Two Photon microscopy
Final lab report
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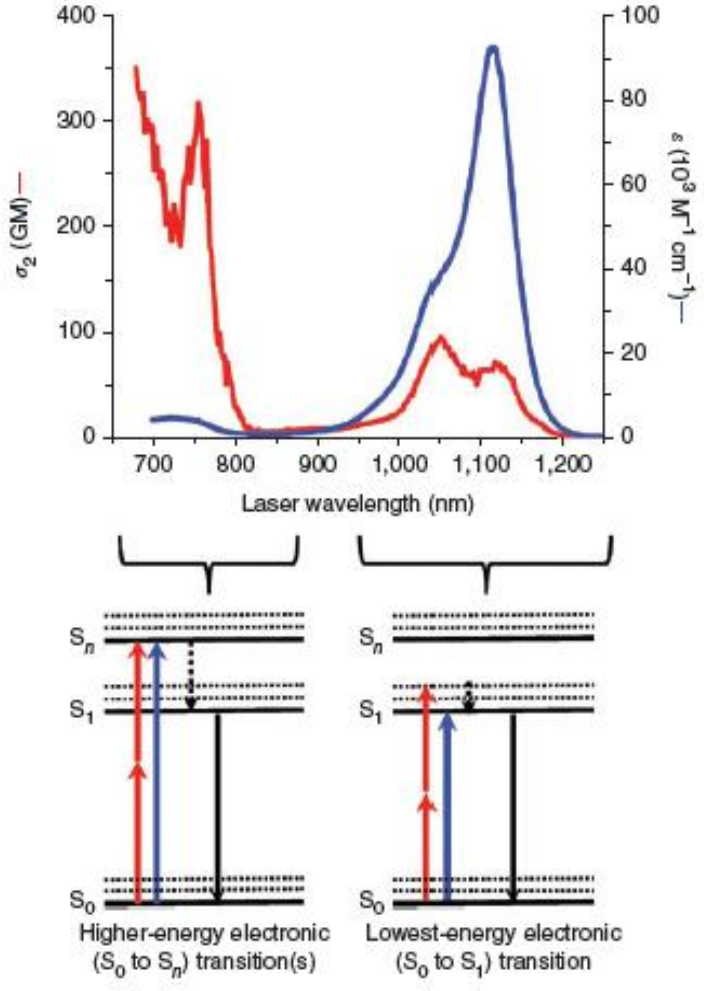
Introduction

In two-photon absorption, a molecule simultaneously absorbs two low energy photons to transition to a higher energy state. The absorption rates are low compared to the rate of single photon absorption; therefore it is only observed over stimulus spectrum that doesn't overlap with single photon absorption. After a pair of photons is absorbed, the emission process involves a fast decay to the first excited state and then emission of a photon when the molecule decays to the ground state.



Two-photon absorption properties of fluorescent proteins

Mikhail Drobizhev[1], Nikolay S Makarov[1,4], Shane E Tillo[2,4], Thomas E Hughes[2] & Aleksander Rebane[1,3]

Figure 1: Two photon cross section (red) overlayed on single photon absorption (blue) spectrum of the same flourophore. The single photon spectrum is plotted at ½ wavelength. The two Jablonski diagrams show the transitions corresponding to the peaks. [1]

Two photon microscopy is advantageous for optical sectioning, since the absorption is a non-linear process. As figure two shows, the quadratic dependence of the absorption rate results in selection of a single plane in which absorption occurs. In contrast, in single photon absorption, a constant number of molecules in each plane are excited, thus an additional aperture is required for collection.

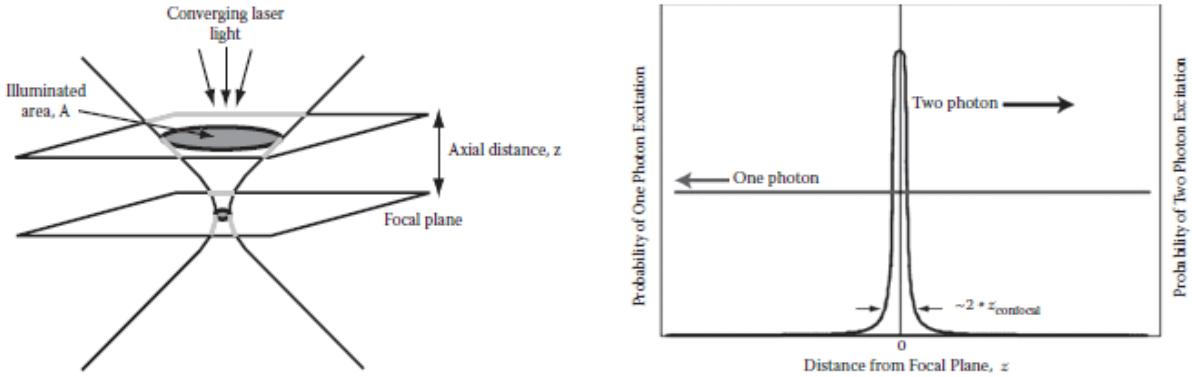


Figure 2: Due to the Quadratic Relationship between Intensity and Absorption, two photon has innately high z-resolution.

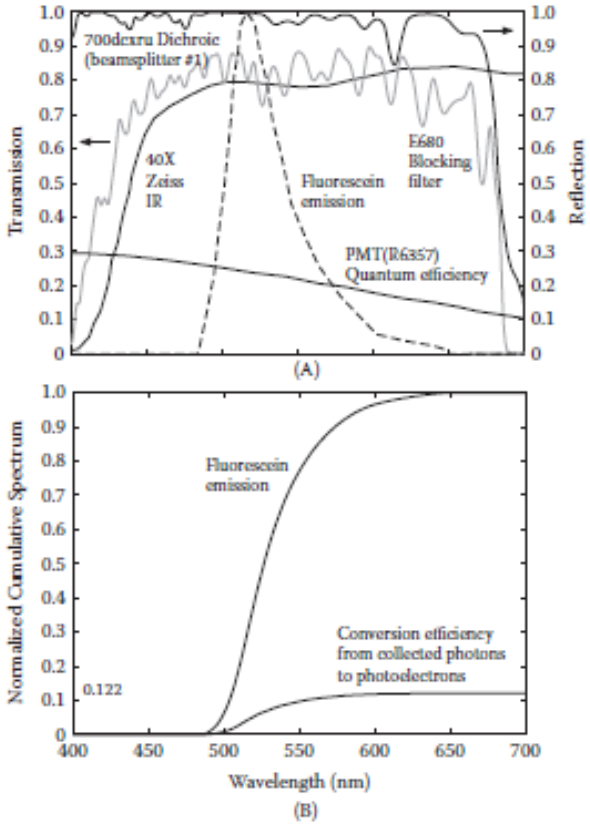


Figure 3: Photons emitted via fluorescence is much less than the reflected photon count from the incoming laser. Collection filters are necessary.

The intensity of the incident laser beam is high compared to the intensity of emitted light, thus filtering before photo detection is required to detect the low intensity signal. Since the light emitted is at a shorter wavelength than the incident light, it is possible to select a filter that selectively blocks the laser light. Figure 3 shows an absorption and emission spectrum for fluorescein, and the transmission wavelengths of an appropriate filter.

Results

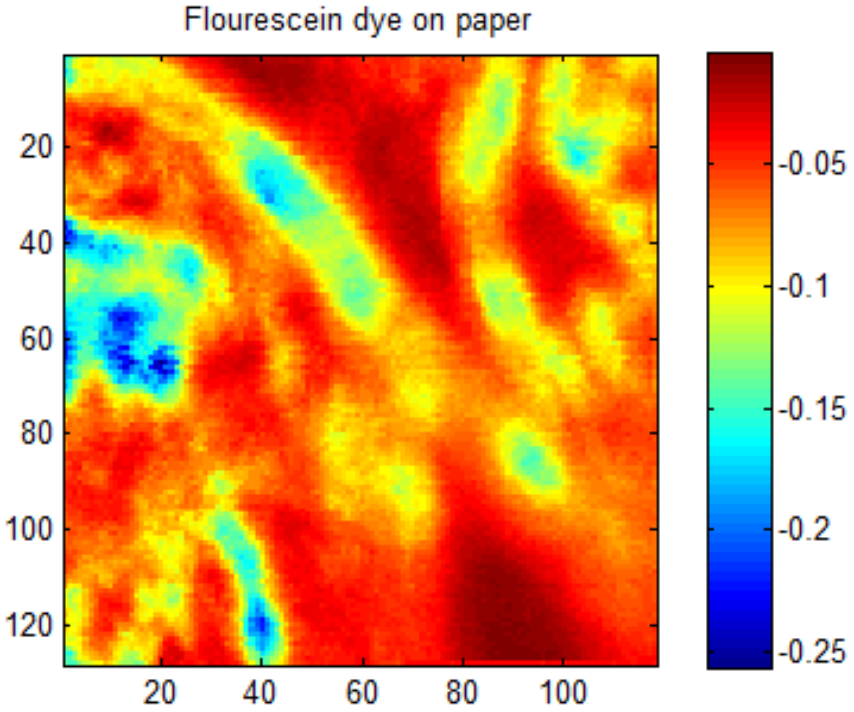


Figure 4: We were first able to see a signal using a strong fluorescent dye on paper. Structure is clearly evident.

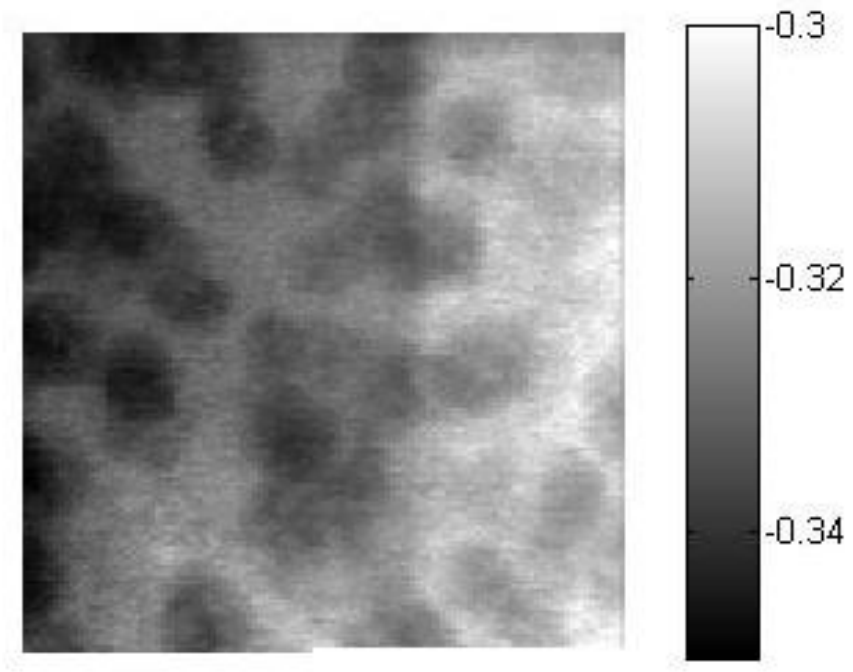


Figure 5: Succesful imaging of Mouse brain slice with Nuclear Stain of Alexa. Negative values denote higher intensity.

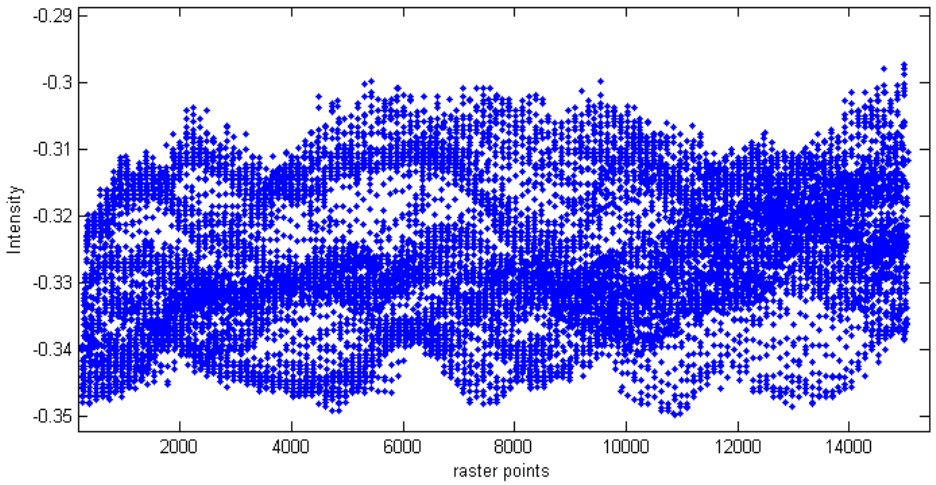


Figure 6: Corresponding raw data from the scanning system. Figure 5 generated by rastering.

Discussion

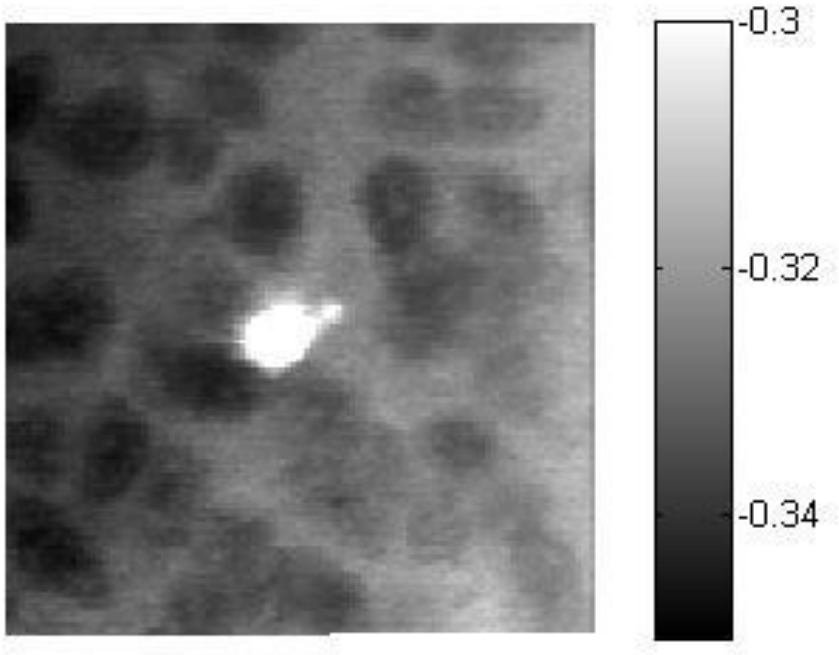


Figure 7: Prolonged exposure to laser resulted in Bleaching. Image taken at the same location as figure 5.

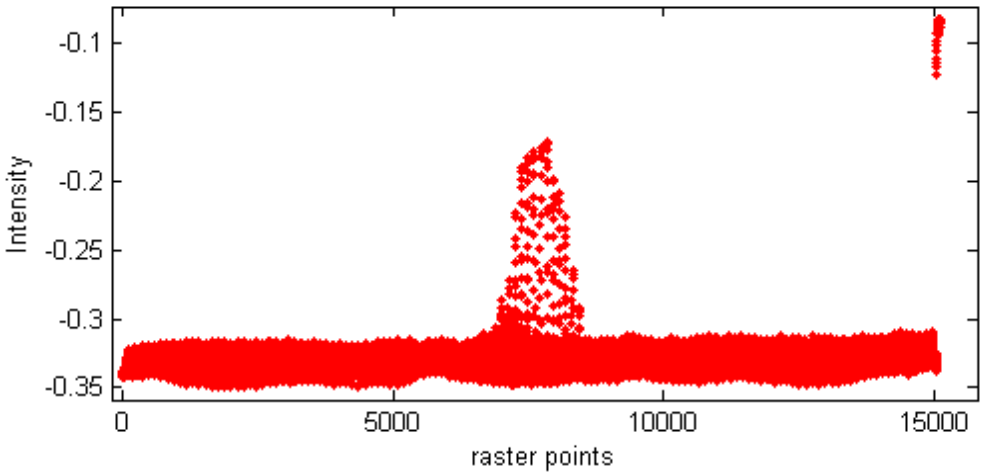


Figure 8: Bleaching. Raster plot of the image in Figure 7

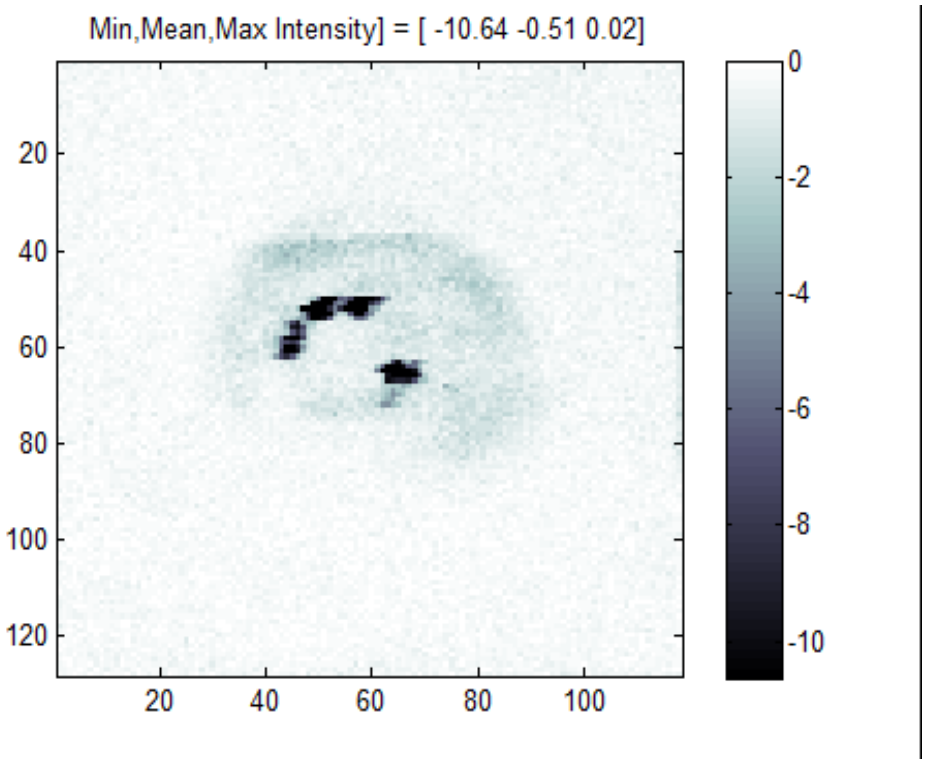


Figure 9: Image after maximally prolonged exposure to the laser. The spots of higher intensity may be a result of distortion of the sample.

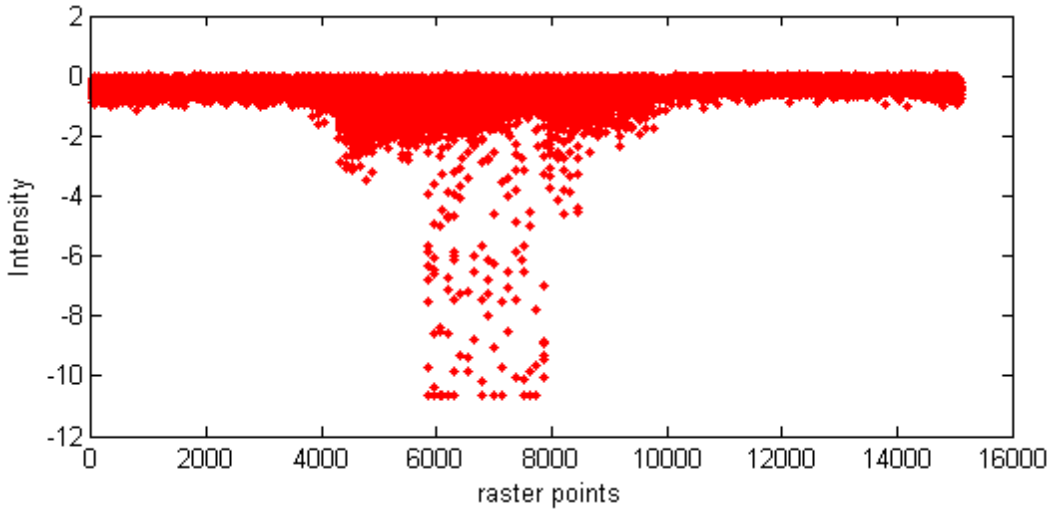


Figure 10 : Raster of figure 9, showing the saturation of the signal intensity at the peak values.

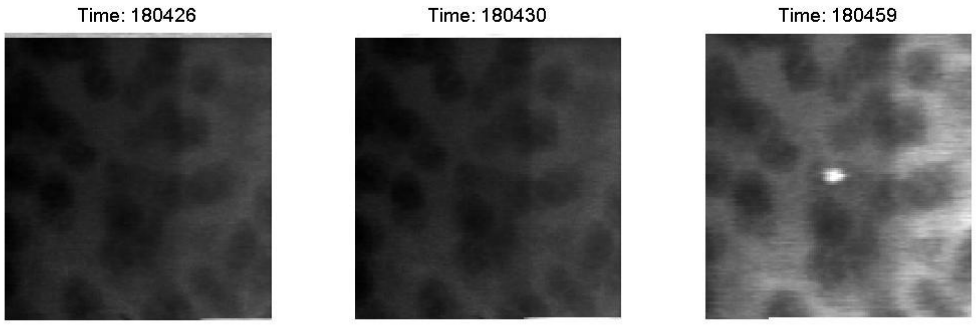


Figure 11: Three scans showing the time course of photobleaching. All images are taken at the same spot. Rastering was stopped between the second and third image. Titles are times in HHMMSS. Photobleaching occurs within 30 seconds upon exposure.

References:

1) Two-Photon absorption properties of fluorescent proteins. M Drobizhev, NS Makarov, SE Tillo, TE Hughes, A Rebane Nature Methods (2011)

2) *In vivo* two-photon laser scanning microscopy with concurrent plasma-mediated ablation: Principles and hardware realization. Tsai & Kleinfeld.