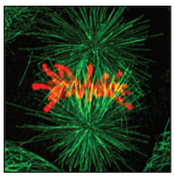

Contents

CHAPTER TEN

OMX: A New Platform for Multimodal, Multichannel Wide-Field Imaging

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BIOIMAGING IS CURRENTLY UNDERGOING AN EXCITING revolution. This includes all aspects of imaging from probe development, specimen preparation, and instrumentation to image analysis and quantitation. Perhaps the most exciting developments are new platforms for imaging that radically advance the capabilities for collecting high spatial and high temporal resolution data. In many cases, the standard microscope has been replaced with new purpose-built platforms that are much more flexible and enable the implementation of new imaging modalities such as particular single-molecule and super-resolution imaging methods.

For live cell imaging, there are a number of competing critical requirements. Any live cell imaging system must be physically stable so that vibrations and temperature shifts do not move the sample or the optical path. This requirement is undermined by the need to change focus and to collect images as rapidly as possible. Fast live cell imaging thus requires a very stable, rapidly, and accurately moving imaging system with little vibration or temperature change. Photobleaching and photodamage limit the photon budget, the number of photons transmitted through the microscope. When working at photon-limited levels, any additional sources of background and noise, such as stray light or noise from camera electronics, must be avoided. A recent study has highlighted the presence of additional non-Poisson noise in all tested commercial imaging platforms (Murray et al. 2007). Thus, photon-limited imaging is extremely challenging on traditional microscope platforms.

In this chapter, we discuss the design principles and applications of the OMX microscope, a new platform that provides unprecedented mechanical and thermal stabilities coupled with a photon budget that is dramatically improved over traditional microscope platforms. These characteristics make the OMX microscope outstanding for fast live cell imaging and super-resolution imaging. Moreover, its open flexible architecture makes it particularly amenable to adding other modes of microscopy to the platform.

HISTORY AND DESIGN OF OMX

OMX was designed and built by John Sedat in collaboration with David Agard (both at the University of California, San Francisco [UCSF]) and a number of coworkers including Mats

[4]Joint first authors.

Gustafsson (now at Janelia Farm Research Center), Lukman Winoto, and Peter Carlton. The microscope's name is derived from the original name that Agard and Sedat gave to their first wide-field deconvolution microscope, Optical Microscope 0 (OM0). OM0 was based on a Zeiss Axiomat, with an LN2-cooled charge-coupled device (CCD) camera from Texas Instruments, and was run from a VAX 8650 mainframe computer. OM0 was used to acquire the first three-dimensional (3D) fluorescence images of cellular structures (Agard and Sedat 1983). There followed OM1, a turnkey, Silicon Graphics, Inc., workstation-controlled microscope that included fiber-optic illumination and stage-based focusing on an inverted microscope, and this provided some of the first 3D fluorescence images of living cells passing through the cell cycle (Minden et al. 1989). This was later commercialized as the DeltaVision microscope (Applied Precision, Inc. [API]), which continued its own path of development. The X in OMX stands for "eXperimental," as the microscope is a continuously evolving platform that allows further development and improvement.

Traditional microscope stands have to take into account optical performance, cost, and ease of use. The optical path is designed to accommodate a range of additional components as well as the microscopist sitting at the microscope using the eyepieces and controls. Consequently, the efficiency of light transmission and the suppression of stray light are compromised. Additionally, it is difficult to rapidly and efficiently capture two channels simultaneously in wide-field microscopes, and capturing three or four channels simultaneously is nearly impossible. Although laser-scanning confocals can often capture multiple channels simultaneously, they are relatively slow, taking seconds to minutes per image stack, and have much lower photoefficiency (Murray et al. 2007). OMX was designed and built to provide a flexible platform that would be a foundation for many different modes of microscopy and to reduce the limitations found in conventional microscope platforms.

OMX includes separate modules for illumination, imaging, microscope control, and locating the sample. The layout and individual components are shown in Figure 1. For illumination, OMX uses solid-state continuous-wave lasers to provide bright monochromatic illumination. At least five separate lasers can be installed in the laser bed. Shuttering is achieved using individual solenoids that can operate reliably down to 1-msec opening time, and six-position wheels carrying neutral-density filters provide attenuation to control the excitation light intensity very precisely. At any time, all active lasers are focused into one of two fibers that provide alternative light paths into the microscope body. In the current implementation of OMX, one path is used for fast live cell imaging using conventional optics, and the other is used for three-dimensional structured illumination microscopy (3D-SIM). As described below, the flexible open design of OMX allows many alternative configurations.

The microscope is based on a solid-metal-block platform drilled to allow fitting of components. The block holds a kinematically mounted drawer that contains the elements of the fluorescence light path. This base is mounted on an antivibration table, and the whole assembly is housed in an acoustically isolated thermally controlled room. For fluorescence, the light path has been optimized to collect emission light, somewhat at the expense of excitation light. This block-and-drawer configuration replaces the standard microscope stand. The stage and lens mounting are made of Invar, a nickel–steel alloy with a very low coefficient of thermal expansion. Instead of using a rotating turret with a focus knob to raise, lower, and quickly change objectives, OMX objectives are fixed into kinematically mounted Invar plates adding stability and reproducibility. Focus change for optical sectioning is achieved by a piezoelectric device that changes the position of the stage and sample while leaving the lens fixed in place. Two Nanomover motors (Melles Griot) are used for translation in the image plane. There are no binoculars, and the microscope is kept isolated from the user in a filtered temperature-regulated environment. Up to four separate cameras can be mounted on OMX enabling fast and simultaneous multichannel data acquisition.

The fluorescence drawer used in OMX contains four beam splitters (BS1–4) that direct emitted light to the cameras (Fig. 1). The central beam splitters (BS1 and BS2) permit all wavelengths of excitation light to pass through virtually unreflected; the very small amount of reflected light

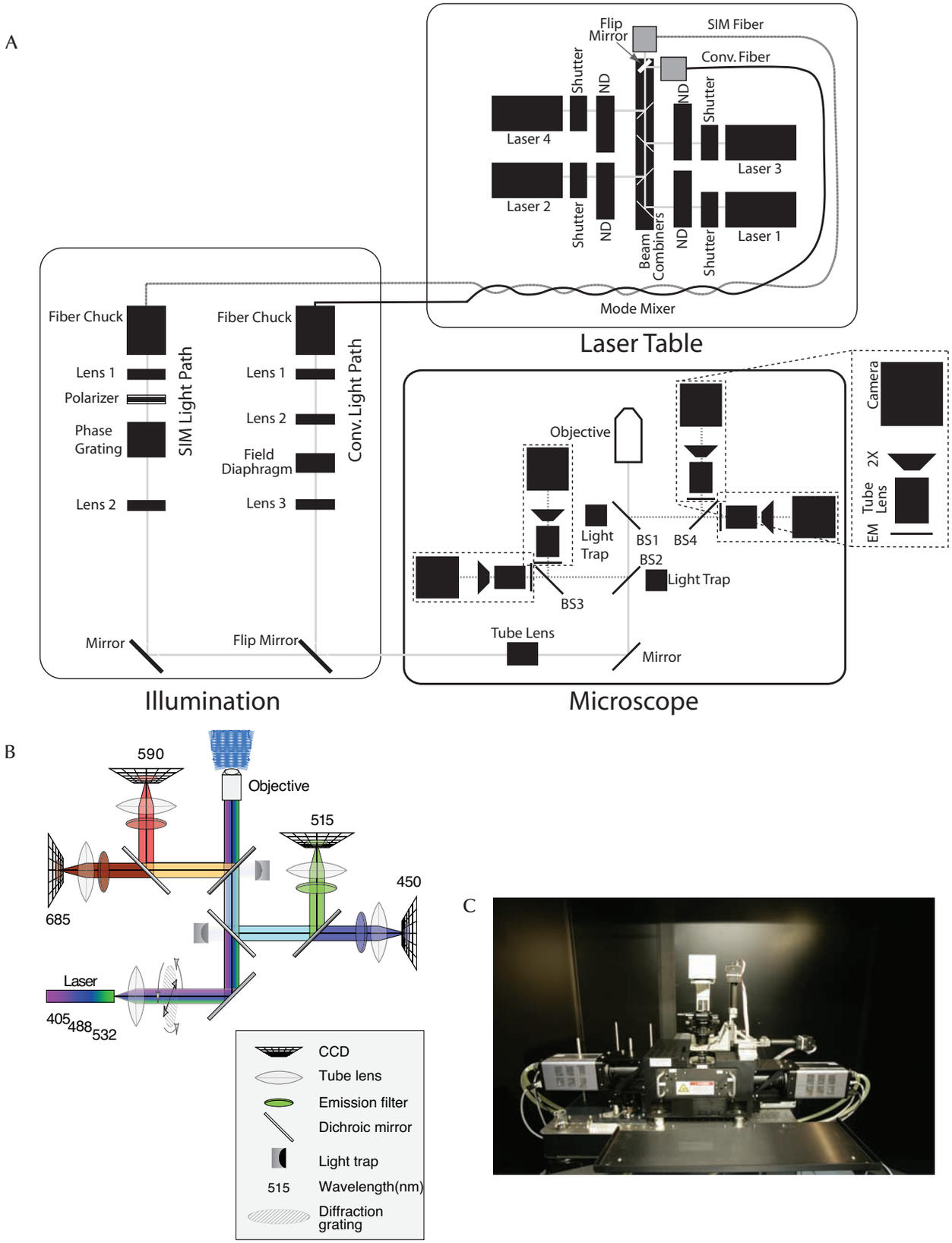

FIGURE 1. OMX components and layout. (*A*) Schematic of the optical setup of OMX. (*B*) The light path for the live cell drawer configuration to enable the use of simultaneous imaging of fluorescent combinations including CFP/YFP, GFP/mCherry, and CFP/YFP/mCherry. (*C*) A photograph of the OMX microscope body. (*A*, Courtesy of Paul Goodwin, API. *B*, © 2008 American Association for the Advancement of Science. Used by permission.)

is blocked by light traps suppressing stray light and, hence, reducing background. Emission light returning from the sample is reflected by the beam splitters to the appropriate cameras. First, both red and far-red light are reflected by BS2 to the left, where BS3 reflects red light to one camera and allows far-red light to pass through to another. Green and blue light pass through BS2, but both are reflected by BS1 to BS4, which directs green light to one camera and allows blue light to pass through. Drawers containing different beam-splitter arrangements may be easily switched in and out to allow simultaneous imaging of various combinations of fluorophores. Multiple cameras and a simply exchanged filter assembly allow the emitted light to be split in other ways such as by polarization.

OMX is controlled by a group of Windows-based computers that provide all user interfaces and control of the cameras, shutters, and other devices. The software controlling the system is written in C++ and Python. The Python source code is directly accessible on the computer during active operation and can be modified and reloaded at will. Users can issue commands or create new scripts via the built-in Python interpreter to control operation of the microscope at many levels, at any degree of sophistication desired. This flexibility allows complex illumination strategies such as those required for PALM-type experiments to be easily implemented. All functions running on auxiliary computers are accessible from the main control computer over the network via the Pyro distributed-object system (http://pyro.sourceforge.net/). Most user actions are saved to logs, so imaging sessions can be exactly recreated or debugged in case of problems. Furthermore, the temperature of the stage, the microscope body, the motors, and the entire room is continuously monitored and saved to disk, so users are aware of any possible irregularities.

To make sample finding and setup easy, the OMX uses a dedicated conventional auxiliary microscope, the low-magnification microscope (LMX). The LMX has a range of high-working-distance objectives with both transmission and epifluorescence illumination. The LMX has a high-precision motorized stage used for sample location and mapping. This stage is cross-indexed to the OMX stage to allow sharing of coordinates between the two systems. The LMX is configured to enable tile scanning of the entire region of a conventional slide that is within the stage travel of OMX. This tile scan can then be exported to the OMX control computer and used as a location map for finding specific features or locations once the slide has been mounted on OMX.

The workflow for using OMX for fixed samples involves preparing the samples, imaging them first on the LMX using a tile-scan mode in either bright field or fluorescence, and then moving the sample to the OMX stage. The tile scan is transferred to the OMX control computer, and the OMX software is used to locate regions for further imaging. For live cells, a prescan can be performed on the LMX and then used as a basis for finding living cells, or the live cell chamber can be directly mounted on OMX and then scanned using the OMX control software. The tile scan is displayed on the screen using texture mapping, allowing very fast panning and zooming over an entire slide's worth of image data at high resolution. This arrangement allows the operator to scan the slide at leisure without either illuminating the sample or needing to use eyepieces.

The first OMX prototype, OMX v1, was designed and built in John Sedat's laboratory at UCSF. API has licensed the OMX design and has built a number of beta systems referred to collectively as OMX v2. The data in Figures 2–6 are from two OMX v2 systems based at the Universities of Oxford and Dundee.

IMAGING APPLICATIONS USING OMX

The first versions of OMX have been outfitted for two specific applications: fast multichannel microscopy for high sensitivity and high temporal resolution analysis of living cells and 3D-SIM for high spatial resolution imaging of fixed cells. In the next sections, we detail the use of these different modes and show examples of the results that can be achieved.

Fast Live Imaging for High Temporal Resolution Analysis

Biological processes operate on a wide range of timescales from submillisecond to many minutes. The drive to develop mathematical models of molecular interactions presumes the availability of high-quality data sets that properly sample these events. Currently, conventional microscopes can record many single images per second. However, when sampling across space (optical sections) and spectral range (as in multichannel fluorescence microscopy), the practical limit of temporal sampling is on the order of ~1 3D image/sec. Any process that occurs on a subsecond timescale is thus subject to temporal aliasing. Furthermore, the raw speed of time-lapse acquisition is not necessarily the key parameter to consider because the efficiency of light transmission and detection determine critically whether useful images can be acquired in any particular set of conditions.

OMX offers two distinct advantages for live cell imaging. First, up to four channels can be recorded simultaneously, or in the case of fluorophores with some spectral overlap, in very rapid sequence such that the delay between sampling at different wavelengths is no more than 1–2 msec. Second, the very bright laser-light sources, fast shutter, rapid and stable focusing, and integration of electronic control mean that very rapid, accurate, and precise 3D imaging is achievable. The current implementations of OMX can record 2–10 3D images/sec for each channel. The fastest we have run our microscopes, with 1-msec exposure, is 93 or 107 images/sec depending on the precise individual prototype. The major speed bottleneck is the read time of the cameras, which for a full-frame image (512 × 512-pixels, 10 MHz, 16 bits) is 13 msec. Reducing the imaging area on the CCD substantially reduces this time.

Most live cell imaging applications make use of fluorescent proteins (FPs). The choice of FP is dependent on the laser lines present on the system and on the brightness and photostablity of the proteins. For simultaneous acquisition of multiple wavelengths, well spectrally separated FPs work best. For example, green fluorescent protein (GFP) and mCherry are compatible with the 488 nm and 593 nm laser lines, do not spectrally overlap significantly, and are relatively photostable. Figures 2 and 3 show examples of using this mode of imaging FP-labeled living yeast, *Drosophila,* and human cells

To date, OMX uses the Bioptechs FCS2 closed live cell chamber. Samples are grown on or adhered to 40-mm circular (no. 1.5 thickness) coverslips either by their own adherent properties or by coating slides with substances such as polylysine or concanavalin A. The design of the FCS2 system permits temperature control and media exchange but not an environment in which CO_2 is regulated. A critical advance for the future is the design and building of an environmental chamber for live cell imaging.

3D-SIM for High Spatial Resolution Analysis

The achievable resolution in light microscopy has been limited to ~$\lambda/2$ by diffraction since the development of the modern microscope in the late 1800s. Over the past few years, a number of methods for overcoming this diffraction limit have been developed. These methods are collectively known as super-resolution techniques (Hell 2009).

3D-SIM uses structure within the illumination and multiple images per sample section combined with postacquisition analysis to double the achievable resolution in all three spatial dimensions. By illuminating a fluorescent sample with a sinusoidal striped pattern, additional information from the sample is encoded in the fluorescent emissions (Gustafsson 2000; Schermelleh et al. 2008). The phase of the striped pattern is shifted over a full cycle in five steps and also rotated to three positions at 60° intervals. The resulting 15 images per *z* section are processed to produce the final super-resolution image. Images of fluorescent beads in both conventional widefield microscopy and 3D-SIM, with profiles showing the resolution improvement, are shown in

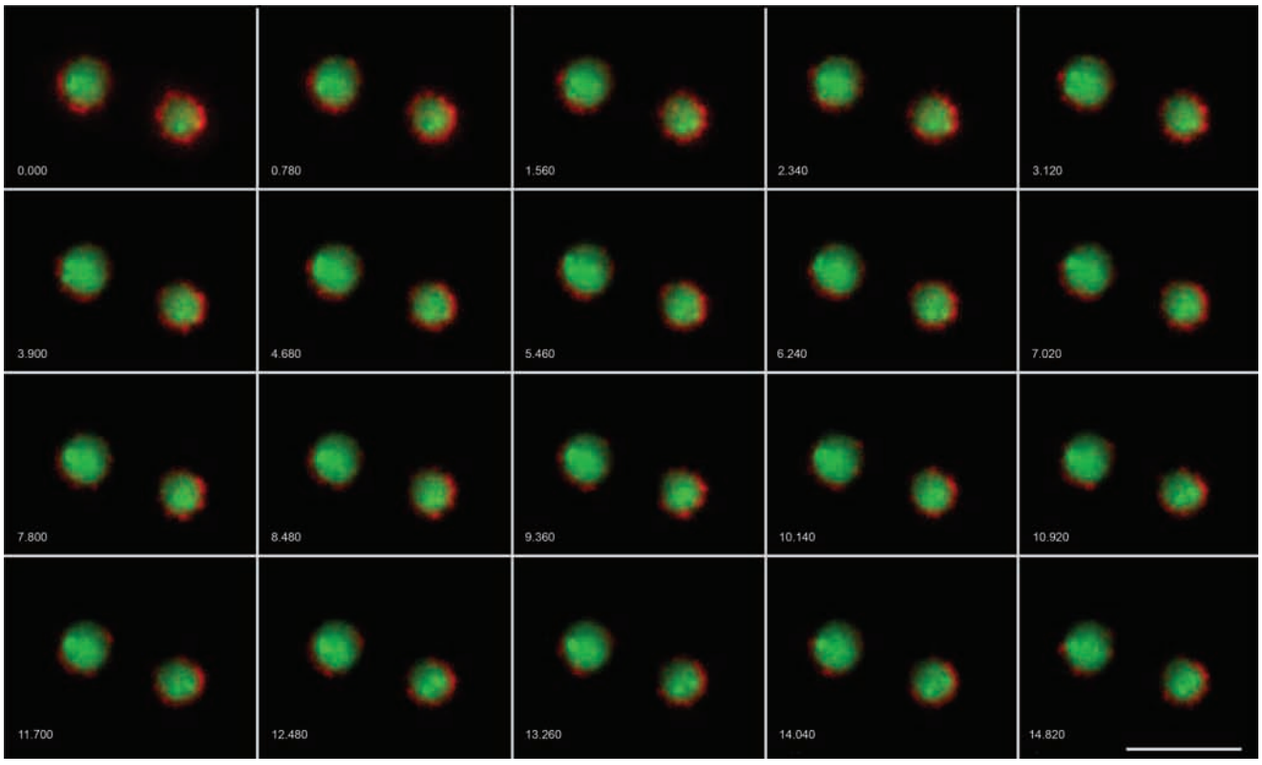

FIGURE 2. Live cell imaging with OMX showing *Saccharomyces cerevisiae* with mCherry-labeled nuclear pore complexes and GFP-TetO-tagged chromosome IV (13 z sections, 0.3 µm apart, time lapse 780 msec). Wavelengths were acquired simultaneously on OMX v2 and then aligned and fused. The images have been deconvolved, and the maximum intensity is projected. Numbers indicate elapsed time in seconds. Scale bar, 5 µm. (Courtesy of Emma King and David Dickerson, University of Dundee, and Paul Goodwin, API.)

Figure 4. The achievable resolution varies from 105 nm with 405 nm illumination to 165 nm with 593 nm resolution (Gustafsson 2000).

As implemented on the current versions of OMX, 3D-SIM is realistically useful for fixed specimens. With that caveat, 3D-SIM on OMX has been successfully applied to a broad range of specimens—microorganisms, vertebrate cells, tissue sections, and even whole embryos. Our own microscopes have been used by a variety of collaborators, and these data will be published elsewhere. Figures 5 and 6 show two examples of the application of OMX in cultured *Drosophila* macrophages and HeLa cells (Fig. 5) and a section of fixed mouse colon (Fig. 6). The improvement in axial resolution in comparison with conventional deconvolution or confocal imaging is apparent in the subcellular structures visible in these images. For example, the diameters of microtubule fibers are closer to their true sizes, and overlapping and dense fields of microtubules or actin networks can be resolved into individual fibers much more readily.

Sample Preparation and Imaging Protocols for 3D-SIM

Sample preparation for imaging using the structured illumination (SI) protocol on OMX does not vary greatly from that used for image acquisition on other systems. However, emphasis needs to be placed on good practice to ensure a specific, bright, and photostable fluorescent signal as well as good morphological preservation. Fixation should be of the highest quality that is consistent with preserving epitopes and the fluorescence of FPs as appropriate.

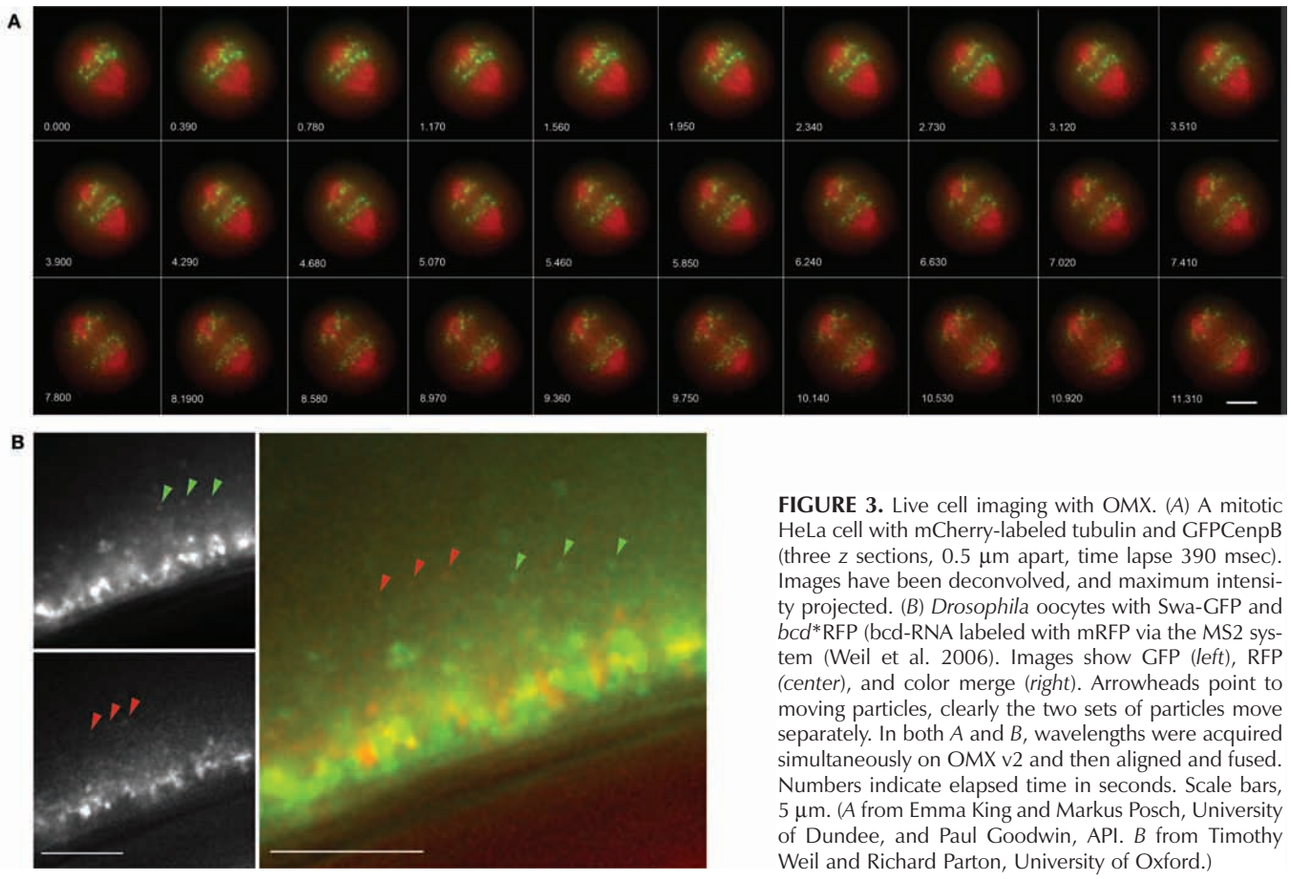

FIGURE 3. Live cell imaging with OMX. (*A*) A mitotic HeLa cell with mCherry-labeled tubulin and GFPCenpB (three *z* sections, 0.5 µm apart, time lapse 390 msec). Images have been deconvolved, and maximum intensity projected. (*B*) *Drosophila* oocytes with Swa-GFP and *bcd**RFP (bcd-RNA labeled with mRFP via the MS2 system (Weil et al. 2006). Images show GFP (*left*), RFP (*center*), and color merge (*right*). Arrowheads point to moving particles, clearly the two sets of particles move separately. In both *A* and *B*, wavelengths were acquired simultaneously on OMX v2 and then aligned and fused. Numbers indicate elapsed time in seconds. Scale bars, 5 µm. (*A* from Emma King and Markus Posch, University of Dundee, and Paul Goodwin, API. *B* from Timothy Weil and Richard Parton, University of Oxford.)

The system is designed for use with scrupulously clean no. 1.5 (0.17 mm thick) coverslips. So far the system has been used to image material within 16 µm of the surface of the coverslip. The fixation conditions and the choice of primary antibody (one that localizes strongly to the structure of interest) need to be optimized to achieve a good signal-to-background ratio, thus optimizing reconstruction output and minimizing the generation of reconstruction artifacts. A broad range of samples has been successfully imaged using the SI protocol of OMX—from cell monolayers to mouse-gut wax sections to plant-leaf peels.

There is a wide choice of bright photostable secondary antibodies to match the laser lines available on the system allowing excellent image quality by optimizing the match between the filter systems and the excitation and emission characteristics of the fluorochromes. We have successfully utilized Alexa Fluor–conjugated secondary antibodies (Invitrogen), and recently, Jackson ImmunoResearch, Inc. has released a new DyLight collection that further expands the availability of wavelength-specific antibodies to target a broader range of primary antibodies raised in different species. The 593 nm laser line is compatible with both 568 and 594 nm excitable dyes. Protein fusions of a bright FP, such as GFP, can be used for imaging samples that require a small number of *z* sections and/or exposure to a limited number of excitation wavelengths. However, the photostability of FPs has so far limited their use in 3D-SIM. Acquisition of images using the 3D-SIM protocol is possible using cameras with electron-multiplying CCD amplification enabled, which has opened the door to the imaging of dimmer samples with low background fluorescence. Furthermore, to protect the fluorescence emitted and to minimize

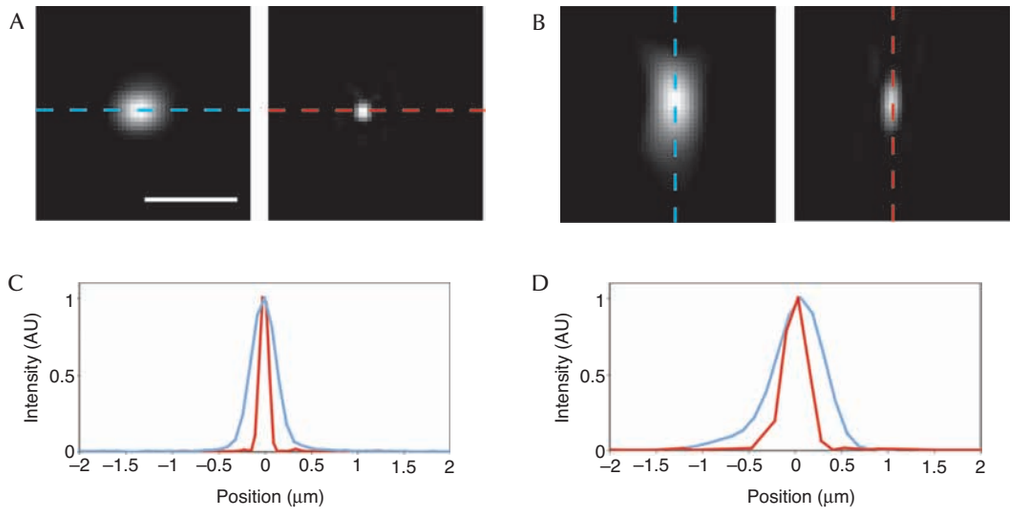

FIGURE 4. OMX point-spread functions with and without 3D-SIM. Images (*A,B*) and line scans (*C,D*) of 89-nm fluorescent beads illuminated at 488 nm and detected at 510 nm in conventional wide-field- and 3D-SIM-imaging modes. *x-y* (*A*) and *x-z* (*B*) are shown in conventional wide-field mode (*left* images) and in a 3D-SIM reconstruction (*right* images). Profiles through the center of a bead clearly show the increase in resolution between conventional wide-field microscopy (blue) and 3D-SIM (red), in both *x-y* (*C*) and *z* (*D*), demonstrating the increase in resolution is achieved in *z* as well as in *x-y*. Scale bar, 1 μm. (Courtesy of Ian Dobbie, University of Oxford.)

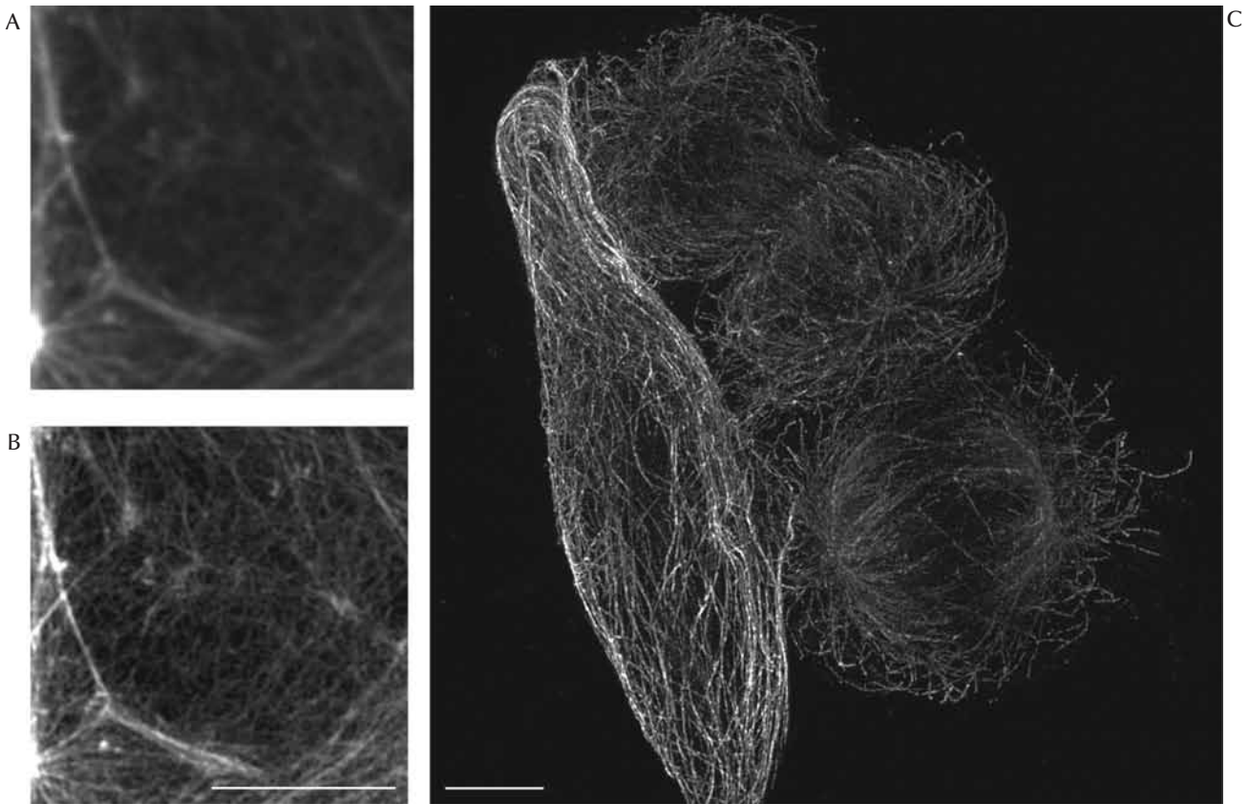

FIGURE 5. 3D-SIM imaging of cultured cells on OMX. *Drosophila* macrophage cells that have been fixed and stained for F-actin with fluorescein-isothiocyanate (FITC)-phalloidin are shown (*A*) in a conventional wide-field image and (*B*) in a 3D-SIM reconstruction; the latter clearly shows the dramatic increase in detail achieved. (*C*) A formaldehyde-fixed HeLa cell-stained antitubulin and an Alexa Fluor 488 secondary antibody are also shown; these images were acquired using the 3D-SIM protocol on OMX v2. For each panel, the image shown is a maximum-intensity projection through the full volume of the cells. Scale bars, 5 μm. (Parts *A* and *B* are from Ian Dobbie and Ilan Davis, University of Oxford. Part *C* is from Jason Swedlow, University of Dundee, and Paul Goodwin, API.)

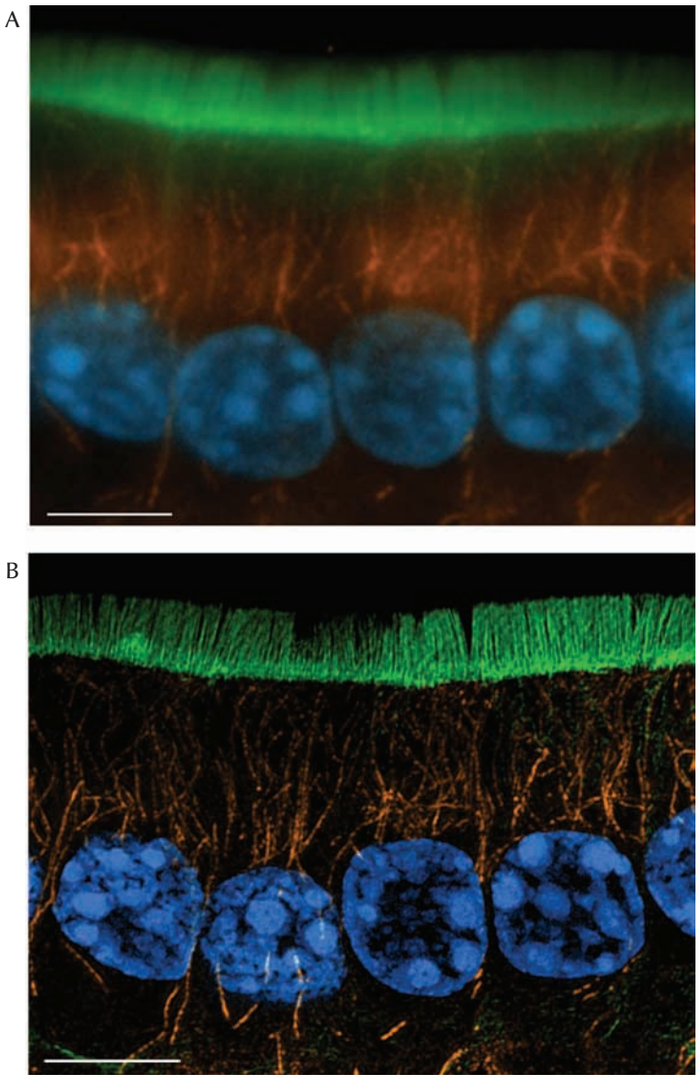

FIGURE 6. 3D-SIM imaging of cryosections on OMX. A 10-µm cryosection of formaldehyde-fixed mouse small intestine, stained with DAPI (4′,6-diamidino-2-phenylindole) to show nuclei (blue), antitubulin/Alexa Fluor 568 to show microtubules (orange), and FITC-phalloidin to show F-actin (green), is shown as (*A*) a deconvolved conventional wide-field image with five *z* sections and maximum-intensity projection and as (*B*) an image acquired using the 3D-SIM protocol on an OMX v2. In *B*, the wavelengths were acquired sequentially, reconstructed, and then aligned and fused to form a five-*z*-section maximum intensity projection. Scale bars, 5 µm. (Courtesy of Emma King and Paul Appleton, University of Dundee.)

changes of refractive index in the light path, samples should be mounted in a medium that matches the refractive index of the objective lens fitted. The mountant should contain antifade agents such as 1,4-phenylenediamine in a buffer of 90% glycerol and 10% Tris; we have found this to give optimal results, especially with 594 nm excitable dyes.

FUTURE DIRECTIONS

OMX is fundamentally a research instrument and is still under development. Its basic premise of reworking the fluorescence light microscope from the ground up provides a very flexible new platform. Currently, the commercial OMX sold by API is set up for fast live multichannel imag-

ing and super-resolution 3D-SIM. Adding novel functionality is relatively easy because of the open nature of the excitation light paths and the flexibility of the hardware and software controls. Some of the possible extensions in functionality are discussed below, although this list is far from exhaustive and is continuously evolving.

Two-Dimensional SI Microscopy in Living Cells

3D-SIM on OMX is able to double the resolution compared with a conventional fluorescence microscope. However, there are two major drawbacks to the technique. To perform 3D SI, at least five illumination phases must be recorded at three different angles. Therefore, for each reconstructed image section, 15 images must be recorded. This can cause substantial photobleaching and very slow acquisition because of the large number of images required. Data collection is further slowed by the fact that the current implementation of OMX rotates a physical diffraction grating to take images with the SI at different angles. This process requires ~2 sec per angle change. To properly perform reconstructions to provide the higher-resolution output image, any structure of interest must not move during imaging by a significant fraction of the 100 nm resolution. In the current generation of OMX, the minimum time to take a full SI *z* series is ~10 sec, long enough for the internal contents of the cell to move substantially more than 100 nm. This situation can be improved in two complimentary ways. First, by providing *z* sectioning via total internal reflection fluorescence (TIRF), only a single *z* section is required, and the number of images that are captured is reduced to 9, three phases at three angles. It should be noted that this provides a single-section super-resolution image rather than a 3D-image stack. Second, using a spatial light modulator, the diffraction grating angle can be changed without physically moving the optical elements. Combining these two approaches allows single two-dimensional (2D) TIRF super-resolution images to be collected in <100 msec (Kner et al. 2009).

TIRF

TIRF is a method for achieving 100 nm *z* resolution combined with very low fluorescence background. This is achieved by illuminating the sample with light above the critical angle for complete reflection between the coverslip and the sample. This produces an evanescent wave parallel to the coverslip that falls exponentially with distance from the interface (see Chap. 36). Multiwavelength objective-based TIRF has been implemented on the Sedat laboratory OMX by redirecting the standard wide-field illumination into a multiwavelength single-mode fiber. This fiber is then translated to shift the position of the beam path in the back focal plane. This allows rapid changes of the angle of incidence to achieve TIRF at different wavelengths for multicolor live TIRF imaging.

3D TIRF

The precise control of the angle of incidence on OMX also allows the acquisition of TIRF images at a range of angles all above the critical angle. In this way, an image stack can be created by steadily imaging further into the sample. Image processing allows calculation of the depth of objects within the image with very high *z* resolution. This technique allows imaging with 100 nm *z* resolution to depths up to 1 μm.

Image Processing for Live Cell Imaging

A primary design aim of OMX is to maximize the signal-to-noise ratio (SNR) to gather as much data as possible from limited illumination intensity and short exposures that occur in fast imaging of highly photosensitive living cells. Analyzing the data generated in fast live imaging requires

the implementation of object identification and tracking software that can track rapidly moving particles in images with limited SNR (Jaqaman et al. 2008). These tools are critical for delivering quantitative measurements of objects recorded in live cell imaging (see Chaps. 13, 15, and 16).

A promising area for future improvement in fast live cell imaging is the use of image-processing tools to improve SNR and thus the performance of subsequent analysis tools. Deconvolution, used on 3D-data stacks from OMX, is able to improve the SNR using the point-spread function to develop an estimate of the in-focus object in the sample (Swedlow et al. 1997; Wallace et al. 2001; Parton and Davis 2006). In addition, the application of denoising algorithms can substantially improve the appearance of image data. Denoising relies upon the fact that signal but not noise is correlated between adjacent pixels in 2D or 3D. In 2D, denoising is relatively simple and widely applied using processes such as a Gaussian or a median filter. Extending this to 3D further improves the SNR because extra information is available. Recently, more advanced algorithms have become available. By applying denoising techniques to four-dimensional image sets, 3D data in time, even more SNR improvement can be gained.

Photoactivation Localization Microscopy

A second technique for generating so-called super-resolution images, with a precision >20 nm, is photoactivation localization microscopy (PALM) (Betzig et al. 2006). This technique works by iteratively building up an image from the fluorescence emission of individual dye molecules imaged in a stochastic manner, a small number at a time. Basic 2D PALM, as described above, can be achieved easily on OMX, as it simply requires a custom illumination pattern, low-intensity activation pulse, followed by multiple frames of standard excitation imaging. This can either bleach out the active molecules or be followed by a deactivation pulse. *z*-position information can be added to this by either taking images at two *z* positions (Juette et al. 2008) or by using an imaging system with astigmatism (Huang et al. 2008). The multiple cameras and simple filter arrangement on OMX allow multiple *z* positions to be simultaneously acquired using a 50–50 beam splitter between the two channels and introducing an extra lens into one channel producing a focus shift relative to the other channel. Alternatively, astigmatism can be easily introduced by having a cylindrical lens in the illumination path. On OMX, this is easily achieved because of the open access to the illumination beam path.

CONCLUDING REMARKS

Because of its flexibility, the OMX platform is still rapidly evolving. We anticipate that many more functionalities will be added to the platform, as essentially all applications of wide-field fluorescence microscopy can be improved by taking advantage of the improved light budget, stability, and simultaneous acquisition characteristics of the system.

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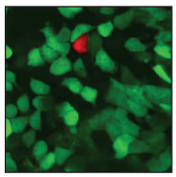

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