1 List of abbreviations

APD	Avalanche photodetector
AOM	Acousto-optical modulator
HWP	Half-wave plate
PMT	Photomultiplier tube
PSF	Point spread function
pSTED	Polarisation-resolved stimulated emission depletion microscopy
QWP	Quarter-wave plate
SLM	Spatial light modulator
STED	Stimulated emission depletion
TCSPC	Time-correlated single photon counter

2 Results (outline)

2.1 Validating the laser modules

For every laser in the setup (561 nm, 640 nm, and 775 nm), I measured the polarisation state of the light at different points in the beam path and at the sample plane, by measuring the power transmitted by a linearly polarising filter at different angles relative to the beam. At the sample location, the 640 nm beam is quite well-polarised when set to a linear polarisation. When rotating a polariser, the transmitted power drops to about one fifth of the maximum. At the circular setting, though, the light is quite elliptical, with a minimum power transmitted through the polariser being about half as bright as the maximum, see Figure 5. The quality of the 561 nm polarisation is higher. When set to a linear polarisation, the power transmitted through a polariser drop from about 25 µW to a value almost equal to background levels around 0.1 µW, while the amplitude variations of the circularly polarised light are withing 14% of the mean, see Figure 6. In conventional STED, the depletion beam is circularly polarised in order to achieve polarisation-independent quenching, so I also measured its polarisation state, see Figure 7. The minimum power transmitted is about 63% of the max. (Calculate ellipticity angle, or introduce the E_{max}/E_{min} characteristic in the beginning.) (Put this stuff into a table?)

There are two other things we can take away from those figures: the power of the 640 nm laser is not very constant. This can be explained by a slow ramp up to the set power every time the laser is turned on. Secondly, the noise on the signal from the 640 laser is higher. I believe this is due to the same effect. Therefore, I also measured the power profiles of the different lasers, see (figure). The conclusion from those measurements is that the response of the lasers to the desired power set in software is not linear at low powers. If experiments need to be done at low power, a neutral density filter is required. This is luckily not the case for biological specimens with a low density of fluorophores.

Finally, we also measured the PSFs of the different lasers as a sanity check. This is shown in Figure 8. This data looks good. (Why does it look good?) (Mention how we got this data: PMT and gold beads.)

2.2 Validating the detection module

First off, I checked the polarisation dependence of the APDs. To do so, I ensured the light coming into the APDs was unpolarised by illuminating a sample of TetraSpec beads with circularly polarised 561 nm light, since that was the closest to circular that we could get. Then I put a polarising filter after the detection waveplates and measured the signal from the APD as a function of the incoming polarisation, see Figure 9. For both APDs, there is a difference of about 4%.

To do:

- Should I do min/max like before? That would also be closer to a G factor, I guess.
- APD2 and power meter pol sensitivity.
- Correct for ellipticity of 561 polarisation

Second, I tested the effect of the detection waveplates on the polarisation state of incoming light. Linearly polarised input light was achieved by putting a polariser (P1) in the sample location and illuminating it with the microscope's top lamp. Then the intensity was measured by the APDs, which was dependent on the angle of a polariser located after the last waveplate (P2). Using Jones calculus, we know that two QWPs and a HWP can form a proper polarisation rotator (ref methods), but the default calibration (a function that maps the total rotation angle to the angles the waveplates have to take on) is unable to do so. I determined that a better calibration sets the (non-fixed) QWP to an angle of 50° and the HWP to half the rotation angle. The angle of the QWP was chosen such that it is aligned with the fixed QWP at the microscope end. The behaviour of both calibrations is shown in Figure 1. The new calibration works as expected.

Finally, I also measured the polarisation characteristics of the POL cube. I generated linearly polarised light as in the previous paragraph, but left out P2. Then I rotated the polarisation using the detection waveplates and measured the intensity of light reflected and transmitted by the POL cube into APD1 and APD2 respectively. Apparently, the degree to which the POL cube can split the signal is strongly dependent on the polarisation of the light at the sample (the angle of P1), Figure 2. The cause of this effect is not immediately obvious, but is most likely due to some birefringence present in the POL cube. (Is that true?)

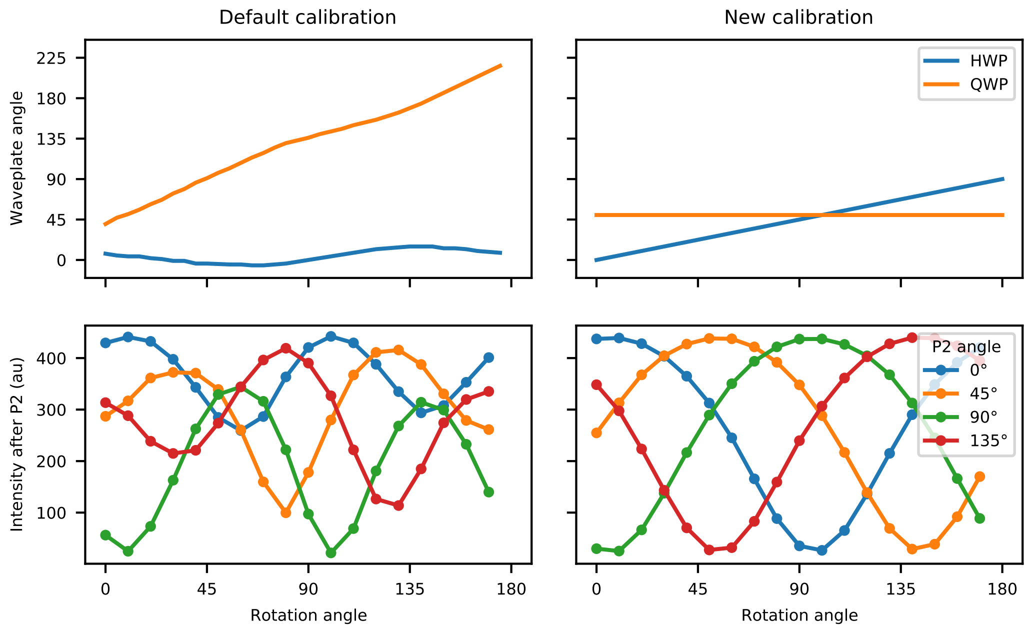

Figure 1: text

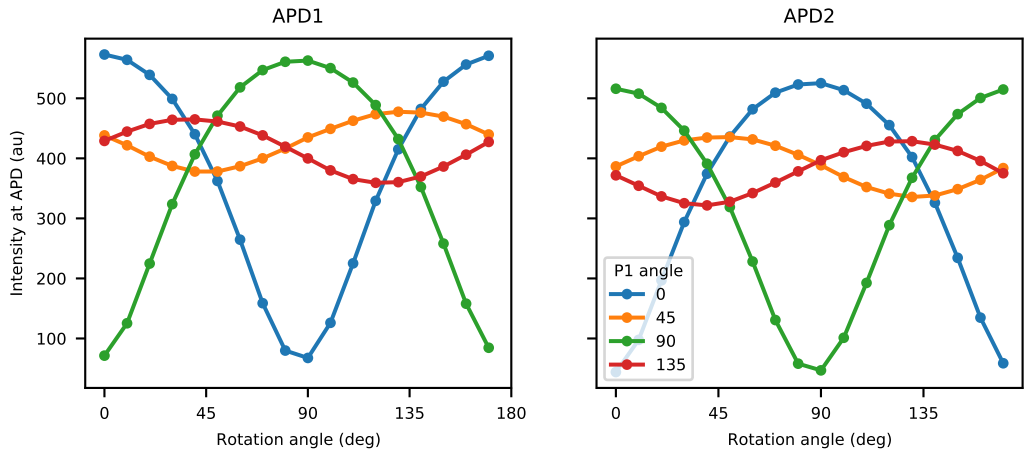

Figure 2: The POL cube distorts the polarisation state of incoming light. APD should measure the vertical component of the light (reflected by the POL cube), and APD2 should measure the horizontal component (transmitted). The detection waveplates were used to rotate incoming light, which was polarised along an angle shown in the legend.

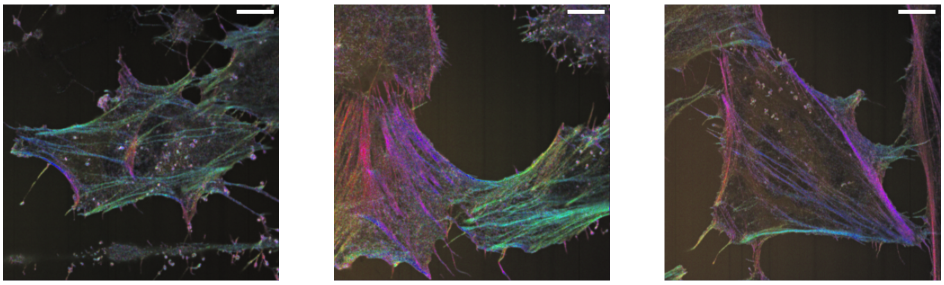

Figure 3: Polarisation microscopy images of three different cells. Scale bars 10 μm.

2.3 Demonstration of conventional polarisation microscopy

See Figure 3.

To do:

- Colour wheels in the images
- sSTED + polarisation
- Can I get some quantitative results? (e.g. histograms of orientation/degree of polarisation, weighted by intensity)

2.4 Polarisation-resolved STED microscopy

- Aligning the QWP
- Finding the HWP zero point
- pSTED PSF as a function of polarisation
  - Power
  - Ellipse orientation
  - eccentricity
  - displacement
- pSTED controls:
  - beads: circular excitation, linear quenching (March 23)
  - beads: linear excitation, linear quenching

3 A note on laser safety

The 775 nm line is a class 4 laser source. Under normal operation, the user is protected from it. However, when calibrating the STED beam or placing new components in the beam path, it is theoretically possible for the collimated laser beam to be reflected into the user's eyes. A high-powered laser beam can do permanent damage to the skin and retina, so we have to make sure we stay below the limits imposed by the Work Environment Agency's (Arbetsmiljöverkets) limits [?]. These regulations set forth three main conditions to calculate the Maximum Permissible Exposure (MPE) of a pulsed laser, see table 2.6 of the regulations. Important values and formulas about our setup, as well as the limits provided by the Work Environment Agency are provided in Table 1 and in the text below.

Table 1: Operating characteristics of the 775 laser line and relevant safety parameters.

Quantity	Symbol	Value
Beam radius	r	0.5 mm
Pulse width (FWHM)	τ	1.3 ns
Pulse repetition frequency	f	40 MHz
Pulse energy	E_{pulse}	31 nJ
Average power	P_{avg}	1.25 W
Thermal correction time	T_{min}	18 µs
Ca	C_a	1.41
Cc	C_c	1
Ce	C_e	1

Rule 1: The dose of a single pulse must not exceed the single-pulse MPE. The pulse dose H_{pulse} of the 775 nm laser at full power is

$$H_{pulse} = \frac{E_{pulse}}{2\pi r^2} \approx 39\,\mathrm{mJ/m^2}, \tag{1}$$

whereas the MPE equals

$$H_{pulse}^{MPE} = 5 \times 10^{-3} C_a C_e = 7.1\,\mathrm{mJ/m^2}. \tag{2}$$

This formula is found in table 2.2 of the regulations.

Rule 2: The dose of a single pulse may not exceed the thermally-corrected MPE. This weighs the pulse MPE with the amount of pulses in an interval T_{min}. The number of pulses in such an interval is $n = f \cdot T_{min}$, so

$$H_{thermal}^{MPE} = n^{-1/4} H_{pulse}^{MPE} = 1.3\,\mathrm{mJ/m^2}. \tag{3}$$

Rule 2 is therefore more strict than the rule 1. For safe operation, the laser must be ran at a power below 3.3% ($= H_{thermal}^{MPE}/H_{pulse}$).

Rule 3: The cumulative dose for a group of pulses in an interval of time t must not exceed the MPE for a single pulse of that time. Taking the necessary values from tables 2.2 and 2.3, the cumulative MPE is defined as

$$H_{cum}^{MPE}(t) = \begin{cases} 5 \times 10^{-3}\, C_a C_e & t < 18\,\mu\mathrm{s}, \\ 18 t^{0.75}\, C_a C_e & 18\,\mu\mathrm{s} < t < 10\,\mathrm{s}, \\ 10t\, C_a C_c & t > 10\,\mathrm{s}. \end{cases} \tag{4}$$

The actual dose, on the other hand, is

$$H(t) = \lfloor ft \rfloor H_{pulse}, \tag{5}$$

where $\lfloor x \rfloor$ is the flooring function, which rounds down numbers to the nearest integer less than x. This function is plotted in Figure 4, from which it can be seen that the laser is only safe to use at 0.0005% capacity. Since the minimum laser power offered by the software is .05%, OD2 goggles should be worn to guarantee safe operation of the 775 nm laser.

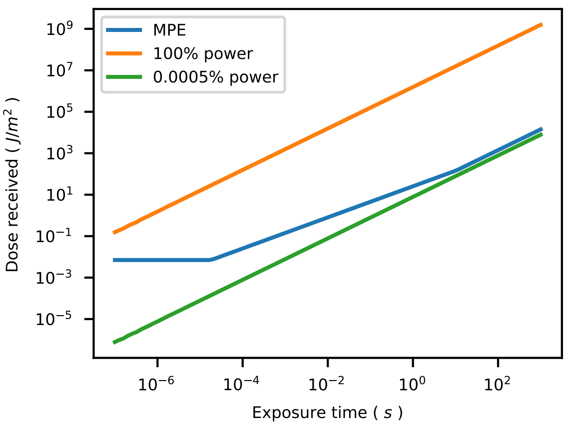

Figure 4: Maximum permissible and actual exposure to the collimated STED beam as a function of exposure time.

4 Supplemental figures

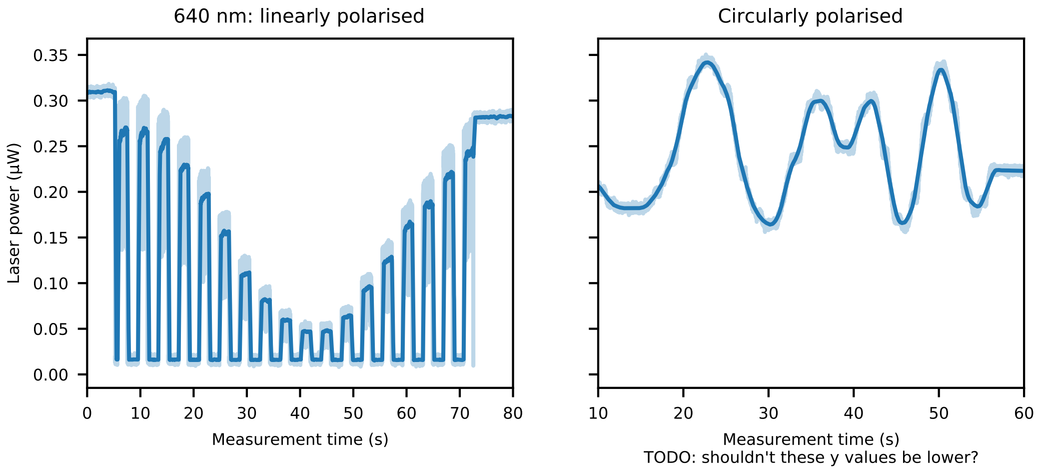

Figure 5: Polarisation characteristics at the sample plane of the 640 laser. **Left:** power transmitted through a stationary polariser aligned to maximise transmission of the 640 laser set to an polarisation of 0° (vertical in the sample plane), while the laser beam rotates. **Right:** power transmitted through a manually rotating polariser, while the beam is set to circular polarisation.

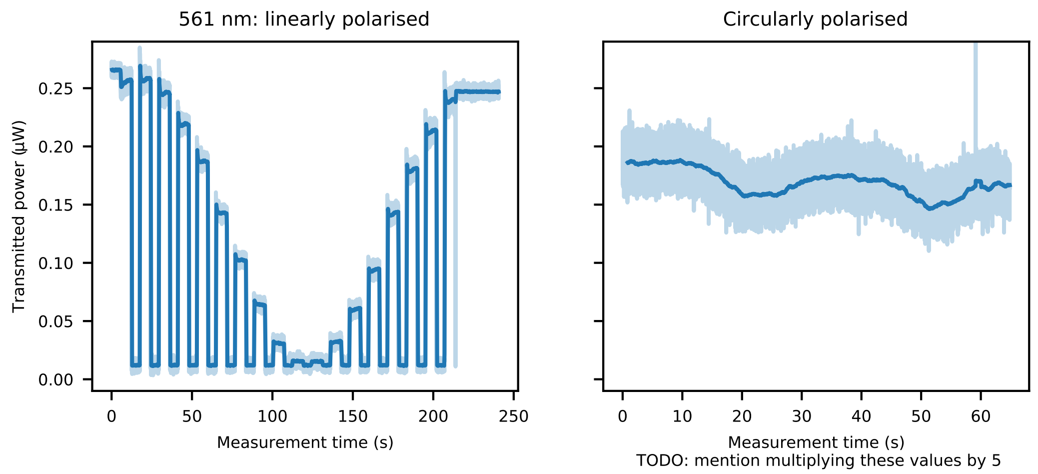

Figure 6: Polarisation characteristics at the sample plane of the 561 laser. Left and right panes are the same as Figure 5. (As the data on the left was acquired at 50% laser power to increase the signal at minimum transmission, but the data on the right was acquired at 10% laser power for safety reasons, the data on the right has been multiplied by a factor of 5 to allow comparison between the two figures. This also increased the noise.)

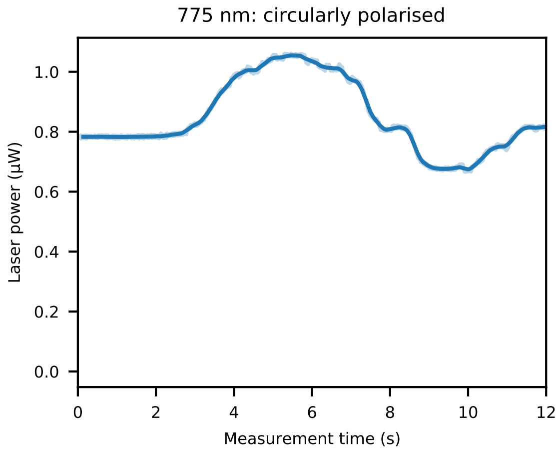

Figure 7: Polarisation characteristics at the sample plane of the depletion beam, without pSTED optics. As its polarisation state cannot be manipulated through the software, only circular polarisation is characterised by manually rotating a polariser at the sample plane and measuring the transmitted power.

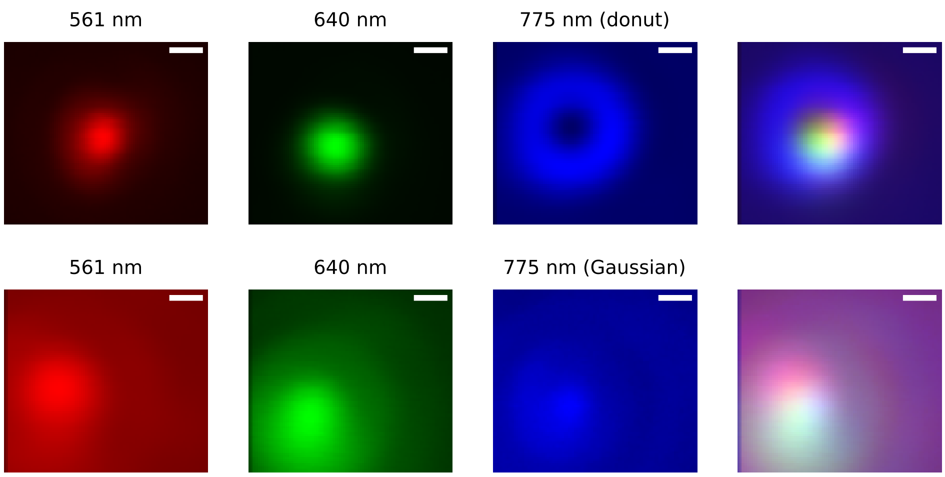

Figure 8: Point spread functions of the different lasers at different SLM configurations, by measuring the reflection from 100 nm wide gold beads. Scale bars 200 nm. (show better data from 26 march)

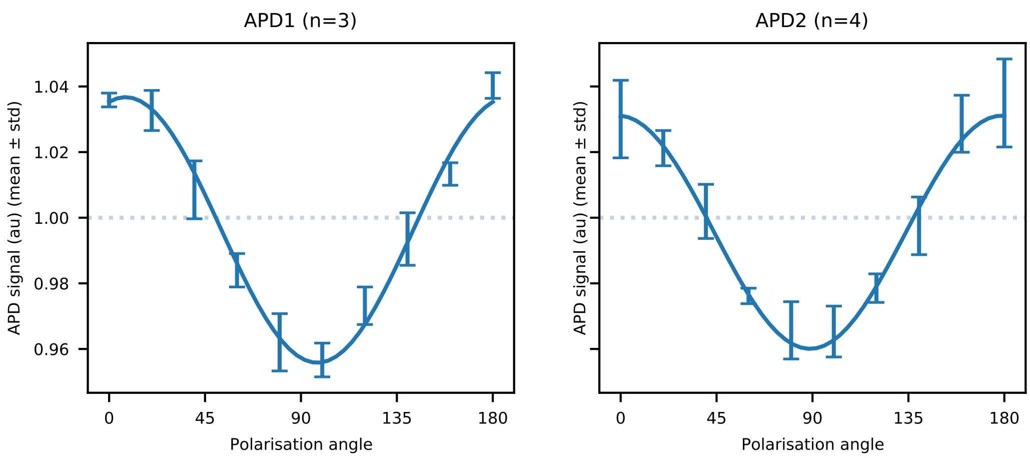

Figure 9: Dependence of the signal from APD1 on the angle of polarisation of incoming light.