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Post-processing strategies in image scanning microscopy

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ABSTRACT

Image scanning microscopy (ISM) coupled with pixel reassignment offers a resolution improvement of 1.41 over standard widefield imaging. By scanning point-wise across the specimen and capturing an image of the fluorescent signal generated at each scan position, additional information about specimen structure is recorded and the highest accessible spatial frequency is doubled. Pixel reassignment can be achieved optically in real time or computationally *a posteriori* and is frequently combined with the use of a physical or digital pinhole to reject out of focus light. Here, we simulate an ISM dataset using a test image and apply standard and non-standard processing methods to address problems typically encountered in computational pixel reassignment and pinholing. We demonstrate that the predicted improvement in resolution is achieved by applying standard pixel reassignment to a simulated dataset and explore the effect of realistic displacements between the reference and true excitation positions. By identifying the position of the detected fluorescence maximum using localisation software and centring the digital pinhole on this co-ordinate before scaling around translated excitation positions, we can recover signal that would otherwise be degraded by the use of a pinhole aligned to an inaccurate excitation reference. This strategy is demonstrated using experimental data from a multiphoton ISM instrument. Finally we investigate the effect that imaging through tissue has on the positions of excitation foci at depth and observe a global scaling with respect to the applied reference grid. Using simulated and experimental data we explore the impact of a globally scaled reference on the ISM image and by pinholing around the detected maxima, recover the signal across the whole field of view.

1. Introduction

'Image scanning microscopy' (ISM) differs from conventional scanning microscopy techniques in that an image of the emission produced by each excitation focus is recorded rather than a single value from a photo multiplier tube. This approach offers an improved signal to noise ratio and increased resolution with relatively little modification to the existing hardware of a laser-scanning microscope. There are now several different implementations of ISM, underpinned by the concept of 'pixel reassignment' [1–3], achieved either by optical (ISIM [4,5], OPRA [6]) or computational means (MSIM [7] & spinning disk ISM [8]. This manuscript will explore the potential of computational approaches utilising methods drawn from single molecule localisation microscopy for *a posteriori* pixel reassignment.

To understand the concept of pixel reassignment, it is helpful to consider a single image of the fluorescence generated by a single excitation focus. Each pixel on the camera can be considered as a 'micropinhole' [4], displaced by some distance from the excitation axis. Like a pinhole camera, each pixel 'micropinhole' detects an image of the emitted fluorescence; the smaller the pinhole, the sharper the image. It is these multiple copies of the signal, each detected by a single, point-like pixel acting as a stopped down confocal pinhole, which underlies the improved resolution offered by this approach. If each displaced copy of the image can be correctly overlaid, the fluorescent image formed by a single excitation focus will become sharper and higher intensity. The images of all the excitation foci are then summed together to form a complete image of the specimen with enhanced resolution.

Fig. 1 illustrates the principle of pixel reassignment in terms of excitation and detection point spread functions. The further a pixel is from an excitation source, the dimmer the intensity of the image it detects, although the resolution is not degraded [7,9]. This is because the probability of detecting a photon at the displaced pixel depends on the overlap (multiplication) between the detection point spread function (PSF_{det}) and the excitation point spread function (PSF_{ex}). PSF_{det} is centred on the detection axis and

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characterises the probability of a photon being collected by that pixel. PSF_{em} is centred on the excitation axis and characterises the probability of a photon being emitted. To detect a fluorescent signal, both excitation and detection are required so the probability distributions are multiplied; the result of this multiplication is the 'effective' PSF (PSF_{eff}) of the imaging system. The further apart these PSFs are, the smaller the overlap and the lower the probability of detecting a signal. This results in a lower intensity image. However there is also a spatial consequence of imaging with a displaced pinhole. Assuming that both emission and excitation point spread functions are identical, as would be the case for single photon fluorescence with no Stokes shift, the probability of an excitation and detection event is maximal at the position midway between the excitation and detection maxima. Because the detected light is therefore most likely to have originated from this position, it can be 'reassigned' to a location half the original distance from the excitation focus [1,6,8]. Performing this for each pixel corresponds to scaling the image by a factor of ½ around the excitation focus. In general the excitation and emission wavelengths are different, resulting in excitation and detection PSFs of different widths, meaning that the position of the maximum of the resulting multiplication, and therefore the appropriate scaling factor (m), is slightly larger than ½ [6]. Approximating the PSFs as Gaussian, the appropriate theoretical scaling factor is given by Eq. (1) [6]. In practice, a factor of ½ is used [see 5,6,8].

$$m = \frac{\sigma_{ex}^2}{\sigma_{ex}^2 + \sigma_{em}^2} \quad (1)$$

Before scaling, the image generated by each excitation focus may be multiplied by a Gaussian function to simulate a 'macropinhole' [7]. This process provides axial sectioning, removing out of focus light by mimicking the effect of a confocal pinhole. Additionally, it suppresses background noise and pixel cross-talk between neighbouring excitation foci in a single exposure. The final image is the sum of the images generated at each excitation focus as the laser scans across the specimen. The width of the effective PSF can also be calculated using the standard deviations of the emission and excitation PSFs using Eq. (2) [6]; this value defines the resolution of the final ISM image and can be used to inform any subsequent deconvolution. The processes involved in ISM are summarised in Fig. 2.

$$\sigma_{eff}^2 = m^2\sigma_{em}^2 + (m-1)^2\sigma_{ex}^2 \quad (2)$$

It is possible to scale each image around the excitation focus optically by descanning the emitted light and demagnifying the image of each excitation focus [4,6] or doubling the spacing between excitation foci [5,10]. This has the advantage of performing the scaling step in real time rather than at the post-processing stage. Unfortunately not all microscopes are amenable to this fully optical approach. Acousto-optic devices, used in random access microscopes, have wavelength-dependent and inefficient transmission characteristics making it impractical to use the same

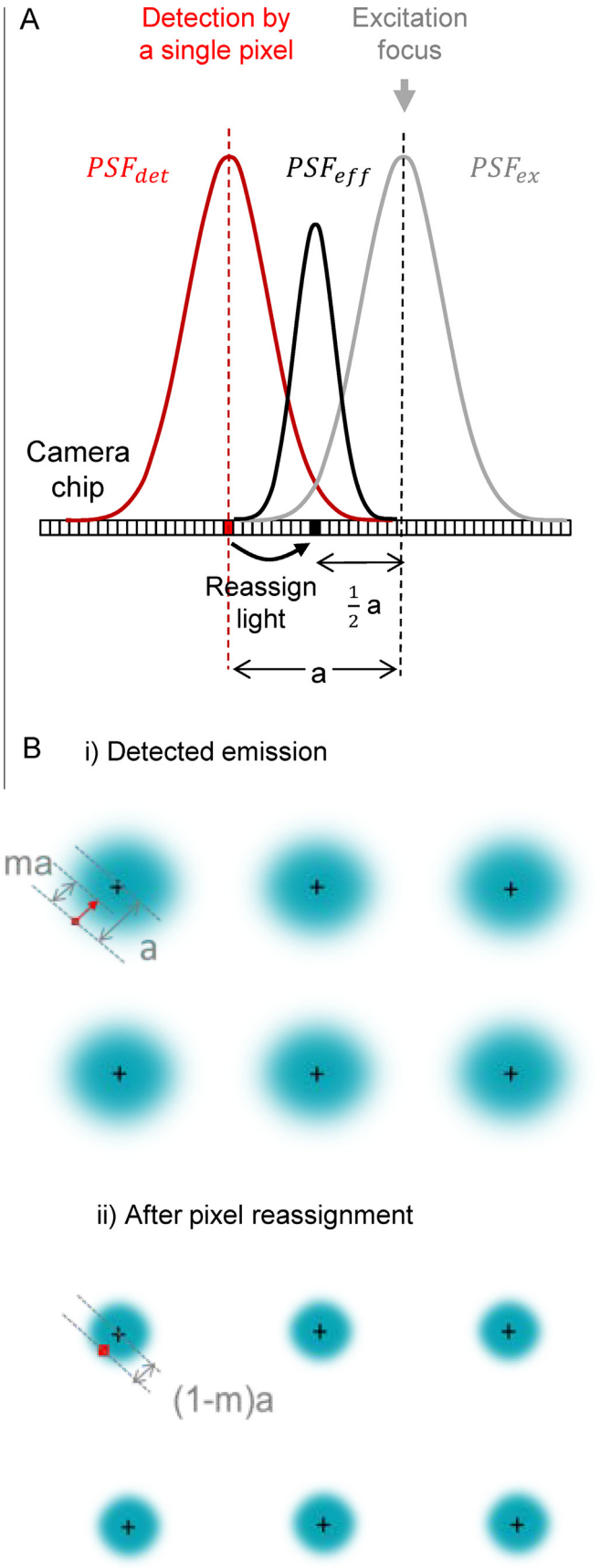

Fig. 1. Schematic diagram illustrating the principles of pixel reassignment. (A) Shown is a 1D representation of the concept underlying pixel reassignment. The light detected by a single pixel (red) displaced by a distance 'a' from the excitation focus is most likely to have originated from the location of the peak of the product (PSF_{eff}) of $PSF_{det}(x - a)$ and $PSF_{ex}(x)$. In the case that PSF_{det} and PSF_{ex} are equal in width, (i.e. neglecting the Stokes shift) the maximum in PSF_{eff} occurs at a distance of a/2 from the excitation focus. The light from the pixel represented in red is thus reassigned to the position midway between the excitation and emission focus (black). For emission and excitation PSFs of differing widths, the scaling factor, m, is given by Eq. (1). In this representation $m = 1/2$. (B) Pixel reassignment in 2D: (i) a single image frame for a uniformly fluorescing sample showing emission generated by multiple excitation foci. (ii) After pixel reassignment, the image produced at each excitation focus appears locally contracted toward the excitation focus. Light detected at position 'a' is reassigned to a position $(1 - m)$a from the excitation focus.