20 mL of SPEM (consisting of 1 M D-sorbitol, 10 mM EDTA, and 10 mM sodium phosphate) buffer was added followed by 40 μL zymolyase 20 T (Amsbio, UK) and 30 μL β -mercaptoethanol (Sigma, Zwijndrecht, The Netherlands). Cells were incubated at 30°C while shaking at 75 rpm for 30 min. Twenty milliliters of 1 M D-sorbitol was added to stop the spheroplasting process, and the cells were centrifuged for 5 min at $1000 \times g$ at 10°C . After the supernatant was discarded, 2 mL of STC (1 M sorbitol, 10 mM Tris HCl, and 10 mM CaCl_2 and 2.5mM MgCl_2) buffer was added and the mixture was incubated for 20 min at room temperature. In the end, 50 μL of $2 \mu\text{g mL}^{-1}$ FNDs at a size of 70 nm were added to the 200 μL yeast spheroplast suspension, followed by 5 min incubation at room temperature. Finally, the treated yeast cells were put in SD complete medium supplemented with 1 M D-sorbitol for 1 h at 30°C to regrow their cell wall.

3.2.3 Immobilizing yeast cells

To monitor single cells during and after cell division they were immobilized using the following protocol; glass-bottom dishes with 4 compartments were coated with 0.1 mg mL^{-1} concanavalin A (Sigma, Zwijndrecht, The Netherlands). The coating process was followed by a washing step with sterilized demineralized water and a drying step in a 37°C incubator. After the coated dish dried, 300 μL SD medium and 4 μL of cell suspension (strain

BY4741, approximately $2.4 \times 10^7 \text{ cells mL}^{-1}$) with internalized FNDs from the previous step were added in each compartment and the dish was sealed by parafilm to avoid evaporation of the medium.

3.2.4 Equipment

Imaging was performed on a home-built confocal microscope operating with a 532 nm excitation laser. The confocal microscope is similar to what is typically used in the diamond magnetometry community^{30,31}. Below we shortly describe the most important specifications. A detailed description including a drawing (Figures S4 and S5) and a list with all the parts of our equipment can be found in the supplementary material. We have a homebuilt system because it allows for flexibility to perform diamond magnetometry. However, this functionality was not used in this article, and the measurements could have also been performed on a commercial system with similar capabilities. For detection, our instrument has an avalanche photodiode implemented for detection, which is capable of single photon counting. The fluorescent counts we receive for 70 nm diamond particles are typically $\sim 1,000,000$ per second for a single particle. These values are close to what we expect for this number of NV centers per particle. The instrument has built-in microwaves (which we do not use in this article) and uses sensitive detection with avalanche photodiodes. The set-up is equipped with a green laser at 532 nm, and we have the ability to track particles in 3D. The sample stage is designed in a way that allows for standard glass-bottom petri dishes to be measured. For the measurement, the sample suspension was dropped onto a microscope cover slide and evaporated at room temperature. The instrument was set to -12 dBm of microwave power and 1 mW of laser power. One hundred repetitions were performed to obtain a sufficient signal-to-noise ratio¹⁸. To better identify the cells, the confocal microscope is equipped with a bright-field

microscope, which is used to collect images simultaneously. Bright-field illumination is achieved with a 470 nm Fiber coupled LED supplied with T-Cube LED Driver. The images are collected using a Compact USB 2.0 CMOS Camera from Thorlabs, and an Olympus PLN 4x objective to focus the blue light with NA 0.1.

3.2.5 Tracking FND movement during cell division

To separate the FND signal from other fluorescence, a 550 nm long-pass filter was used. A signal above 550 nm was attributed to the FNDs. It is also possible to use a filter above 600 nm (or higher), but there is a trade-off. If one uses a higher filter, the technique is more specific for ND. However, one also loses part of the signal and thus sensitivity. Therefore, if the background is comparably low, it is possible to choose a lower wavelength filter to gain sensitivity. We detect on average $90,000 \pm 10,000$ counts per second for the background of the cell, whereas the FNDs are $1,000,000 \pm 500,000$ counts per second (for control images without particles see supplementary information Figure S6). A laser power of 60 μ W at the laser power output was chosen to limit potential damage to the cells from high laser power. First, we scanned an area ($50 \times 50 \mu\text{m}$ field of view) with cells. Then, we identified diamonds by observing stability of their fluorescence intensities, as diamonds are not bleaching. Usually, we observe a particle for ~ 10 min. If the fluorescence does not drop, it is most likely a diamond. Images were acquired every hour. Light intensity was measured using an Olympus UPLanSApo40x NA = 0.95 air objective and an Avalanche photodiode (SPCM-AQRF-15-FC) in single photon counting mode. Simultaneously, bright-field time series images were recorded continuously to give a better view of cell division. Confocal images were processed in Fiji software using specific plugins³¹. Deconvolution was performed to get clearer particle locations and

lower background using Diffraction point spread function (PSF) 3D and Iterative deconvolve 3D plugins.

3.2.6 FND quantification during cell division

While following the FNDs during cell division (using the above mentioned confocal microscope in Figures S4 and S5), FND quantification was performed after re-growing the cell walls. Four microliters of yeast spheroplast suspension contained approximately 9.6×10^4 cells (strain HxT6-GFP) that were fixed using 1% paraformaldehyde in PBS buffer of pH 7.4. The cell suspension was put between a glass slide and the cover glass and was imaged with Zeiss LSM 780 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). FNDs were imaged at excitation/emission wavelength 561/650 nm and GFP was imaged at 488/525 nm. A homemade Fiji program was used for determine the number of particles that have been ingested by the cells¹⁶ before and after cell division. To this end, a specific, custom-made FND quantification plugin was used to approximate the amount of internalized FNDs. The analysis was divided into three phases: cell selection, masking, and particle analysis. During the first phase, images are visually inspected and random cells are selected. The images were composed of several slices (Z-stacks), and the cellular region was defined in all the three dimensions. In the horizontal plane, the selection considered an area containing only the cell of interest. In the height, the first and last slices containing the cell were identified. As a result, the first phase defines a volume that holds only the cell of interest. In the masking phase, that volume is molded to resemble the shape of the cell. The GFP signal (staining the cell membrane) is converted to binary using the Isodata algorithm to calculate the threshold³², and the cell's perimeter is detected in every slice. To avoid counting particles on the surface, the program excludes the outer micrometer of the volume. (As a result,

the program slightly underestimates the real number of particles.) In the third step, a special function of Fiji analyses the particles, which are found in a selected region. Applying this function to the masked image, it is possible to directly obtain the number of objects (connected positive pixels) in the specified region. A threshold is used to separate the background light from the signal emitted by the FNDs. Every pixel with intensity less than the threshold is assumed as background and set as black, whereas every pixel with an intensity greater than or equal to the threshold is assumed as part of a particle. To find an adequate value for this parameter, the image was visually inspected and different values were probed. Finally, we chose the lowest possible value, which gives zero for a negative control image. In the end, this method gives two important values: the number of objects and the number of particles. An object here is defined as the amount of adjacent FND positive pixels. This means an object can be composed of a single diamond or can be an aggregate of multiple diamond particles. The difference between the number of objects and the number of particles reveals the aggregation status of the sample in the intracellular environment (rather than in a test tube as in DLS for instance).

3.2.7 Single particle tracking

To better understand the intracellular processes a nanodiamond can be subjected to, we performed single particle tracking. This was done for both uncoated (bare FNDs) and lipid-coated nanodiamonds (FND-lip) to study the impact of enclosing nanodiamonds in liposome vesicles inside the cell. As a liposome is larger (the thickness of liposome on FND surface was determined by TEM to be 4.8 ± 1.2 nm¹⁸) than an uncoated nanodiamond, we would expect it to move slower, according to Fick's laws. If the particle is, on the other hand, actively moved to a specific compartment, a directed motion can be observed.

Using a home-built confocal microscope (for details see supplementary material Figures S4 and S5), the sample was excited with a 532 nm laser. Matching the confocal image with a bright-field image was done to confirm if the diamond is inside the cell. After finding a particle, it was tracked for ~45 min. The custom-made software recorded 2×2 μ m images of the particles throughout the tracks to make sure we are following the same particles. By applying a Gaussian fitting of the intensity profiles, the software calculates the position of the particle center. Cells were put in the coated glass-bottom dishes and, to keep the cells alive, we performed the experiments only for 45 min. This time was chosen conservatively after several preliminary experiments were cells died after longer exposure times. Temperature in the samples was ~25°C.

Recording images of a particle took 1.8 s per image. In total, we performed 1500 repetitions. To interpret these trajectories, mean square displacement (MSD) curves³³ were used. This value provides a measure on how far a particle travels within a certain time period (τ). These trajectories were divided into subtracks using a rolling window (200 repetitions) to gain more information about each segment of the trajectory.

The MSD curves were calculated from the coordinates of the recorded trajectories. The typical logarithmic scaling of a graphical representation of the MSD versus the time interval allows differentiating between three typical cases: purely random motion (diffusion), ballistic motion overlaying random diffusion, and confined diffusion. For all three cases we have simulated trajectories and calculated the corresponding MSD curves (see Figure 2), using Python.

For the simulated trajectories, a random generator drew 2000 displacements with varying step length between -1 and 1. To better simulate the actual movement of the microscopic particles, we assumed that we do not record every single displacement but only every third, by cumulating three

displacements into one. This also ensures that the distribution of step sizes and displacements follow a normal distribution. For the ballistic motion, one additional drawing with a directed motion (only positive values for x and y) was added to the three random displacements. A confined displacement trajectory was generated like the random motion with an additional step: whenever the trajectory of the particle left a predefined area (here: $30 \times 30 \mu\text{m}$), the particle was reflected into the area — according to a particle hitting the wall of its confinement. However, this means that the particle becomes faster when it is reflected from the border, which can be seen in the slightly higher slope of the left side of the graph for confined motion in Figure 2 (green). The full Python script used to generate the trajectories and to calculate the mean squared displacements can be found in the supplementary material.

Figure 2. Three possible types of the particle movement generate three distinct trajectory types: random (red), ballistic (blue), and confined (green). The trajectories (a) represent the movements a particle may execute over 2000 steps:

random (red), random diffusion with overlying ballistic motion (blue), and confined (green), wherein the trajectory of the particle is limited by a predefined border (here: $30 \times 30 \mu\text{m}$) that reflects the particle. (b) The mean squared displacements of the trajectories.

Simple diffusion is shown in red — the particle performs a random walk, slowly moving away from the original position, and can theoretically explore every point of the volume. Similar behavior can be observed for a particle floating in suspension. The green trajectory illustrates confined diffusion — the particle is walking randomly within a certain limited volume (e.g. a vesicle, a cytoskeletal “cage”, or even an entire cell) and cannot leave it, no matter how much time has passed. The blue trajectory shows the case of simple diffusion combined with directional motion. The particle has a preferred direction, in which it moves with a certain speed (e.g. being transported along the cytoskeleton by molecular motors). At the same time, the diffusional component results in deviations from the shortest straight line between the initial and the final positions.

At high τ , less data points contribute to the calculation, and thus the MSD calculation has a larger statistical uncertainty. To compensate for this, we used only the first 75% of the resulting curves for fitting.

3.2.8 Statistical analysis

All statistical analyses were performed with GraphPad prism with 95% confidence interval. One-way analysis of variance (ANOVA) was performed to analyze significance between particle uptake experiments. Correlation assessment was performed for comparing cell size and number of particles, and the Student T-test was used to analyze single particle tracking parameters inside cells.

3.3 Results

3.3.1 FNDs movement during cell division

The particles inside cells have been followed between 0 and 6 h after the particle uptake process. These times were used because according to Broach et al.³⁴, yeast cells proliferate for about 6 h in fermentable carbon sources (in this experiment, SD medium supplemented with 2% of glucose). Hu et al. used the same time span³⁵. The microscopic images resulting from this experiment are shown in Figure 3. Observing the movement of FNDs in living cells during cell division, we found three different cases of what the cell does with the particles. To display the different cases that we differentiate, we show three cells that behave differently under the same conditions. There are several possible explanations for the biological variety leading to these different behaviors. The most important difference is probably that the cells are in different stages of their cell cycle when the experiment is started. Additionally, there are genetic differences as well as differences in the metabolic state of cells.

Figure 3. Different cases for particle fate during cell division. The red particles show the position of the fluorescent nanodiamonds, whereas green color indicates the cell stained with GFP. The first row shows images at 0 h while the second row shows the same cells at 6 h. In panels (a,b), the particles have remained in the mother cells over time. In panels (c,d), the particle has moved to the daughter cell. In panels (e,f), the particle that was in the cell at time zero has completely moved out of the cell after 6 h. The scale bars are 2 μm long.

3.3.2 FND quantification after cell division

As mentioned earlier, there exists biological variation between cells. Therefore, we measured 100 cells per condition to get a more complete picture of what happens and to obtain statistics. To analyze the distribution of FNDs over mother and daughter cells, a particle counting protocol¹⁶ was performed before and after cell division (see Figure 4).

Figure 4. Comparison of number of objects and particles in FND-lip and bare FND group before (a) and after (b) cell division. The correlation between particle number and cell volume is shown in panel (c) for FND-lip and in panel (d) for bare FNDs. The red line represents the cutting point between mother and daughter cells. One-hundred cells have been used for these experiments, and error bars represent standard deviation with significance level 0.05.

Comparing Figure 4a,b, we see that the overall number of particles/objects per cell is smaller after division than before. Thus, we surmise that for both particle types a large proportion of diamond particles is excreted from the cells. Excretion in yeast has been observed for diamond¹⁷ as well as for other materials. The process has been described to happen when the cells are not in a balanced condition. This can be, for example, when the cells are transferred to a medium lacking a required compound. As a consequence, they are producing unusually large amounts of amino acids³⁶. We do not find a significant difference in the number of objects per cell and number of particles per cell

between FND-lip and bare FNDs. This is the case both before cell division and after cell division (P value < 0.05). Correlation analysis was performed for both groups, and the results show no correlation between cell size and number of particles in FND-lip ($R^2 = 0.0007$) or FND ($R^2 = 0.0684$). This finding suggests that particles move randomly during cell division.

Based on Jorgensen et al. (2002)³⁷, the volume of mother cells in yeast is approximately 420–820 nm³. We categorized the cells based on the volume. Cells with a volume greater than 420 nm³ were assigned to the mother group, whereas cells with smaller size are counted in the daughter group. Figure 5 shows the distribution of particles in mother and daughter during the measurement. We divided groups based on cell volume and the number of particles the cells contain.

Figure 5. Particle distribution after cell division in yeast cells (in percentages). From the FND-lip and bare FND groups. There are more daughter cells that are containing particles than mother cells.

3.3.3 Single particle tracking

In this study, we wanted to observe if the presence of liposomes has an effect on the particle behavior inside living cells. A control experiment with FNDs has been performed as a comparison with FND-lip. We compared the tracked pattern of particle movement in 3-dimensional trajectories and mean square displacement for both FNDs (Figure 6) and FND-lip (Figure 7). Due to the small

size of yeast and the limited optical resolution, it is tricky to conclude whether a particle is inside or outside of a cell. However, we also observed the movement of particles in the medium alone and in glycerol. The medium alone is much less viscous than the cellular content, and the movement of the particles is too rapid to be tracked with our set-up. FNDs moving freely in glycerol explore substantially larger volumes than the particles tracked in the experiments with yeast cells (Supplementary Figure S1). We observe the displacements of 10^2 – 10^3 μm^2 and largely unconstrained (simple) diffusion, with the values of α being 0.58 on average and not dropping below 0.32 (Supplementary Figure S2). Thus, the confined movement we observe in this set of experiments is much more consistent with a movement inside cells. For a comparison with the simulated data see supplementary information. The 3D trajectories suggest that liposome coating has only little effect on the movement of the particles. Both coated and uncoated particles were in a confined diffusion type of movement. Comparison of MSD curves showed that the displacement area was not statistically different between the particles with and without lipid coating.

Figure 6. Movement of bare diamonds in cells. The mean square displacement (MSD) curves of bare FNDs on top (a) show only confined diffusion. Panel (b) shows the complete three-dimensional trajectory, showing the types of movement detected at each segment of the trajectory. The only types of movement observed were confined diffusion (blue) and ultraconfined diffusion (magenta), for which the calculated α approaches 0, and no displacement can be detected. The final part of the trajectory, for which no analysis can be performed, is shown in black. The right lower panel (c) shows the values of α observed at different segments of the trajectory.

Figure 7. Movement of FND-lip in cells. (a) MSD curves of lipid-coated FNDs. MSD analysis reveals that they only move in confined diffusion and ultraconfined diffusion modes. The image on the left side of the bottom panel (b) is the three-dimensional trajectory, colored to show the modes of motion that have been detected at different segments of the track. Just like in bare FNDs, no other types than confined and ultraconfined diffusion were observed. The 3D trajectory on the right (c) shows the fluctuations of estimated α , reflecting the degree of confinement. Note that the overall volume explored by the particle is smaller, compared to the bare FNDs, resulting in lower α values.

A statistical analysis has been done to compare the displacement between bare and lipid-coated diamonds. It indicates that there is no significant

difference between the two groups with P value 0.1131 (Figure 8a). Alpha indicates the how freely a particle moves. The higher the value of alpha, the larger is the volume available to the particle. The results showed a significant difference in alpha (P value 0.007) between the two groups (Figure 8b). The presence of liposomes surrounding FNDs facilitates aggregate formation and thus results in change in the volume and limitation of the movement. This difference is reflected in a lower alpha value in FNDs-lip (Figure 8b). The diffusion coefficient Figure 8c shows no significant difference (P value 0.972) between uncoated and FND-lip.

Figure 8. Statistical comparison between bare-FNDs and FNDs-liposome. Panel (a) compares the displacement of each particle. Panel (b) is a comparison between alphas of each particle. A significant decrease was found in the case of FND-lip. Panel (c) is a comparison between the diffusion coefficient. All of these experiments have been done in triplicates, error bars represent standard deviation, and statistical differences are tested at significance level 0.05. * indicates $p \leq 0.05$.

3.4 Discussion and conclusion

Here we provide the first analysis of the fate of FNDs during and after yeast cell division. We found that there are multiple routes that the particles can take and in total we observed three different fates of particles during cell division.

For bare FNDs, 14.9% of particles stayed in the mother cell, compared to 21.6% for the FND-lip (Figure 3a). Twenty-one percent of the bare FNDs and 28.4% for the FND-lip ended up in the daughter cells (Figure 3b). Finally, 98.35% of particles for FNDs and 98.38% of the particles for FND-lip were most likely excreted from the cell (Figure 3c).

Surprisingly, the distribution of particles between mother and daughter cells (result in Figure 5) shows that more daughter cells in both groups (both bare FND and FND-lip) contain more particles than mother cells (Figure 5). There is a large body of literature available where nanoparticles interact with yeast. However, these articles are almost exclusively concerned with the toxic effect that nanoparticles could have on yeast. The distribution between mother and daughter cells is so far investigated for heat-induced protein aggregates (80 nm)³⁸. The size of FNDs used for this study (70 nm) is comparable to these particles. However, in the case of FNDs, we did not observe more particles being retained in the mother cells, which was typical for protein aggregates in dividing yeast cells. This discrepancy can be explained in different ways.

A first explanation is related to the size of the particles. For heat-induced protein aggregates, passive aggregate formation, fusion, growth, and exclusion from the bud was observed³⁸. As the protein aggregates grow, they can reach sizes of 600 nm³⁹. We do not see this for FNDs. In this case, one might assume that non-aggregated FND particles are substantially smaller, thus it is easier for them to cross the bud neck and end up in the daughter cell.

On the other hand, there are a number of models that suggest the impact of certain active control mechanisms on the particle distribution in the dividing cells. The protein aggregates are thought to be retained in mother cells due to their tethering to actin cables, retrogradely moving to the mother cell⁴⁰, attachment to the nucleus and the vacuole⁴¹, or to the surface of endoplasmic

reticulum and anchored mitochondria⁴². The first model suggests active transport of the aggregates out of the bud, whereas the second and third models explain the asymmetric distribution of the aggregates by general decrease in their mobility. If any of these models are true, it might mean that FNDs simply lack certain features of protein aggregates and are thus “invisible” for those mechanisms of active control. Molecules that are adsorbed on the diamond surface could also influence how they interact with the cells. It has been shown for other nanoparticles that the protein corona can dictate the interaction with cells. Drug-loaded nanodiamonds, on the other hand, behave more like bare diamond particles in mammalian cells than drug molecules alone. The fact that FND and FND-lip behave similarly, despite the different surface chemistry, indicates that here the behavior might also be dictated by the diamond particle. It can be useful that both mother cells and daughter cells contain diamonds. This way it is possible to observe and track both. Additionally, FNDs might also be considered “safe” for the cells and are thus not actively hindered from entering daughter cells.

The spheroplasting process might also affect the fate of FNDs by giving a stress condition to the cells after being treated by zymolyase affect cell wall integrity⁴³, which is used for intracellular transport⁴⁴. In this case, changing of cell wall components (especially actin) due to spheroplasting process will affect particle movement during cell division. Both yeast and spheroplasts have a cell membrane; in spheroplasts, only the cell wall is removed. When FND-lip (which have a positive zeta potential) are taken up by the cells, they bind to negatively charged components of the plasma membrane. Another study also showed that positively charged particles can be ingested by the cells better than negatively charged particles⁴⁵.

Finally, we also observed and quantified movement of particles during cell division. We mainly find confined diffusion for both coated and uncoated particles without showing significant differences between displacement and diffusion coefficient. On the other hand, we have noticed a significant difference in α . This is due to the fact that coated particles have the tendency to aggregate. Thus, this movement is more confined.

The results from this study might in the future be useful for labeling yeasts with diamonds or magnetometry in yeast cells. Additionally, this work might be interesting to compare with how other nanoparticles behave during cell division.

References

1. Balasubramanian, G. *et al.* Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature*. **455**, 648-653 (2008).
2. Bradac, C. *et al.* Observation and control of blinking nitrogen-vacancy centres in discrete nanodiamonds. *Nat Nanotechnol*. **5**, 345-349 (2010).
3. Dolde, F. *et al.* Electric-field sensing using single diamond spins. *Lett Nat Phys*. **7**, 459-463 (2011).
4. Laube, C. *et al.* Controlling the fluorescence properties of nitrogen vacancy centers in nanodiamonds. *Nanoscale*. **11**, 1770-1783 (2019).
5. Hickey, D. P., Jones, K. S., & Elliman, R. G. Amorphization and graphitization of single-crystal diamond - A transmission electron microscopy study. *Diamond and Related Materials*. **18(11)**, 1353-1350 (2009).
6. Bosia, F. *et al.* Modification of the structure of diamond with MeV ion implantation. *Diamond and Related Materials*. **20(5)**, 774-778 (2011).
7. Dantelle, G. *et al.* Efficient production of NV colour centres in nanodiamonds using high-energy electron irradiation. *Journal of Luminescence*. **130(9)**, 1655-1658 (2010).
8. Boudou, J.P. *et al.* High yield fabrication of fluorescent nanodiamonds. *Nanotechnology*. **20(23)**, 1-11 (2009).
9. Chipaux, M. *et al.* Nanodiamonds and their Applications in cells. *Small*. **14(19)**, 1704263 (2018).
10. Mitura, K.A., Włodarczyk, E. Fluorescent nanodiamonds in biomedical applications. *J AOAC Int*. **101**, 1297-1307 (2018).
11. Hemelaar, S.R. *et al.* The response of HeLa cells to fluorescent nanodiamond uptake. *Sensors (Switzerland)*. **18**, 355 (2018).
12. Vijayanthimala, V., *et al.* The biocompatibility of fluorescent nanodiamonds and their mechanism of cellular uptake. *Nanotechnology*. **20**, 425103 (2009).
13. Chu, Z. *et al.* Rapid endosomal escape of prickly nanodiamonds: Implications for gene delivery. *Sci Rep*. **5**, 1161 (2015).
14. Fang, C.Y. *et al.* The Exocytosis of fluorescent nanodiamond and its use as a long-term cell tracker. *Small*. **7**, 3363-3370 (2011).
15. Liu, K.K., *et al.* Endocytic carboxylated nanodiamond for the labeling and tracking of cell division and differentiation in cancer and stem cells. *Biomaterials*. **30**, 4249-4259 (2009).
16. Hemelaar, S.R. *et al.* Generally applicable transformation protocols for fluorescent nanodiamond internalization into cells. *Sci Rep*. **7**, 5862 (2017).
17. van der Laan K. J. *et al.* Towards using fluorescent nanodiamonds to study chronological ageing in *Saccharomyces cerevisiae*. *Anal Chem*. **90**, 13506-13513 (2018).
18. Morita, A. *et al.* Cell uptake of lipid-coated diamond. *Part. Part. Syst. Charact.* **1900116**, 1-8 (2019).
19. Neiman, A.M. Sporulation in budding yeast. *Genetics*. **189**, 737-765 (2011).
20. Baldi, S. *et al.* Heat stress promotes longevity in budding yeast by relaxing the confinement of age-promoting factors in the mother cell. *Elife*. **6**, e28329 (2017).
21. Kaeberlein, M., Burtner, C.R. & Kennedy, B.K. Recent developments in yeast aging. *PLoS Genet*. **3**, 655-660 (2007).
22. Hou, J. *et al.* Engineering of *Saccharomyces cerevisiae* for the efficient co-utilization of glucose and xylose. *FEMS Yeast Res*. **17**, 1-11 (2017).
23. Walker, G., Hill, A. *Saccharomyces cerevisiae* in the Production of Whisk(e)y. *Beverages*. **2**, 38 (2016).
24. Karas, B.J. *et al.* Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat Protoc*. **9**, 743-750 (2014).

25. Hemelaar, S.R. *et al.* Nanodiamonds as multi-purpose labels for microscopy. *Sci Rep.* **7**, 720 (2017).
26. Shenderova, O. A. *et al.* Synthesis, properties, and applications of fluorescent diamond particles. *Journal of Vacuum Science & Technology B.* **37(3)**, 030802 (2019).
27. Fu, C.C. *et al.* Characterization and application of single fluorescent nanodiamonds as cellular biomarkers. *Proc Natl Acad Sci.* **104(3)**, 727-732 (2007).
28. Rondin, L. *et al.* Surface-induced charge state conversion of nitrogen-vacancy defects in nanodiamonds. *Phys Rev B.* **82(11)**, 115449 (2010).
29. Laan, K. J. *et al.* Nanodiamonds for in vivo applications. *Small.* **14(24)**, 1703838 (2018).
30. Loretz, M. *et al.* Nanoscale nuclear magnetic resonance with a 1.9-nm-deep nitrogen-vacancy sensor. *Appl. Phys. Lett.* **104**, 033102 (2014).
31. Zheng, T. *et al.* Recombinant protein polymers for colloidal stabilization and improvement of cellular uptake of diamond nanosensors. *Anal Chem.* **89**, 12812-12820 (2017).
32. Torrano, A.A. *et al.* A fast analysis method to quantify nanoparticle uptake on a single cell level. *Nanomedicine.* **8**, 1815-1828 (2013).
33. Dougherty, R. Extensions of DAMAS and Benefits and Limitations of Deconvolution in Beamforming. *11th AIAA/CEAS Aeroacoustics Conference*; (2005).
34. Broach, J.R. Nutritional control of growth and development in yeast. *Genetics.* **192**, 73-105 (2012).
35. Hu, J. *et al.* Two programmed replicative lifespans of *Saccharomyces cerevisiae* formed by the endogenous molecular-cellular network. *J. Theor. Biol.* **362**, 69-75 (2014).
36. Velasco I. *et al.* *Saccharomyces cerevisiae* Aqr1 Is an internal-membrane transporter involved in excretion of amino acids. *Eukaryot Cell.* **3**, 1492-1503 (2004).
37. Jorgensen, P. *et al.* Systematic identification of pathways that couple cell growth and division in yeast. *Science.* **297**, 1070850 (2002).
38. Andrade-Restrepo, M. Is aggregate-dependent yeast aging fortuitous? A model of damage segregation and aggregate dynamics. *Biophys J.* **113**, 2464-2476 (2017).
39. Paoletti, C. *et al.* Kinetics of formation and asymmetrical distribution of Hsp104-bound protein aggregates in yeast. *Biophys J.* **110**, 1605-1614 (2016).
40. Liu, B. *et al.* The polarisome is required for segregation and retrograde transport of protein aggregates. *Cell.* **140**, 257-267 (2010).
41. Spokoini, R. *et al.* Confinement to organelle-associated inclusion structures mediates asymmetric inheritance of aggregated protein in budding yeast. *Cell Rep.* **2**, 738-747 (2012).
42. Zhou, C. *et al.* Organelle-based aggregation and retention of damaged proteins in asymmetrically dividing cells. *Cell.* **159**, 530-542 (2014).
43. Rodríguez-peña, J.M. *et al.* Activation of the yeast cell wall integrity MAPK pathway by zymolyase depends on protease and glucanase activities and requires the mucin-like protein Hkr1 but not Msb2. *FEBS Lett.* **587**, 3675-3680 (2013).
44. Lesage, G., Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol MolBiol Rev.* **70**, 317-343 (2006).
45. Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int.J. Nanomedicine.* **7**, 5577-5591 (2012).

Supplementary information

Figure S1. 3D trajectories of FNDs: (A) Bare FND moving in glycerol. (B) Bare FND in the yeast cell. (C) Liposome-coated FND moving in the yeast cell. The scale is preserved for all trajectories. The colors change, as the particle progress over the trajectory. Note that the movement of FNDs in yeast cells occurs in a substantially smaller volume, as compared to the FND, freely diffusing in glycerol.

Figure S2. Estimated values of α over the 3D trajectories of FNDs: (A) Bare FND moving in glycerol. (B) Bare FND in the yeast cell. (C) Liposome-coated FND moving in the yeast cell. The color scale is preserved for all trajectories. Note that the calculated values of α is substantially lower for FNDs moving in yeast cells, reflecting the smaller volume available to the particles.

Figure S3. Spectrum from FNDs. The data points (in blue) are well fitted by the sum (in red) of seven Lorentzians (in black). This reveals that the NV centers are mostly in there negatively charged state as characterized by a zero phonon lines at 637 nm and of seven phonon bands spaced by 63meV at lower energies¹. Supporting Information on the python script to generate typical trajectories representing ballistic, random and confined motility, and to analyze their mean squared displacement (MSD).

This code is optimized for python 3.6 and higher, using the Spyder distribution 3.3.4.

The code has the following parts:

Loading of the needed modules:	Line 1 – 11
Definition of Parameters:	Line 12 - 15
Generation of trajectory depicting random motility:	Line 16 -41
Preparing a common plot for all trajectories	Line 42 - 51
MSD calculation and plot, random trajectory:	Line 52 – 111
Preparing a common plot for all MSDs:	Line 112 - 120
Generation of trajectory depicting ballistic motility:	Line 121 -143
MSD calculation and plot, ballistic trajectory:	Line 144 – 191
Generation of trajectory depicting confined motility:	Line 192 - 295
MSD calculation and plot, confined trajectory:	Line 296 – 337
Finalizing a common plot for all MSDs:	Line 338 - 343

Description of the home built diamond magnetometer:

Figure S4 shows a schematic representation of the first part where the green laser beam is generated.

Figure S4. First part of the optical setup: (1) laser, (2) filter wheel to tune laser intensity, (3) beam splitter cube, (4) Acoustooptical modulator which allows for laser pulsing, (5) $\lambda/4$ plate, (6) lenses increase beam size to ease fiber coupling, (7) coupling into a fiber.

The setup consists of a Neodym YAG laser, which emits green light at 532 nm. To tune laser intensities the beam is directed through a manually adjustable filter wheel. If pulsing is desired, the laser is directed through the path between (3) and (5) in Fig 4S This path consists of a beam splitter cube which directs the beam through an acoustooptical modulator, through a $\lambda/4$ plate and is then reflected back through the same aperture, which allows fast and precise laser pulsing. Finally, the beam is coupled into a fiber and directed to the second part of the setup, which is shown in Figure S5. There the laser is directed through a dichroic mirror (3) which divides the incoming laser light from the red fluorescence which is emitted from the NV centers. The next element in the path is a scanning mirror (4) which allows addressing different parts of the sample. Different heights can be scanned by moving an objective with a piezo-stage.

Figure S5. Second part of the optical setup: (1) fiber port, (2) lenses to change beam size (3) dichroic mirror to direct only the NV fluorescence to the detector, (4) scanning mirror allows to move the laser to different locations on the sample, (5) lenses (6) objective that allows for focus scanning, (7) homemade micro fabricated microwave strip line with sample on top (8) 3D stage for positioning a small permanent magnet close to the sample.

The sample, containing the diamonds, is located on top of a micro-fabricated (standard lift off lithography) microwave strip line. The strip line allows for microwave sweeping in order to find magnetic resonance line. After excitation the defect emits red shifted fluorescence, which is directed back the same path until the dichroic mirror. There it is separated from reflected green light and enters the detection path (3)-(10). After passing a pinhole the light is detected by an avalanche photo-diode with single photon sensitivity. To reduce counts from reflected light the detection path is contained within a dark box (which for simplicity is not shown in the drawing.) While the basic optical setup has already been built in our laboratory, additional parts will be implemented to improve sensitivity and spectral resolution.

Figure S6. Background from yeast without diamond particles. (a) shows a typical confocal image of yeast at time zero and (b) shows a brightfield image of the same sample. (c) is a confocal image at the 6 hour time point and (d) a bright field image of the same sample. As expected, no particles are visible if the blue light for bright field imaging is on the background is around 90.000 counts/second and 50.000 counts per second when the light is off

Reference

1. Su, Z.C. *et al.* Luminescence landscapes of nitrogen-vacancy centers in diamond: Quasi-localized vibrational resonances and selective coupling. *J Mater Chem C*. **7**, 8086-8091 (2019).

4. Targeting nanodiamonds to the nucleus in yeast yells

Aryan Morita^{1,2#}, Thamir Hamoh^{1#}, Alina Sigaeva¹, Neda Norouzi¹, Andreas Nagl¹, Kiran J van der Laan¹, Emily P. P. Evans¹, Romana Schirhagl¹

1. Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
2. Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

Published in *Nanomaterials* 10(1962); 1-10 (2020)

Chapter 4

Abstract

Nanodiamonds are widely used for drug delivery, labelling or nanoscale sensing. For all these applications it is highly beneficial to have control over the intracellular location of the particles. For the first time, we have achieved targeting the nucleus of yeast cells. In terms of particle uptake, these cells are challenging due to their rigid cell wall. Thus, we used a spheroplasting protocol to remove the cell wall prior to uptake. To achieve nuclear targeting we used nanodiamonds, which were attached to antibodies. When using non-targeted particles, only 20% end up at the nucleus. In comparison, by using diamonds linked to antibodies, 70% of the diamond particles reach the nucleus.

Keywords: fluorescence nanodiamonds, nucleus targeting, yeast cells

4.1 Introduction

Fluorescent nanodiamonds (FNDs) show perfectly stable fluorescence^{1,2,3}. Thus they are potentially useful for long term fluorescent labelling and tracking^{4,5,6}. Additionally, it is particularly useful that FNDs are very well visible for multiple imaging techniques⁷. Furthermore, FNDs are interesting carriers for drugs in drug delivery. Their small size, inertness and biocompatibility leads to longer circulation times or sustained release of the drug^{8,9}. Additionally, nanodiamonds can help to dissolve otherwise non-soluble drugs and thus increase bioavailability¹⁰. Another possible benefit is that nanodiamonds can help drug delivery particles to evade macrophages¹¹. Benefits for drug delivery have been demonstrated for a number of drug candidates^{12,13}. FNDs also function as a tool for nanoscale intracellular sensing of magnetic fields. They change their optical properties based on their magnetic surrounding. Thus, they have been used to sense temperatures^{14,15}, pH¹⁶, or magnetic fluctuations^{17,18}. They have been introduced to the intracellular environments of many types of cells^{19,20} and show excellent biocompatibility²¹.

For all the above-mentioned applications, it is essential to not just bring nanodiamonds to random locations, but to control their fate. A limited number of studies have attempted this. In these articles, the nanodiamonds were placed in specific organelles in mammalian cells^{22–26}.

Targeting fluorescent nanodiamonds has been done for mostly cancer/tumor diagnostic applications^{24,27}. As a first, we have been able to achieve targeting in *Saccharomyces cerevisiae* (yeast cells). For this purpose, we used FNDs linked to antibodies. Linking FNDs to antibodies has already been achieved several times before for different applications^{28,29,22}. Yeasts were chosen for this study due to their relevance as a model organism for aging. It is also frequently used in food processing as well as in biotechnology and biosynthesis. Since yeast

cells are surrounded by a rigid cell wall, it is important to overcome this barrier. There are several options to achieve this. Firstly, one can either treat yeast cells chemically or with electroporation¹⁹. This induces pores in the surface or even removes the cell wall altogether³⁰. The second process, called spheroplasting, was chosen here because the relatively large particles might be challenging for uptake through small pores. Several different methods exist to achieve spheroplasting³¹⁻³³. They might all work for this application, however, we opted for a method that has already been used successfully for nanodiamonds³⁰. Within yeast cells, we chose to target the nuclear pore complex (NPC), which is the port of entry to the nucleus. In mammalian cells FND targeting to the nucleus has been achieved before³⁴. This is an important organelle since it contains the genetic blue print and plays a key role in the cell cycle³⁵. Hence, it is an important target area for drug delivery. In addition to this, DNA damage that occurs in the nucleus causes cancer. This makes the nucleus a compelling location for sensing applications using nanodiamonds. Labelling specific molecules on the nucleus is also of interest for imaging. Lastly, the nucleus is relatively easy to identify which makes it an interesting model system to evaluate and compare targeting strategies. Schematic scheme of targeting strategy is represented in figure 1.

Figure 1. Schematic representation of the targeting strategy in yeast cell. An antibody with a binding domain to the nuclear pore complex (NPC) was linked with FNDs. After using a spheroplasting protocol, FND-antibody particles are ingested and will accumulate on the nucleus.

4.2 Materials and methods

4.2.1. Fluorescent nanodiamonds starting material

Fluorescent nanodiamonds with a hydrodynamic diameter of 70 nm from Adamas Nanotechnology (Raleigh, NC, USA) were used for this study. They have a relatively broad size distribution and irregular shape. According to the vendor, these particles are irradiated to host approximately 500 nitrogen vacancy (NV) centers per particle. Since they undergo a cleaning process in oxidizing acid, their surface is oxygen terminated. They have been extensively characterized in previous works^{7,36}.

4.2.2. Preparation of FNDs conjugated antibody (FND-AB)

Monoclonal anti-nuclear pore complex (anti-NPC) antibodies (Pro Sci, Poway, CA, USA) were used for preparing FND-AB. According to the vendor, these antibodies have reactivity to yeasts and are produced in mice. Four microliters of anti-NPC antibodies (1 mg mL⁻¹) were added to 4 µL of FNDs (1

mg mL⁻¹) and were incubated for 20 min at room temperature. We chose to have approximately 7 antibodies per FND. During this process, the antibodies most likely adsorb non-specifically to the diamond surface. Such adsorption has already been reported for a large number of different proteins including antibodies^{37,38}. The mixture was vortexed and stored at 4 °C before being used.

4.2.3. Zeta potential and size measurements

Zeta potential and size measurements of FND-AB were performed with a Malvern Zetasizer Nanosystem (Westborough, MA, USA). The FND-AB were diluted in ultrapure water to a total volume of 1 mL. The samples were measured in triplicate and all the measurements were done at 25 °C. As a control, bare FNDs were measured. To monitor particle behavior in yeast culture medium, zeta potential and size of FND and FND-AB have been measured in a yeast culture medium.

4.2.4. Fourier-transform infrared spectroscopy (FTIR) measurements

FTIR spectra were collected to evaluate antibody conjugation to the FND surface and performed with an Agilent FTIR spectrometer (Santa Clara, CA, USA). For this experiment, a germanium crystal was used as a sample holder. After measuring the background of the crystal, 15 µL of FND or FND-AB were dropped onto the crystal and spectra were collected. Further characterization of the particles by optically detected magnetic resonance is shown in the supplementary information, Figure S6

4.2.5. Co-localization of FND-AB

To perform a preliminary evaluation of the conditions needed for antibody conjugation we used fluorescein (FITC) labelled antibodies, which were

attached to FNDs. The antibodies we used were monoclonal immunoglobulin G 1 (IgG 1) produced in mice and purchased from ProSci Inc. (Poway, CA, USA). Then, we investigated colocalization. Using this data, we evaluated the stability of the conjugate. If the conjugate was stable, the FITC signal co-localizes with the red signal from FNDs. To perform the assay, the mixture was diluted in ultrapure water and dropped on top of a glass surface. After air-drying, the sample was observed using a Zeiss LSM 780 confocal laser scanning microscope (Zeiss, Jena, Germany).

For FITC labelled antibodies, a 488 nm Argon (Ar) laser with 2% power was used. Since the samples were diluted in water, acquisition was performed between 508–597 nm. The FNDs were imaged using a 561 nm DPSS (diode-pumped solid state) laser (Roseville, CA, USA), and the photons were collected from 606–694 nm. Identification of FNDs and FITC labelled antibodies was confirmed by measuring spectra in the instrument's lambda mode. Since the final application of this work is in magnetometry where background light is detrimental, we only used the labelled antibodies for the preliminary evaluation (in Figure 2).

Figure 2. Confocal microscope images of FND-AB. Fluorescein (FITC) labelled antibodies (A), FNDs (B), and merged images (C). (D–F) show FITC, FND and merged images in magnification.

4.2.6. Particle uptake in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae BY4741 was used in this study. These cells were also used in our previous work where we established the behavior of pristine particles during uptake and cell division³⁰. Yeast cells were grown in synthetic dextrose (SD) complete medium until mid-log phase ($OD_{600} = 1.05$). Since yeast cells have a thick cell wall, it had to be made permeable¹⁹ or removed entirely³⁰. Here, we chose to remove the cell wall entirely and form so-called spheroplasts. The spheroplasting protocol was adapted from Karas et al.³⁹. To obtain spheroplasts, the cells were washed with sterile demineralized water and centrifuged for 5 min at 2500 xg at 10 °C. The supernatant was discarded, and 20 mL of 1 M D-sorbitol was added to the cells. The cells were again centrifuged for 5 min at 2500 xg at 10 °C. After discarding the supernatant, the pellet was diluted in 20 mL of SPEM (consisting of 1 M D-sorbitol, 10mM EDTA, and 10mM sodium phosphate) buffer and 40 μ L zymolyase 20T (Amsbio, Abingdon, UK). Hereafter,

30 μ L of β -mercaptoethanol (Sigma, Zwijndrecht, Netherlands) was added. The suspension was then incubated at 30 °C while shaking at 75 rpm for 30 min. Cells were monitored with a light microscope to determine when the spheroplasting process was completed (spheroplasts are brighter than normal yeast cells). The spheroplasting process was stopped by adding 20 mL of 1 M D-sorbitol followed by centrifugation for 5 min at 1000 xg at 10 °C. After the supernatant was discarded, 2 mL of STC (1 M sorbitol, 10 mM Tris HCl, 10 mM $CaCl_2$ and 2.5 mM $MgCl_2$) buffer were added to the pellet followed by incubation for 20 min at room temperature. In the end, 50 μ L of bare FNDs or FND-AB in 1 M D-sorbitol were added to the suspension followed by 10 min of incubation time at room temperature.

4.2.7. Immobilizing yeast cells

In order to monitor single cells, they were immobilized using the following protocol: glass bottom dishes with 4 compartments were coated with 0.1 mg mL⁻¹ concanavalin A (Sigma, Zwijndrecht, Netherlands) in sterile demineralized water. This coating process was followed by a washing step with sterilized demineralized water. Then the coated dishes were dried in a 37 °C incubator. After drying, 300 μ L SD medium without glucose and 4 μ L of cell suspension (approximately 2.4×10^7 cells mL⁻¹), with internalized particles from the previous step, were added into each quarter.

4.2.8. Biocompatibility assay

Biocompatibility of FND and FND-AB has been tested by using an 3-(4,5-dymethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay. After creating yeast spheroplasts, cells were placed into 1.5 mL sterile tubes and FND and FND-AB were added into cell suspension. Five milligrams per milliliter of

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Zwijndrecht, Netherlands) in sterile PBS was added. Then we incubated at 30 °C for 2 h. Cells were centrifuged at 1000 xg for 5 min at 10 °C. After discarding the supernatants, 2-propanol (Merck, Amsterdam, Netherlands) was added to dilute formazan and the samples were vortexed for 5 min. Cells were centrifuged at 1000 xg for 5 min at 10 °C; then the supernatants were collected and placed into 96-well plates. Absorbance was measured using a microplate reader (Fluostar optima, Ortenberg, Germany) at 540 nm. One percent of H₂O₂ (Merck, Amsterdam, Netherlands) was used as positive control.

4.2.9. Distance measurement from the nucleus

All images were analyzed using Fiji. Z-stacks were recorded starting from the bottom border of the cell to the upper side. By finding the middle layer of the cell with clear cell border and bright particle the images were selected. A scale line was drawn multiple times for statistical purposes between the center of the particle to the border of the nucleus to measure the distance. Cells were chosen randomly. However, we did inspect the cells that were chosen for their size to exclude systematic errors due to this. We did not observe any significant differences in the cell sizes of the respective groups.

4.2.10. Statistical analysis

Distance measurements were analyzed using a two-way ANOVA. If the results were significant, a Sidak post hoc test was performed to determine the significance within groups. The results from the MTT assay were analyzed using a one-way ANOVA and followed by Tukey as a post hoc test. Significance level was set at 0.05 and the analysis was performed by using Graph Pad v.8 software (GraphPad Software, San Diego, CA, USA).

4.3. Results

3.1. Characterization of FND–AB

Characterization of FND–AB was performed by measuring zeta potential and particle size for both groups. We found that the zeta potential of FND–AB was $+23.03 \pm 0.5$ mV while FNDs was -17.87 ± 0.2 mV. Particle size for FND–AB was 523.87 ± 14.8 nm and 83.59 ± 1.2 nm for the FND group, with a particle dispersion index (PDI) of 0.15 ± 0.002 (FND group) and 0.35 ± 0.03 (FND–AB) (see the supplementary information of zeta potential and size distribution graphs, Figure S1 and S2. Additionally, we performed co-localization measurements between fluorescent antibodies and diamonds. From co-localization data, we conclude that approximately 73% of antibodies bind to FNDs. Confocal microscope images of FND–AB are presented in Figure 2.

For the experiments in cells we used non-fluorescent antibodies from which we assume that the binding characteristics to FNDs are comparable. Since free antibodies were invisible in our experiments in cells and did not disturb the targeting (unless they are so abundant that they hinder the conjugated FNDs to bind), there was no need to remove the unreacted antibodies.

4.3.2. Biocompatibility of FND–AB

While the biocompatibility of bare FND has already been shown in the literature, this hasn't been tested for FND–AB. As expected, the presence of antibody on the FND surface does not have a negative effect on the metabolic activity level of yeasts. Results from MTT assay are presented in Figure 3. Our data also confirms the excellent biocompatibility of FNDs.

Figure 3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for analyzing metabolic activity of yeast cells. Yeasts without any treatment were used as a comparison (grey dots) and 1% of H₂O₂ was used as positive control. Compared to yeasts without any treatment, both FND and FND-AB group have no significant effect on metabolic activity in yeast cells. Both groups only show significant difference with the positive control. Data were analyzed by using one-way ANOVA and followed by Tukey post hoc test. The significance level was set at 0.05 and **** indicated $P < 0.0001$.

4.3.3. Position of FNDs and FND-AB inside cells

Positions of particles were followed by confocal microscopy between 0 and 24 h after uptake by the cells. It has to be noted here that we did not follow specific cells during 24 h continuously, but that we collected data on the average location. Representative images are shown in Figure 4. Particles in the FND group were mostly located inside the cell, however, they were still only close to the cell wall although they had been followed for 24 h. Contrarily, the particles from the FND-AB group are on average closer to the nucleus with increasing incubation time. The reason is likely that the FND-AB remain attached (longer) to the NPC once they arrive there. It should be noted that there was not much difference in the number of FNDs that were inside the cells. This is due to the

excretion of nanodiamonds from yeast over time, which has previously been reported⁴⁰.

Figure 4. Position of particle (red) inside the cell at different time points. FND (a) and FND-AB (b) groups were followed between 0 and 24 h after the particles were taken up by the cells. Scale bars in all images are 0.75 μm .

The successful rate of nanodiamond targeting was evaluated by calculating the distance from the particles to the nucleus (Figure 5), and the proportion of targeted and non-targeted particles from both groups (Figure 6).

Figure 5. Distance measurement from particles to the border of the nucleus. Experiments were performed in 10 cells. Significance level was set at 0.05. **** indicates $P < 0.0001$. Error bars represent standard errors.

According to Figure 5, particles from the FND-AB group were on average closer to the nucleus with increasing incubation time, whilst particles from the FND group remained in the same position. Figure 6 shows that FND-AB were mostly targeted on NPC (70%) compared to FNDs which only had 20% of particles targeted.

4.4 Discussion

We provided a targeting strategy of FNDs in yeast cells using antibodies. Comparing with the literature we can speculate that the antibodies bind primarily via electrostatic interactions. Kong et al.²⁸ have reported that adsorption of proteins on nanodiamonds generally comes from the interplay of ionic interaction, hydrogen bonding, van der Waals interaction, and hydrophobic interaction between protein and surface. Smith et al.⁴¹ report that primarily hydrostatic interactions play a role in antibody binding. Although their article is on detonation nanodiamonds, this might be the case here as well. We found that using the antibody gave a higher success rate of targeting compared to the bare

FND particles. FND-AB were on average closer to the nucleus after the 24 h of incubation (Figure 5). The FNDs, however, remained in the same position (mostly close to the border of the cells (Figure 4)) within the same incubation time. After calculating the proportion of targeted particles (see Figure 6), FND-AB had more cells with targeted particles (70%) compared to the FND group (20%). This means that using a combination of antibody and FND helps to achieve better targeting.

Targeting nanoparticles to the nucleus has been studied for years. Previous studies mentioned that nuclear targeting for nanoparticles had several barriers like selectivity of cell membrane, endosomal entrapment, lysosomal degradation, cytoplasmic trafficking, and most importantly nuclear entry⁴². According to Chu and co-workers (2015)⁴³ FNDs have a sharp edged shape which can break the endosomal membrane. It means particles can escape the endosome and degradation of chemical component on the surface (antibody) can be minimized.

Apart from the antibodies we used there are some alternative strategies for targeting. These include controlling nanoparticle size or modifying the surface charge⁴⁴. In this study, using antibodies can modify the surface charge of FND particles. Additionally, since most cell membranes are negatively charged, shifting the surface charge from negative to positive helps cells take up particles more easily.

In this study, we used FND particles larger than 70 nm. This means the particles could not pass the NPC to enter the nucleus. However, for many applications it is already useful to target the nucleus surface or the NPC.

4.5. Conclusions

Based on the results, we conclude that using antibody linked FNDs helps to achieve intracellular targeting. For further study, controlling the particle size

and surface charge might improve the efficacy of targeting FNDs through specific organelles. There are two applications that we foresee for this material. Firstly, the FND-AB could function as long-term fluorescent label. Secondly, and arguably the most interesting, is their possibility to perform nanoscale MRI. The latter concept has been demonstrated earlier this year for random locations⁴⁵. However, the particles demonstrated in this study would allow magnetometry specifically at the nucleus.

References

1. Vijayanthimala V, Cheng P, Yeh S, *et al.*, Biomaterials the long-term stability and biocompatibility of fluorescent nanodiamond as an in vivo contrast agent, *Biomaterials*. **33**, 7794-7802 (2012).
2. Su L, Wu M, Hui YY, *et al.*, (2017) Fluorescent nanodiamonds enable quantitative tracking of human mesenchymal stem cells in miniature pigs, *Sci Rep*. **7**, 1-11 (2017).
3. Hui YY, Su LJ, Chen OY, *et al.*, Wide-field imaging and flow cytometric analysis of cancer cells in blood by fluorescent nanodiamond labeling and time gating. *Sci Rep*. **4**, 1-7 (2014).
4. Haziza S, Mohan N, Loe-mie Y, *et al.*, Fluorescent nanodiamond tracking reveals intraneuronal transport abnormalities induced by brain-disease-related genetic risk factors. *Nat Nanotechnol*. **12**, 322-328 (2016).
5. Liu K, Wang C, Cheng C, *et al.*, Endocytic carboxylated nanodiamond for the labeling and tracking of cell division and differentiation in cancer and stem cells. *Biomaterials*. **30**, 4249-4259 (2009).
6. Lien Z, Hsu T, Liu K, *et al.*, Cancer cell labeling and tracking using fluorescent and magnetic nanodiamond. *Biomaterials*. **33**, 6172-6185 (2012).
7. Hemelaar SR, de Boer P, Chipaux M, *et al.*, Nanodiamonds as multi-purpose labels for microscopy. *Sci Rep*. **7**, 1-9 (2017).
8. Li Y, Tong Y, Cao R, *et al.*, In vivo enhancement of anticancer therapy using bare or chemotherapeutic drug-bearing nanodiamond particles. *Int J Nanomedicine*. **9**, 1065-1082 (2014).
9. Cui Z, Zhang Y, Zhang J, *et al.*, Sodium alginate-functionalized nanodiamonds as sustained chemotherapeutic drug-release vectors. *Carbon*. **97**, 78-86 (2016).
10. Wang D, Tong Y, Li Y, *et al.*, PEGylated nanodiamond for chemotherapeutic drug delivery. *Diam Relat Mater*. **36**, 26-34 (2013).

11. Zhao L, Xu Y, Akasaka T, *et al.*, Polyglycerol-coated nanodiamond as a macrophage-evading platform for selective drug delivery in cancer cells. *Biomaterials*. **35**, 5393-5406 (2014).
12. Kossovsky N, Gelman A, Hnatyszyn HJ, *et al.*, Surface-modified diamond nanoparticles as antigen delivery vehicles. *Bioconjug Chem*. **6**, 507-511 (1995).
13. Laan KJ, Hasani M, Zheng T, *et al.*, Nanodiamonds for in vivo applications. *Small*. **1703838**, 1-17 (2018).
14. Kucsko G, Maurer PC, Yao NY, *et al.*, Nanometre-scale thermometry in a living cell. *Nature*. **500**, 54-58 (2013).
15. Simpson DA, Morrisroe E, McCoey JM, *et al.*, Non-neurotoxic nanodiamond probes for intraneuronal temperature mapping. *ACS Nano*. **11**, 12077-12086 (2017).
16. Rendler T, Neburkova J, Zemek O, *et al.*, Optical imaging of localized chemical events using programmable diamond quantum nanosensors. *Nat Commun*. **8**, 1-9 (2017).
17. Steinert S, Ziem F, Hall LT, *et al.*, Magnetic spin imaging under ambient conditions with sub-cellular resolution. *Nat Commun*. **4**, 1-6 (2013).
18. Glenn DR, Lee K, Park H, *et al.*, Single-cell magnetic imaging using a quantum diamond microscope. *Nat Methods*. **12**, 736-738 (2015).
19. Hemelaar SR, van der Laan KJ, Hinterding SR, *et al.*, Generally applicable transformation protocols for fluorescent nanodiamond internalization into cells. *Sci Rep*. **7**, 5862 (2017).
20. Chipaux M, van der Laan KJ, Hemelaar SR, *et al.*, Nanodiamonds and their applications in cells. *Small*. **1704236**, 1-25 (2018).
21. Hemelaar SR, Saspaanithy B, L'Hommelet SRM, *et al.*, The response of HeLa cells to fluorescent nanodiamond uptake. *Sensors*. **18**, 1-15 (2018).
22. Mkandawire M, Pohl A, Gubarevich T, *et al.*, Selective targeting of green fluorescent nanodiamond conjugates to mitochondria in HeLa cells. *J Biophotonics*. **2**, 596-606 (2009).
23. Chang B, Lin H, Su L, *et al.*, Highly fluorescent nanodiamonds protein-functionalized for cell labeling and targeting. *Adv Funct Mater*. **23**, 5737-5745 (2013).
24. Sleggerova J, Hajek Mi, Rehor I, *et al.*, Designing the nanobiointerface of fluorescent nanodiamonds: highly selective targeting of glioma cancer cells. *Nanoscale*. **7**, 415-420 (2015).
25. Zhang B, Li Y, Fang C, *et al.*, Receptor-mediated cellular uptake of folate-conjugated fluorescent nanodiamonds: A combined ensemble and single-particle study. *Small*. **5**, 2716-2721 (2009).
26. Zurbuchen MA, Lake MP, Kohan SA, *et al.*, Nanodiamond landmarks for subcellular multimodal optical and electron imaging. *Sci Rep*. **3**, 1-5 (2013).
27. Torelli MD, Rickard AG, Backer MV, *et al.*, Targeting fluorescent nanodiamonds to vascular endothelial growth factor receptors in tumor. *Bioconjug Chem*. **30**, 604-613 (2019).
28. Kong XL, Huang LCL, Hsu CM, *et al.*, High-affinity capture of proteins by diamond nanoparticles for mass spectrometric analysis. *Anal Chem*. **77**, 259-265 (2005).
29. Zhang W, Patel K, Schexnider A, *et al.*, Nanostructuring of biosensing electrodes with nanodiamonds for antibody immobilization. *ACS Nano*. **8**, 1419-1428 (2014).
30. Morita A, Martinez FPP, Chipaux M, *et al.*, Cell uptake of lipid-coated diamond. *Part Syst Charact*. **1900116**, 1-8 (2019).
31. Burgers PMJ and Percival KJ, Transformation of yeast spheroplasts without cell fusion. *Anal Biochem*. **163**, 391-397 (1987).
32. Russell BI and Stewart GG, Spheroplast fusion of Brewer's yeast strains. *J Inst Brew*. **85**, 95-98 (1979).
33. Ovalle R, Lim ST, Holder B, *et al.*, A spheroplast rate assay for determination of cell wall integrity in yeast. *Yeast*, **14**, 1159-1166 (1998).

34. Lake MP and Bouchard LS, Targeted nanodiamonds for identification of subcellular protein assemblies in mammalian cells. *PLoS One*. **12**, 1-18 (2017).
35. Spector DL, The dynamics of chromosome organization and gene regulation. *Annu Rev Biochem*. **72**, 573-608 (2013).
36. Ong SY, van Harmelen RJJ, Norouzi N, *et al*, Interaction of nanodiamonds with bacteria. *Nanoscale*. **10**, 17117-17124 (2018).
37. Martinez FPP, Nagl A, Guluzade S, Schirhagl R, Nanodiamond for sample preparation in proteomics. *Anal Chem*, **91**, 9800-9805 (2019).
38. Hemelaar SR, Nagl A, Bigot F, *et al.*, The interaction of fluorescent nanodiamond probes with cellular media. *Microchim Acta*, **184**, 1001-1009 (2017).
39. Karas BJ, Jablanovic J, Irvine E, *et al.*, Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat Protoc*. **9**, 743-750 (2014).
40. Laan KJ, Naulleau J, Damle VG, *et al.*, Towards using fluorescent nanodiamonds to study chronological ageing in *Saccharomyces cerevisiae*. *Anal Chem*, **90**, 13506-13513 (2018).
41. Smith AH, Robinson EM, Zhang XQ, *et al.*, Triggered release of therapeutic antibodies from nanodiamond complexes. *Nanoscale*. **3**, 2844-2848 (2011).
42. Tammam SN, Azzazy HME, Lamprecht A, How successful is nuclear targeting by nanocarriers. *J Control Release*. **229**, 140-153 (2016).
43. Chu Z, Miu K, Lung P, *et al.*, Rapid endosomal escape of prickly nanodiamonds: Implications for gene delivery. *Sci Rep*. **5**, 1-8 (2015).
44. Heitz F, Morris MC, Divita G, Twenty years of cell - penetrating peptides : from molecular mechanisms to therapeutics. *Br J Pharmacol*. **157**, 195-206 (2009).
45. Morita A, Nusantara AC, Martinez FPP, *et al.*, Quantum monitoring the metabolism of individual yeast mutant strain cells when aged, stressed or treated with antioxidant. (2020), <https://arxiv.org/abs/2007.16130>

Supplementary information

Figure S1. Average of value of zeta potential from FND and FND-AB particles in water. FND particles originally have a zeta potential ($-17,87 \pm 0,2$ mV) (left curves) and when they combine with the antibody, the zeta potential becomes positive ($+23,03 \pm 0,5$ mV) (right curves). All measurements were performed at 25°C and in triplicates.

Figure S2. Average value of particle size distribution from FND and FND-AB in water. FND particles have an average size of $83,59 \pm 1,2$ nm and FND-AB of $523,87 \pm 14,8$ nm. All measurements were performed at 25°C and in triplicates.

Figure S3. Average values of zeta potential from FND and FND-AB in yeast medium. Yeast medium, which contain yeast nitrogen base and 2% D-Glucose change the zeta potential of FND-AB particles. FND-AB become negative while the FND particles remain negative. All measurement were performed at 25°C and in triplicates.

Figure S4. Average value of particle size from FND and FND-AB in yeast medium. Adding both FND and FND-AB into yeast culture medium affects the particle size. Both groups increase drastically in size. All measurements were performed at 25°C and in triplicates.

Figure S5. FTIR spectra of FND and FND-AB particles. The difference between the two spectra can be attributed to the presence of antibodies (which are proteins). The broad peak at 3369 cm^{-1} can be attributed to OH stretching in FND and OH and NH stretching in FND AB. The small side peak at 3300 cm^{-1} comes from CH stretching and is absent in the diamond spectrum. The band at 1639 cm^{-1} can be attributed to C=C stretching. The band at 1463 cm^{-1} comes from CH bending and is also only present in FND-AB. The bands around 1103 cm^{-1} are due to CN and CO stretching.

Optically detected magnetic resonance (ODMR) measurements

ODMR allows to use FNDs to read out their magnetic surrounding, to differentiate nanodiamonds from other particles. After selecting an FND or FND-AB we recorded an optically detected magnetic resonance with a home-built microscope¹. The frequency was swept around the expected resonance frequency of the NV-centre at 2.87GHz .

Figure S6. Comparing between ODMR measurements from bare FND versus FND-AB, the results are similar. (The difference in intensity is FND dependent and varies with distance from the microwave wire). This confirms that FND-AB are suitable for magnetic sensing experiments.

Reference

1. Hemelaar SR, de Boer P, Chipaux M, *et al.*, Nanodiamonds as multi-purpose labels for microscopy. *Sci Rep.* **7**, 1-9 (2017).

Chapter 5

5. Quantum monitoring the metabolism of individual yeast mutant strain cells when aged, stressed or treated with antioxidant

Aryan Morita^{1,2+}, Anggrek C. Nusantara¹⁺, Felipe P Perona Martinez¹, Thamir Hamoh¹, Viraj G. Damle¹, Kiran J. van der Laan¹, Alina Sigaeva¹, Thea Vedelaar¹, Michael Chang³, Mayeul Chipaux⁴, Romana Schirhagl^{1*}

1. Department of Biomedical Engineering, Groningen University, University Medical Center Groningen, Antonius Deusinglaan 1 9713AV Groningen, The Netherlands
2. Department of Dental Biomedical Sciences, Universitas Gadjah Mada, Jalan Denta 1 55281 Yogyakarta, Indonesia
3. European Research Institute for Biology of Aging, University Medical Center Groningen, Antonius Deusinglaan 1 9713 AV Groningen, The Netherlands
4. Institute of Physics, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

+ These authors contributed equally

Submitted to *Nature Nanotechnology*

(Available on ArVix <https://arxiv.org/abs/2007.16130>)

Abstract

Free radicals play a key role in the ageing process. The strongly debated free radical theory of ageing even states that damage caused by free radicals is the main cause of aging on a cellular level. However, free radicals are small, reactive and short lived and thus challenging to measure.

*We utilize a new technique called diamond magnetometry for this purpose. We make use of nitrogen vacancy centers in nanodiamonds. Via a quantum effect these defects convert a magnetic resonance signal into an optical signal. While this method is increasingly popular for its unprecedented sensitivity in physics, we use this technique here for the first time to measure free radicals in living cells. Our signals are equivalent to T1 signals in conventional MRI but from nanoscale voxels from single cells with sub-cellular resolution. With this powerful tool we are able to follow free radical generation after chemically inducing stress. In addition, we can observe free radical reduction in presence of an antioxidant. We were able to clearly differentiate between mutant strains with altered metabolism. Finally, the excellent stability of our diamond particles allowed us to follow the ageing process and differentiate between young and old cells. We could confirm the expected increase of free radical load in old wild type and *sod1Δ* mutants. We further applied this new technique to investigate *tor1Δ* and *pex19Δ* cells. For these mutants an increased lifespan has been reported but the exact mechanism is unclear. We find a decreased free radical load in, which might offer an explanation for the increased lifespan in these cells.*

5.1 Introduction

Free radicals are involved in many processes in healthy cells and organisms, such as mitochondrial metabolism, cell death and signaling of cells. They are also part of the working mechanism of many drugs. Additionally, they are suspected to play a crucial role in numerous pathogenic conditions including the diseases responsible for most deaths worldwide: cardiovascular diseases, cancer or immune responses to pathogens¹. Free radicals also play a critical role in ageing. The free radical theory of ageing states that free radicals are the major cause of ageing on the cellular level². Despite their relevance, we know fairly little about them. The reason is that they are produced in low concentrations (ranging from nanomolar to micromolar concentrations)^{3,4} and have short lifetimes. Quantifying, identifying and localizing free radicals has been recognized as main bottlenecks to translate free radical biology into biomedical advances⁵.

There are several ways to detect free radicals in cells. The different methods can be divided in indirect and direct methods. The indirect ones measure the cellular response towards a certain species. This is done for instance by detecting the expression of certain genes which encode enzymes involved in coping with stress (e.g. superoxide dismutase or catalase)⁶. One way to achieve this goal is via quantitative reverse transcription polymerase chain reaction (RT-qPCR). The major advantage is that these enzymes are specific and one can differentiate between different species. However, one needs to know in advance which enzymes or molecules are involved (which is often unknown). Additionally, the actual radical species are converted into each other⁷. So if you for instance have more catalase, it doesn't necessarily mean that it is the hydrogen peroxide production that went up. Furthermore, it takes some time for a cell to change the gene expression patterns and even more time is needed to adjust proteins expression. Thus, these data always represent the samples history rather

than the current situation. Furthermore, spatial and temporal information on where in the cell and when the chemicals were generated is lost.

In contrast imaging is a more direct approach and provides spatially resolved data. Fluorescence imaging allows direct detection of free radicals. Additionally, it is relatively easy and photons can be detected very sensitively (in principle single photon counting is possible). Thus, the spatial resolution that is achieved is only limited by diffraction, down to a few hundreds of nm, which can be even be improved by super-resolution techniques. The assays are based on organic dyes (like 2,7-dichlorofluorescein diacetate (H₂DCFDA) which is used for comparison in this work), which fluoresce when reacting with reactive oxygen species (ROS). However, there are several problems with this approach. First, although there is some overlap between ROS and free radicals they are not the same (there are some ROS that are not radicals as for instance H₂O₂ while there are also some radicals that are not ROS as for instance nitrogen based radicals or radical degradation products of biomolecules). Secondly, the ROS-specific dyes are often consumed in the process and thus distinct time points and no real time curves are obtained. These dyes mostly are not specific to radicals and react with a wide range of different reactive molecules. Only a limited number of specific dyes for radicals are available. There is currently no probe available, which can detect the sum of all radical. Additionally, organic dyes which are used in these techniques usually suffer from photo-bleaching and thus can only be used for short term studies^{8,9}. These organic dyes are especially in high concentrations also often somewhat toxic. Another drawback is that organic dyes always measure the history of the sample rather than the current status and the detected value can only increase. So detecting inhibition or the effect of an antioxidant in real-time is not possible.

Furthermore, direct techniques also include conventional magnetic resonance imaging (MRI) and electron spin resonance spectroscopy (ESR) that are the gold standard in many different scientific disciplines. These techniques allow for functional contrast. However, especially when high spatial resolution is required or only limited amounts of sample are available, the methods approach their limits. In conventional MRI, approximately 10¹²-10¹⁸ atomic nuclei (or a factor of 1000 less electron spins) are needed to generate an observable signal. This limits the resolution to about 3 μm³ at its best^{10,11}. In addition, these techniques require large and powerful magnets, which reflect on the price, complexity and availability of the instruments.

Solutions to circumvent these problems are emerging from the field of diamond magnetometry that makes use of defects in diamonds which change their optical properties based on their magnetic surroundings^{12,13,14}.

The most prominent of these defects, the so-called nitrogen vacancy center (NV center), is so sensitive that even the small magnetic signal of single electrons¹⁵ or nuclear spins^{16,17} can be detected. In physics, this technique has successfully been used for a number of applications including magnetic characterization of materials under high hydrostatic pressures^{18,19,20}, 2-dimensional materials magnetic nanoscale microscopy²¹ or high speed study of magnetic domain walls²², magnetic fields of nanoparticles²³ or the presence of molecules on the diamond surface^{24,16,25}. Diamond magnetometry has also been used to measure spin labels in solution or even in a biological environment²⁶. A study from Ermakova et al (2013)²⁷ showed the detection of iron in ferritin (a protein family found in many living organisms). Steinert et al. were able to record data from cells that were fixed, sliced and labeled with contrast agents²⁸. Le Sage et al. recorded the magnetic signature of magnetosomes (naturally occurring iron nanoparticles) in living magnetotactic bacteria²³. Glenn et al. investigated cells

that were labeled with magnetical nanoparticles²⁹. This way they were able to quantify biomarker expression at a single cell level.

Here we use diamond magnetometry for the first time for measuring the natural metabolism in living yeast cells. Such yeast cells are popular in ageing research for several reasons. Many processes in ageing and coping with stress are highly conserved between different organisms including yeast. Unlike in most other cells is possible to differentiate between young and old cells by size. Additionally, yeast can age in two different ways: replicatively (by dividing) and chronologically (by spending time in a non-replicative stage). Both are used extensively to model ageing in different types of human cells.

In our article, we detect the free radical formation during stress responses or the addition of antioxidants and chronological ageing of yeast cells. Despite the relevance, such information remained unavailable so far.

5.2 Results and discussions

In this study, we provide a new approach for detecting free radicals during stress conditions and the aging process in yeast. Figure 1 shows a typical workflow for a magnetometry experiment. After uptake into yeast cells as described earlier³⁰, we first collect confocal images of yeast cells. By imaging Z-stacks (Figure 1 (A)) we determined if a particle was indeed inside the cell. Once a diamond nanoparticle was identified, we performed a magnetometry measurement. More specifically we used a so-called relaxation or T1 measurement of the NV center spins. This technique, which requires optical manipulation only, is sensitive to the spin noise generated by free radicals³¹. The pulsing sequence that is needed is shown in Fig 1 (B) and a typical measurement in Fig 1 (C). During this sequence the NV centers are brought into the (bright, polarized) ground state. After different amounts of time (=dark time τ) we then probed whether or not the NV

centers remained in this state. If there is spin noise (in our case from free radicals) present, this process occurs faster. The characteristic time this process takes is called relaxation time or T1 time. It gives a measure for the concentration of radicals in the surrounding of the diamond particle. After an initial measurement of the T1 of a nanodiamond in a cell, we perform a biological intervention on the cell and observe the effect on the T1 (on the same nanodiamond, in the same cell). Each set of experiment was reproduced 4 or 5 times.

Figure 1. Concept of detecting radical formation in yeast. (A) shows a z-stack of yeast cells with FNDs inside (red: FNDs, green: green fluorescent protein (GFP)) (B) shows a schematic of a yeast cell triggered by H_2O_2 to induce the oxidative stress response by inducing free radical generation. FNDs with NV center sense the magnetic noise that is produced by free radicals. (C) shows the pulsing sequence that is produced in a home-built diamond magnetometry setup. We use laser pulses at a varying distance to pump NV defect in the ground state. After different dark times we record the photoluminescence signals to determine how long the NV center remains in this polarized state. In presence of noise from radicals surrounding the NV centers this process occurs faster. Thus, this T_1 time gives a measure for the concentration of radicals in the surrounding. (D).

5.2.1 Comparing wild-type and knock out strains before and after ageing

We used 4 different yeast strains and observed the free radical load during the aging process. We chose wild type cells and three knockout mutant *sod1Δ*, *tor1Δ*, and *pex19Δ*, which are expected to have an impaired metabolism. Superoxide dismutase (SOD) is an enzyme that is found in mitochondria (MnSOD (encoded by the *SOD2* gene)) and also in the cytoplasm (Cu/ZnSOD (encoded by *SOD1* gene))³². SOD controls the level of H_2O_2 and engages in metabolic regulation as a response to the presence of oxygen and glucose^{33,34}. SOD also binds to two casein kinases through localized production of H_2O_2 . In short, SOD is very crucial for dismutating O_2^- free radicals³⁵. Deletion of the *SOD1* gene in yeast (*sod1Δ*) causes extensive damage of DNA and oxidative stress in cells³⁶. This means free radical production cannot be inhibited and the cells cannot degrade free radicals. As consequence, intracellular free radical levels are expected to increase.

The *tor1Δ* mutant was chosen because *TOR1* (target of rapamycin) regulates cell growth and metabolism. It has been found in several cellular locations but mainly on the vacuolar membrane^{37,38}. It also regulates the transcription factors Msn2 and Msn4, which are responsible for different stress responses^{39,40}. Deletion of *TOR1* in yeast (*tor1Δ*) decreases cell metabolism.

The *PEX19* (peroxin 19) is gene that is responsible for peroxisome membrane biogenesis⁴¹. Such a defect on peroxisome membrane biogenesis will affect the metabolism of peroxisomes which are responsible for the generation of superoxide and nitric oxide⁴². However, for both *TOR1* and *PEX19* it is unknown how this exactly influences the free radical load. The differences in T_1 values of the 4 yeast strains can be found in Figure 2.

Figure 2. Representative T1 curves in cells before any interventions **(A)** and average values **(B)** in 4 different yeast strains. Wild type strain (BY4741) (red) has the highest T1 signal (meaning the lowest free radical load) compared with the 3 isogenic mutants (*sod1Δ* (green), *pex19Δ* (orange), and *tor1Δ* (blue)). All measurements were performed at room temperature in living yeast cells. The data were analyzed by using one way ANOVA followed by Tukey post hoc test. Analyses were done against the wild type group. Significance level was set to 0.05. ** indicates $P \leq 0.01$.

All mutant strains have lower T1 values and thus higher free radical loads compared to the wild type strain. The lowest value was detected in *tor1Δ*. We also compared all our measurements with 3 conventional methods, the MTT assay (a measure for metabolic activity), the H₂DCFDA assay (measuring ROS production) and the HPF assay (measuring hydroxyl radicals (*OH)). In Figure S1 of the supplementary information we show the results for the standard techniques. While all other trends are the same we find that the metabolic activity in *tor1Δ* was lowest. While comparing ROS production level, *sod1Δ* showed the highest ROS production compared to others. Looking at [*OH] level between those 4 strains, *sod1Δ* also showed the higher level of [*OH] and *tor1Δ* has the lowest [*OH]. From the data in Figure S1 (and even more so in the detailed comparison of the methods in Figure S11) it is clear that the data that T1 provides is unique.

While the trends are often correlated, none of them measures the exact same as relaxometry (the local concentration of all radicals at a given moment). The H₂DCFDA and MTT measurements are dominated by the non-radical species, which are way more abundant. HPF on the other end only measures a small subset of the radicals that are created.

Additionally, the existing methods do not allow following the same cell during the entire experiment. Thus, these results are always an average of a population of cells whereas T1 signals are from one specific cell. As mentioned earlier there is also a fundamental difference in what kind of data one obtains. The existing methods always reveal the history of the sample and capture everything that has been generated between adding the compound and the measurement. Since these compounds can diffuse, the location (especially for long measurements) is very inaccurate. Our relaxometry based approach on the other hand measures the current situation.

5.2.2 Investigating stress responses and ageing

Next, we observed the yeast's free radical response to the addition of a chemical stress. To this end 1% H₂O₂ was used because it is highly diffusible and below a lethal concentration for yeast. Additionally, it is commonly used as a bleaching agent and also to induce stress in cells^{43,44}. We first measured the initial condition of the cells (before any intervention). We then triggered them with H₂O₂ and measure in the same cells at the same location again. As expected, the 4 strains responded differently when they were exposed to stress (see Fig. 3). In Figure 3 A, B, C, and D we display the evolution of T1 caused by H₂O₂. We also compared these results with metabolic activity, ROS production and *OH radical production (supplementary information figure 2,3, 4 and 12).

We next investigated the effect of 2 stress factors: ageing and H₂O₂ as chemical stress. Here, we compare between T1 before and after H₂O₂ in aged cells (see Figure 3 (E), (F), (G), (H)). It is worth mentioning that the high photostability of diamonds allows for a very powerful control, as the reference measurements can be performed on the same particle in the same location within the same cell. In contrast, with organic dyes/other nanoparticles, one usually has to use the data from different cells as a baseline for the experiments.

Figure 3. Aging and stressed cells. We plot the T1 response (left black axis) of internalized nanodiamond in 4 yeast strains. As an indication, the left grey axis represents the approximated concentration determined from a calibration with ^{*}OH radicals in solution from previous work⁴⁵. For young cells we compare the T1 in the cell before (blue) and after adding 1% H₂O₂ to promote oxidative stress (red) in (A), (B), (C) and (D). Then we compare young cells (blue) with themselves after 24 h of ageing (yellow) in (E), (F), (G), (H). In the same figures we also show the effect of 1% H₂O₂ in addition to ageing. The error bars represent standard errors. The experiments were repeated 4 times. The grey lines follow individual experiments. Data were analyzed by using paired T-test against the previous group. Significance level was set at 0.05 (*P ≤ 0.05; **P ≤ 0.01)

In young cells, both wild-type and *sod1Δ* have lower T1 after adding H₂O₂ while *tor1Δ* and *pex19Δ* show slightly higher T1 values. This can be seen

from the average values but also from the grey data points generated each from a single cell experiment. We can explain these findings in the following way. *Sod1Δ* cells already contain a high amount of free radicals as a response to deletion of *SOD1*. When 1% H₂O₂ was added to the cells, it triggered mitochondria to produce more radicals. This can be concluded from the decreasing of T1 values. On the contrary, *tor1Δ* and *pex19Δ* behave differently than the wild type and *sod1Δ* strains. A previous study showed that *TOR1* inhibits transcription of stress-responsive elements and controls a starvation response³⁹. Semchyshyn and Valishkevych (2016)⁴⁶ showed that deletion of the *TOR1* gene activates glyoxalase 1 (*GLO1*), which is associated with the metabolism of carbohydrates and H₂O₂. As a result, H₂O₂ is metabolized and yeast cells become more resistant to oxidative stress. Mutation of the *PEX19* gene in yeasts means a lack of peroxisomes⁴⁷. Peroxisomes are cell organelles which function as pro and antioxidants. According to Pascual-Ahuir and coworkers (2017)⁴⁸, peroxisomes are producing oxidase and catalase to combat oxidative stress. In this study, *pex19Δ* revealed that there is lower radical production after adding 1% H₂O₂. This means that the mutation of the *PEX19* gene increases the resistance of yeasts to oxidative stress.

We then focused on chronological aging where the cells entered the stationary phase of growth and are deprived of nutrients. Accumulation of oxidatively damaged proteins and ROS has been observed during chronological ageing^{49,50}. To increase the longevity of the cells and stress response during nutrition scarce conditions, several pathways (Sch9, TOR, and Ras/cAMP-dependent PKA) are down-regulated⁵¹. In our experiments, wild type, *sod1Δ*, and *tor1Δ* have T1 value lowered with aging, which indicates a higher free radical load. According to the free radical theory of aging⁵² these results are expected. Compared to wild type and *sod1Δ*, *tor1Δ*, which has an increased chronological

lifespan⁵³, shows a slightly lower T1 value after aging, and when aging cells were stressed by H₂O₂, T1 values slightly increase. Comparing T1 values with metabolic activity (supplementary Figure S2), both *sod1Δ* and *tor1Δ* in young and aged stages decreased their metabolic activity profile in the presence of 1% H₂O₂. Interestingly, aged *pex19Δ* has even a slightly lower radical load compared to their initial young condition. This finding provides an explanation for the increased chronological lifespan which has been found for these cells⁵⁴. We attribute this finding to the elevation of hydrogen peroxide, which activates superoxide dismutase (SOD) as scavenger of superoxide anions. A detailed comparison with the existing methods is shown in Figure S12 of the supplementary.

5.2.3 Effect of adding antioxidant on free radical loads

In yeasts, there are enzymatic and non-enzymatic defense systems against chemical stress. In enzymatic systems, several enzymes (e.g. SOD and catalase) are involved in removing oxygen radicals and repair the damage from oxidative stress³². Non-enzymatic systems involve glutathione (GSH) and endogenous antioxidants⁵⁵. The non-enzymatic system acts as radical scavenger, which maintains the cellular redox state. Although yeast cells produce natural radical scavengers, adding external anti-oxidant supplements helps them to survive oxidative stress. Here, we investigate how the radical production changes after antioxidant treatment. Figure 4 shows the effect of the common anti-oxidant L-ascorbic acid.

Figure 4. Free radical response to ageing in presence or absence of an antioxidant/stressor. In figure (A), (B), (C), and (D) we first compare T1 values (left black axis). The left grey axis represents the approximated concentration determined from a calibration with *OH radicals in solution from previous work⁴⁵. We investigated wild type, *sod1Δ*, *tor1Δ*, and *pex19Δ* cells before (blue) and after antioxidant treatment (green). We also show the response after subsequent addition of 1% H₂O₂. In (E), (F), (G) and (H), we additionally display the evolution of T1 values after ageing. We compare the initial condition with young cells to antioxidant treated cells followed by ageing and adding 1% H₂O₂. The error bars represent the standard error, above and below the average. The experiments were repeated 4 times. Data were analyzed by using paired T-test against the previous group. Significance level was set at 0.05 (*P≤0.05; **P≤0.01; ***P≤0.001).

In young cells, adding anti-oxidant increases the T1 values (i.e. reduces the free radical load in cells). In the wild-type (A) and *sod1Δ* (B), adding anti-oxidant before adding 1% H₂O₂ does not inhibit radical production in cells. This is indicated by decreasing of T1 values after 1% H₂O₂ has been added. On the other hand, adding anti-oxidant in *tor1Δ* (C) and *pex19Δ* (D) reduces free radicals formation. In aged cells, anti-oxidant was added before the cells were allowed to age. In all cases, the aged cells that were treated with the antioxidant are better off after the addition of H₂O₂, as compared to the results in Fig 3. Since all the strains have been grown in glucose supplemented medium, they are not able to

produce L-ascorbic acid⁵⁶ but they can accumulate external ascorbate through a hexose uptake system⁵⁷. Our findings thus offer an explanation for the findings of Saffi and coworkers (2006)⁵⁸ who reported that adding L-ascorbic acid supplement in wild type and *SOD1* deficient strains before oxidative stress conditions increases survival. L-ascorbic acid can restore the cellular activity of yeast due to the scavenging of superoxide radicals. In supplementary Figure S5 and S6, adding anti-oxidant increases metabolic activity and decreases ROS concentration in all strains compared to the situation without anti-oxidant groups. However, when the cells are aged, anti-oxidant supplement has no significant effect for improving metabolic activity levels. Anti-oxidant supplement also only slightly reduces [$\text{OH}^\bullet$] in all strains both in young and age stage (Figure S7 and for a more detailed comparison of methods see Figure S13).

5.3 Conclusion

Free radicals are central in oxidative stress responses and the ageing process. Since these molecules are highly reactive and have short life times, there are limited techniques that can be used to measure the free radical load. Our study offers a new and unique technique, which can measure the productions of free radical by single cells in real time with high spatial resolution. With the T1 signal, we were able to clearly differentiate the response of mutant strains, young and aged cells, in the presence or absence of H_2O_2 and anti-oxidants.

Using NV centers in FNDs as a tool to convert magnetic noise from free radicals into optical signals provides a better understanding about stress responses of the cells during oxidative stress conditions and the ageing process. Using this new tool we are able to provide an explanation for increased longevity that has been reported for *tor1Δ* and *pex19Δ*.

We believe that this work paves the way to faster and more efficient free radicals studies in living cells, reducing assay size down to the single cells level. This new information is not just fundamentally interesting but also opens up opportunities to evaluate the effect of mutation as well as new drugs and their working mechanisms.

5.4 Methods

Diamond starting material. All experiments in this article were done using commercially available fluorescent nanodiamonds (FNDs) with a hydrodynamic diameter of 70 nm from Adamas Nanotechnology (USA), which seem to be a good compromise. There are two factors that are important to balance here. The larger the particle, the more NV centers can contribute to the measurement. Since every measurement is already an average of many centers at different locations within the particle, the particles are very similar to each other and thus the smaller the error bars are. However, if the particle becomes too large, the NV centers in the center of the particle do not feel the signal of the radicals any more due to their large distance from the surface. These particles are produced by grinding HPHT diamonds and irradiation with 3MeV electrons at a fluence of $5 \times 10^{19} \text{ e/cm}^2$. As a result these particles contain an average of 500 NV centers per diamond (determined by EPR by the manufacturer)⁵⁹. The surface chemistry of the FNDs is oxygen-terminated, as a result of an acid treatment by the manufacturer. These particles have already been characterized extensively in previous literature^{60,61}.

Diamond uptake in yeast cells. Budding yeast *Saccharomyces cerevisiae* cells of the BY4741 strain background⁶² were grown in synthetic dextrose (SD) medium (Formedium, UK) until they reached midlog phase (optical density 600 nm

(OD₆₀₀) approximately 1.05). Since yeast cells are surrounded by a rigid cell wall and do not naturally take up diamond particles, it is necessary to remove or permeabilize the cell wall³⁰. Removing the cell wall leads to a so-called spheroplast.

First, the cells were washed with sterile demineralized water for 5 minutes at 2500 xg at 10°C. After the supernatant was discarded, 20 mL of sterile 1 M D-sorbitol were added to the cells followed by centrifugation for 5 minutes at 2500 xg at 10°C. Sorbitol is a reduced medium for yeast cells, which does not cause diamond aggregation⁶³. Then the supernatant was discarded. The cell pellet was diluted in 20 mL of sterile SPEM buffer (consisting of 1 M D-sorbitol, 10 mM EDTA, and 10 mM sodium phosphate) and 40 µL of zymolyase 20T (Amsbio, UK). Then 30 µL of β-mercaptoethanol (Sigma, Netherlands) was added. The mixture was incubated at 30°C for 30 minutes while shaking at 75 rpm. The spheroplasting process was monitored with a light microscope (native yeast cells are darker than spheroplast cells). The process was stopped by adding 20 mL of sterile 1 M D-sorbitol followed by centrifugation at 1000 xg for 5 minutes at 10°C. The pellet then was mixed with 2 mL of sterile STC buffer (consisting of 1 M D-sorbitol, 10 mM Tris HCl, 10 mM CaCl₂, and 2,5 mM MgCl₂). The mixture was incubated for 20 minutes at room temperature⁶⁴. Finally, 50 µL of 4 µg mL⁻¹ FNDs in 1 M D-sorbitol was added to the suspension followed by a 10 minute incubation at room temperature.

The isogenic mutant strains *sod1Δ*, *tor1Δ*, and *pex19Δ* were obtained from the Yeast Knockout Collection⁶⁵. They were maintained on solid yeast extract-peptone-dextrose (YPD) supplemented with 2% D-glucose. For all the experiments, the cells were grown in SD medium supplemented with 2% D-glucose followed by spheroplasting for the diamond uptake protocol.

Sample preparation for diamond magnetometry. Yeast cells with internalized FNDs were seeded in concanavalin-A coated glass-bottom dishes. The function of concanavalin A is to promote cell adhesion to the glass surface and thus limiting cell movement. For coating, the glass surface of the dishes (Grainer) were submerged in 0,1 mg mL⁻¹ concanavalin-A (Sigma, The Netherlands) in sterile demineralized water. The dishes were incubated at 37°C overnight in static condition. On the following day, the dish was washed using sterile demineralized water followed by drying in a 37°C incubator⁶⁶.

The first step of the experiment was finding the FNDs and confirming their location inside the cell using Z-stack imaging.

Diamond magnetometry. To perform diamond magnetometry we utilized fluorescent defects in diamond called NV centers. Using this technique, the magnetic noise of the surrounding medium is read by optical means. These experiments were done using a home-made magnetometry setup. The setup, which has been described before⁶⁶ is in principle a confocal microscope with a few changes which are described below. For light collection we used a 100x magnification oil objective (Olympus, UPLSAPO 100XO).

We implemented the ability to pulse the laser with an acousto-optical modulator (Gooch & Housego, model 3350-199) to conduct the pulsing sequence that is shown in Figure 1.

There are several modes that can be used in diamond magnetometry. These modes (including the rotating frame magnetometry⁶⁷, T2¹³, CPMG⁶⁸) require different pulsing sequences. These sequences require an alignment of an external magnetic field with the NV centers. This would be problematic for a rotating particle. Here we chose the relaxometry or T1 sequence. Probably the biggest advantage of this sequence is that it does not require microwaves. In

biological samples the water in the medium absorbs the microwaves. This not just deteriorates the signal but also causes heating. During a T1 measurement the NV center is pumped with a laser pulse into the (bright) ground state (see Figure 1 (C)). Then we probe after different dark times, if the NV centers are still in this ground state. The time it takes to reach the equilibrium condition (relaxation time) is linked to the presence of the concentration of free radicals. To estimate which concentration of radicals causes a certain T1 time we used a calibration where $\cdot\text{OH}$ radicals were created in a controlled way⁴⁵.

To perform a T1 measurement, a train of 5 μs green laser pulses (532 nm) with dark times (τ) between 200 ns to 10 ms was used to excite the NV centers. The detection is done using an avalanche photodiode (APD) (Excelitas, SPCM-AQRH) after passing through a 550 nm long-pass filter. To obtain a sufficient signal to noise ratio we repeated the pulse sequence 10000 times for each T1 measurement. Each T1 measurement took around 16 minutes. The optically detected T1 measurements are equivalent to T1 signals in conventional magnetic resonance imaging (MRI) but for nanoscale voxels. There are several considerations that determine the choice of laser power. The laser power has to be low enough to not damage the cells. On the other hand the power needs to be high enough to polarize the NV centers sufficiently. A good compromise was found at 50 μWatt measured on top of the objective lens. A common strategy in the field is using diamonds, which contain single defects^{69,31}. This has several advantages. The particles can be small and single defects can be very precisely manipulated. However, this approach usually requires spending significant amounts of time on searching for a superb defect. These highly preselected defects have fantastic sensing properties. Once the perfect defect is found it is typically reused for a long time. Unfortunately, this approach does not work in a biological system. We need to use whichever particle we can get in the cells and

if we would preselect from these, we could only use the particle once and discard it with the biological samples, which need to be disposed after each experiment. Even worse is the large spread one can observe in defect quality for single defects. This would make it impossible to compare different experiments on different cells and different particles. To circumvent this problem we used nanodiamonds with ensembles. These are easier to find on top of the background fluorescence. Since every particle contains around 500 NV centers we always measure the sum of many different NV centers, which are more or less close to the surface and to each other. The result is, that the measurements with different particles are way more reproducible than with single centers at the expense of relaxation time.

Since diamond particles move inside the cells it is crucial to use a tracking algorithm. The purpose of the tracking algorithm is to find the new position of the particle and move the laser there. The procedure has to be repeated more often if the particle is moving faster. For the conditions in this article we found that it is necessary to track the position of FND every 5 seconds during a T1 measurement. If the particle is lost this results in a sudden drop in fluorescent counts.

Magnetometry data analysis.

The model that was used to fit the T1 data is described in equation (1):

$$PL(\tau) = I_{\text{inf}} + C_a e^{-\tau/T_a} + C_b e^{-\tau/T_b} \quad (1)$$

$$T1 = \max(T_a, T_b)$$

This model is a bit different than the fitting model for single NV³¹ because we are using FNDs containing ensembles of NV centers. The relaxation time of an ensemble is approximated from two component which are the NV centers that

are closer to the surface (T_a) and the NV centers that are deeper in the crystal (T_b)⁷⁰. Between these two constants, the longer T_1 time was selected for quantification because it is more sensitive to changes of the NVs' surrounding. Data were processed with MatLab software version R2018b.

Cell aging behavior. The behavior of aging cells was monitored in this study. After spheroplasting, cells were washed with sterile phosphate buffered saline (PBS) pH 7.4 followed by centrifugation at 1000 xg at 10°C for 5 minutes. Cells were then kept in sterile water for 24 hours at 30°C in an incubator. At the desired time, cells were washed and tested with magnetometry or conventional methods (MTT, H₂DCFDA, and HPF).

Effect of adding antioxidant. An antioxidant was used for preventing or reducing radical formation during stress conditions and during the aging process in yeast cells. In this study, 0.025 mM of L-ascorbic acid (Sigma, Netherlands) has been used as antioxidant. The radical load was determined by magnetometry while conventional methods (MTT, H₂DCFDA, and HPF assays) were used for comparison.

Comparison with established techniques. To compare magnetometry data with existing methods several assays were performed which are described in the following.

Morphological changes after stress condition. Morphological changes on the surface of yeast spheroplast cells that have been exposed with 1% H₂O₂ compared to cells without any treatment were visualized by using a bioscope catalyst atomic force microscope (AFM) (Bruker, USA).

To immobilize yeast spheroplasts on a hydrophilic glass slide, 1x1 cm glass slides were sterilized by using 70% ethanol⁷¹. Then 0,1 mg mL⁻¹ concanavalin- A (Sigma, Netherlands) per mL sterile water was spread on the surface of the glass slides. After the drying process, they were placed in sterile 12-well plates (Grainer). The cells were incubated at 37°C overnight and washed with sterile demineralized water. Then yeast spheroplast cells were seeded on the concanavalin-A coated glass slides and incubated at 30°C for 3 hours. After that, cells were exposed to 1% H₂O₂ and incubated for 1 hour at 30°C. Glass slides with cells on top were taken from the well plates and dried at room temperature for 3 hours before observing by AFM. The results are shown in the supplementary (Fig. S10)

Metabolic activity measurement. Metabolic activity was observed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenylterazolium bromide] (Sigma, Netherlands). The assay was modified from the protocol established by Teparić et al (2004)⁷². To perform the assay, spheroplast cells were washed with sterile PBS pH 7.4. Cells were divided into 3 groups and placed in a sterile tube with a volume of 1 ml each tube. Cells were mixed with 1% hydrogen peroxide (H₂O₂) (Merck, Netherlands) and 4µg mL⁻¹ FNDs. In the control group only sterile demineralized water was added. Then 100 µl of 5 mg mL⁻¹ MTT solution in sterile PBS was added to the cells from the different groups. They were wrapped with tin foil and incubated for 1 hour at 30°C. After that, cells were resuspended with 1 mL 2-propanol (Merck, The Netherlands), agitated for 10 minutes, and centrifuged at 1000 xg for 5 minutes at 10°C. 1 mL of supernatant was taken from all groups and placed in sterile 96-well plates (Grainer). Finally, the plates were measured with a microplate reader (Fluostar optima, Germany) at 540 nm. All the measurements were performed in triplicates.

Measuring reactive oxygen species (ROS) with fluorescence based probes. ROS in yeast spheroplasts were measured by using a H₂DCFDA kit (ThermoFisher, Netherlands). The cells were placed into 1,5 mL sterile tubes and centrifuged at 1000 xg for 5 minutes. The supernatant was discarded and the cells were washed with sterile PBS pH 7.4 followed by centrifugation at 1000 xg for 5 minutes. After discarding the supernatant, cells were diluted with sterile PBS pH 7.4 and transferred to sterile 96- well plates. The H₂DCFDA with a concentration of 10 µg mL⁻¹ was added in each well and the cells were incubated for 2 hours at 30°C (the plates were wrapped with aluminium foil to avoid the light exposure). The cells were treated with 1% H₂O₂; 4 µg mL⁻¹ FNDs; and cells without treatment were used as control. Finally, the fluorescence intensity was measured by a microplate reader (Fluostar optima, Germany) at 540 nm as describe by Gallardo and co-workers (2013)⁷³. All the measurements were performed in triplicates.

Measuring hydroxyl radical (*OH) with fluorescence based probes. Hydroxyl radical generation was measured by HPF (hydroxyphenyl fluorescein) (ThermoFisher, Netherlands). Yeast cells were put in 1,5 mL sterile tubes then centrifuged at 1000 xg for 5 minutes at 10°C. After discarding the supernatant, cells were washed with sterile PBS pH 7.4 followed by centrifugation at 1000 xg for 5 minutes. 10 µM HPF probes in sterile PBS were mixed with the cells and incubated for 1 hour at 30°C (all tubes were wrapped with aluminum foil to avoid light exposure). Cells were centrifuged at 1000 xg for 5 minutes to remove free HPF followed by diluting the cells into sterile PBS. After placing the cells into 96- well plates (100 µL/well), they were treated with 1% H₂O₂; 4 µg mL⁻¹ FNDs; and cells without treatment were used as control. Fluorescence intensity was

measured by a microplate reader (Fluostar optima, Germany) at 495/520 nm. All the measurements were performed in triplicates.

Statistical analysis. Continuous data are presented as mean ± standard errors and analyzed by paired T-Test against the previous group. For MTT, H₂DCFDA, and HPF without anti-oxidant data were analyzed by using one-way ANOVA followed by Tukey post hoc test and anti-oxidant effect for all measurements were analyzed by using two-way ANOVA followed by Sidak post hoc test. Statistical analysis was performed using GraphPad Prism version 8 software (GraphPad Inc.). P value <0.05 was considered to indicate significance statistic value.

References

1. Nathan, C. & Cunningham-Bussel, A. Beyond oxidative stress: An immunologist's guide to reactive oxygen species. *Nat. Rev. Immunol.* **13**, 349–361 (2013).
2. Harman, D. The free radical theory of aging. *Antioxid. Redox Signal.* **5**, 557–561 (2003).
3. Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet.* **344**, 721–724 (1994).
4. Paardekooper, L. M. *et al.* Human monocyte-derived dendritic cells produce millimolar concentrations of ROS in phagosomes per second. *Front. Immunol.* **10**, 1–9 (2019).
5. Phaniendra, A., Jestadi, D. B. & Periyasamy, L. Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian J. Clin. Biochem.* **30**, 11–26 (2015).
6. Diguiseppi, J. & Fridovich, I. The toxicology of molecular oxygen. *Crit. Rev. Toxicol.* **12**, 315–342 (1984).
7. Nimse, S. B. & Pal, D. Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Adv.* **5**, 27986–28006 (2015).
8. Mason, R. P. Imaging free radicals in organelles, cells, tissue, and in vivo with immuno-spin trapping. *Redox Biol.* **8**, 422–429 (2016).
9. Wardman, P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. *Free Radic. Biol. Med.* **43**, 995–1022 (2007).
10. Ciobanu, L., Seeber, D. A. & Pennington, C. H. 3D MR microscopy with resolution 3.7 μm by 3.3 μm by 3.3 μm . *J. Magn. Reson.* **158**, 178–182 (2002).
11. Glover, P. & Mansfield, P. Limits to magnetic resonance microscopy. *Rep. Prog. Phys.* **65**, 1489–1511 (2002).
12. Gali, A., Fyta, M. & Kaxiras, E. Ab initio supercell calculations on nitrogen-vacancy center in diamond: Electronic structure and hyperfine tensors. *Phys. Rev. B - Condens. Matter Mater. Phys.* **77**, 1–28 (2008).
13. Schirhagl, R., Chang, K., Loretz, M. & Degen, C. L. Nitrogen-Vacancy Centers in Diamond: Nanoscale Sensors for Physics and Biology. *Annu. Rev. Phys. Chem.* **65**, 83–105 (2014).
14. Chipaux, M. *et al.* Nanodiamonds and their applications in cells. *Small* **1704263**, 1–25 (2018).
15. Grinolds, M. S. *et al.* Nanoscale magnetic imaging of a single electron spin under ambient conditions. *Nat. Phys.* **9**, 215–219 (2013).
16. Mamin, H. J. *et al.* Nanoscale nuclear magnetic resonance with a nitrogen-vacancy spin sensor. *Science (80-.)*. **339**, 557–560 (2013).
17. Cujia, K. S. *et al.* Tracking the precession of single nuclear spins by weak measurements. *Nature* **571**, 230–233 (2019).
18. Hsieh, S. *et al.* Imaging stress and magnetism at high pressures using a nanoscale quantum sensor. *Science (80-.)*. **366**, 1349–1354 (2019).
19. Yip, K. Y. *et al.* Measuring magnetic field texture in correlated electron systems under extreme conditions. *Science (80-.)*. **366**, 1355–1359 (2019).
20. Lesik, M. *et al.* Magnetic measurements on micrometer-sized samples under high pressure using designed NV centers. *Science (80-.)*. **366**, 1359–1362 (2019).
21. Thiel, L. *et al.* Probing magnetism in 2D materials at the nanoscale with single-spin microscopy. *Science.* **364**, 973–976 (2019).
22. Juraschek, D. M. *et al.* Dynamical magnetic field accompanying the motion of ferroelectric domain walls. *Phys. Rev. Lett.* **123**, 127601 (2019).
23. Le Sage, D. *et al.* Optical magnetic imaging of living cells. *Nature.* **496**,

- 486–489 (2013).
24. Loretz, M. *et al.* Nanoscale nuclear magnetic resonance with a 1.9-nm-deep nitrogen-vacancy sensor. *Appl. Phys. Lett.* **104**, 3–8 (2014).
 25. Staudacher, T. *et al.* Nuclear magnetic resonance spectroscopy on a (5-nanometer)³ sample volume. *Science*. **339**, 561–563 (2013).
 26. Davis, H. C. *et al.* Mapping the microscale origins of magnetic resonance image contrast with subcellular diamond. *Nat. Commun.* **9**, 1–9 (2018).
 27. Ermakova, A. *et al.* Detection of a few metallo-protein molecules using color centers in nanodiamonds. *Nano Lett.* **13**, 3305–3309 (2013).
 28. Steinert, S. *et al.* Magnetic spin imaging under ambient conditions with sub-cellular resolution. *Nat. Commun.* **4**, (2013).
 29. Glenn, D. R. *et al.* Single-cell magnetic imaging using a quantum diamond microscope. *Nat. Methods.* **12**, 736–738 (2015).
 30. Morita, A. *et al.* Cell uptake of lipid-coated diamond. *Part. Part. Syst. Charact.* **1900116**, 1–8 (2019).
 31. Tetienne, J. P. *et al.* Spin relaxometry of single nitrogen-vacancy defects in diamond nanocrystals for magnetic noise sensing. *Phys. Rev. B - Condens. Matter Mater. Phys.* **87**, 1–5 (2013).
 32. Jamieson, D. J. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast*. **14**, 1511–1527 (1998).
 33. Juarez, J. C. *et al.* Superoxide dismutase 1 (SOD1) is essential for H₂O₂-mediated oxidation and inactivation of phosphatases in growth factor signaling. *Proc. Natl. Acad. Sci.* **105**, 7147–7152 (2008).
 34. Reddi, A. R. & Culotta, V. C. SOD1 integrates signals from oxygen and glucose to repress respiration. *Cell*. **152**, 224–235 (2013).
 35. Che, M. *et al.* Expanding roles of superoxide dismutases in cell regulation and cancer. *Drug Discov. Today*. **21**, 143–149 (2016).
 36. Tsang, C. K. *et al.* Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat. Commun.* **5**, 3446 (2014).
 37. Betz, C. & Hall, M. N. Where is mTOR and what is it doing there? *J. Cell Biol.* **203**, 563–574 (2013).
 38. Li, H. *et al.* Nutrient regulates TOR1 nuclear localization and association with rDNA promoter. *Nature*. **442**, 1058–1061 (2006).
 39. Beck, T. & Hall, M. N. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **402**, 689–692 (1999).
 40. Crespo, J. L. & Hall, M. N. Elucidating TOR signaling and rapamycin action: Lessons from *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **66**, 579–591 (2002).
 41. Smith, J. J. & Aitchison, J. D. Peroxisomes take shape. *Nat. Rev. Mol. Cell Biol.* **14**, 803–817 (2013).
 42. Sacksteder, K. A. *et al.* PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell Biol.* **148**, 931–944 (2000).
 43. Jamieson, D. J. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. *J. Bacteriol.* **174**, 6678–6681 (1992).
 44. Flattery-O'Brien, J., Collinson, L. P. & Dawes, I. W. *Saccharomyces cerevisiae* has an inducible response to menadione which differs from that to hydrogen peroxide. *J. Gen. Microbiol.* **139**, 501–507 (1993).
 45. Martínez, F.P. *et al.* Comparing nanodiamond based relaxometry under biologically relevant conditions and determining free radicals produced in chemical reactions. (*submitted*), (2020).
 46. Semchyshyn, H. M. & Valishkevych, B. V. Hormetic effect of H₂O₂ in

- Saccharomyces cerevisiae*: Involvement of TOR and glutathione reductase. *Dose-Response*. **14**, 1–12 (2016).
47. Götte, K. *et al.* Pex19p, a farnesylated protein essential for peroxisome biogenesis. *Mol. Cell. Biol.* **18**, 616–628 (1998).
 48. Pascual-Ahuir, A., Manzanares-Estreder, S. & Proft, M. Pro- and antioxidant functions of the peroxisome-mitochondria connection and its impact on aging and disease. *Oxid. Med. Cell. Longev.* **2017**, 1–17 (2017).
 49. Herker, E. *et al.* Chronological aging leads to apoptosis in yeast. *J. Cell Biol.* **164**, 501–507 (2004).
 50. Reverter-Branchat, G. *et al.* Oxidative damage to specific proteins in replicative and chronological-aged *Saccharomyces cerevisiae*. Common targets and prevention by calorie restriction. *J. Biol. Chem.* **279**, 31983–31989 (2004).
 51. Fabrizio, P. & Longo, V. D. Chronological aging-induced apoptosis in yeast. *Biochim. Biophys. Acta - Mol. Cell Res.* **1783**, 1280–1285 (2008).
 52. Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300 (1956).
 53. Gladyshev, V. N. The free radical theory of aging is dead. Long live the damage theory. *Antioxidants Redox Signal.* **20**, 727–731 (2014).
 54. Mesquita, A. *et al.* Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H₂O₂ and superoxide dismutase activity. *Proc. Natl. Acad. Sci.* **107**, 15123–15128 (2010).
 55. Marchler, G. *et al.* *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* **12**, 1997–2003 (1993).
 56. Hancock, R. D., Galpin, J. R. & Viola, R. Biosynthesis of L-ascorbic acid (vitamin C) by *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **186**, 245–250 (2000).
 57. Özcan, S. & Johnston, M. Function and regulation of yeast hexose transporters. *Microbiol. Mol. Biol. Rev.* **63**, 554–569 (1999).
 58. Saffi, J. *et al.* Antioxidant activity of L-ascorbic acid in wild-type and superoxide dismutase deficient strains of *Saccharomyces cerevisiae*. *Redox Rep.* **11**, 179–184 (2006).
 59. Shenderova, O. A. *et al.* Synthesis, properties, and applications of fluorescent diamond particles. *J. Vac. Sci. Technol. B* **37**, 030802 (2019).
 60. Ong, S. Y. *et al.* Shape and crystallographic orientation of nanodiamonds for quantum sensing. *Phys. Chem. Chem. Phys.* **19**, 10748–10752 (2017).
 61. Hemelaar, S. R. *et al.* Nanodiamonds as multi-purpose labels for microscopy. *Sci. Rep.* 1–9 (2017).
 62. Brachmann, C. B. *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. **14**, 115–132 (1998).
 63. Hemelaar, S. R. *et al.* Generally applicable transformation protocols for fluorescent nanodiamond internalization into cells. *Sci. Rep.* **7**, 5862 (2017).
 64. Karas, B. J. *et al.* Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat. Protoc.* **9**, 743–750 (2014).
 65. Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 1–5 (2002).
 66. Morita, A. *et al.* The fate of lipid-coated and uncoated fluorescent nanodiamonds during cell division in yeast. *Nanomaterials* **10**, 1–15

- (2020).
67. Loretz, M., Rosskopf, T. & Degen, C. L. Radio-frequency magnetometry using a single electron spin. *Phys. Rev. Lett.* **110**, 1–5 (2013).
 68. Bar-Gill, N. *et al.* Suppression of spin-bath dynamics for improved coherence of multi-spin-qubit systems. *Nat. Commun.* **3**, (2012).
 69. Rondin, L. *et al.* Stray-field imaging of magnetic vortices with a single diamond spin. *Nat. Commun.* **4**, 1–5 (2013).
 70. Rioux, J. A., Levesque, I. R. & Rutt, B. K. Biexponential longitudinal relaxation in white matter: Characterization and impact on T1 mapping with IR-FSE and MP2RAGE. *Magn. Reson. Med.* **75**, 2265–2277 (2016).
 71. Canetta, E., Walker, G. M. & Adya, A. K. Nanoscopic morphological changes in yeast cell surfaces caused by oxidative stress : An atomic force microscopic study. *J. Microbiol. Biotechnol.* **19**, 547–555 (2009).
 72. Teparić, R. *et al.* Increased mortality of *Saccharomyces cerevisiae* cell wall protein mutants. *Microbiology.* **150**, 3145–3150 (2004).
 73. Pe´rez-Gallardo, R. V. *et al.* Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron – sulfur cluster assembly system. *FEMS Yeast Res.* **13**, 804–819 (2013).

Supplementary informations

Testing stress conditions with conventional methods

To validate and compare our magnetometry data we have conducted measurements with conventional methods under the same conditions.

Supplementary Fig. 1 Characterization of 4 different strains in young stage. MTT result **(A)** shows that all mutant strains have lower metabolic activity compared to the wild-type strain. Those strains then were tested with H₂DCFDA to show total ROS production levels **(B)**. As consequence of lacking SOD1 gene, *sod1Δ* produces more ROS than the wild type strain while lacking the TOR1 gene (*tor1Δ*) decreases ROS production. Hydroxyl radical ([OH•]) levels in all strains **(C)** were tested by using an HPF probe. As expected, *sod1Δ* has a higher radical load than the other strains. Wild type yeast was used as a control and set to 100%. The data were analyzed by using a one-way ANOVA followed by a Tukey post hoc test. The measurements were repeated 5 times. The significance level was set to 0.05. P values ≤ 0.001 was indicated with *** and P values ≤ 0.0001 was indicated with ****.

Supplementary Fig.2 Metabolic activity profile of 4 different strains. All strains were tested at 2 stages (young and aged). The values for cells without treatment (represented in red) were calculated from each strain in their young stage. These were used as control and set to 100%. In the young stage, exposure to FNDs (orange) only has mild effect (A). In *sod1Δ* (B), *tor1Δ* (C), and *pex19Δ* (D), the presence of FNDs has no significant effect on metabolic activity levels of the cells. When the cells were triggered with 1% H_2O_2 (blue), all strains are showing decreasing metabolic activity. When entering the aged stage, metabolic activity in wild type (E), *sod1Δ* (F), *pex19Δ* (H) decrease while *tor1Δ* (G) is increasing their metabolic activity compared to young stage. Data were analyzed by using a one-way ANOVA followed by a Tukey post hoc test. The group with cells only was used as a comparison. The measurements were repeated 5 times. Significance level was set to 0.05. (** P values ≤ 0.01 ; *** P values ≤ 0.001 ; and **** P values ≤ 0.0001).

Supplementary Fig.3 ROS production of 4 different strains. All strains were tested in young and aged conditions. The groups with cells only at young stage from each strain were used as control and set to 100% (red). In the young stage, FNDs (orange) has no effect on the ROS production of any of the strains (wild type (A), *sod1Δ* (B), *tor1Δ* (C), and *pex19Δ* (D)). Triggering cells with 1% H_2O_2 (blue) increases the ROS production inside the cells. When the cells aged, the ROS values increase in wild type (E), *sod1Δ*(F), and *tor1Δ*(G) while in *pex19Δ*(H) ROS values decrease. The data were analyzed by using a one way ANOVA followed by a Tukey post hoc test. The measurements were repeated 5 times. Significance level was set at 0.05. (** P values ≤ 0.001 and **** P values ≤ 0.0001).

Supplementary Fig.4 [OH] level of 4 different strains. All strains were tested in young and aged conditions. The groups with cells only at young stage from each strain were used as control and set to 100% (red). In the young stage FNDs (orange) have no significant effect on [OH] production on any strain (wild type (A), *sod1Δ* (B), *tor1Δ* (C), and *pex19Δ* (D)). Triggering cells with 1% H_2O_2 (blue) increases [OH] production inside the cells. When the cells aged (grey), only *pex19Δ* (H) shows increased [OH] levels while wild type (E), *sod1Δ* (F), and *tor1Δ* (G) remain the same. When aged cells were triggered with 1% H_2O_2 (blue), only the wild type has significant elevation of [OH]. The data were analyzed by using a one way ANOVA followed by a Tukey post hoc test. The measurements were repeated 5 times. Significance level was set at 0.05. (* P values ≤ 0.05; ** P values ≤ 0.01; *** P values ≤ 0.001 and **** P values ≤ 0.0001).

Supplementary Fig.5 Effect of antioxidant supplement in metabolic activity profile. Yeasts were induced with antioxidant to prevent oxidative stress during the aging process. In the young stage, adding antioxidant (blue) in wild type (A), *sod1Δ* (B), and *tor1Δ* (C) can increase the metabolic activity. On the other hand, in *pex19Δ* (D), adding antioxidant almost has no effect on improving metabolic activity. When entering the aging stage, antioxidant only has an effect (increasing metabolic activity) on cell wild type cells (E), *sod1Δ* (F), and *tor1Δ* (G). However, the antioxidant had no significant effect on *pex19Δ* (H). The data were analyzed by using two-way ANOVA followed by a Sidak post hoc test. The significance level was set at 0.05. The measurements were repeated 5 times. (ns means not significant).

Supplementary Fig.6 Effect of antioxidant supplement on ROS production. All strains were induced with antioxidant. In the young stage, adding antioxidant (blue) in wild type (A), *sod1Δ* (B), *tor1Δ* (C), and *pex19Δ* (D) can decrease ROS production. When entering the aging stage, antioxidant has a significant effect on reducing ROS production in wild type (E), *sod1Δ* (F), and *tor1Δ* (G). The effect of antioxidant in aged *pex19Δ* (H) is not significant. The data were analyzed by using a two-way ANOVA followed by a Sidak post hoc test. The significance level was set at 0.05. The measurements were repeated 5 times. (ns means not significant).

Supplementary Fig.7 Effect of antioxidant supplement in [%OH] production. All strains were induced with antioxidant. In the young stage, adding antioxidant (blue) in wild type (A), *sod1Δ* (B), *tor1Δ* (C), and *pex19Δ* (D) has no effect on the [%OH] concentrations. Effect of antioxidant on reducing [%OH] can be seen in cells with 1% H₂O₂ treatment for all strains. In the aging stage, antioxidant has almost no effect on reducing [%OH] production in the wild type (E), *tor1Δ* (G), and *pex19Δ* (H) while in *sod1Δ* (F), adding antioxidant slightly reduces [%OH]. The data were analyzed by using two-way ANOVA followed by a Sidak post hoc test. The significance level was set at 0.05. The measurements were repeated 5 times. (ns means not significant).

The effect of chemicals on bare diamonds

To exclude that the used chemicals induce a change in T1 by themselves we tested FNDs with the chemicals alone without the involvement of cells.

Supplementary Fig.8 (A) shows a confocal image of bare FNDs taken from the home-build magnetometry setup. The scale bar is 1 μm. The initial T1 values of FNDs were recorded then 1% H₂O₂ was added to the particles. (B) shows representative T1 curves. T1 values for both groups were compared in (C). There is no significant difference between the measurements before and after adding 1% H₂O₂. Data were analyzed using a paired T-test and significance level was set at 0.05.

Supplementary Fig.9 Effect of anti-oxidant on bare FNDs without cells. (A) Representative T1 curves were obtained from bare FNDs with anti-oxidant followed by 1% H₂O₂. FNDs were measured before (in green) adding any chemicals and used as a control group. The T1 was measured after adding 0.025 mM L-ascorbic acid (blue) followed by adding 1% H₂O₂ (red). (B) T1 values were collected and analyzed by using a one-way ANOVA followed by a Tukey post hoc test. According to the statistical test, the differences between the groups were not significant (P value 0.53). Data were analyzed by a paired T-test against previous group and P value < 0.05 was considered has significant difference. AO means anti-oxidant.

Supplementary Fig.10 Morphological changes during oxidative stress in yeasts. Yeast cells were stimulated by 1% H₂O₂ then we observed the morphological changes using AFM. Yeasts without any stress inducer have a smooth surface while stressed yeasts have a rougher (wrinkly) surface.

Comparison between different methods

Supplementary Fig.11: Comparison between different methods. (A) shows what the respective techniques in this work are measuring exactly and how they are related. The colored circles stand for different groups of molecules that are detected. They do have a certain overlap but there is currently no technique, which is able to measure the exact same signal as we do. (B) compares the data in Fig 2 (B) of the main manuscript with the existing techniques. For a better comparison we have normalized all the data to the average value for the wild type strain with each technique (indicated by the grey line). Further, we are showing 1/T1 and 1/MTT. As a result, in this graph the depicted values are all proportional to the concentration of measured molecules.

Supplementary Fig.12: Comparison between different methods. Here we compare the data in Fig 3 of the main manuscript with the existing techniques. Again all the data are normalized all but this time to the initial measurements with each technique (indicated by the grey line). Also here, we are showing 1/T1 and 1/MTT. As a result, in this graph the depicted values are all proportional to the concentration of measured molecules.

Supplementary Fig.13: Comparison between different methods. Here we compare the data in Fig 4 of the main manuscript with the existing techniques. Again all the data are normalized all but this time to the initial measurements with each technique (indicated by the grey line). Also here, we are showing 1/T1 and 1/MTT. As a result, in this graph the depicted values are all proportional to the concentration of measured molecules.

Chapter 6

6.1 FNDs inside cells

Nowadays, fluorescent nanodiamonds (FNDs) attract a lot of attention in the biomedical field. They have superior physical and chemical properties and are also biocompatible for biological application^{1,2}. Due to particular defect (NV centers) in FNDs, they become potential free radical biosensor.

To measure free radicals inside the cells, FNDs have to be ingested. There are many techniques that can be used for particle uptake for example using gene transfection, a gene gun, surface modification, and many more³. In **Chapter 2**, we used 2 different cells for testing particle uptake protocol. We used yeast cells that have a cell wall and colon adenocarcinoma cells (HT-29). None of these cells can ingest the particles spontaneously.

Positively charged lipids allowed us to cover FNDs particle. For yeasts cells, we permeabilized the cells by removing cell wall with a spheroplasting technique then cells were mixed with lipid-FNDs mixture. For HT-29 cells, we just simply mixed the cells with lipid-FNDs. According to the result, we confirmed that using cationic lipids to modify FNDs can help the cells to take up the particles easily. These particles also biocompatible for all the cells and have no significant effect to FNDs spectrum. While we have so far shown that the coating can increase particle uptake in the future such coating might be helpful to improve targeting efficiency. In mammalian cells such coatings might be useful to achieve endosomal escape. In yeast, it might be interesting to combine this coating approach with antibody coating in **Chapter 4**. As a next step it could be interesting to incorporate specific lipids in the coating, which lead to targeting.

After successfully bringing FNDs to the cells, we investigated the fate of FNDs during cell division in **Chapter 3**. Since we used yeast cells as a model organism, the cell division is different compared to mammalian cells. We proposed 4 possibilities of FNDs fate during cell division: 1) the particles stays in

the mother cells, 2) moves to daughter cells, 3) is excreted, or 4) is found in both mother and daughter cells. Intracellular fate of FNDs also was monitored using particle displacement parameters. We found that during cell division, most of FNDs moved from mother cells and we assumed that the particles could be in daughter cells or excreted from the cells. In the future this might open up possibilities to study free radical generation during cell division with diamond magnetometry.

To control the FNDs position, in **Chapter 4** we attached antibodies to FNDs. We chose nuclear pore complex (NPC) antibodies specific for yeast cells. This antibody has been chosen because of the key role of the nuclear pore complex in transporting to the nucleus. Additionally, targeting the nucleus is of relevance to understand DNA damage. Finally, targeting the nucleus is also practicable since the nucleus is comparably easy to find with different imaging modalities. Cells then were incubated with FNDs linked antibody. Intracellular fate of these particles was monitored while performing incubation. The successful rate of targeting process was achieved by measuring the distance between particles and yeast NPC. We noticed that attaching antibody to FNDs helps to control the position of FNDs inside the cells. As mentioned earlier, this paves the way to perform labeling of nucleus but also localized magnetometry experiments.

6.2 Diamond magnetometry in yeast

In healthy cells, free radicals concentrations inside the cell are around nanomolar to micromolar^{4,5}. Yeasts have been chosen as model organism because of their importance as a model for aging. We investigated both oxidative stress response and aging process, which produce free radicals. In **Chapter 5** we treated yeasts cells, which have nanodiamond particles with low concentration of

hydrogen peroxide and let them age. Both conditions affect the free radicals concentration inside cells. Fluctuation of free radicals concentration was monitored by diamond magnetometry. Along with that technique, some fluorescent based probes like H₂DCFDA and HPF (hydroxylphenyl fluorescein) were also used as comparison. We also tried to get a better understanding about the influences of some genes like superoxide dismutase 1 (*SOD1*), target of rapamycin 1 (*TOR1*), and peroxin 19 (*PEX19*) regarding stress responses and aging process by using knock out yeast mutant strains.

Diamond magnetometry provides unique data compared to the state of the art methods. While all the ROS dyes we investigate give some information on the oxidative stress in the cells, it is clear that they all provide different information. Diamond magnetometry is currently the only technique, which reveals the overall concentration of radicals. Additionally, it is fundamentally different from ROS dyes. While the conventional dyes measure the history of the samples, we are able to measure the current status. Furthermore, we are able to perform measurements at single cell level. Given how versatile this technique is we foresee many biomedical applications to profit from this technique.

6.3 Future directions

FNDs have showed promising properties as biological nanosensors. However, there is still some work to be done. In the future, we would like to know if changes in the cellular stage can affect performance of FNDs. Potential influences on the relaxometry readout including temperature, viscosity, or electric field fluctuations need to be excluded. While we so far only measure the sum of all radicals present, it is theoretically possible to differentiate between different radicals using pulsing sequences, which are analogous to the ones used in NMR spectroscopy.

References

1. Mochalin, V. N., Shenderova, O., Ho, D. & Gogotsi, Y. The properties and applications of nanodiamonds. *Nat. Nanotechnol.* **7**, 11–23 (2011).
2. Chipaux, M. *et al.* Nanodiamonds and their applications in cells. *Small.* **17**, 4263, 1–25 (2018).
3. Webster, A. *et al.* The delivery of PEBBLE nanosensors to measure the intracellular environment. *Biochem. Soc. Trans.* **35**, 538–543 (2007).
4. Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet.* **344**, 721–724 (1994).
5. Paardekooper, L. M. *et al.* Human monocyte-derived dendritic cells produce millimolar concentrations of ROS in phagosomes per second. *Front. Immunol.* **10**, 1–9 (2019).

Summary

Thesis summary

Free radicals are produced naturally during cell metabolism. Cells also have a specific mechanism to convert those chemicals to be less reactive and easy to eliminate. However, disturbance of the cell system, which leads to unbalanced levels of free radicals inside the cells results in an oxidative stress condition and an aging process. Developing free radical biosensors is challenging since all existing methods have some issues with sensitivity and specificity. Fluorescent nanodiamonds (FNDs) containing defects called NV centers are promising biosensors. A technique called diamond magnetometry, combines the advantages of fluorescence techniques with magnetic resonance methods. It uses NV centers that can convert magnetic signals into an optical signal. A specific type of magnetometry measurements that we used in this thesis are called T1 measurements. These are specific for spin noise and thus great candidates for free radical sensing.

Chapter 1 introduces 5 important topics that were involved in this research. First, an overview about free radicals generation and their elimination process by the cells are discussed. The chapter includes a discussion on how it is related to oxidative stress response and aging. Second, I briefly discuss antioxidants used against free radicals. These can be naturally produced by the cells or supplemented from external sources. Third, some existing methods for detecting free radicals have been investigated together with their limitations. Then I shortly introduce diamond magnetometry as a new approach for detecting free radicals inside the cells. Finally, in this chapter a thesis objective is clearly stated.

FNDs have to be as close as possible with the source of the molecules. This means that FNDs have to be inside the cells. In **Chapter 2**, we developed a spheroplasting method for internalizing FNDs into yeast cells which are enclosed

by a thick cell wall. By using this method, the cell wall has been removed to increase the permeability without damaging the cells. Moreover, we modified FNDs using cationic lipid to increase the uptake capacity especially for cells that cannot ingest FNDs easily like colon cancer cells (HT 29 cells). Both spheroplasting and the lipid coating process greatly increased particle uptake.

Chapter 3 is concerned with the fate of FNDs during the yeast division process. Yeast cell division occurs asymmetrically and is correlated with the aging process. Thus, it is important how FNDs behave during this process. There are 4 possible fates of FNDs during yeast divisions: the particles stay in the mother cells, move to daughter cells, are excreted, or end up in both mother and daughter cells. In this chapter we evaluate 2 different FNDs (bare FNDs and FNDs lipid from chapter 2). We found that most of the particles were either excreted or transferred to daughter cells. The initial position of the particles is considered as a factor that influences the fate.

While it is already very useful to measure free radical generation at the location where they end up naturally, it is more useful if the particle can be targeted to a specific location. Thus in **Chapter 4**, we modify the surface of FNDs by attaching antibodies to direct the FNDs to the nucleus. A nuclear pore complex (NPC) antibody has been chosen to target the nuclear surface. Due to their size FNDs cannot penetrate into the nucleus so sensing has to be done from the surface. Antibody-coated FNDs have been characterized and incubated with the cells. Their intracellular fate was monitored at specific time points. An evaluation of the targeting has been done by measuring the distance between particles and nucleus. We concluded that attaching antibodies to FNDs helps to control the position of FNDs inside the cells.

In **Chapter 5**, we implemented diamond magnetometry to detect free radical generation in yeast cells during oxidative stress conditions and aging. An

oxidative stress condition has been achieved by triggering the cells with hydrogen peroxide and putting the cells into sterile demineralized water to mimic chronological aging. With FNDs inside the cells, free radicals generation was evaluated by using diamond magnetometry. As a comparison, ROS generation was monitored by using fluorescence based probes. We also tested the ability of diamond magnetometry to detect free radical scavenging by antioxidants. We find that both free radicals generation and antioxidant effect could be detected by diamond magnetometry. Then in the last chapter (**Chapter 6**) we shortly discuss future applications of diamond magnetometry.

Samenvatting

Vrije radicalen worden van nature geproduceerd tijdens het celmetabolisme. Cellen hebben ook een specifiek mechanisme om deze radicalen om te zetten naar radicalen die minder reactief zijn en die makkelijker te elimineren zijn. Een verstoring van dit cel systeem, dat leidt tot verstoorde balans van vrije radicalen in de cel, resulteert in oxidatieve stress en veroudering. De ontwikkeling van een biosensor voor vrije radicalen is uitdagend; alle huidige methoden hebben moeite met sensitiviteit en specificiteit. Fluorescerende nanodiamanten (FNDs) met zogenoemde NV center defecten, vormen een veelbelovende biosensor. Een techniek genaamd diamant magnetometrie, combineert de voordelen van fluorescentie technieken, met de voordelen van magnetische resonantie methoden. Het gebruikt FNDs met NV centers die magnetische signalen kunnen omzetten in een optisch signaal. Een specifiek type magnetometrie metingen zijn de T1 metingen die in dit proefschrift gebruikt zijn. Deze metingen zijn specifiek voor “spin noise” en zijn dus een goede kandidaat voor vrije radicalen sensors. Dit werk presenteert meer informatie over hoe FNDs gebruikt kunnen worden als biosensors voor vrije radicalen.

Hoofdstuk 1 introduceert 5 belangrijke onderwerpen die van belang zijn in dit onderzoek. Als eerste wordt een overzicht gegeven van het ontstaan van vrije radicalen en van de eliminatie processen van de cel. Dit hoofdstuk bevat ook een discussie over de relatie tot de oxidatieve stress reactie en veroudering. Ten tweede worden kort de antioxidanten besproken, die gebruikt worden tegen vrije radicalen. Deze kunnen van nature door de cel worden geproduceerd of als supplementie worden toegediend. Ten derde volgt een verslag van bestaande methoden, en hun beperkingen, met de detectie van vrije radicalen. Hierbij wordt diamant magnetometrie geïntroduceerd als een nieuwe manier om vrije

radicalen in de cel te detecteren. Als laatste wordt in dit hoofdstuk duidelijk het doel van dit proefschrift geformuleerd.

Als vrije radicaal sensors moeten de FNDs zo dicht mogelijk bij de bron van deze moleculen zijn. Dit betekent dat de FNDs in de cellen moeten zijn. In **hoofdstuk 2** hebben we een sferoplast methode ontwikkelt voor de opname van FNDs in gistcellen, welke omgeven worden dooreen relatief dikke celwand. Met deze methode wordt de celwand verwijderden de permeabiliteit te verhogen, zonder de cellen te beschadigen. Daarnaast hebben we de FNDs bewerkt met kationische lipiden om de opname capaciteit van cellen die moeilijk FNDs ingestemd, zoals dikke darm kankercellen (HT-29 cellen), te verbeteren. Zowel de sferoplast methode als het costing proces met lipiden verhoogt de FND opname enorm.

Hoofdstuk 3 gaat over de bestemming van FNDs tijdens het proces van het gist delingsproces. Gist celdeling is een asymmetrisch proces, dat in verband kan worden gebracht met veroudering. Daarom is het belangrijk te weten hoe FNDs zich gedragen tijdens dit proces. Er zijn 4 mogelijke bestemmingen van FNDs tijdens gistdeling: de FNDs blijven in de moeder cel, verplaatsen naar de dochter cel, worden uitgescheiden, of worden verdeeld over zowel de moeder als de dochter cellen. In dit hoofdstuk evalueren we de bestemming van twee soorten FNDs: naakte FNDs en de gecoatete FNDs uit hoofdstuk 2. We observeerden dat de meeste FNDs ofwel werden uitgescheiden ofwel werden verplaatst naar de dochter cellen. De oorspronkelijke positie van de FNDs wordt gezien als een belangrijke factor die van invloed is op de bestemming.

Alhoewel het al zeer zinvol is om het ontstaan van vrije radicalen te kunnen meten op de locatie waar FNDs zich vanzelf bevinden, zou het nog meer zinvol zijn als de FNDs naar een specifieke locatie gestuurd kunnen worden. In **hoofdstuk 4** modificeren we daarom het oppervlakte van de FNDs door

antilichamen te laten binden om de FNDs naar de nucleus te navigeren. Hiervoor is een antilichaam tegen het kernporiecomplex (NPC) gekozen om op het kernoppervlak te richten. Door hun formaat kunnen FNDs niet in de kern penetreren, dus detectie moet plaatsvinden vanaf het oppervlak. Met antilichaam gecoatete FNDs zijn gekarakteriseerd en met de cellen geïncubeerd. Hun intracellulaire bestemming is gemonitord op specifieke tijdstippen. Een evaluatie van de succesvolle sturing is gedaan door de afstand te meten van de FNDs tot het NPC. We hebben geconcludeerd dat binding van antilichamen aan de FNDs helpt om de locatie van FNDs in de cel te sturen.

In **hoofdstuk 5** implementeerden we diamant magnetometrie om het ontstaan van vrije radicalen in gistcellen te detecteren tijdens omstandigheden van oxidatieve stress. Oxidatieve stress werd veroorzaakt door de cellen met waterstofperoxide te activeren en door de cellen in steriel gedemineraliseerd water te plaatsen om chronologische veroudering na te bootsen. Met de FNDs in de cel, werd het ontstaan van de vrije radicalen gedetecteerd door diamant magnetometrie te gebruiken. Ter vergelijking, werd het ontstaan van ROS gemonitord met fluorescentie methoden. We hebben ook getest of diamant magnetometrie het opruimen van vrije radicalen door antioxidanten kan meten. We observeerden dat zowel het ontstaan van vrije radicalen als het antioxidant effect gedetecteerd kunnen worden met behulp van diamant magnetometrie.

Tot slot bediscussieren we in **hoofdstuk 6** kort de toekomstige toepassingen van diamant magnetometrie.

Acknowledgement

All praise to GOD, the Almighty! It has been a long journey until the day I officially finish my PhD. This whole 4 years PhD was crazily meaningful, frustratingly beautiful, and yet it was an endearingly adventurous research experience for me at the same time. This PhD quest has lead me to meet wonderful people who support me during difficult and joyous times. For the lady who I adore the most throughout my PhD, **Romana**, thank you for everything. For the last 4 years you always support any ideas that came from my mind and teach me to improve myself to be confident (although I still always doubting myself). Having you as my boss and mentor is a great treasure.

I would like to express my gratitude to **Prof. Andreas Hermann**, **Prof. Thorben Cordes**, and **Prof. Cor Calkhoven** for their willingness to be my assessment committees. Thank you for giving valuable feedback and input for this dissertation.

During my stays in BME, I spent every single day with my beloved roomies. **Simon**, it is a pleasure to have you as my roomie for 3 years. Without your companion, I would be already lost my way on my first day in BME. Thank you for trusting me as your cat sitter (especially for Chedar, you always be in my heart) and for visiting my parents when you were in Indonesia. I will miss you and Marthe so bad when I leave the Netherlands. **Kiran**, my yeast buddy, thank you for always helping me with everything. You are an amazing planner and my private editor. I enjoy every time we spent together and our random chats about your kitties. **Felipe**, a great problem solver, your brilliant ideas always serve as lifesaver for all the projects and papers. Thank you for introducing me to Chilean cola de mono and pisco (I am now addicted to these boozes) and sending my favorite LOACKER directly from Berlin. And **Linyan**, being your real neighbor and sharing our meals were amazing experiences (Thank you for accepting my offer to be one of my paranymphs). I enjoy every single time we spent together.

For the rest of diamond members, you guys own a special place in my life. Dear **Alina**, without your help, I would not be able to finish my last manuscript. I also enjoyed spending time with your princess (Vesta) and still looking forward to meet her again. **Thamir**, I appreciated our collaborations and wishing you all the best for the next. **Viraj**, who always comes with brilliant ideas, it is such a great pleasure knowing and working together with you. **Yori**, I have never imagine having an awesome colleague with similar background as half-blood Indonesian. You often come with crazy ideas when we talk about diamond and teeth. **Citra**,

my Indonesian little sister, who never complain about working in the weekends and the only person I can talk in my mother tongue when I am in doubt. Don't worry too much about your future and please take care of your health. Everything will be okay. **Charles**, **Neda**, **Rokshana**, **Sandeep**, **Yuchen**, **Yue**, **Kaiqi**, **Runrun**, **Claudia**, **Ari**, and **Thea**, thank you for being supportive colleagues. Dear **Aldona**, thank you for being my co-promotor.

Dear **Mayeul**, a very first person who introduced me to diamond setup, thank you for your endless helps and supports for all my papers. I am always inspired by your passion and dedication for your work. Thank you as well for introducing delicious homemade French pastries. I wish you all the best for your future with Adeline.

I would like to acknowledge all my co-authors **Michael Chan**, **Andreas Nagl**, **Axel Hochstetter**, and **Nicolas Jamot** for their contributions in my papers. Without their help, these papers will not be finished by this time.

To all scientific staff at BME (**Henk**, **Henny**, **Jelmer**, **Prashant**, **Theo**, **Patrick**, **Inge**, **Chris**, and **Brandon**), it is really nice to interact and share knowledge during coffee break and sleutel meeting. For **Wya** and **Ina**, thank you for helping me with all paper works since I was still in Indonesia. For all technicians: **Gesinda**, **Jelly**, **Betsy**, **Reinier**, **Joop**, **Willem**, **Marja**, **Willy**, **Hans**, **Rene**, and **Ed**, thank you for giving TAR, solving lots of technical problems in the lab, and thank you for all the cheerful conversations during the lab works.

To all PhDs at BME, thank you for always being nice and welcoming me. Especially for **Damla**, I will always remember you as fellowcrazy cat lady in our department. Thank you for inviting me to your home and letting me play with Pera. For **Can**, being your lab mate is always a wonderful experience. Thank you for sharing your story during our weekend works. I hope we will get an opportunity to meet in another time. For **Lu Yuan (Sara)**, thank you for the hotpot dinner and play time with Xiao Hei. I enjoyed those times a lot. Good luck for your future. For **Yanyan**, thank a lot for helping me with pre culture stuff. I wish you all the best.

For Indonesian community (**Mbak Tania**, **Zildji**, **Mbak Erna**, **Mas Yoga**, **Ina**, **Mas Tri**, **Azka**, **Panji**, **Mbak Tiwi**) thank you for everything. It is my pleasure to know you all. For **Mas Didik** and **Mbak Rosel**, thank you for letting me stay at your house during my first week in Groningen. Dear **Mbak Isti**, having you as a

sister, crazy cat partner, and also travel mate is wonderful. Thank you for always available when I am in doubt and also for sharing “the art of doing nothing” even though I can only implement it for 3 hours maximum. Thank you for being my paranympth!

My next door neighbor, **Apostolos**, thank you so much. I had lots of fun from our talks during laundry time. Thank you for always sharing your Greek sweets with me and being a nice and caring brother when I was sick. Best wishes for you.

During the pandemic and writing season, I would like to thank all Groningen cats (especially **Oyen**, **Lica**, **Pure**, **Jutek**) for always being around and help me to get rid all the negative though. For **Hyun Bin**, **So Ji Sub**, **Jo In Sung**, **Takeru Satoh**, and **Takahiro Moriuchi**, you guys really made my day!

For my seniors and colleagues from Faculty of Dentistry Universitas Gadjah Mada, thank you for giving me the opportunity and support me through this whole PhD trajectory Especially for **Prof. Bundi** and family, thank you for your advices about learning Dutch culture. It helps me a lot to survive in Netherlands.

My dear parents, **Bapak** and **Ibu**, I might not be your perfect devoted daughter. I cannot say anything but thank you for raising me and teaching me to be a brave and independent woman. I dedicate this dissertation for you. My lil brother and sister, **Iwa** and **Dewi**, thank you for all your supports and prays. Looking forward to meet you soon with your 2 little angels **Aurora** and **Freya**.

The last but not least, **Andri**. Your endless supports and patience especially for the last 4 years (no matter how many times I ignored you over my stuffs but you admirably never get upset about that) really prove that you are an amazing partner for me. Thank you for taking care of everything and thank you for being my shelter. あなたを本当に愛しています

Groningen, November 2020

Aryan Morita

Curriculum vitae

Aryan Morita was born on 23rd August 1987 in Yogyakarta, Indonesia. In 2005, she started her undergraduate degree in Faculty of Dentistry Universitas Gadjah Mada Indonesia and completed her degree as a dentist in 2011. During her clinical clerkship, she also started her master degree in Biomedical

Sciences (majoring in Pharmacology and Toxicology) from the same university and received the degree in 2012.

Since 2011, she becomes a teaching staff and junior researcher in Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada Indonesia. In 2016, she received doctoral scholarship from Ministry of Finance Republic Indonesia under LPDP scheme and started her PhD program in Bioanalysis and Imaging group of Romana Schirhagl at Department Biomedical Engineering, University Medical Center Groningen, Netherlands. Her project focused on application of nanodiamonds as biological sensor for detecting free radicals formation during stress and aging process. During her PhD, she received De Cock grant (2019) and travel grant from Nano Today, Elsevier (2019). She published her papers and contributed as a co-author in several papers.

List of publications:

1. **Morita, A.**, Nusantara AC., Martinez FPP., et al., Quantum Monitoring The Metabolism of Individual Yeast Mutant Strain Cells when Aged, Stressed or Treated with Antioxidant. **2020** (Submitted to *Nature Nanotechnology*).<https://arxiv.org/abs/2007.16130>
2. Damle, VG., Vedelaar, TA., Wu, K., **Morita, A.**, et al., Applying NV center-based quantum sensing to study intracellular free radical response upon viral infections. **2020** (Submitted to *Nature Communication*).
3. **Morita, A.**, Hamoh, T., Sigaeva, A., et al., Targeting Nanodiamonds to The Nucleus in Yeast Cells. *Nanomaterials*, 10 (1962), 1-10 (2020).
4. Damle, V. G., Sharmin, R., **Morita, A.**, et.al., Micro Versus Macro – The Effect of Environmental Confinement on Cellular Nanoparticle Uptake. *Front Bioeng Biotechnol.* 8, 1–13 (2020).
5. **Morita, A.**, Hamoh, T., Martinez, F. P. P. et al., The Fate of Lipid-Coated and Uncoated Fluorescent Nanodiamonds during Cell Division in Yeast. *Nanomaterials*, 10 (3), 1–15 (2020).
6. Laan, K. J. Van Der, **Morita, A.**, Perona-martinez, F. P. & Schirhagl, R. Evaluation of the Oxidative Stress Response of Aging Yeast Cells in Response to Internalization of Fluorescent Nanodiamond Biosensors. *Nanomaterials*, 10, 1–13 (2020).
7. Damle, V., Wu, K., De Luca, O., Ortí-Casañ, N., Norouzi, N., **Morita, A.**, ... Schirhagl, R. Influence of Diamond Crystal Orientation on The Interaction with Biological Matter. *Carbon*, 162, 1–12 (2020).
8. **Morita A.**, Martinez FPP, Chipaux M, et.al., Cell Uptake of Lipid-Coated Diamond. *Part Part Syst Charact* 1900116:1–8 (2019).
9. Sigaeva A, Ong Y, Damle VG, **Morita A.**, et.al., Optical Detection of Intracellular Quantities Using Nanoscale Technologies. *Acc Chem Res* 52:1739–1749 (2019).
10. Sigaeva, A., **Morita, A.**, Hemelaar, S. R., & Schirhagl, R. Nanodiamond uptake in colon cancer cells: the influence of direction and trypsin-EDTA treatment. *Nanoscale*. (2019)