Imaging depth extension via structured light

Temporal focusing, invented independently by the groups of Silberberg and Xu is a method for performing axially-resolved multiphoton excitation of a sample without the need for scanning a focused spot [4, 5]. With sufficient power, large planes can be illuminated simultaneously, allowing high-resolution depth-resolved multiphoton microscopy to be performed with unprecedented frame rates. Owing to the need to image the resulting fluorescence onto a camera, temporal focusing cannot achieve the same tissue penetration depths as conventional point-scanning multiphoton microscopy, however the extent of this deficit has not been quantified in real tissues until our recent publication [6].

In order to perform the comparison, focal stacks were taken of various fixed mouse organs which had been stained with Hoechst 34580, a nuclear stain which provided a consistent image with high frequency content throughout all the tissues under comparison. The imaging systems were as similar to each other possible, sharing the objective, piezo actuator, dichroic and emission filter, and while the systems were physically separate, they were built around the same design of microscope body. Excitation wavelength was 800nm in both cases.

Given the large number of images that will require comparison, we set out to develop an automated method that would be based on accepted optical imaging theory, without the need for arbitrary thresholds or parameters to be set. The algorithm is described as follows: first, the image is cropped so that it is square. The 2D FFT is then taken, and a 'radial average' is then performed, whereby all spatial frequencies with the same vector magnitude are averaged together to form a crude estimate of the MTF at a given spatial frequency. The resulting plot of estimated MTF vs spatial frequency should have an approximately monotonic decay. This procedure is repeated for each image in the focal stack, and the penetration depth is defined for a particular spatial frequency (arbitrarily selected as 100lp/mm for the purposes of this study) as the point at which the signal drops within three standard deviations of the background. The entire algorithm is illustrated in **Figure 1**.

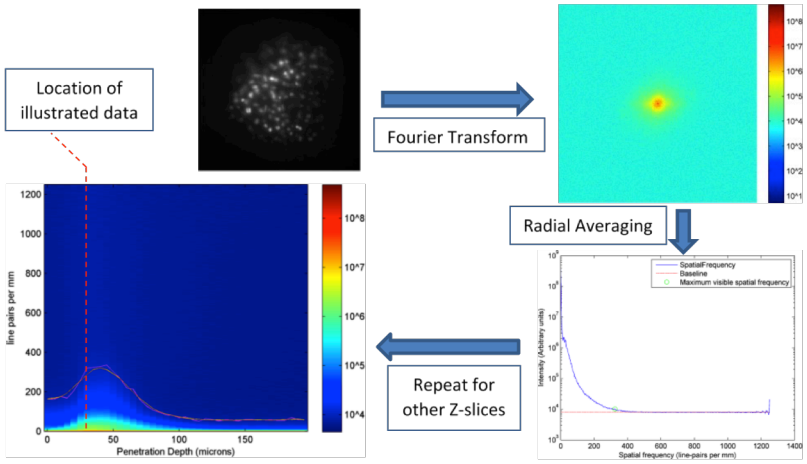

Figure 1: Illustration of how the penetration depth is estimated. From each image in the Z-stack, the Fourier transform is taken, a radial average is performed and the baseline is estimated. The highest observable spatial frequency is then found and plotted as a function of penetration depth. The data for all the other Z-positions is then combined, and the maximum observable frequency plotted as a function of depth (magenta line). A 5-element moving average filter is applied in order to minimize the effect of any unusual outlier results (yellow line).

It is clear from the data in **Figure 2** that, depending on the sample, scanning two-photon microscopy can achieve approximately twice the penetration depth of temporal focusing when subject to practical issues such as sample damage. It was also possible to compare organs in terms of the achievable penetration depth; for the previously mentioned maximum spatial frequency of 100 line-pairs per mm, the heart consistently demonstrated the lowest penetration depth, around 50μm for temporal focusing and 120μm for scanning two-photon microscopy. The lungs, liver and kidneys were all very similar, with penetration depths of approximately 90μm for temporal focusing and 150μm for scanning two-photon

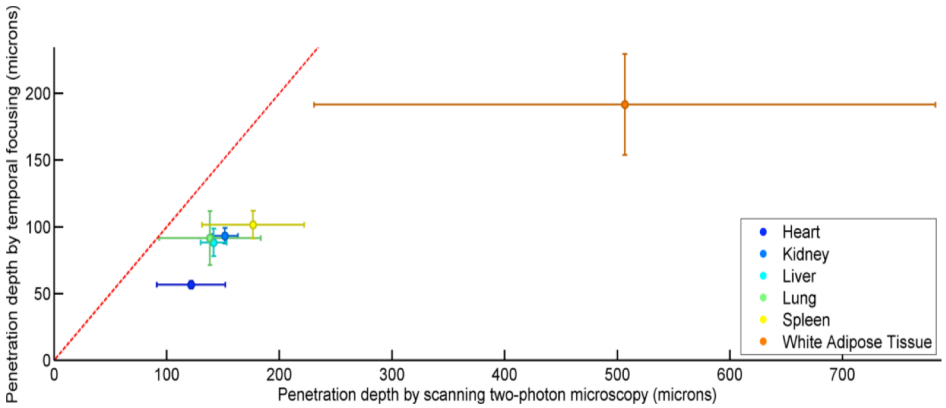

Figure 2: Comparison between temporal focusing microscopy and scanning two-photon microscopy. Each point corresponds to a different organ. The horizontal coordinate of each point is the mean penetration depth of three Z-stacks obtained using scanning two-photon microscopy, and the error bars indicate the standard deviation of these points. The vertical coordinate and error bars are determined from three Z-stacks obtained in the same manner, but using temporal focusing microscopy. A dashed line with a gradient of unity and an intercept of zero indicates where points would lie if the penetration depths of temporal focusing microscopy and two-photon microscopy were equal.

microscopy. The spleen is slightly easier to penetrate, at around 100μm for temporal focusing and 180μm for scanning two-photon microscopy, but the low absorption and scattering due to the large lipid droplets in white adipose tissue meant that penetration depths of nearly 200μm for temporal focusing and over 500μm for scanning two-photon microscopy were possible. The especially large error bars were due to the large variation in measured scanning two-photon penetration depths; values of 190μm, 635μm and 695μm were recorded, and the 190μm result strongly skewed the results. This extremely large variation was, in turn, caused primarily by the sparse staining of the sample; the higher resolution of the temporal focusing image made it possible to locate the nuclei more readily, whereas the lower resolution and smaller field-of-view of the raster-scanning two-photon image meant that the compressed nuclei were harder to distinguish.

In conclusion, we have created a method to automatically determine the achievable penetration depth in both point-scanning and temporal focusing microscopy, and used the technique to determine the tissue penetration depth for a number of tissues. While the speed increase of temporal focusing is considerable, it is clear that the achievable penetration depth is approximately one half of that achievable with point-scanning two-photon microscopy.