Real-time slit scanning microscopy in the meridional plane

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The standard microscope architecture around which confocal microscopes are built imposes fundamental restrictions on the speed with which images (three-dimensional data sets) can be obtained. Commercially available slit scanning confocal microscopes are able to produce optically sectioned images at frame rates well in excess of 100 Hz. However only the focal $(x-y)$ plane can be imaged at this speed. To image a volume specimen it is necessary to physically change the distance between the objective lens and the specimen. This refocusing process is often necessarily slow and represents a bottleneck to the speed of image acquisition. We describe the construction of a slit scanning confocal microscope based on what we know to be a novel microscope architecture, which permits images of other planes and, particular, the meridional $(x-z)$ plane to be acquired in real time.

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In recent years, the confocal fluorescence microscope has become an indispensable imaging tool in the biological sciences, providing high resolution sectioned images of the focal plane. This optical sectioning is achieved by placing a pinhole aperture in front of the photodetector to discriminate against out-of-focus fluorescence. Images are constructed, pixel by pixel, by scanning the focal spot relative to the specimen, typically using galvanometer mirrors. In this way, three-dimensionally resolved images can be acquired at rates up to a few frames per second. An improvement in acquisition speed can be obtained using a slit scanning confocal fluorescence microscope [1,2]. In this system the pinhole is replaced with a slit and the photodetector replaced with a linear array. Such systems can be operated with frame rates in excess of 100 frames per second, an increase in speed that comes at the expense of a slight decrease in image resolution and optical sectioning [3,4].

It is important to realize, however, that slit scanning microscopes image only the focal $(x-y)$ plane at such high frame rates. This is because scanning along the axial (z) direction involves physically changing the distance between the objective lens and specimen, which cannot be done as quickly as galvanometer scanning of the lateral direction. The fastest frame rates can only be achieved, therefore, if we restrict ourselves to imaging the focal plane. In some applications this is not a problem as the specimen can simply be oriented so as to force the plane of interest to lie in the focal plane. However, this is not always possible as the objective lens sometimes obstructs the specimen from being reoriented appropriately. This is the case, for instance, when dealing with bulk tissue samples, where the primary plane of interest lies perpendicular to the tissue surface [5]. Here, reorientation is extremely impractical, and it would be far more useful if the meridional $(x-z)$ plane could be imaged directly at high speed. Other examples where reorientation is impractical can be found in microfiltration studies [6], image cytometry, and live cell imaging.

It is clear that the key to imaging the meridional plane (MP), or indeed any plane other than the focal plane, at high speed is to develop an axial scan method that does not require the specimen or objective lens to be moved. Unfortunately, any attempt to do this optically on the standard microscope architecture leads to the introduction of optical aberrations, which degrade image quality [7], and this undermines the benefits gained by such methods. We have recently proposed a microscope architecture that permits optical scanning along the axis without introducing aberrations [7,8]. The benefits of this architecture are that: (i) it does not require the objective lens or specimen to be moved, (ii) it does not introduce optical aberrations that degrade image quality while refocusing, and (iii) axial scanning can be performed at high speeds. In this Letter, we demonstrate the application of this microscope architecture to slit scanning confocal microscopy and show how the MP can be imaged in real time using a standard CCD camera without moving the specimen and without introducing optical aberrations.

A schematic of the system we built is shown in Fig. 1. The key feature of this setup is the second high-NA objective lens L2 that is inserted into the beam path of the microscope to cancel out aberrations introduced by the imaging objective L1 during the axial scan. Scanning was performed by mirror M1, which was mounted on a piezo translation stage, in the focal region of L2. In this design, L1 and L2 were an Olympus 1.4 NA 60× oil immersion objective and Olympus 0.95 NA 40× dry objective, respectively. A dry lens was specifically chosen for L2 to ensure that there was no mechanical interference between the scanning mirror and the objective via the immersion medium. The pupil planes of L1 and L2 were mapped together by a $4f$ imaging system having unit magnification, which comprised two achromatic dou-

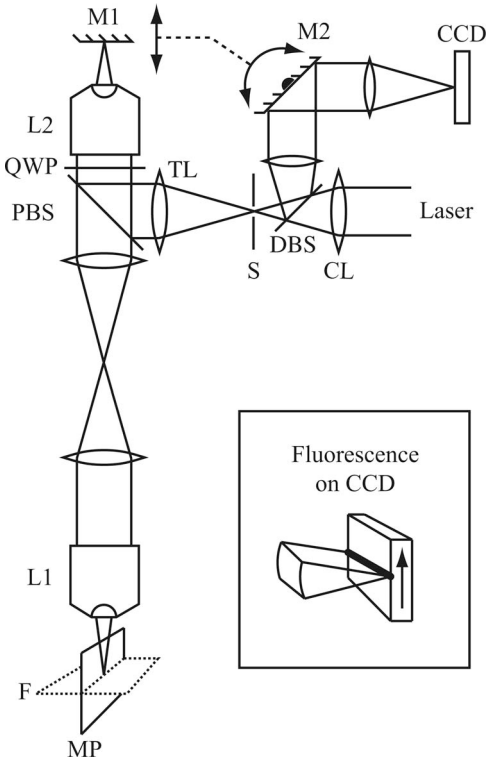

Fig. 1. Slit scanning confocal microscope that images the MP directly with a CCD camera. Inset, line information from different specimen depths is imaged onto different rows of pixels during each frame.

blet lenses with focal length of 160 mm. We note that this choice of magnification is not trivial and refer the reader elsewhere for a full explanation of this parameter [7].

In operation, an expanded beam from a diode-pumped solid-state laser (Calypso, Colbolt, Sweden), with a maximum power of 50 mW and wavelength of 491 nm, was coupled into the system via a cylindrical lens (CL) having a focal length of 100 mm. This produced a linear illumination pattern on a slit aperture S, with width of 10 μm and length of 10 mm (Comar, UK), placed in the image plane of the microscope system. The light passing through the slit traveled through the tube lens (TL), which was an achromatic doublet of focal length of 200 mm, and was directed into objective lens L2 by a polarizing beam splitter (PBS). A quarter-wave plate (QWP) was also inserted between PBS and L2 to ensure that all light reflected off the scan mirror (M1) would travel back through the PBS and into the final stage of the system. In this way a diffraction-limited line of light was formed, perpendicular to the optical axis, in the focal region of L1 where the specimen was placed. Moving mirror M1 a distance Z along the axis had the effect of moving this illumination line along the axis by a distance $z=2Zn_2/n_1$, where $n_{1,2}$ are the refractive indices of the immersion media of two objective lenses. The illumination line was therefore swept along the MP of the specimen by scanning mirror M1. Scanning the illumination in this manner, using objective lens L2, ensured that no aberrations were introduced during the scan.

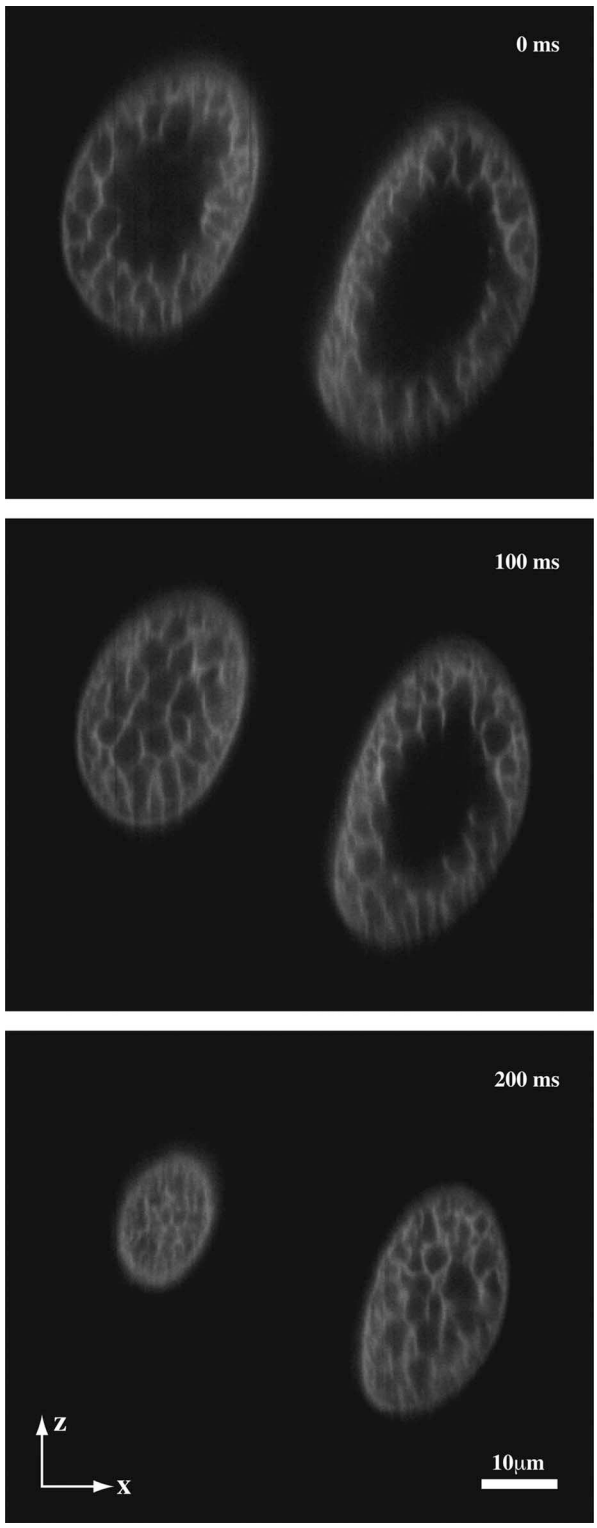

Fig. 2. Image of the parts of a fluorescent pollen grain lying in the MP of our slit scanning confocal microscope. These are frames from a real-time movie obtained of the specimen.

Fluorescence light generated in the specimen passed back through the system reciprocally to the slit, which provided optical sectioning by obscuring out of focus light and letting through only fluorescence from the effective focus. The fluorescence light passing through the slit was then reimaged onto a row of pixels of a low noise peltier cooled CCD cam-

era (ORCA-ER, Hamamatsu, Japan) using a 4*f* imaging system with unit magnification. This comprised a pair of achromatic doublet lenses with focal length of 100 mm. A dichroic beam splitter (DBS) with cutoff wavelength of 493 nm and emission filter with a bandpass of 500–550 nm (not shown) were also used to remove backscattered laser light. A galvanometer mirror M2 was mounted in the intermediate plane of the 4*f* system. This could be tilted to move the image of the slit laterally on the CCD camera. This is shown in more detail in the figure inset. It was therefore possible to image line information from different specimen depths onto different rows of pixels on the CCD camera by driving both mirrors synchronously. The mirrors were driven over the full range during the frame time of the camera. As such, the image registered on each camera frame corresponded to the "image" of the specimen section lying in the MP. The imaging rate was limited by the frame rate of the CCD camera to 10 Hz. Movies were acquired of a pollen specimen [Carolina w.m. 30–4264 (B690)]. To simulate a dynamic behavior of the specimen, the pollen grains were moved manually with a translation stage so as to make them pass through the MP. Three images from adjacent time points in one such movie are shown in Fig. 2 from which it is possible to see a clear image detail over a large range of specimen depths. This demonstrates that no significant amount of optical aberration has been introduced by this scanning method.

The frame rate of this system was not limited by the axial scan speed of M1 but rather by the frame rate of the CCD camera used. Improved frame rates on this system could therefore be achieved with a faster camera.

It should be pointed out that the fluorescence generated in the specimen here was unpolarized, so half the signal was reflected and lost while traveling back through the PBS toward the slit. In some situations this loss may be unacceptable, for example, when fluorescence levels are low. In such cases as these we would suggest introducing a further objective lens, QWP, and a mirror in the reflected arm of the PBS to process this part of the signal and pass it back toward the slit as well. If the motion of this extra mirror is synchronized to the motion of M1 then twice as much fluorescence should reach the CCD. Further optimization of the signal levels could also be achieved by using achromatic versions of the PBS and QWP to handle the wide fluorescence bandwidth generated by the specimen.

It is also an important point to note that the resolution NA of this imaging system is determined by objective lens L1 and had a value of 1.4. At first glance, this might seem counterintuitive, because L2 had a lower NA and would therefore appear to limit the resolution. This is not the case, however, because it is the objective lens with the lowest *angular* aperture that determines the resolution in such systems [7]. As L1 had a lower acceptance angle (α=67.3°) than L2 (α=71.8°), it was the NA of L1 that determined the resolution of the system.

We should like to emphasize once more that imaging in the MP using this technique is carried out without moving the objective lens or specimen. As a result, this system architecture could be particularly useful for imaging specimens that are sensitive to mechanical perturbations, for example, when imaging a floating specimen with a water dipping objective lens.

Although the system described here images only the MP, it could easily be adapted to image the focal plane as well by placing a single galvanometer mirror in the pupil plane of the system so as to scan the illumination line laterally in the specimen. The usefulness of this system could then be extended by combining both axial and lateral scanning motions so as to image any plane, or curved surface, that possesses a principle component along the direction of the illumination line.

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