Improved sectioning in a slit scanning confocal microscope

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Optical sectioning is achieved in a laser-scanning confocal microscope by illuminating the sample with a point source and by blocking the light originating from out-of-focus parts of the sample using a pinhole detector. Scanning the illumination and detection across the sample forms an optically sectioned image

Similarly, line-scanning confocal microscopes [2], employing a slit or line illumination source and detector, can provide much faster image acquisition, as only one-dimensional scanning is required. However, they do not block out-of-focus light traveling along the slit direction, resulting in a poorer FWHM sectioning strength and a much slower attenuation of signal away from the focal plane. Out-of-focus subtraction techniques have already been proven to increase the spatial resolution of various confocal techniques [3–5], and here we investigate the use of what are called multiple detection slits to allow the detection of out-of-focus light to improve both FWHM axial response and far from focus signal suppression.

We consider an optical system consisting of a conventional line-scanning microscope where the detection slit and one-dimensional array detector have been replaced by a two-dimensional array detector, typically a CCD camera. As the beam (or sample) is scanned, an image is acquired for each line position. We implement a virtual detection mask, that is to say, we attribute each pixel of the image to be within a set of \( N \) detection slits matched and aligned to a set of \( N \) illumination stripe positions. The intensity on the CCD in the \( j \)th stripe position when illuminating the sample at the \( i \)th slit position can be written as

\[
I_{ij}(x) = I_{i,j}^{\text{out}}(x) + I_{i,j}^{\text{in}}(x),
\]

where \( x \) is a given image pixel, \( I_{i,j}^{\text{out}}(x) \) is the out-of-focus light, and \( I_{i,j}^{\text{in}}(x) \) is the in-focus light when illuminated at the \( i \)th illumination position. The latter is detected only by this detector stripe position and attenuates strongly with defocus. While the in-focus signal will certainly be different for different illumination positions, the out-of-focus signal attenuates much more slowly with defocus and contributes to a significant background in the final image. We note that this signal will be very similar when illuminating adjacent stripes, thus

\[
I_{i,j-1}(x) = I_{i,j+1}(x) \approx I_{i,j}^{\text{out}}(x),
\]

and we can use these adjacent signals as an estimate of the out-of-focus signal and subtract it from the central stripe position image, \( I_{i,j} \), to obtain an improved confocal image \( I_{\text{sec},j} \):

\[
I_{\text{sec},j} = \left[ I_{i,j} - \frac{I_{i,j-1} + I_{i,j+1}}{2} \right] = I_{i,j}^{\text{in}}.
\]

To demonstrate the subtraction technique experimentally we used the microscope arrangement depicted in Fig. 1. Illumination was provided by a single 17 \( \mu \)m wide, 3.6 mm long stripe LED from a 120 element array [6,7], which was projected onto the sample using a standard 180 mm tube lens and a 20 \( \times \) 0.5 NA dry objective. Fluorescent emission from

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the sample was imaged by an identical system onto a CCD camera (Orca ER, Hamamatsu) and acquired as a set of 576 × 64 pixel images, each with 100 ms exposure. The sample was scanned across the beam using an automated X–Y stage. The distance between line positions at the sample was set to 0.6 μm, corresponding to four optical units at λ = 470 nm, and 100 scan positions were used to generate the images in Fig. 2. The central virtual detection slit was calculated from a single image of an in-focus thin fluorescent sheet, using Canny edge detection [8]. The slit had a width of about 34 μm (6 pixels or about 11 optical units) at the camera. This virtual slit pattern was laterally shifted by ±2 pixels (about four optical units) during postprocessing to provide the images \( I_{i=1} \) and \( I_{i=+1} \) for subtraction.

Figure 2 shows images of pollen grains using wide-field, conventional slit-scanning confocal and our subtraction technique. Figures 2(a) and 2(b) represent the wide-field (produced by summing the shifted raw camera data) and conventional slit-scanning images, respectively, the latter showing only a partial suppression of out-of-focus features. By contrast, the subtraction image in Fig. 2(c) shows very good suppression of out-of-focus features. Small details such as pollen grain spikes are clearly best resolved in the autoscaled image in Fig. 2(e), where the out-of-focus blur is almost completely removed.

We consider the performance of such a microscope in terms of how it images an infinitely thin fluorescent sheet in a plane orthogonal to the optic axis. With finite width, infinite length illumination, and detection slits aligned parallel to the y axis and incoherent illumination, the response to defocus of a slit-scanning microscope at low NA is

\[
I(u, \beta) = C \int_{-\infty}^{+\infty} T^2(s_x, 0, u) \frac{ps_x}{2\pi} \frac{qs_x}{2\pi} \exp(-is_x\beta) ds_x. \tag{4}
\]

The lateral shift between illumination and detection slits, given by the distance \( \beta \), and their widths, given by \( p \) and \( q \), respectively, are measured in normalized lateral optical units. The defocus, \( u \), is measured in normalized axial optical units, and \( s_x \) is the normalized spatial frequency associated with the direction perpendicular to the slit. \( C \) is a constant. These normalized variables are related to real coordinates, \( x \) and \( z \), and spatial frequency \( f_x \) by

\[
\beta = \frac{2m}{\lambda} x \sin(\alpha), \quad u = \frac{8m}{\lambda} z \sin^2\left(\frac{\alpha}{2}\right), \quad s_x = \frac{\lambda}{n \sin \alpha} f_x, \tag{5}
\]

where the NA of the system is given by \( NA = n \sin(\alpha) \) and \( n \) is the refractive index.

The incoherent optical transfer function, \( T(s, u) = T(S_z, S_y, u) \) (where \( s = \sqrt{s_x^2 + s_y^2} \)), is assumed to be identical for the illumination and detection optical paths (i.e., there is no Stokes’s shift in the fluorescence process). Stokseth’s approximation [9] is used to estimate the variation in \( T \) with defocus

\[
T(s, u) = \begin{cases} 
    g(s) \left( \frac{J_1[u(s-1)/2)]}{u(s-1)/2} \right), & \text{if } 0 < s < 2 \\
    0, & \text{if } 2 \leq s 
\end{cases},
\]

\[
g(s) = 1 - 0.69s + 0.0076s^2 + 0.0437s^3. \tag{6}
\]

Substituting Eq. (4) into Eq. (3), we can write the defocus response of our improved slit

\[
I_{\text{sub}}(u) = \int_{-\infty}^{+\infty} I(u, \beta) + I(u, -\beta) ds_x,
\]

\[
= C \int_{-\infty}^{+\infty} \frac{1}{2} T^2(s_x, 0, u) \frac{ps_x}{2\pi} \frac{qs_x}{2\pi} \exp(-is_x\beta) ds_x. \tag{7}
\]

Similarly we can write the simple slit-scanning confocal response, \( I_{\text{slit}}(u) \), and the simple point-scanning confocal response, \( I_{\text{point}}(u) \), as

\[
I_{\text{slit}}(u) = I(u, 0)
\]

\[
= C \int_{-\infty}^{+\infty} T^2(s_x, 0, u) \frac{ps_x}{2\pi} \frac{qs_x}{2\pi} ds_x, \tag{8}
\]

\[
I_{\text{point}}(u) = C \int_{0}^{+\infty} T^2(s, u) \frac{ps}{2} \frac{qs}{2} ds, \tag{9}
\]

where \( \sin(x) = \sin(x)/x \), \( \sin(x) = J_1(x)/x \), and \( p \) and \( q \) are also the diameter of the illumination and detection pinholes in the point-scanning case.

Figure 3 shows the normalized response with defocus for the slit-scanning, point-scanning, and subtraction microscopes. Each is modeled for the same parameters as the experimental setup described above, with the same pinhole diameters for point scanning as slit widths for line scanning. Also shown are experimental measurements made for the slit-
scanning and the subtraction case using a thin (≤90 nm) fluorescent sheet of polyfluorene F8BT spin coated onto a glass slide as the object. The FWHMs of the simulations were found to be 7.2, 3.4, and 3.3 μm, respectively, implying a sectioning capability of the subtraction system 2.2 times better than for slit scanning and marginally improved over point scanning in this particular configuration.

The performance of the three systems far from focus is illustrated in the log–log plots shown in Fig. 4, where much improved rejection of out-of-focus light can be seen with the subtraction technique. We estimate slopes of the residual signal on the log–log plots as −1.0, −2.0, and −2.9 for the slit, point, and subtraction cases, respectively. A simple analysis of the asymptotic form of Eqs. (7)–(9), noting that $T(s, u)$ has significant values only at low spatial frequencies for large defocus, gives

$$I_{\text{slit}}(u) \propto \int_0^2 \left( \frac{J_1(us)}{us} \right)^2 ds = o(1/u),$$

$$I_{\text{point}}(u) \propto \int_0^2 \left( \frac{J_1(us)}{us} \right)^2 sds = o(1/u^2),$$

$$I_{\text{sub}}(u) \propto \int_0^2 \left( \frac{J_1(us)}{us} \right)^2 s^2ds = o(\ln(u)/u^2), \quad (10)$$

Supporting the empirical observations from Fig. 4.

In conclusion, we have presented a simple modification of a slit-scanning confocal microscope that produces improved confocal images by using an array detector and reducing residual out-of-focus background by signal subtraction. We see a higher order of suppression of signal with defocus than both slit- and point-scanning confocal microscopes, although we note that as a subtraction technique the noise is limited by the noise in the acquired images. This results in a noise floor declining as $o(u^{-3/2})$ (equivalent to the slit-scanning case) in this particular subtraction microscope, compared to a noise floor declining as $o(u^{-1})$ in the point confocal case. As can be seen from the device used in Fig. 1, line LEDs can be produced in an array format. Thus, in principle, and providing sufficient device uniformity, these concepts can be applied with fully solid-state confocal scanning techniques, where the illumination is scanned using an array source and the sample is kept fixed [7].

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References