Low-temperature plasmonically enhanced single-molecule spectroscopy of fluorescent proteins

O. Evans[1], **V. Singh**[1], **O. Aksakal**[2], **D. Jones**[2], **P. Borri**[2], **W. Langbein**[1]

1. *Cardiff University, School of Physics and Astronomy, The Parade, CF24 3AA Cardiff, UK*
2. *Cardiff University, School of Biosciences, Museum Avenue, CF10 3AX Cardiff, UK*

Ideal bioimaging probes in fluorescence microscopy are bright, biocompatible, offer a large signal-to-noise ratio against autofluorescence, and can be specifically attached to target biomolecules of interest. Fluorescent proteins have been engineered towards this goal. However, a limitation of fluorescent proteins is the quantum yield; the low brightness of the probes limits the contrast between the structure of interest and the background. This limitation can be overcome using plasmonic enhancement by coupling the electronic resonances of the molecule to the plasmonic resonances of a metal nanoparticle with a dominant radiative broadening [1]. This allows an enhanced fluorescence emission of the molecule by increased absorption via near-field enhancement and increased radiative decay rate of the molecule by the Purcell effect, with photons emitted to the far field. Gold nanorods (GNRs) have a surface plasmon resonance which can be tuned by changing their aspect ratio to match the absorption and emission wavelengths of the fluorophore. Therefore, coupling GNRs to fluorescent proteins offers a route toward developing improved imaging probes. Moreover, understanding the photophysical properties of the fluorescent probe used is vital in their application. These properties can be probed at low temperatures where the coupling between the vibrations and electronic transitions is not masked by dominant homogenous broadening.

We present low temperature (5 K) and high resolution (15 pm) micro-spectroscopy of plasmonically enhanced fluorescent proteins, mRhubarb720, embedded in a thin film of polyvinyl alcohol under 632 nm excitation. mRhubarb720, a recently developed monomeric fluorescent protein, is derived from bacterial phytochrome photoreceptors and utilises the chromophore biliverdin [2]. It is the farthest red emitting fluorescent protein but has a low quantum yield. We show spectroscopic data of individual mRhubarb720 by the local excitation and emission enhancement in the 'hot spots' at the GNR tips. The zero-phonon line (ZPL) and phonon replicas of single mRhubarb720 can clearly be distinguished from the background ensemble emission. We present data using a jitter scan, as shown in figure 1.

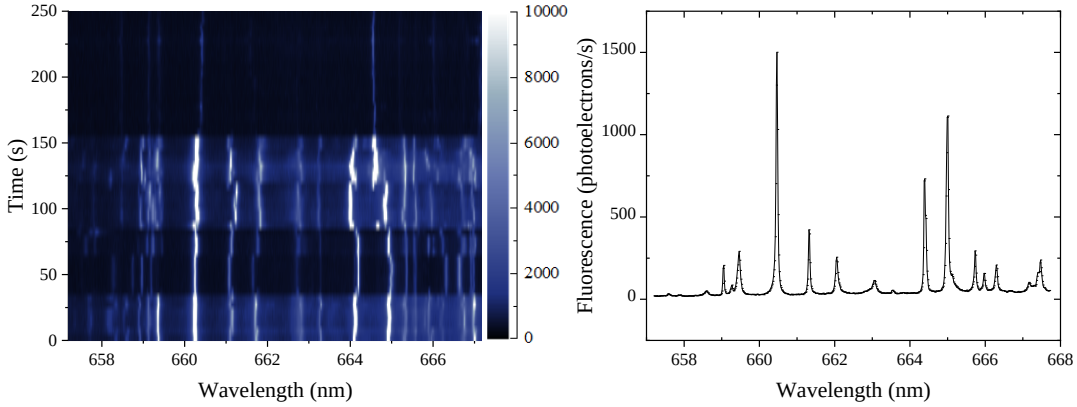

Fig. 1 Left: Jitter scan showing photoluminescence of plasmonically enhanced mRhubarb720 at 5 K over 250 seconds (5 s exposure, 50 acquisitions). The scale bar shows the photoelectron count. Right: spectrum at 100 s.

We find multiple closely spaced emission lines for a single emitter, which are switching on a longer time scale in intensity. Analysing this behaviour provides information on the spatial dynamics of the fluorescent protein within the polyvinyl alcohol polymer matrix and of the chromophore within the molecule. We speculate that the set of strong peaks are ZPLs at different conformations of the chromophore in the protein, while the switching is due to protein orientation and position changes within the GNR hotspot. This work demonstrates the ability of this method to provide spectroscopic data of individual molecules with high signal-to-noise ratio and opens avenues towards enhanced single-molecule imaging.

Acknowledgement: V. Singh has received funding by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 812992.

References

[1] Tam, F., Goodrich, G.P., Johnson, B.R. and Halas, N.J., 'Plasmonic enhancement of molecular fluorescence.' *Nano letters*, *7*(2), pp.496-501 (2007)

[2] Rogers, O.C., Johnson, D.M. and Firnberg, E., 'mRhubarb: Engineering of monomeric, red-shifted, and brighter variants of iRFP using structure-guided multi-site mutagenesis.' *Scientific reports*, *9*(1), pp.1-8. (2019)