Super Resolution Imaging of Mitotic Cells using Stimulated Depletion Emission Microscopy

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Introduction

All life starts out as a single cell, which, by the time the average human is fully grown, will increase to over 32,000,000,000,000 cells. To do this, cells must divide; replicating their DNA and content. Results can be catastrophic if this process goes wrong, and can result in diseases such as cancer or birth defects. In order to understand the complexity of cell division we must observe it using high-resolution microscopy techniques.

STED (STimulated Emission Depletion) microscopy, a novel imaging technique - for which its inventor won the Nobel Prize in 2014 - allows us to see cellular detail at a resolution of 60 nm; 1000 times thinner than a human hair. Previously, this was only achievable using electron microscopy and disruptive preparatory techniques that damaged the cell. The aim of this project was firstly to optimize STED microscopy by finding the ideal combination of fluorescent tags and preparatory techniques.

Aims

1) To find optimal conditions of cell fixation in order to maximize the resolution of STED microscopy

2) To find computable antibodies that can be used with STED

3) To understand the particular uses and limitations of STED in regards to mitotic cells, with a particular focus of phosphorylation patterns across the kinetochore.

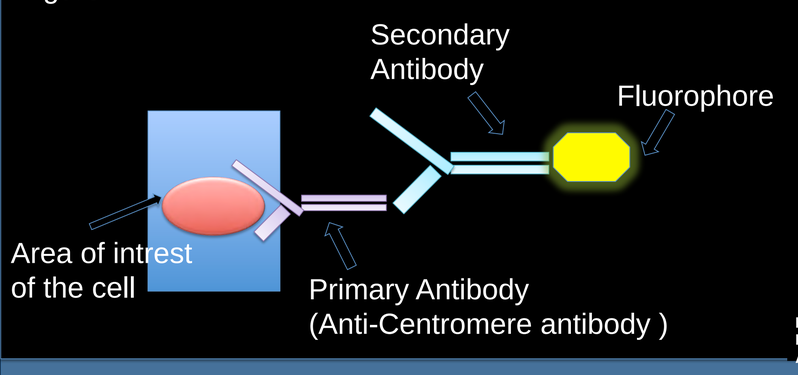
Immunofluorescence

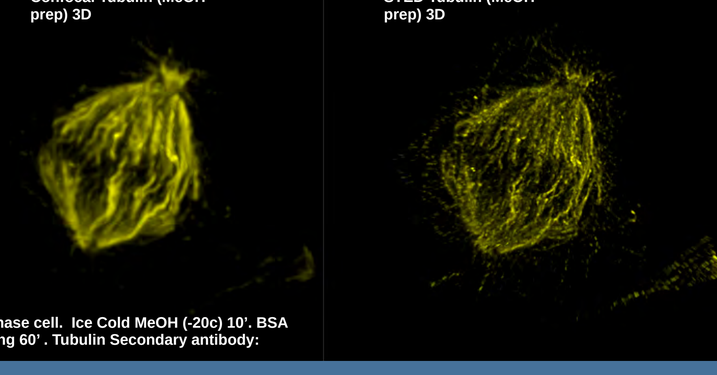
In order to visualise cellular structure under fluorescent microscopy, we need to label the structure of the cell we want to see. We do this by using two types of antibodies. Antibodies are able to bind and stick to very specific structures. We can exploit this concept by attaching fluorophores to specific antibodies. A fluorophore is a molecule that emits light (fluoresce) when a laser is shone on to it. We do this by adding a primary antibody that binds to the thing we want to see (see fig.1). We then add a secondary antibody which specifically binds to the primary antibody. This secondary antibody has a fluorophore attached to it. This means, when we look at the slide under a fluorescent microscope, we can see the part of interest as it illuminates. This principle can be used to see any part of the cell as long as there is a primary antibody for it.

Cell Fixation

The next step was to identify optimal cell fixation techniques in order to maximise the resolution of STED. Fixation is when we take live cells, and use a chemical to kill them in a static state. There are many way we can do this, and the fixation can impact on the resoultion of the images we acquire. We tried a variety of methods including, but not limited to; PFA fixation, PFA fixation with glutaraldehyde, and ice cold methanol. We also tested several primary antibodies with different fixation techniques. We found that most of the primary antibodies that we used, including Aurora B, CENP-A, ACA, and CENP-A upstate S7ph, were extremely sensitive to the fixation conditions. This meant that with certain techniques, the primary antibody was unable to bind to the cell. This resulted in us changing the fixation technique depending on the cell component we wanted to visualise.

Figure 1



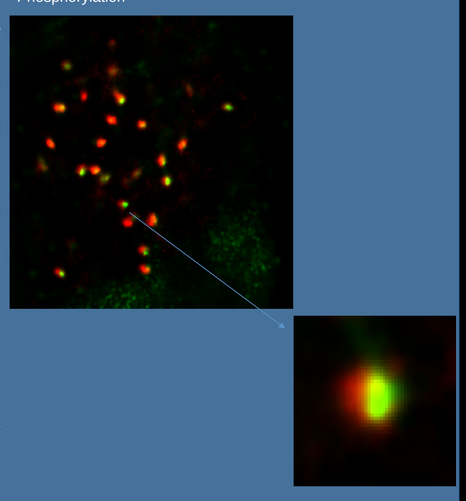


Metaphase cell. Ice Cold MeOH (-20c) 10'. BSA Blocking 60' . Tubulin Secondary antibody: AF594

Uses of STED

We then decided to look at the co-localisation of certain molecules on the kinetochore. The kinetochore is part of a chromosome and is important in the segregation of chromosomes in cell division. The area of particular interest was CENP-A, and the phosphorylated version of CENP-A. It was believed that these molecules should be co-localised, however, the STED images we acquired appeared to show that this was not the case. In order to confirm this observation, we needed to rule out the possibility that the visual shift was caused by the fluorophores, or by the difference in wavelength of light that the primary, and depletion laser emit.

Figure 2 shows the non-co-localization of CENPA, and CENPA Phosphorylation



We tested several different molecules that should also be co-localised, and found that there was no visual shift. This meant that there was a good chance that CENP-A, and the phosphorylated version of CENP-A are not always co-localised on the kinetochore. This will be looked at future in future projects.

Overall, the project lead to several conclusions and areas of future interest. We were able to optimise STED for future projects, not only within the field of cell division but also in other areas of interest. I would like to thank that the Wellcome Trust (funding body), Newcastle University Bio-Imaging Unit, and the Higgins' Lab Newcastle University for all of thier support with this project.

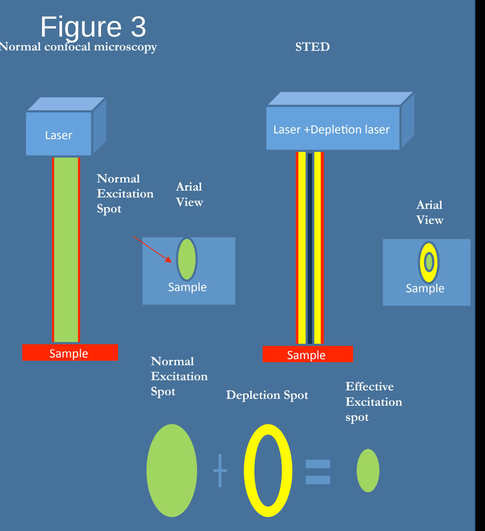
How STED works.

Normal Confocal Microscopy uses a single laser to excite a flourophore, as well as dichromic mirrors (two way) mirror. This allows us to acquire super resolution images but creates a larger excitation spot (see figure 2).

STED microscopy uses two different lasers. The first is a standard laser that excites the fluorophores which then emit light. The second is a depletion laser, which produces a donut shaped ring that depletes the emitted light from the primary laser (see figure 3) . This results in a smaller excitation spot which allows us to see small objects in better resolution.

When using two powerful lasers it is very easy to bleach the sample. This occurs when the fluorophores become over exposed to light, and no longer emit fluorescents. When this happens we can no longer acquire images using that cell.

Figure 3



Tubulin z-stack acquired in confocal microscopy

The same image in STED, note significant bleaching

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