Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Two photon spectroscopy and microscopy of the fluorescent flavoprotein, iLOV

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Supporting Figures

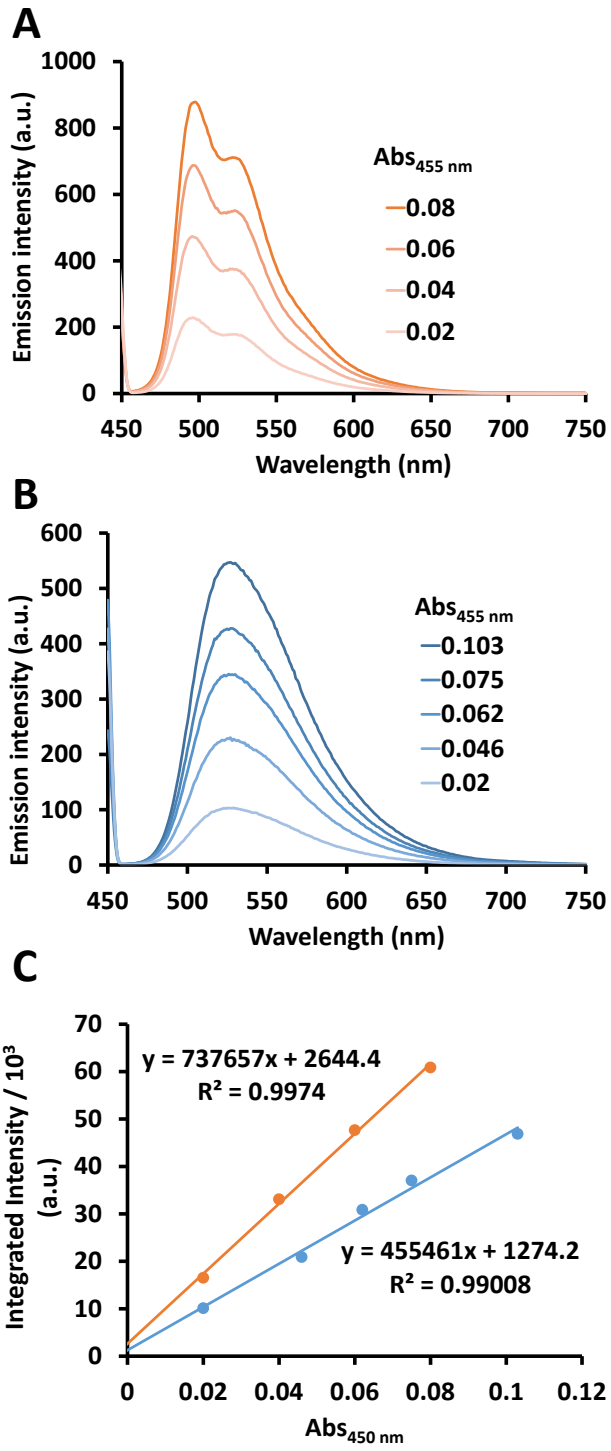

Figure S1. Fluorescence quantum yield for iLOV. Emission spectra of iLOV (**A**) and free flavin mononucleotide (FMN, **B**) as a function of absorbance at excitation wavelength, 455 nm ($Abs_{455\ nm}$). **C.** Integrated emission intensity and corresponding linear fit for iLOV (orange) and free FMN (blue). A fluorescence quantum yield of 0.35 at excitation wavelength 450 nm was calculated for iLOV using the comparative method described in the Detailed Experimental Methods (above) and adapted from.[3] For this calculation a quantum yield of 0.246 was used for FMN,[2] and an extinction coefficient, ε, for FMN and iLOV at 450 nm of 12.2 and 17.6 $mM^{-1}\ cm^{-1}$, respectively.

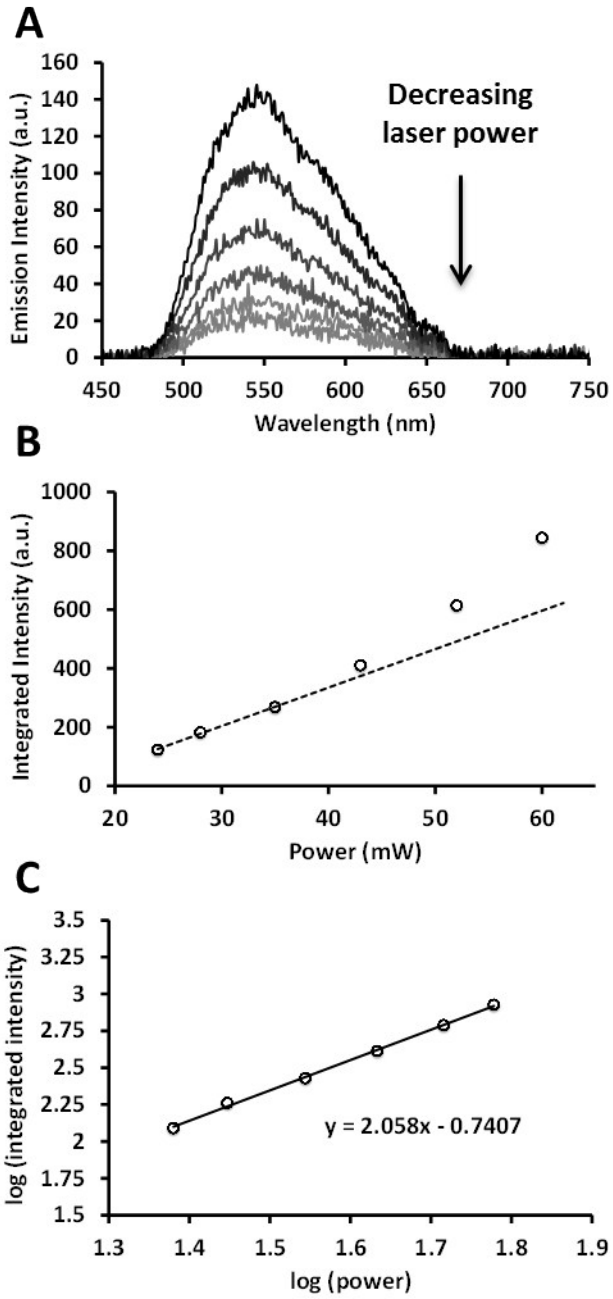

Figure S2. Two photon (860 nm) excitation of FMN. FMN emission spectra (**A**) and integrated emission intensity (**B**) as a function of laser power. The dashed line in **B** serves to illustrate deviation from a linear-dependence. **C.** $\log_{10}$(integrated emission intensity) as a function of $\log_{10}$(laser power), with corresponding linear fit. The gradient of ~ 2 confirms that the excitation is predominantly from the absorption of two non-resonant photons of 860 nm.

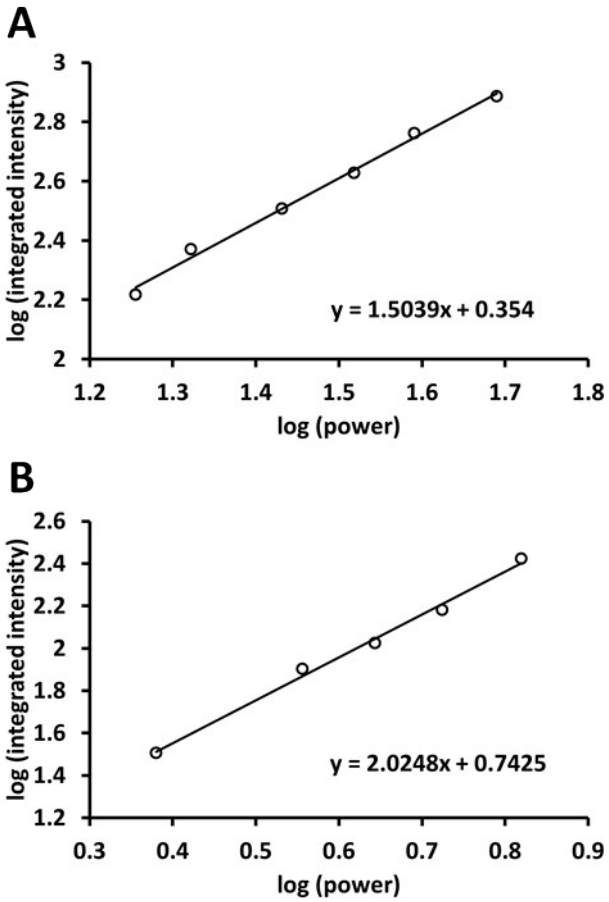

Figure S3. Two photon (730 nm) excitation of iLOV. A. $\log_{10}$(integrated emission intensity) as a function of $\log_{10}$(laser power), with corresponding linear fit. The laser power range was 18 – 49 mW. The fact that the gradient is 1.5 (*i.e.*, < 2) is most likely owing to stimulated emission by the focussed beam at higher laser powers. Whilst the direction of spontaneous emission is random, photons from stimulated emission are always directed along the incident laser path and therefore never reach the orthogonal detector. The effect is a sub-quadratic dependence. **B.** $\log_{10}$(integrated emission intensity) as a function of $\log_{10}$(laser power), with corresponding linear fit. The laser power range was 2.4 – 6.6 mW. The gradient of ~ 2 confirms that the excitation is predominantly from the absorption of two non-resonant photons of 730 nm.

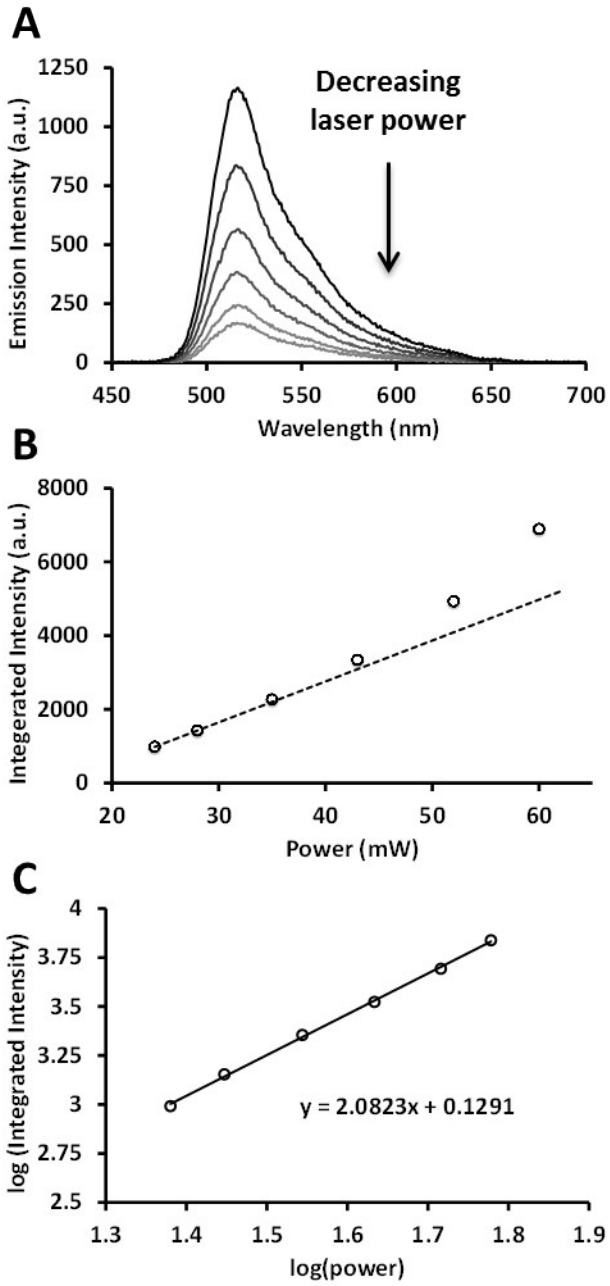

Figure S4. Two photon (860 nm) excitation of fluorescein. Fluorescein emission spectra (**A**) and integrated emission intensity (**B**) as a function of laser power. The dashed line in **B** serves to illustrate deviation from a linear-dependence. **C.** $\log_{10}$(integrated emission intensity) as a function of $\log_{10}$(laser power), with corresponding linear fit. The gradient of ~ 2 confirms that the excitation is predominantly from the absorption of two non-resonant photons of 860 nm. Equivalent data from various excitation wavelengths were used to calculate the two photon absorption cross sections for FMN and iLOV (Figure 3D of the main article).

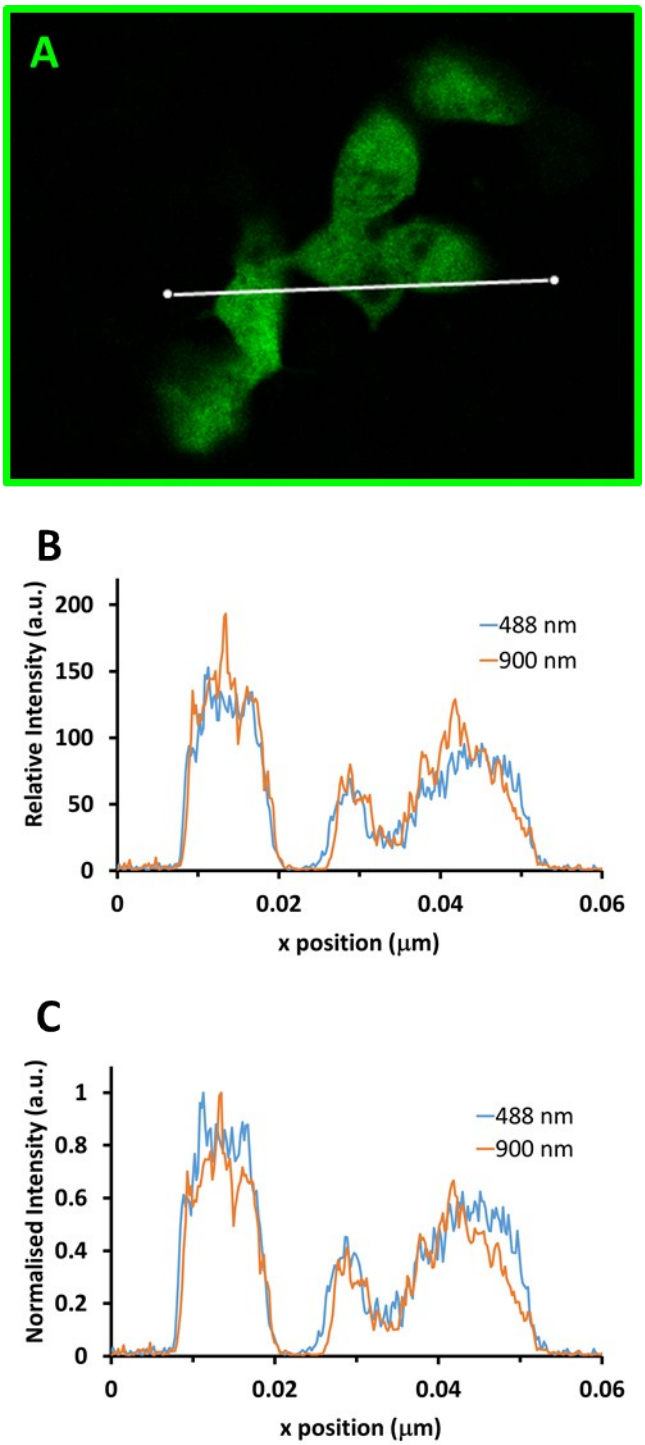

Figure S5. Fluorescence intensity profiles. A. HEK293 cells expressing iLOV (from Figure 4 of the main article). Fluorescence intensity profiles were analysed along a line of pixels (white line) for both one (488 nm) and two (900 nm) photon images. This line was chosen because it intersects several features on the image where differences in contrast are most evident. **B.** Relative fluorescence intensity as a function of x position along the pixel line in panel **A**, for both one (blue) and two (orange) photon images. **C.** The data from panel **B** normalised to the peak of the profile for each image. In panels **B** and **C** the intensity profile from the two photon image appears to have slightly sharper, narrower features than corresponding one photon image. The additional 'noise' in the one photon image could be owing to more out of focus light.

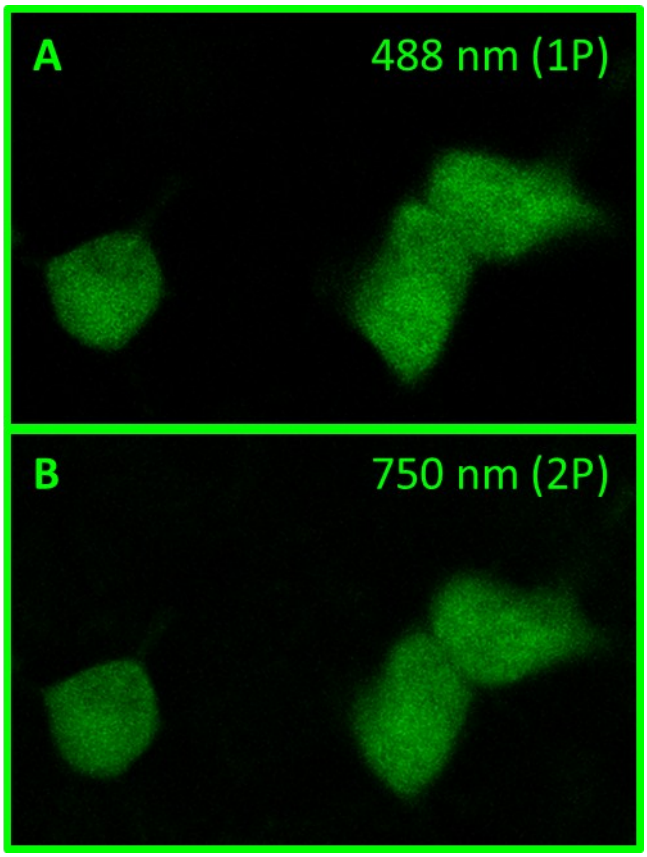

Figure S6. One (488 nm, **A**) and two (750 nm, **B**) photon images of HEK293 cells expressing iLOV. The slight gain in clarity apparent at longer wavelengths (Figure 4 of the main article) for the two photon over the one photon images is not in evidence here, despite the higher two photon cross section for iLOV at 750 nm (Figure 3D).

References

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