Developing a new green photoactivatable fluorophore HbGFP

Tina Sarapa.
Supervisor: Dr Colin Rickman, IB3 institute

1. Introduction: Super-resolution microscopy (SRM) has revolutionised microscopy, allowing scientists to observe events at a much smaller scale, e.g. at the molecular level in the cellular organisation of biological structures. At present there is only one photoactivatable green fluorophore (PA-GFP), which has been used in photoactivation localisation microscopy (PALM). However, it is not ideal as it is also readily activated by both room lights and its excitation wavelengths. The protein HbGFP was initially isolated from a marine comb jelly organism – and is a potential candidate for a green fluorophore used in PALM. Previous work has indicated that HbGFP can change its oligomeric state upon illumination.

To make HbGFP a good candidate for use in PALM, spectrofluorimetric characterisation needed to be performed to determine its nature of excitation and emission, as well as PALM in different environments (different bacteria lines, different media etc)

2. Project aims: To develop and study the biophysical and biochemical properties of a new green photoactivatable fluorophore HbGFP.

3. Key techniques used in the project:

- Recombinant protein purification
- Protein expression in different bacteria lines
- Spectrofluorimetry
- Single molecule localisation microscopy (PALM)
- Image analysis

Figure 1: The structure of HbGFP from the AlphaFold prediction software.

References: Haddock, Steven H D et al. “A photoactivatable green-fluorescent protein from the phylum Ctenophora.” Proceedings. Biological sciences vol. 277,1685 (2010): 1155-60. doi:10.1098/rspb.2009.1774

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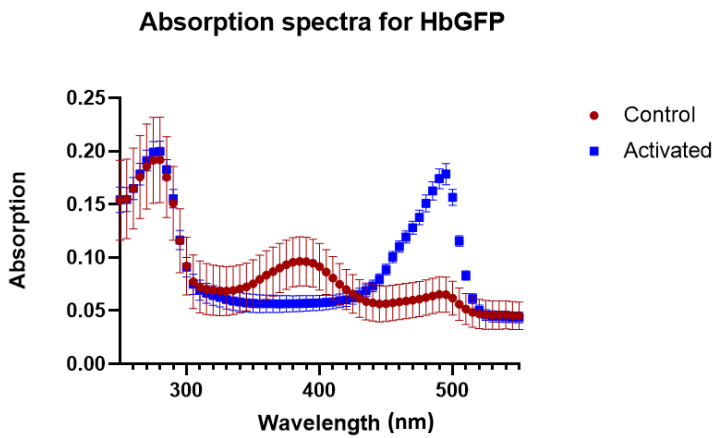
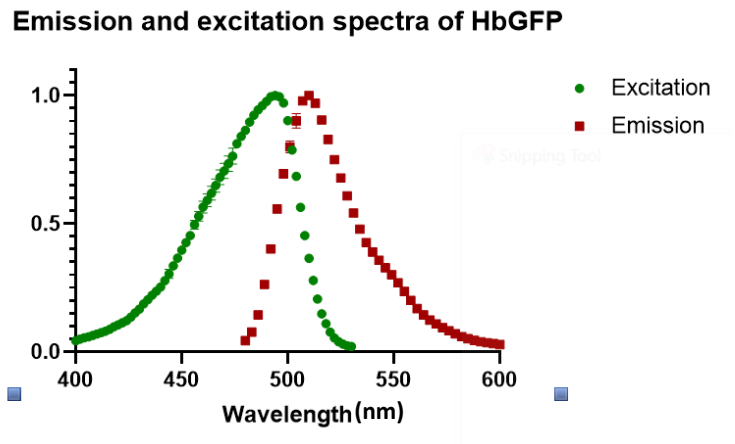
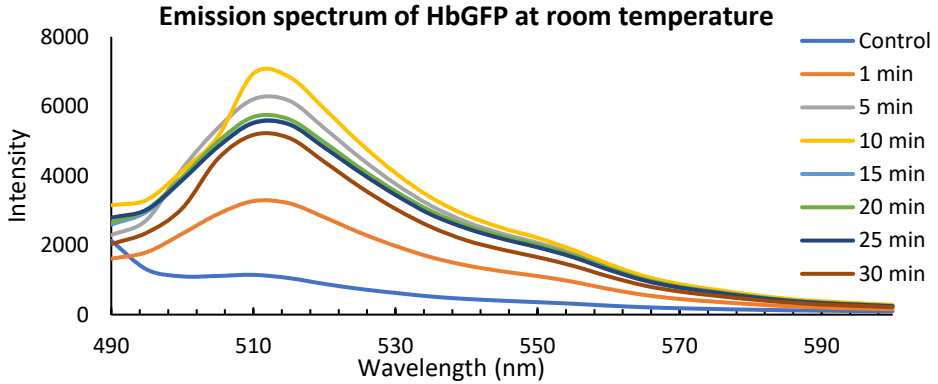


5. Absorption spectra for HbGFP – clear that the fluorophore shows shift in absorption upon activation (from 390nm to 490nm)



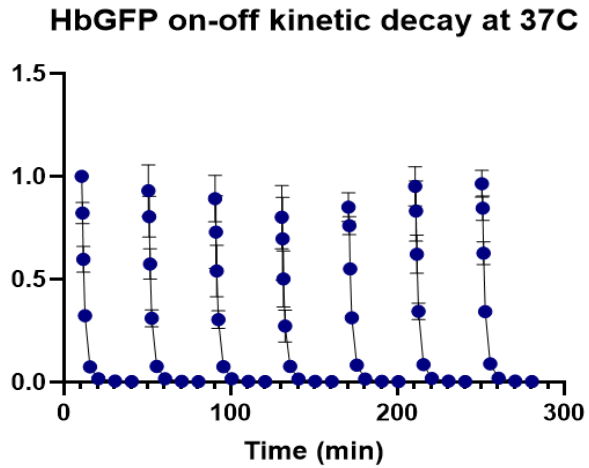
6. The emission and excitation fluorescence spectra for HbGFP – shows that the fluorophore reaches its maximum excitation at 495nm and maximum emission at 510nm.



8. Emission spectrum for HbGFP with variable activation – the fluorophore was activated at 405nm for varying lengths of time. This data set shows that the fluorophore reaches its maximum activation state after roughly 10 minutes. The experiments at 37°C showed similar results.

9. Spectrofluorimetry conclusions:
The fluorophore displays a shift in absorption upon activation (from 390nm to 490nm).
The emission/excitation spectra shows excitation at about 495nm and emission at 515 nm. This shows that HbGFP is ideally suited for detection on the microscope using an eGFP channel.

Graph 7 shows the on/off switching experiment done at 37C when the protein was switched on with 405nm laser for 10min, then its fluorescence decay was measured for 30 min, then switched back on – cycle was repeated 7 times. Interestingly, HbGFP can repeatedly cycle between max on and off states with no loss of fluorescence.



7. Repeated maximal activation and decay of HbGFP fluorescence - graph of the exponential decay in fluorescence of the HbGFP- taken at 37°C, with the fluorophore activated for 10mins under the 405nm light, then decay in fluorescence recorded (repeated 7 times). Provided below is the table for activation temperature vs the half-life of the fluorescence for HbGFP – shows that the protein stays fluorescent for much longer. at room temperature.

Activation temperature	Half-life (s)
21	1956
37	77.76

References: Haddock, Steven H D et al. "A photoactivatable green-fluorescent protein from the phylum Ctenophora." Proceedings. Biological sciences vol. 277,1685 (2010): 1155-60. doi:10.1098/rspb.2009.1774

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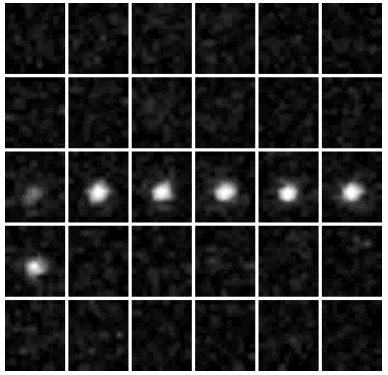
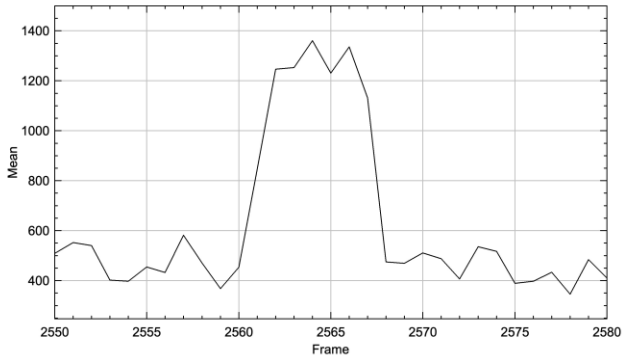
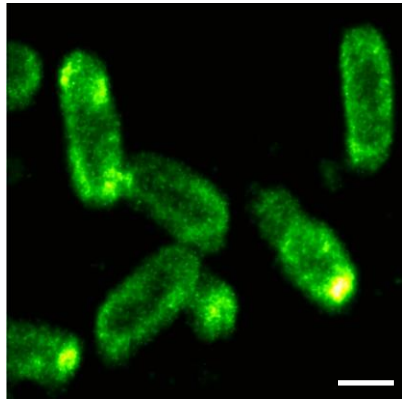
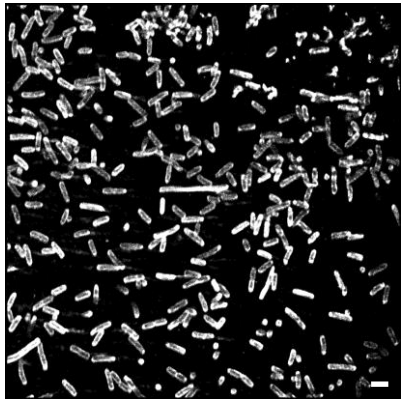




10. A flash of a single molecule captured in 30 frames – HbGFP fluorophore on glass (*left panel*, immobilised on poly-D-lysine). The intensity profile over time is shown (*right panel*).





11. PALM of HbGFP expressed in BL21 cells - BL21 were grown in M63 media, immobilised on glass using poly-D-lysine and imaged under PALM acquisition settings. Zoomed in on the individual cells (*left panel*, false coloured) with each spot representing a single protein molecule. The whole field of view is shown (*right panel*). Scale bars are 500 nm (*left panel*) and 1 μm (*right panel*)

12. Microscopy conclusions:

HbGFP was successfully detected on the microscope using the GFP excitation/emission settings. The exact location of a single molecule was pinpointed and its intensity was traced, as shown in section 10.
HbGFP was successfully expressed in BL21 cells, grown in M63 media and imaged on the Olympus Cell Excellence. There was some spectral bleed through to the red Cherry channel however, which may be a potential problem. The same cells, when grown in LB media, showed a lot of inclusion bodies due to over expression.
The fluorophore was also expressed in XL10 Gold cells, grown in M63 and LB media, but the expression was too poor and slow for it to be useful on the microscope.

13. Summary:

HbGFP was synthesised, purified and expressed in different types of cells – BL21 and XL10 Gold. It was imaged in on the glass as a pure protein, then in BL-21 cells – both experiments were successful.
Spectrofluorimetry experiments showed that HbGFP does not lose its fluorescence when cycled between on and off stages – something that would be very useful in microscopy imaging.

14. Future work:

It would be interesting to perform random mutagenesis on the HbGFP to see if it will improve the brightness or cause a shift to another wavelength – perhaps, a random mutation would shift its wavelength, or improve its brightness or biochemical properties. Expression and study of HbGFP in mammalian cells will also be highly beneficial for its further development.