Investigating the Use of Fluorescent Nanodiamonds as Imaging Probes for Super-Resolution Microscopy

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Lay Summary:

Microscopy is a scientific technique that allows us to look at the building blocks of life. One of the biggest challenges in the field is that the components of cells intrinsically lack colour, so to be able to visualise different parts of the cell, common practice is to tag them with labels. This labels the structures we are interested in with a fluorescent tag, which glows in the dark and can be seen easily through the microscope. Using this strategy, we can now see components as small as individual molecules such as proteins and DNA! However, there is a big stumbling block - these traditional fluorescent dyes are not durable, and during imaging they can degrade and reach a state where they no longer glow in the dark. This means that images must be taken very quickly and in the dark to protect samples from exposure to light and degradation, limiting the quality of the images obtained. In this project I explored strategies to use tiny diamonds (nanodiamonds) as alternatives to fluorescent dyes – as diamonds are indestructible, they can glow permanently. I developed nanodiamond tags to label certain parts of the cell, using two different types of super resolution microscopy. I focused on labelling a structure called the nuclear pore complex, a protein channel that makes up the main gateways to the nucleus (the brain of the cell), and successfully obtained images showing the location of these channels.

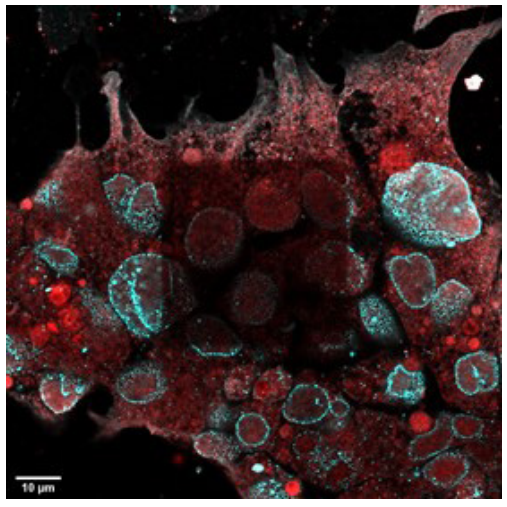


Figure 1. HEK 293T cell imaged using an Airyscan Confocal Microscope, illustrating the phenomenon of photobleaching. The outer part of the image was taken using a low-powered laser, however the dim square in the centre was imaged using a very powerful laser to try to achieve a better image, so became photobleached. The red channel shows SERCA dyed with Alexa Fluor 488, and the cyan channel shows the nuclear pore complex (Nup-98) dyed with Janelia Fluor 549.

Introduction:

The field of super-resolution microscopy has grown rapidly over the last few years due to the ability to take images that bypass the diffraction limit - a development recognised by the Nobel prize in Chemistry in 2014. A key bottleneck preventing further utilisation of these techniques, however, is the durability of the fluorescent dyes that are used to tag cell structures. Unfortunately, traditional aromatic dyes are susceptible to photobleaching under intense laser light, so degrade quickly during imaging, as seen in *Figure 1*. A more durable alternative to these are nanoparticles such as carbon dots, quantum dots, and fluorescent nanodiamonds (FNDs). FNDs are ideal for this purpose as they undergo zero photobleaching, are not toxic to the cells, and can be surface functionalised to adapt to various applications. Using FNDs for super-resolution imaging combines the advantages of such a high resolution with an unlimited imaging duration. This project focuses on assessing the viability of a FND conjugation protocol for this brand-new optical super-resolution modality known as *self-activated nanodiamond-based Stochastic Optical Reconstruction Microscopy* (sandSTORM). The current gap in this field is the limited understanding of the chemistries that allow nanodiamonds to be conjugated to molecular probes such as antibodies, which is something I investigated over the course of this project.

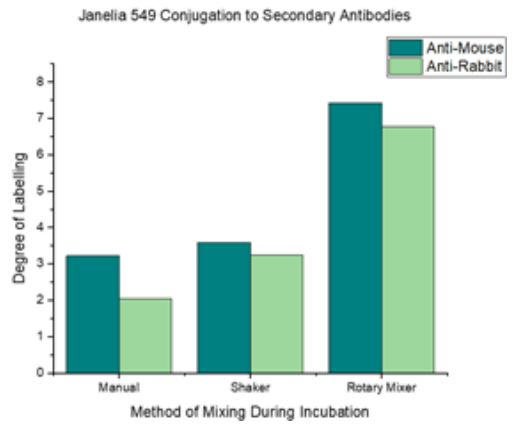


Figure 2. Graph showing the effect of changing the method used to mix the sample during incubation, on the degree of labelling achieved.

Aim:

The aim of this project was to trial a protocol for the N-Hydroxysuccinimide (NHS)-ester based conjugation of FNDs and benchmark this against an NHS-ester based conjugation of an aromatic fluorophore, Janelia 549, then to assess the efficacy of this conjugate as a probe for performing high-resolution (Airyscan Confocal Microscopy) and super-resolution (sandSTORM) imaging of molecular targets in cells, namely the nuclear pores and markers of the endoplasmic reticulum.

Methods and Approach:

We conjugated FNDs to secondary antibodies, ensuring minimal clumping by using a bath sonicator, as well as conjugating Janelia Fluor 549 to secondary antibodies as a control probe. The conjugation efficiency was quantified by measuring the degree of labelling obtained using a nanodrop spectrophotometer, and the protocol was altered to result in improved labelling efficiencies. We then used these optimised conjugates as secondary antibodies to label various cell structures in human embryonic kidney (HEK 293T) cells including: the nuclear pore complex sub-unit Nup-98, an ionic pump on the endoplasmic reticulum called the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA), a peptide target in the endoplasmic reticulum called KDEL, actin anchor α-Actinin, microtubule monomer Tubulin, the membrane protein Cav-3, and a giant calcium channel called the ryanodine receptor RyR-1. We then used these samples for imaging using a Zeiss Airyscan confocal microscope and a Nikon n-STORM microscope.

Key Findings:

To optimise the conjugation protocol for Janelia 549, various methods were tested including altering the concentration of reagents, the length of the incubation period, the temperature during incubation and the method of mixing. As shown in *Figure 2* the limiting factor for achieving the desired degree of labelling appeared to be the method of mixing during incubation. A degree of labelling of 10.015 was achieved for the Anti-mouse Janelia Fluor 549 conjugate, after the protocol was optimised.

Only one nanodiamond conjugation experiment was successful, due to clumping of the nanoparticles and degradation of reagents. The batch that succeeded

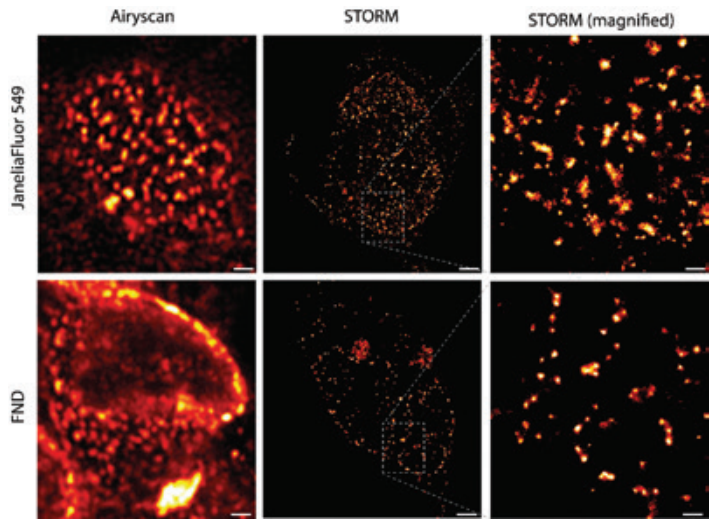


Figure 3. Table comparing images taken of the nuclear pore complex dyed with FNDs and Janelia Fluor 549, taken on a Zeiss Airyscan confocal microscope and on a Nikon nSTORM microscope. The scale bars show 1 μm for the left and middle columns, and 200 nm for the right column.

had a degree of labelling of 0.918. This was very close to the value of 1 degree of labelling that was expected, due to the steric hindrance caused by the large size of the nanoparticle (100 nm) binding to the much smaller antibody (~10 nm) preventing further labelling.

The ratio of the concentration of FNDs to antibodies was calculated to be 1666:1, far larger than that for the Janelia 549 conjugations which was 10:1. The scope, and short timescale, of this project unfortunately did not give me time to tweak the protocol, but if more time was available, I would have adjusted this ratio to be 10:1 instead, and assessed if this affected the resulting degree of labelling.

To decide which target would be best to showcase the FND probe, many targets were tested using different commercial dyes, to identify which had the most unique, identifiable, and clear distribution in HEK 293T cells.

This method allowed a direct comparison of the staining of the nuclear pore complex with different types of probes across two different imaging modalities, as seen in *Figure 3*.

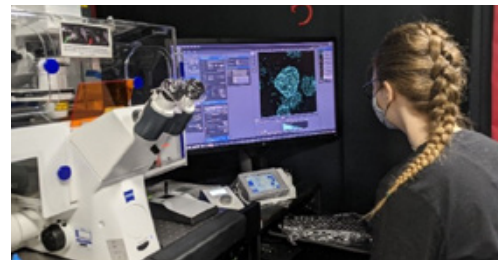
In each image we can see punctate labelling, of similar density, on the nuclear envelope. The FND labelling is of the same high quality to the established label, Janelia Fluor 549. Unlike Janelia Fluor 549, which is significantly less effective for STORM imaging, the FND probe is an effective dye for both confocal and STORM imaging. Another benefit of using FNDs instead of traditional dyes is that during STORM imaging the sample does not need to be immersed in a reducing environment to ensure oxygen depletion. This makes the imaging process simpler, faster, and more accessible for portable microscopes - for applications from monitoring marine environments in situ, to diagnostics. In conclusion, the FND probes are very versatile dyes for super resolution microscopy.

What did you learn from participating in this project?

I really enjoyed the practical elements of the project and being immersed in the laboratory work was exciting. I learnt a great deal about cell culture and using sterile techniques – and as a chemistry student this was all new to me, so was a steep learning curve! I enjoyed the freedom of being able to design and carry out my own experiments, tweaking them as I went along. I also learnt patience and resilience when things didn't work out as I expected them to, whether that be reagents being delayed at customs or cell culture batches failing. I learnt how to use an Airyscan confocal microscope proficiently and how to analyse the images obtained in ImageJ, and I even got the chance to use an n-STORM microscope on one occasion. During the project I also attended the Microscience Microscopy Congress (MMC) conference and joined their virtual super-resolution workshop. This was the first conference I have attended; it was eye opening to see such a breadth of research presented and was a great introduction to the field of super-resolution microscopy. Despite the limitations that COVID-19 restrictions placed on the project, something that really stood out to me was the community aspect, which is so important to the research process. Working as part of a such a cutting-edge research group and being part of such a thriving scientific community in the department of molecular biology and biotechnology was an amazing opportunity, which I am very grateful for.

How has this project affected your long-term goals?

I really enjoyed the microscopy component of the project – having such visual feedback on experiments is very exciting. My long-term goals before I started the project were to continue with research in inorganic chemistry, either by doing a PhD or going into industry. However, after completing the project, they have shifted slightly, as I would love to be involved with research that incorporates microscopy in the future. I am interested in research which combines material science with imaging, for example in crystallography. In addition to this, the technical aspect of the microscopes, such as understanding the alignment of the lasers intrigued me, as well as the biological aspects which I hadn't had much previous exposure to – cell culture was a very new field for me, and I would like to continue to develop my skills in this area. This project has opened up a whole host of potential career avenues that I am excited to explore further.



Photograph of me in the lab.