fluorescence anisotropy decay than from the fluorescence decay of a GFP-tagged protein. The homotransfer is detected by a fast fluorescence depolarization, much faster than the fluorescence depolarization due to rotation of the dimeric protein, whereas a weak hetero-FRET efficiency is detected by a small decrease in fluorescence lifetime.

Here we show that, although FRET-based interaction determination is a widely used method, its application to living cell studies requires the performance of extensive quantitative analyses of fluorescence properties (kinetics of fluorescence decays and fluorescence anisotropy decays). In addition, according to the type of interaction, hetero- or homodimer, the methodology, hetero- or homo-FRET, must be judiciously chosen to obtain the best information about structural data within the macromolecular complex.

Acknowledgments

We are indebted to Dr. Robert B. Pansu and Patrick Denjean for technical advice, to Prof. Jean-Claude Nicolas for fruitful discussion, and to Dr. Fabien Gerbal for critical reading of the manuscript. This work was supported by grants from the European Union (BIO4 CT97 2177), the Association pour la Recherche sur le Cancer (Grants 9222 and 5632 to J.C. and Grants 9518 and 5936 to V.M.), the Groupement des Entreprises Francaises de Lutte contre le Cancer, and le Laboratoire Glaxo Wellcome. M. Tramier and T. Piolot were supported by European Union fellowships.

[26] Spinning Disk Confocal Microscope System for Rapid High-Resolution, Multimode, Fluorescence Speckle Microscopy and Green Fluorescent Protein Imaging in Living Cells

By Paul S. Maddox, Ben Moree, Julie C. Canman, and E. D. Salmon

Introduction

We describe here a spinning disk confocal fluorescence microscope system we initially assembled for fluorescent speckle microscopy (FSM) of the assembly dynamics and movements of individual microtubules and actin filament arrays within cells.1–4 In this application, image contrast is generated by polymer assembly from

a cytoplasmic subunit pool containing a small fraction of fluorescently labeled sub-units (typically 1% or less). The random nature of subunit association creates a nonuniform fluorescent "speckle" pattern along the polymer lattice during polymerization. FSM can also be used to record the binding and release of microtubule-associated proteins (MAPs) on the microtubule lattice. Optimum contrast is obtained for fluorescent speckles containing only a few fluorophores, often five or fewer, within the maximum resolution limits of the light microscope. This requires a high-resolution imaging system designed for maximum sensitivity to detect few fluorophores without significant photobleaching problems. Previously, we have used wide-field epifluorescence microscopy and a cooled charge-coupled device (CCD) camera with high quantum efficiency and low noise. Similar instrumentation has worked well for thin specimens such as the lamella of mammalian tissue culture cells, neuronal growth cones, flattened axons, and newt cells in mitosis, which remain flat. However, for thicker polymer arrays such as the mitotic spindle in most tissue cells and embryos, better depth discrimination is needed to permit resolution of specific fibers, such as bundles of kinetochore microtubules, and to prevent the loss of speckle contrast produced by out-of-focus fluorescence from speckles in polymers above and below the plane of focus.

Inoué and co-workers have described the advantages of the Yokogawa CSU-10 Nipkow spinning disk confocal scanning unit for obtaining high-quality fluorescent images with brief exposures and low fluorescence bleaching. Stimulated by these studies, we have combined the CSU-10 unit with efficient microscope optics and a panchromatic CCD camera with the high quantum efficiency and speed to facilitate high spatial and temporal resolution fluorescent imaging at multiple wavelengths. In addition to applications in FSM, the resolution and sensitivity of this instrument have proved valuable for live cell imaging of green fluorescent protein (GFP) fusion proteins. Also, the high signal-to-noise ratio of images obtained with this instrument has provided the opportunity to obtain three-dimensional (3-D) immunofluorescent images of extraordinary resolution and image quality by constrained iterative deconvolution.

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The major features of the Yokogawa CSU-10 spinning disk confocal unit have been described in detail elsewhere.\textsuperscript{14–16} Here we describe our system integration and its important features for FSM and other applications. Waterman-Storer \textit{et al.}\textsuperscript{17–19} provide detailed methods for FSM analysis of microtubule and actin filament assembly dynamics and motility within cells, including biochemical methods for obtaining fluorescent tubulins and actins, procedures for their incorporation into living cells, and kymograph analysis of fluorescent speckle motility.

Overview of Instrument Components

Table I lists the major components, accessories, and sources for our spinning disk microscope and the deconvolution systems. Figure 1 shows the major components of the spinning disk microscope system. A Nikon TE-300 inverted microscope equipped with epifluorescence optics, phase-contrast optics, and a transmitted light shutter from Vincent Associates (Rochester, NY) is mounted on a vibration isolation table. A Nikon (Melville, NY) remote-focusing device uses a stepping motor to control microscope z-axis focus. The Yokogawa CSU-10 is fastened to a standard “C-mount” adaptor on the side port of the microscope as seen in more detail in Fig. 2. The CSU-10 obtained from PerkinElmer Wallac (Bethesda, MD) has a Sutter filter wheel mounted on the back for controlling wavelength selection. Excitation light is supplied from an optical fiber connected to a 100-W argon–krypton air-cooled laser. A Hamamatsu Orca ER cooled CCD camera (Hamamatsu Photonics, Bridgewater, NJ) is connected to the C-mount connector at the output of the CSU-10. Shutter control, wave-length selection, focus, time-lapse image acquisition, image storage, and routine image processing are controlled by MetaMorph digital imaging software (Universal Imaging, Downingtown, PA) in a PC computer system (Fig. 1, left). Image deconvolution from a stack of optical sections through a specimen is performed with an off-line DeltaVision deconvolution system and Softworx software (not shown; Applied Precision, Issaquah, WA).

\begin{itemize}
\item \textsuperscript{17} C. M. Waterman-Storer, A. Desai, and E. D. Salmon, \textit{Methods Cell Biol.} \textbf{61}, 155 (1999).
\end{itemize}
<table>
<thead>
<tr>
<th>Instrument Component</th>
<th>Source/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nikon TE-300 epifluorescence inverted microscope (Nikon USA, Melville, NY)</td>
<td>Lens options: x100/1.4-NA Planachromatic bright field, ×100/1.4-NA Planachromatic phase 3, ×60/1.4-NA Planachromatic bright field</td>
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<td>Epi-fluorescence filter sets</td>
<td>DAPI, HiQ FITC, and HiQ Texas Red (Nikon USA, Melville, NY)</td>
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<tr>
<td>Air table</td>
<td>6 ft by 4 ft by 8 inches (Technical Manufacturing, Peabody, MA)</td>
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<td>Transmitted light</td>
<td>Lamp house (HMX-2)</td>
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<td></td>
<td>Unibliz shutter for transmitted light (Vincent Associates, Rochester, NY)</td>
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<td></td>
<td>IR-cut filter</td>
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<tr>
<td></td>
<td>Phase optics in condenser</td>
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<td></td>
<td>0.85-NA dry condenser lens</td>
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<td>Nikon focus accessory</td>
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<td>Spinning disk confocal unit</td>
<td>Yokogawa CSU-10 (PerkinElmer Wallac, Bethesda, MD)</td>
</tr>
<tr>
<td></td>
<td>488 single-line filter set</td>
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<td></td>
<td>568 single-line filter set</td>
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<tr>
<td></td>
<td>Sutter Lambda 10.2 filter wheel (PerkinElmer Wallac)</td>
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<tr>
<td>Custom dual-dichroic and emission filter set (488- and 568-nm excitation; Chroma Technology, Brattleboro, VT)</td>
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<td>Laser</td>
<td>Omnichrome 100-mW argon–krypton mixed gas air-cooled laser (488- and 568-nm laser lines; PerkinElmer Wallac)</td>
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<td></td>
<td>Single-mode fiber optic cable with manipulator (PerkinElmer Wallac)</td>
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<tr>
<td></td>
<td>Power meter</td>
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<td>Orion-PD (Ophir Optronics, Danvers, MA)</td>
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<td>Cooled CCD camera</td>
<td>Hamamatsu Photonics CCD Orca ER (Hamamatsu Photonics, Bridgewater, NJ)</td>
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<tr>
<td>Digital image acquisition and processing system</td>
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<td>MuTech mv-1500 digitizing board (CCD interface)</td>
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<td>Deconvolution system</td>
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<td></td>
<td>IRIX64 release 6.5 operating system</td>
</tr>
<tr>
<td></td>
<td>Softworx software version 2.5 (Applied Precision, Issaquah, WA)</td>
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</table>
FIG. 1. Photograph of the spinning disk confocal microscope system in the Salmon laboratory. Key parts are labeled. See Table I for details.

FIG. 2. Close-up view of the Yokogawa CSU-10 mounted at the output port of the Nikon TE-300 inverted microscope. The prototype Hamamatsu Orca ER camera is attached to the output port of the CSU-10. Note that the fiber optic cable from the laser attaches to the CSU-10 on the lower left-hand side. The Sutter excitation filter wheel is mounted to the CSU-10 on the back side (not in view).
Rationale for Component Selection

**Inverted Epifluorescence Microscope**

Our imaging system is based on a Nikon TE-300 inverted microscope stand. We chose an inverted stand for several reasons. First, the stage on the Nikon inverted microscope is stable and resists focal drift (less than 2 μm/hr). A stable stage is extremely important for time lapse imaging of living specimens and accurate z series. Second, manipulations of samples (for instance microinjection) are facilitated with the use of a long working distance condenser. Finally, the inverted stand allows for easy access to the optical paths of the microscope. Focus is controlled by using a Nikon stepper motor, capable of accurate steps of 100 nm or greater for optical sections. We use standard epifluorescence optics and filter cubes for locating specimens by eye and then switch to an open filter slot for confocal imaging.

We typically use a Nikon ×100/1.4-NA (numerical aperture) Plan Apochromat objective for maximum confocal effect. It is light efficient and the ×100 magnification is needed to make a 0.5-μm-diameter illumination spot on the specimen by light passing through the 50-μm-diameter pinholes in the spinning disk. Lower magnification objectives increase proportionally spot size and potential out-of-focus light.

The ×100 magnification is also needed to match the optical resolution of a 1.4-NA objective to the 50-μm diameter of the pinhole in the spinning disk.\(^\text{15,16}\) Fluorescent point sources in the specimen are spread out at the image plane by the diffraction of light at the objective aperture (the point-spread function, PSF, of the objective\(^\text{2}\)). The Airy pattern describes the intensity distribution in the \(x-y\) plane of focus as a function of the radial distance from the optical axis. Optical resolution is given by the radius, \(r\), of the first minimum of the central Airy disk by Eq. (1)\(^\text{21}\):

\[
r = 0.61\lambda / N A_{\text{obj}}
\]

For GFP fluorescence at 510 nm, and \(N A_{\text{obj}} = 1.4\), then \(r = 0.22 \mu m\) and the predicted diameter of the Airy disk is 0.44 μm. For a ×100 objective, this Airy disk diameter at the spinning disk will be 44 μm, slightly smaller than the 50-μm pinhole diameter. For 600-nm wavelength red fluorescence excited by the 563-nm laser line, \(r = 0.27 \mu m\), and the diameter of the Airy disk central maximum at the spinning disk is 53 μm, for the ×100 objective, slightly larger than the pinhole diameter.

The ×100 magnification slightly oversamples the CCD we currently use (see Cooled Charge-Coupled Device Camera, below), ensuring that resolution is limited by the optics and not the pixel size of the CCD chip. The optimal magnification, \(M\),

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needed for sufficient contrast to resolve overlapping Airy disks separated by radius \( r \) depends both on the sampling frequency of the pixel detector elements in the detector and the signal noise.\(^{20}\) For FSM we have used the following criteria\(^{1,3}\):

\[
M = \frac{3 \times \text{pixel width}}{\text{optical resolution}}
\] (2)

The optimal magnification from Eq. (2) for \( r = 0.22 \mu m \) is 85, for the 6.45-\( \mu m \) pixel size of the CCD chip in our Orca ER camera.

For samples requiring phase-contrast as well as fluorescence imaging, we use a Nikon \( \times 100/1.4\)-NA Plan Apo Phase 3 objective. Using a phase lens is somewhat problematic for low light-level, high-resolution applications because the phase ring in the back aperture of the objective reduces the signal by 10–15\% and slightly distorts the objective point-spread function (PSF; see Image Acquisition, Storage, and Processing, below). A Vincent shutter is placed in front of the 100-W quartz halogen lamp used for transmitted light illumination and this shutter is used to control illumination for phase-contrast recordings. A heat-reflecting filter is placed in the illumination light path to prevent specimen damage and to remove infrared light from the camera image.

For samples with extremely low fluorescence imaging, we use one of two options to increase the fluorescence signal without overilluminating (bleaching) the sample. The first is to bin the CCD chip \( 2 \times 2 \) (binning increases the signal by 4-fold without increasing the readout noise). Using the \( \times 100/1.4\)-NA objective and binning the CCD result in loss of lateral resolution in the image by a factor of about 2, because of undersampling by the camera detector, but does not decrease the confocal performance of the instrument. Another option that does not sacrifice as much lateral resolution is to use a \( \times 60/1.4\)-NA objective and \( 1 \times 1 \) binning. The \( \times 60 \) lens passes more light than the \( \times 100 \) lens to the image \((I_{\text{image}} \approx 1/M^2)\) but only slightly undersamples the CCD chip. However, the \( \times 60 \) lens reduces the confocal ability of the microscope by creating an illumination spot size larger than optimal and producing Airy disk images of points in the specimen smaller than the 50-\( \mu m \) size of the pinholes in the spinning disk. This effect is not as significant for the larger diameter Airy disks of red fluorescence compared with green.

**Yokogawa CSU-10 Spinning Disk Confocal Scanner**

The CSU-10 mounts between a standard output port of the microscope and the camera. As mentioned above, the CSU-10 is mounted to a C-mount adaptor on the side port of the TE-300 Nikon inverted microscope and the cooled CCD camera is mounted on a C-mount adaptor on the output port of CSU-10 (Fig. 2). A slight adjustment of the ocular focus makes viewing the specimen by eye using the standard epifluorescence illumination in the microscope parfocal with the camera. CSU-10 illumination and imaging is achieved by moving the microscope filter cube carrier to an open position to allow illumination and image formation at the microscope side port without interference from any filter cubes.
The major features of the CSU-10 confocal scanner are diagrammed in Fig. 3. The scanner uses a Nipkow disk positioned at an intermediate image plane so that the holes in the disk are par-focal with the specimen plane of the microscope. Figure 4 shows an image of the Nipkow disk obtained with the Orca ER camera when the motor that spins the disk is turned off. As mentioned above, the pinholes are fixed in size, about 50 μm in diameter. They are spaced in a constant pitch helical array with a spacing between the centers of the holes of about 250 μm. This spacing is needed to significantly prevent fluorescent light excited by illumination through one hole from entering an adjacent hole (Fig. 4). With these dimensions, there are about 1000 beams within a 7 × 10 mm imaging frame at the Nipkow disk. The illumination area on the specimen depends inversely on objective magnification, and for the ×100 objective it is about 70 × 100 μm. In the smaller field of view of our CCD camera, there are about 800 pinholes at any one time (Fig. 4). The disk spins at a constant speed of 1800 rpm. The pinhole array on the Nipkow disk of the CSU-10 is designed to raster scan 12 image frames in one rotation. At 1800 rpm, the disk scans 360 frames/sec.
Fig. 4. Camera image (900 x 900 pixels) of the pinholes. The motor for the spinning disk was turned off and an image was recorded, using a 200-ms exposure with transmitted light illumination of an empty slide–coverslip preparation viewed with a ×100/1.4-NA bright-field objective. Note that the pinholes have about a 50-μm diameter and occupy a small percentage of the disk area, about 5%. Bar: 5 μm.

A novel feature of the CSU-10 is that it has two disks: one is the pinhole Nipkow disk described above and the other is an upper disk that contains microlenses at positions aligned with the pinholes on the lower Nipkow disk (Fig. 3). Both upper and lower disks are mechanically connected and spun at the same rate. Our measurements from images like those in Fig. 4 indicate that the holes in the Nipkow disk represent about 4% of the disk area. A novel feature of the CSU-10 is that the lenses on the upper disk pass about 40% of the light incident on the upper disk through the pinholes. This helps significantly to overcome a major defect of the Nipkow disk design: poor incident light illumination intensity.\(^{10,15,16}\)

The excitation light path within the CSU-10 begins with the output from the fiber optic cable connected to the laser light source. Lenses expand the light from the end of the fiber into a wide beam capable of illuminating the 7 × 10 mm imaging area of the disks (Fig. 3). The illumination light passes an 8-position Sutter Lambda 10.2 filter wheel (provided by PerkinElmer Wallac; Table I) before becoming incident on the spinning disks. The Sutter wheel has variable speeds (adjustable by software) and is attached directly to the confocal unit (see Fig. 2). We typically use two excitation filters in the wheel: 488 and 568 nm. All other positions in the wheel contain aluminum disks to shutter the light. The electronic
shutter within the CSU-10 unit requires about 0.5 sec to open or close. This is much too slow for our applications. Therefore, we use the filter wheel for both excitation wavelength selection as well as to shutter the excitation light between exposures. This filter wheel can switch between positions within 50 ms, but we typically use 100 ms to prevent vibrations from reducing image resolution. To eliminate this vibration problem completely, it would be better to place the device for wavelength selection between the laser and the optical fiber input to the CSU-10.

Another major optical feature of the CSU-10 design is that the dichromatic mirror is placed between the upper and lower disks (Fig. 3). Excitation light is collected by the microlenses on the upper disk and projected straight through the dichromatic mirror to the conjugate pinholes of the lower disk and focused by the objective lens onto the specimen. Fluorescent light from an image point in the specimen excited by light from an individual pinhole is collected by the objective and focused back through the same pinhole. It then is reflected from the dichromatic mirror, redirected by relay optics through an emission filter, and then projected to the output port to the camera (Fig. 3). Note that the emission light does not go through the microlens disk and, unlike conventional Nipkow disk scanners, the excitation light that reflects off the front side of the microlens disk is separated from the emission light pathway. This design helps the CSU-10 achieve the sensitivity and high signal-to-noise ratio (SNR) in images needed to resolve the pattern of fluorescent speckles containing few fluorophores in our FSM applications.

Individual filter sets were available from the manufacturer for maximizing fluorescence for single-wavelength excitations of green or red fluorescence, using 488- and 568-nm laser lines, respectively (Table I). These emission filters have broadband emission spectra. In our living cell FSM studies, we were particularly interested in dual-wavelength imaging using fluorophores excited sequentially by the 488- or 568-nm laser lines by simply changing excitation filters, using the excitation filter wheel as described for wide-field FSM microscopy. For this application, Chroma Technology constructed multiple bandpass dichromatic mirror and emission filters (Table I) that have excellent transmission efficiency within their bandwidths and low levels of "bleedthrough" between the different fluorescent channels. Our measured bleedthrough between green and recording red is 1.4% and between red and green is 0.02%.

To maximize the light throughput of the system, it is crucial to ensure that the spinning disk is lined up on the optical axis of the side port of the microscope. To test this, the spinning disk is switched on and illuminated with either 488- or 568-nm light. An arc lamp focusing device is placed on the objective turret and the position of the illuminating light observed (as if you were focusing the wide-field epifluorescence arc lamp). The circular pattern should be centered on the focusing device. If it is not, the screws on the side port of the microscope are loosened and the confocal head shimmed until it is centered. Then the screws are retightened. We also use stiff foam placed under the camera to support the camera and to dampen the vibration produced by the excitation filter wheel.
Laser Light Source

We currently use a 100-mW argon–krypton air-cooled laser that has most of its energy in either the 488-nm line or the 568-nm line (Table I). The first adjustment is properly aligning the laser to the fiber optic cable. The laser light is coupled by a single-mode fiber optical cable between the laser and the CSU-10 (Table I). The laser can be aligned so that about 50–70 mW passes through the fiber optic cable into the confocal head. Alignment of the fiber should be done by using a high-sensitivity light power meter (Table I) with the laser on the lowest power setting, stand-by, to reduce risk of injury to persons and the tips of the fiber optic cable.

Cooled Charge-Coupled Device Camera

For FSM we needed diffraction-limited resolution and as close as possible to photon-limited sensitivity for resolving and detecting red or green fluorescent speckles containing only a few fluorophores so that excessive photobleaching would not be a major problem. In addition, we also had several applications requiring image acquisition at 3–5 frames/sec.

Fortunately, at the time we were assembling this imaging system, we were able to obtain a prototype camera from Hamamatsu that contained a newly developed CCD chip that meets our needs (Fig. 2). The current camera, an Orca ER, has about 60–70% quantum efficiency over a broad spectrum including the green and red fluorescent wavelengths (Fig. 5). There is no shutter in this camera so that shutter vibration and speed are not a problem. The CCD is an interline chip design, in which alternate columns of pixel elements are masked. To achieve high efficiency
of light collection to the pixels in the unmasked columns, microlenses are placed over each pixel to collect light from both the open and masked regions. A full frame for the imaging system reads \(1280 \times 1024\) light-collecting pixel elements in the chip. These pixels have a 6.45-\(\mu\)m center-to-center spacing along their rows and between columns.

During an exposure, photoelectrons are collected by the unmasked pixels and at the end of the exposure, the collected electrons are rapidly transferred to adjacent pixels in the masked rows and readout to the computer through high-speed digital connections. Exposures can be selected from 1 ms to 10 sec. We typically use between 200- and 1000-ms exposures, depending on sample fluorescence intensity.

Each pixel has a maximum well capacity of about 20,000 photoelectrons. Counting these electrons uses 12-bit analog-to-digital conversion, where \(2^{12}\) is equal to 4096 gray levels in an image. The conversion rate is about 4.9 electrons per digital count, so that 4096 gray levels corresponds approximately to the well capacity of 20,000 electrons.

The readout rate for the camera is 14.7 megapixels/sec, which corresponds to a full frame transfer time to the computer of about 120 ms. Faster rates of image transfer to the computer can be achieved by reading out subarrays within the chip. We often use this feature of the camera for imaging small cells such as budding yeast or regions within larger cells.

The other remarkable feature of this camera is that the readout noise at the 14.7 megapixels/sec readout rate is equivalent to about 8 electrons. This low noise is important for detecting weak fluorescence such as that from a few fluorophores. For a full well capacity of 20,000, the useful dynamic range of the camera is \(20,000/8 = 2500\).

To increase sensitivity with the Orca ER camera, we often use \(2 \times 2\) binning. This combines the electrons collected in four adjacent pixels on the chip and reads them out as 1 pixel. This gives a 4-fold increase in signal-to-readout noise by sacrificing a 2-fold decrease in lateral resolution and no effect on \(z\) axis resolution (see above). In many applications where fluorescence is weak and photobleaching is a problem, \(2 \times 2\) binning gives a sufficient signal-to-noise ratio (SNR) and a proportional reduction in the rate of photobleaching by reducing the intensity of illumination. For FSM, \(2 \times 2\) binning reduces the resolution of fluorescent speckles along microtubules or within bundles of microtubules, so we try to avoid binning if possible.3

**Image Acquisition, Storage, and Processing**

The filter wheel, focus motor, transmitted light shutter, camera settings, and image acquisition are controlled via a PC computer (Table I) running MetaMorph imaging software (Table I). The main features of the computer are 768 MB of RAM memory, a 50-GB hard disk, an \(8 \times\) CD writer, and a 21-inch monitor with a
large-enough viewing screen to display three channels of images as well as windows for controlling image acquisition. The Hamamatsu Orca ER camera is connected to the computer bus through a MuTech digitizing board that is controlled by the MetaMorph imaging software. Image acquisition, display, and storage are done with journal routines similar to those we have described previously for wide-field multimode, multiwavelength digital imaging microscopy. The green fluorescent, red fluorescent, and phase-contrast images are each displayed and stored as an image stack, with each image marked with the time of initial acquisition.

An important feature of the MetaMorph software for live cell applications is that each image stack can be reviewed on the screen in between image acquisitions or by pausing image acquisition during time-lapse mode. This allows the investigator to judge if focus needs correction or to judge when another experimental manipulation is needed. All fluorescent images are stored as 12-bit images while transmitted light (e.g., phase-contrast) images are converted to 8-bit images before storage to save memory. Currently we transfer image data to CD-ROM for permanent storage.

The MetaMorph software also has extensive analysis tools for image intensity measurement, contrast enhancement, arithmetic motion analysis, and color encoding. The software also has subroutines we often use for generating red, green, and blue 24-bit color image stacks from three 12-bit image stacks, aligning images within a stack for analysis by both translation and rotation, making montages of sequential images, and generating kymographs of fluorescent speckle movement.

Delta Vision Off-Line Deconvolution System

We have been performing constrained iterative deconvolution and three-dimensional (3-D) image reconstruction of confocal immunofluorescent images, using a Delta Vision off-line system (Table 13). This software produces significant improvement in z-axis resolution, enhances lateral resolution and, in general, greatly improves the contrast of fine structural detail in the specimen as well as providing 3-D views. We purchased the Softworx software with the ability to directly import MetaMorph image stacks. These image stacks are sent from the PC computer to the Silicon Graphics (Mountain View, CA) workstation (Table I) by file transfer protocol (FTP) through the Internet. To deconvolve the confocal images, the software is calibrated to the microscope as described in detail by Agard et al.13 Briefly, a PSF is experimentally obtained by acquiring a z series of 100 images at 100-nm steps through a 175-nm fluorescent bead (Molecular Probes, Eugene, OR) and stored as a MetaMorph image stack. A different z-series image stack through a fluorescent bead is acquired for each objective, and also for each wavelength. These z-series image stacks are then loaded into the Silicon Graphics computer and a subroutine in a pull-down menu is used to calculate the “optical transfer function” (OTF) for each objective at the different wavelengths used. These
OTFs are then applied to experimental data, producing the deconvolved data by a constrained iterative deconvolution method. The deconvolved data can then be rendered into a 3-D projection for accurate views of complex structures. Deconvolution is also effective in removing the weak "cross-talk" (about 0.5%) that occurs between adjacent pinholes of the spinning disk. Cross-talk means that emission light from a single point is collected by multiple pinholes, causing some loss of contrast. This combination of software and hardware creates a robust platform for high-resolution, high-contrast digital imaging.

Listed below are the specific steps we use for deconvolution and 3-D image reconstruction, because they have not been described in detail elsewhere.

1. Image stacks (.stk) are acquired by MetaMorph. For immunofluorescent specimens we typically acquire 100 images separated by 100-nm steps along the z axis.

2. Files are then transferred by FTP to the Delta Vision computer (SGI Octane Silicon Graphics) in the .stk format.

3. In the SGI, files are converted from MetaMorph Stacks (.stk) to Delta Vision (.dv) files by manually entering x and y pixel spacing as well as z step size, from a pull-down menu.

4. The .stk_dv files are then deconvolved, using a wavelength-specific optical transfer function (OTF) file.

5. The deconvolution method is done by using 15 constrained iterations (the default settings). This "ratio" or "conservative" method will more reliably come to a solution. For finer resolution more iterations can be used.

6. On deconvolution, the files (now called .stk_dv_d3d) are then color combined by using the Image Fusion subroutine in Softworx.

7. The color-combined files are then processed to create a 3-D reconstruction by using the Volume Viewer in Softworx. The standard 3-D reconstruction can be done around either the x or y axis for 180 or 360° with 15 projections or 45° around the normal or z axis. The viewing parameters that are used include maximal intensity method, best quality for optimal z section interpolation, and best z resolution. Custom rotations can be done in which one varies the number of the projections (and thus the angle between each projection), the start angle on the rotation, or the axis of rotation.

8. Quick projections, that form an image by taking the maximal intensity for each point through the stack, can also be made by using the Quick Projection subroutine for the .d3d files.

Examples

Our major applications for the spinning disk confocal microscope are for imaging structural dynamics in living cells, cytoplasmic extracts, or reconstituted preparations. Imaging fluorescently labeled structures in live cells presents several
practical problems. First, living cells do not react well with high-intensity light and photobleaching is a major problem, particularly for FSM, where speckles contain few fluorophores. Second, live cells are highly dynamic. Third, many studies involving live cells focus on subcellular structures and require high resolution. The spinning disk confocal microscope described here uses a sensitive, low-noise CCD camera for detection that allows the use of low-excitation light levels and provides longer observation periods with acceptable image SNR.

We have found our spinning disk instrument to be superior to laser scanning confocals for time-lapse imaging of live cells, including mammalian tissue cells, yeast cells, and *Drosophila* embryos. One advantage is that much less excitation light intensity is required for the same total exposure time. For example, to obtain a 1000 × 1000 pixel image using a laser point scanning instrument requires that fluorescence for each pixel element be recorded within 1 μs. This will require about 50,000 times greater intensity than needed for a 1-sec exposure, using our spinning disk instrument (the spacing between the pinholes reduces the net illumination time during the 1-sec exposure). Lower excitation light intensities also avoid the problem of fluorescence saturation that can occur with high-intensity illumination. The high quantum efficiency and low noise of the CCD camera also appear much better than that of the scanners, photomultipliers, and readout electronics commonly used with the laser scanning confocal instruments. The CCD also has a rapid readout, making it possible to acquire data at 5 frames/sec and that offers insight into dynamic processes. Combining a low-noise, fast-readout CCD with the spinning disk confocal unit and a high-numerical aperture objective lens produces a high-contrast, high-resolution image without the use of image-processing tools. However, postprocessing by deconvolution can enhance image quality if needed. Below is a brief description of several of our applications for the spinning disk confocal microscope that highlight its performance features.

Our spinning disk confocal system has important advantages for *in vitro* assays of molecular dynamics. It can image clearly individual fluorescent speckle microtubules assembled *in vitro* in thick chambers, where out-of-focus fluorescence makes individual microtubules nearly invisible by normal wide-field microscopy. Figure 6 compares images of fluorescent speckled microtubules bound to the inner surface of a coverslip that sits on top of a 70-μm-thick chamber of fluorescent microtubules at steady state assembly with a free tubulin subunit pool. About 2% of the tubulins are labeled with X-rhodamine. Individual microtubules are barely detectable from the background fluorescence by wide-field imaging with the CCD camera. In contrast, views of the same specimen (different region of the coverslip) with the spinning disk and CCD camera system and the same objective yield

FIG. 6. Comparison of (A) wide-field and (B) spinning disk confocal images of fluorescent speckle microtubules attached to the inner surface of a coverslip by kinesin motor proteins in a 70-μm-thick chamber of self-assembled microtubules and unassembled tubulin dimer subunits. About 2% of the tubulins are labeled with X-rhodamine to produce fluorescent speckled microtubules as they polymerize. Images were obtained with the Nikon ×100/1.4-NA Plan Apochromat objective and no binning in the camera. See S. V. Grego, V. Cailliana, and E. D. Salmon, Biophys. J. 81, 66 (2001) for details (reprinted with permission of the Biophysical Society). Bar: 5 μm.

high-contrast images of both microtubules and the fluorescent speckles along their lattice. The ability of the spinning disk confocal system to reject out-of-focus fluorescence is a major feature of the instrument important for FSM of cells, embryos, and extracts.

For live cell imaging, we have used our spinning disk confocal system to rapidly image GFP-tubulin-labeled microtubules and other spindle proteins in small cells such as budding yeast,12 in large embryonic cells such as those of Caenorhabditis elegans (Fig. 7), and in mammalian tissue cells for long periods of time (∼100–1000 images collected) with minimal photobleaching. Figure 7 shows an image of spindle and astral microtubules in a C. elegans embryo in first division. This embryo is expressing low levels of GFP-α tubulin25 and microtubules near the coverslip exhibit fluorescent speckles (data not shown). The image shown is at the center of the 40-μm-thick embryo. Light scattering by the yolk platelets makes imaging microtubule fluorescent speckles nearly impossible, but the rejection of out-of-focus fluorescence still provides images of excellent clarity for spindle and astral microtubule organization within the embryo. The spinning disk confocal allows fast, high-resolution imaging of the rapid embryonic mitoses of this genetic model system.

Drosophila melanogaster is another genetic model organism that can be difficult to image. The first 14 divisions are syncytial and the cell cycle time is short, about 15 min.26 The spinning disk confocal has also provided a fast, high-contrast, high-resolution solution to imaging microtubule dynamics of the early embryonic

Fig. 7. Rejection of out-of-focus fluorescence by the spinning disk confocal system produces clear images of the assembly dynamics of fluorescent microtubules in the first division of *C. elegans* embryos. A frame from a time-lapse record is shown. The worms are constitutively expressing GFP–α-tubulin. Images were obtained with the Nikon ×100/1.4-NA oil immersion objective using 2 × 2 binning in the camera. Bar: 5 μm.

In *Drosophila* embryos, where the mitotic spindles draw close to the embryo surface, we have been able to achieve excellent FSM of microtubule assembly dynamics within the spindles (data not shown) with our spinning disk confocal system.

In budding yeast, which are about 5 μm in diameter, we have been able to image GFP–tubulin fluorescent speckles within individual astral microtubules. Tran et al. have shown that the spinning disk confocal is effective for FSM of interphase microtubule dynamics within fission yeast.

S. Inoué, in pioneering work with polarization microscopy (reviewed in Inoué and Salmon), discovered by the early 1950s that mitotic spindle fibers were assembled in a dynamic equilibrium with a cellular pool of subunits (now known

FIG. 8. High-resolution spinning disk confocal FSM of microtubules relative to kinetochores in a *Xenopus* extract spindle. Shown is an image from a time-lapse series recorded with the Nikon x100/1.4-NA Plan Apochromat objective and no binning in the camera. Red fluorescent speckles on the microtubules were obtained by adding low amounts of X-rhodamine tubulin to the *Xenopus* egg extracts during spindle assembly. Kinetochores were selectively labeled with green fluorescent antibodies to the kinetochore-specific protein CENP-A. In this black-and-white image, the bright green kinetochores are superimposed on the red fluorescent speckle image of spindle microtubules. Bar: 5 μm.

Fluorescence speckle and confocal microscopy with our spinning disk system show with great temporal and spatial resolution the growth dynamics and poleward flux of individual microtubules within the spindle responsible for the spindle dynamic equilibrium. Figure 8 shows an FSM image taken with our spinning disk confocal system of a metaphase spindle assembled in isolated cytoplasmic extracts of *Xenopus* egg extracts. With the spinning disk confocal system we are able to resolve both fluorescent speckles within microtubules and the fibrous structure of the spindle fibers. By wide-field imaging, the density of fluorescence needed to detect individual bundles of microtubules within the spindle, like kinetochore fiber microtubule bundles, makes detection of fluorescent speckle contrast nearly impossible. In Fig. 8, kinetochores are marked by the bright white dots of fluorescence in the black-and-white image.

Fig. 9. Deconvolved confocal images of immunofluorescence specimens yield a view of extraordinary high resolution, clarity, and SNR of immunofluorescently labeled microtubules in a mitotic spindle and surrounding interphase PtK1 mammalian tissue cells. Red fluorescent confocal optical sections were obtained with the Nikon ×100/1.4-NA Plan Apochromat objective at 100-nm z-axis steps through the specimen with 20 planes included in the stack for both regions above and below the specimen. The total image stack was deconvolved in the DeltaVision system and rendered in 3-D. The image shown is a maximum-intensity projection along the z axis through the 3-D volume. Bar: 5 μm.

of the red fluorescent speckles within the spindle and astral microtubules. These dots were obtained from the green fluorescence of a kinetochore marker as described in the caption to Fig. 8. These dots mark the kinetochore ends of bundles of kinetochore microtubules. Time-lapse movies of images similar to that in Fig. 8 show that fluorescent speckles form at kinetochores and flux poleward at the same rate as the majority of microtubules in the spindle. These data were used to show that at metaphase, microtubule polymerization occurs at kinetochores at the rate of poleward microtubule flux (about 2 μm/min at 19°) and that microtubule depolymerization occurs near the spindle poles at the same rate because the spindle maintains a steady state constant length.11

In addition to our live cell and extract studies, we have been able to use our spinning disk confocal system in combination with deconvolution to obtain high-resolution, high-quality, multicolor 3-D images of the distribution of the molecular components of the mitotic spindle, chromosomes, and kinetochores within immunofluorescently stained PtK1 mammalian tissue cells.30 Figure 9 shows the microtubule channel from a multicolor deconvolved image of microtubules (red)

FIG. 10. Comparisons of objective point-spread functions (PSFs) in the $x$–$z$ plane (side view) that were obtained for green fluorescent beads (175 ± 5 nm; Molecular Probes, Eugene, OR) by wide-field (WF) and spinning disk confocal (CF) before (WF, CF) and after deconvolution (WF-DC and CF-DC). Images were obtained with the Nikon ×100/1.4-NA Plan Apochromat objective and no binning in the camera. Bar: 1 μm.

and chromosomes (green, not shown). The remarkable SNR of the spinning disk images makes the exceptional clarity of the microtubules images possible.

Our last example is a comparison of the lateral and $z$-axis image resolution of a 175-nm green fluorescent bead obtained by wide-field, spinning disk confocal and by deconvolution of wide-field and spinning disk $z$-axis image stacks. A Nikon ×100/1.4-NA Plan Apochromat objective was used to obtain 100 images at 100-nm steps though the specimen for both the wide-field and spinning disk image stacks. These stacks were then sent to the DeltaVision system and 3-D views obtained. In Fig. 10, we show the $x$–$z$ intensity pattern of the bead PSF along the optical axis through the center of the bead. The width of the pattern in the $x$ direction at focus is the lateral width of the Airy disk image of the bead, while the width of the pattern along the $z$ axis is the depth of the central maximum of the Airy pattern along the $z$ axis.\(^{20,31}\)

One measure commonly used in confocal microscopy for lateral and longitudinal resolution\(^{31}\) is to take $x$-axis and $z$-axis line scans through the point of focus and measure values for the full width of the profile at half the intensity of the peak (FWHM). Our measured FWHM values from $x$-axis scans were 330 nm for

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FIG. 11. Comparison of full-width half-maximal (FWHM) values obtained from line scans in the x-axis (X) and z-axis (Z) directions through the focal point of the PSFs shown in Fig. 10 for wide-field (WF), confocal (CF), and deconvolved wide-field (WF-DC) and confocal (CF-DC) images.

Wide-field, 200 nm for wide-field with deconvolution, 260 nm for confocal, and 180 nm for confocal with deconvolution. FWHM values from z-axis scans were 680 nm for wide-field, 400 nm for wide-field with deconvolution, 520 nm for confocal, and 290 nm for confocal with deconvolution. These measurements are plotted in Fig. 11 for comparison. The confocal improves both lateral (by 21%) and z-axis resolution (by 24%) compared with wide-field imaging, but most significant is the improvement in z-axis resolution. For both lateral and z-axis resolution, wide-field plus deconvolution gives better performance than the spinning disk confocal without deconvolution, 40 and 41%, respectively. However, deconvolution of the spinning disk images gives the best resolution, by about 9% over wide-field plus deconvolution for lateral and 27% for z-axis resolution.

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