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Image scanning microscopy (ISM) with a single photon avalanche diode (SPAD) array detector

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Abstract

If a scanning illumination spot is combined with a detector array, we acquire a 4 dimensional signal. Unlike confocal microscopy with a small pinhole, we detect all the light from the object, which is particularly important for fluorescence microscopy, when the signal is weak. The image signal is basically a cross-correlation, and is highly redundant. It has more than sufficient information to reconstruct an improved resolution image. A 2D image can be generated from the measured signal by pixel reassignment. The result is improved resolution and signal strength, the system being called image scanning microscopy. A variety of different signal processing techniques can be used to predict the reassignment and deconvolve the partial images. We use an innovative single-photon avalanche diode (SPAD) array detector of 25 detectors (arranged into a 5x 5 matrix). We can simultaneously acquire 25 partial images and process to calculate the final reconstruction online.

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Image Scanning Microscopy (ISM) with a Single-Photon Avalanche Diode (SPAD) array detector

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ABSTRACT

If a scanning illumination spot is combined with a detector array, we acquire a 4 dimensional signal. Unlike confocal microscopy with a small pinhole, we detect all the light from the object, which is particularly important for fluorescence microscopy, when the signal is weak. The image signal is basically a cross-correlation, and is highly redundant. It has more than sufficient information to reconstruct an improved resolution image. A 2D image can be generated from the measured signal by pixel reassignment. The result is improved resolution and signal strength, the system being called *image scanning microscopy*. A variety of different signal processing techniques can be used to predict the reassignment and deconvolve the partial images. We use an innovative single-photon avalanche diode (SPAD) array detector of 25 detectors (arranged into a 5×5 matrix). We can simultaneously acquire 25 partial images and process to calculate the final reconstruction online.

Keywords: Image scanning microscopy, confocal microscopy, structured illumination microscopy, detector array, pixel reassignment, superresolution

1. INTRODUCTION

In a conventional imaging system, a direct image is formed using a lens, and acquired by an array detector. On the other hand, in a scanning system, patterned illumination is used together with a non-imaging single element detector [1] (as in a so-called single-pixel camera). The patterned illumination need not be a simple raster scan, but can be an array of illumination spots [2], or some coded or pseudorandom pattern. In some systems, such as structured illumination microscopy (SIM), patterned illumination is combined with an array detector, giving an increase in image data and improved resolution [3-5]. Basically, the improved resolution comes from the fact that both the patterning (before the object) and the imaging (after the object) contribute to the overall resolution. A confocal microscope, can also be considered as a patterned illumination microscope where the pattern is a scanned, tightly focused spot, and the detector array is the confocal pinhole. Again the resolution is improved [6, 7].

If a scanning illumination spot is combined with a detector array [8], we have a 4 dimensional signal, and unlike in the case of a confocal microscope with a small pinhole (single-pixel detector) we detect almost all the light from the object, which is particularly important for fluorescence microscopy when the signal is weak. The image signal is basically a cross-correlation, and is highly redundant [9, 10]. It has more than sufficient information to reconstruct the image. The most straightforward way to generate a 2D image from the measured signal is by pixel reassignment [8, 11, 12]. Recognizing that the combination of an illumination spot and a detection spot effectively images a point on the object midway between them, the images can be simply summed after reassignment. The result is improved resolution and signal strength, the system being called image scanning microscopy (ISM) [11]. The effective imaging point is midway strictly only for the case when the point spread functions for illumination and detection are identical. Otherwise, the optimum reassignment factor takes a slightly different value [10]. Ref. [8] considers both partially coherent and fluorescence systems. It introduces the concept that a detector element gives information about points of the object other than the illuminated point, introduces the pixel reassignment and summation approach, and explains why a confocal microscope can give superior resolution compared with a conventional one: the signal from an off-axis detector pixel gives information about a different object point.

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Many other papers have reported using a combination of illumination and detection arrays, to give improved resolution and/or optical sectioning. Bertero and Pike, in a series of papers, investigated a point-scanning confocal microscope with a detector array, giving superresolution by singular value decomposition [13-16], but they did not use pixel reassignment, i.e. using information from a particular detector pixel for restoring image points other than the illuminated point. In Ref. [16] they did compare deconvolution applied on pixel reassigned images with their approach, but did not consider multi-image deconvolution. Defrise and De Mol recognized the different nature of restoration using pixel reassignment [17, 18].

Reinholz introduced the technique of illuminating with a scanning spot and using a detector array to measure the spread light distribution. Selecting the signal from the pixel exhibiting the maximum intensity avoids problems caused by movements of the confocal spot [19, 20].

Several papers have investigated subtractive imaging, where the image from a large confocal pinhole is subtracted from that from a small pinhole [21-25]. This operation could be efficiently performed using a detector array [21].

In video confocal microscopy (VCM), spread-field analysis is used with multi-point illumination [26, 27]. As the size of the image of a point object in the detector plane is limited in size, only a small size of detector (around 1 Airy unit) is necessary to collect most of the signal, and as the size of each pixel does not need to be smaller than about 0.3 Airy units, the total number of detector pixels does not have to be very large. In fact, it has been shown that using a quadrant detector gives good performance in terms of both resolution and detection efficiency for point illumination [28]. In a similar way, a small number of illumination patterns can be used with a single detection pinhole to give an improvement in resolution [29, 30]. A variety of different algorithms have been used for image reconstruction in VCM [30]. Taking the maximum intensity for a particular illumination point gives an image similar to a multi-point confocal image. Subtracting the minimum value removes cross-talk and background to give an image close to a point-scanning confocal image. The superconfocal approach generates an image from the maximum plus the minimum minus twice the average value of the detector pixels [31, 32]. Further reconstruction algorithms based on the odd central moments of the detector intensity distribution give even better resolution [32].

In the programmable array microscope [33-35], a series of patterns, either deterministic or pseudorandom, are generated by optical modulators for illumination and/or detection. Using a micromirror array for the detection, simultaneous images can be recorded by detecting the signal from confocal and non-conjugate pixels.

Lu *et al.* introduced the general technique of structured detection, rather than structured illumination [36]. In SPIN, a structured illumination pattern is written by a time-modulated scanning beam, while in SPADE, the detector is switched on and off while the illumination is constant [36]. Lu further investigated use of a digital detection mask [37].

Nonlinear effects such as saturation of the fluorophore can be combined with some imaging methods, for example with structured illumination, to give further improvement in resolution [38, 39]. Combination with structure detection has also been explored [40].

Although the principle of pixel reassignment was proposed back in 1988 [8], it was not demonstrated experimentally until much later, in 2010 [11]. The promise of this technique was immediately recognized [12], but the original implementation was based on single point scanning with a charge-coupled device (CCD) detector, and hence imaging speed was slow [41]. Subsequently, several different experimental implementations have been described, using single point or multi-point illumination, and single- or two-photon excitation [42-48]. The principle is exploited commercially in the AiryScan microscope of Zeiss [47]. As ISM gives the resolution improvement of confocal microscopy, or even better, with a substantially increased signal level, it seems likely that the general principle will replace confocal microscopy with a pinhole for many applications.

Some systems perform the reassignment optically, and integrate the image signal directly on a CCD detector [43, 44]. There is some advantage, however, in acquiring the full 4D signal, as then the full 4D data is available for subsequent image processing. A variety of different signal processing techniques can be used to predict the reassignment and deconvolve the partial images. In order to acquire the full 4D signal we need to record an image for each scanning point, with high sensitivity and speed. We have chosen to use an innovative single-photon avalanche diode (SPAD) array detector of 25 detectors (arranged into a 5×5 matrix), with high fill factor, specifically designed for microscopy. We can simultaneously acquire 25 partial images and perform image processing to calculate the final reconstruction online. It is possible to obtain standard confocal images (the same results that would be obtained with a single detector of different sizes) simply by summing the partial images without processing. Hence, it is possible to investigate improvements in terms of resolution and signal-to-noise ratio of the proposed approach. Experimental results, in

agreement with theoretical simulations, showed an improvement in resolution from ~230nm to ~170nm, in the NIR range.

The imaging performance of ISM has been explored in a few papers [8, 10, 12, 28, 49]. For a confocal fluorescence microscope with a small pinhole, the intensity point spread function (PSF) is given by the product of the illumination and detection PSFs: $H_1(\mathbf{x})H_2(\mathbf{x})$. The OTF is thus given by the convolution of the illumination and detection OTFs, $C(\mathbf{m}) = C_1(\mathbf{m}) \otimes C_2(\mathbf{m})$, and the spatial frequency bandwidth is doubled if a Stokes shift can be neglected [7]. For ISM with a large detector array, the PSF is $H_1(2\mathbf{x}) \otimes H_2(2\mathbf{x})$. The OTF is $C(\mathbf{m}) = C_1(\mathbf{m}/2)C_2(\mathbf{m}/2)$, and again the spatial frequency cutoff is doubled [8]. If the PSFs, and therefore the OTFs, are Gaussian, the PSFs for confocal and ISM are equal to each other, and sharpened up by a factor of $\sqrt{2}$ compared with conventional or scanning (non-confocal) fluorescence. The OTFs for confocal and ISM are also equal to each other, and $\sqrt{2}$ broader than the conventional or scanning OTF.

However, for a confocal microscope with circular pupil apertures, it is found that as the confocal pinhole is offset from the axis, the PSF actually gets narrower [50]. At the same time, the peak intensity decreases, and the side-lobes get stronger [50]. The PSF also moves laterally, so that it images a point of the sample displaced from the illuminated point. In ISM the signal from the displaced detection pixel is reassigned to the correct object point [8]. After summation, the PSF is found to be even narrower than for confocal microscopy, by a factor of 1.1. The PSF for confocal microscopy is 1.39 times narrower than in conventional or scanning microscopy, and for ISM with a large detector array it is 1.53 times narrower [12].

Although the resolution improvement of confocal fluorescence has been recognized for a long time [6, 7], it has not been exploited in practice until recently, as usually a pinhole of around 1 Airy unit in size must be used to detect a strong enough signal. Further, if there is a Stokes shift, a scanning (non-confocal) microscope results in a resolution improved compared with conventional imaging because the excitation wavelength is shorter than the emission wavelength.

ISM with a large detector array does not exhibit an optical sectioning property. In order to retain an optical sectioning effect, we limit the size of the detector array. The optical sectioning for ISM with a finite sized array is approximately equal to that for confocal microscopy with a pinhole of the same size. Figure 1 shows the image of a point object for a conventional microscope and for a confocal microscope, the image for the latter being about $\sqrt{2}$ narrower. We also show the images for ISM with pixel reassignment, for a coherent system and a large array, and for fluorescence with an array size of 1 Airy unit. For a coherent system, we assume coherent summation of the signals after reassignment; the result is a doubling of the resolution [8]. If the signals are summed incoherently after reassignment, the width of the image of a point object is $H_1(2\mathbf{x}) \otimes H_2(2\mathbf{x})$ [8], i.e. narrower than the conventional case by a factor of 1.53.

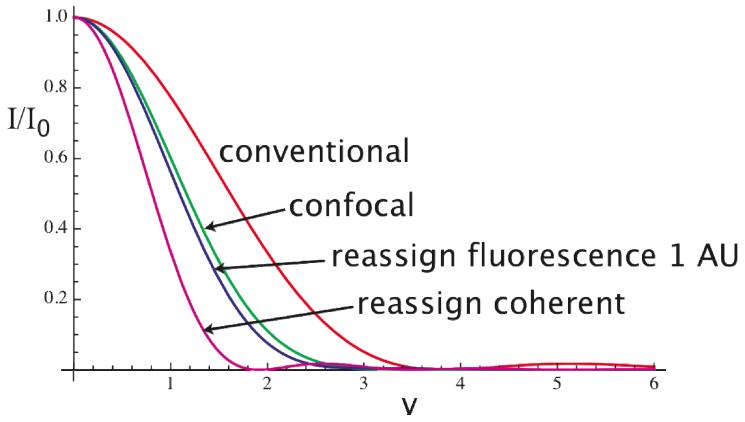

Figure 1. The image of a point object in a conventional microscope, a confocal microscope with a point detector, a coherent microscope with coherent summation after reassignment, and a fluorescence microscope with a detector array of 1 Airy unit and summation after reassignment. Figure based on Ref. [10].

Figure 2 shows the images of a two point object, for four different spacings $2v_0$ of the points, where the normalized distance $v_0 = 2\pi x \mathrm{NA}/\lambda$, where NA is the numerical aperture and x is the true distance. $v_0 = 2$ corresponds to close to the Rayleigh resolution criterion. It can be seen how the dip at the center of the image is deeper for confocal microscopy, and deepest for ISM.

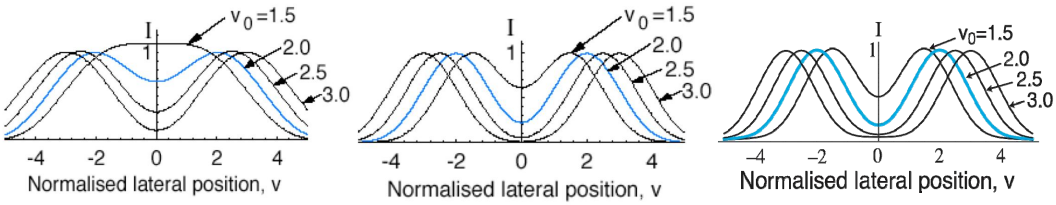

Figure 2. Images of two fluorescent point objects of different spacings, in (left) a conventional microscope, (center) confocal microscope, and (right) ISM with a large detector array.

For ISM with circular pupils, the PSF does not depend strongly on array size. The width of the central lobe gets narrower as the array size increases [12], and the tail of the PSF changes as shown in Fig. 3. For a large detector array, the PSF decays slowly, associated with the cusp of the OTF.

As the width of the PSF is narrower than the conventional Airy disk, but most of the light is detected, the peak intensity of the PSF is greater than in a conventional imaging system [12]. For a large detector array, the peak intensity is 1.84 times stronger, while for an array of 1 Airy unit it is 1.63 times stronger. As this effect overcomes the classical limit of light concentration, it has been called superconcentration [51].

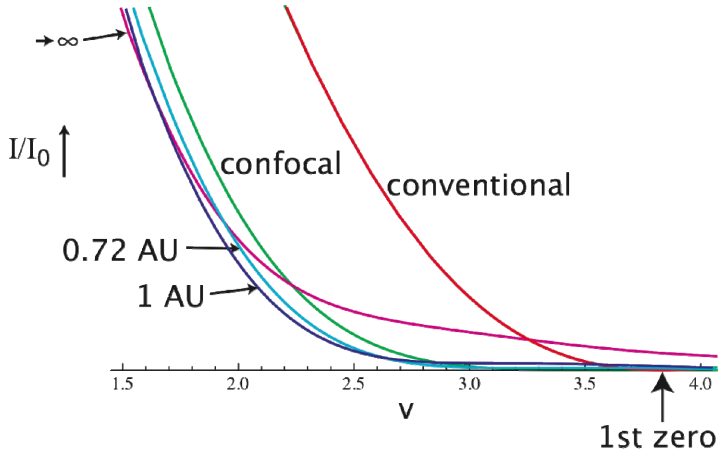

Figure 3. The tails of the PSF for a conventional microscope, a confocal microscope, and ISM with array sizes of 0.72 Airy units, 1 Airy unit, and an infinite array. Figure based on Ref. [10].

The OTF for ISM was explored in Ref. [49]. The OTFs were unnormalized, to take account of the signal level, so that the value of the OTF for confocal and ISM with an array size equal to the pinhole size for zero spatial frequency are equal. The unnormalized OTFs for confocal microscopy and ISM are shown in Fig. 4.

The effect of Stokes shift was explored in Ref. [10]. In fact, any value of reassignment factor can be used, a reassignment factor of zero (back to the scan position) giving a scanning image with an effective pinhole size equal to the array size, and a reassignment factor of unity (i.e. no reassignment) with a large array giving a conventional image.

As the PSF gets narrower as the detector pixel is moved from the confocal point, outer parts of the array give a sharper PSF, and as the central part of the array detects only a small signal, a good combination of signal level and resolution can be achieved using an annular detector array [10]. Also, pupil filters can give a resolution improvement [10].

The ISM principle can also be applied to two-photon fluorescence microscopy [10, 46, 48]. Resolution is improved, and the signal near the conventional two-photon spatial frequency cut-off greatly increased [10].

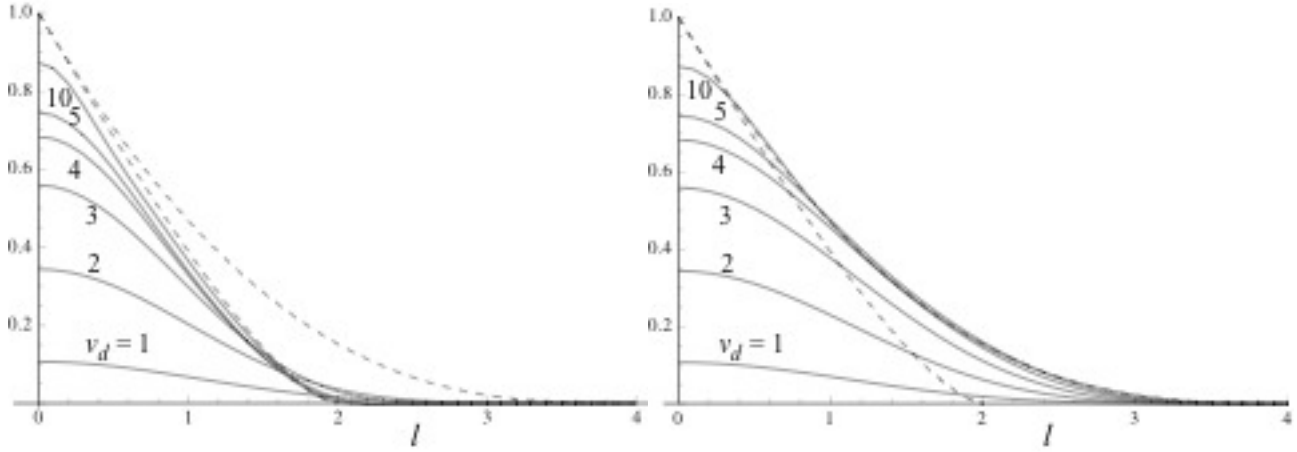

Fig. 4. Unnormalized OTFs for (left) confocal fluorescence microscopes with pinhole radius $v_d = 2\pi r_d NA / M\lambda$, and (right) ISM with array radius $v_d = 2\pi r_d NA / M\lambda$. The dashed curves show the OTFs for a conventional microscope and ISM with a large array.

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