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Advanced photonic tools for hyperspectral imaging in the life sciences

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Hyperspectral methods using acousto-optic and digital micromirror devices have the potential to improve imaging speed and accuracy with an expanding range of uses, including clinical diagnostics.

By the 1950s, optical microscopy in the life sciences had fallen out of favor. The improved electron microscope offered higher resolving power that facilitated the study of new cell substructures, while molecular biologists benefited from chemical and genetic techniques to infer cellular composition and activity. But these new technologies had drawbacks. Electron microscopy required fossilization of specimens, and the tools of molecular biology destroyed cells. Today, light microscopy has undergone a revolution and can address these limitations, thanks to several new technologies, including multicolor fluorescent dyes,[1] high-speed computing for image analysis, and high sensitivity solid-state digital image detectors, such as charge-coupled devices. These technologies have converged to create what is known today as fluorescence and confocal microscopy. New methodologies include real-time imaging, in which the cell becomes a living cuvette for the study of biomolecular and chemical reactions.[2]

Multispectral and hyperspectral techniques have been employed for some time by the remote-sensing community, but recent advances extend their usefulness to microscopy and biomedical imaging, which increasingly depend on simultaneous monitoring of multicolor labels such as fluorophores and quantum dots. Hyperspectral imaging is fundamental, enabling differentiation and interpretation of labels, and providing the basis to study not only cellular components but entire systems.[3] New developments in nonlinear optics have introduced faster, more flexible, sensitive, and precise hyperspectral systems. These will not only provide new insights but also more timely and accurate diagnostic tools for clinical use.

Figure 1. *Acousto-optic tunable filter (AOTF) module (left), schematic of AOTF in operation (middle), and ChromoDynamics HSi-300 Hyperspectral Imaging System (right).*

Current state of the art

A typical wide-field fluorescence microscope comprises an excitation source (often filtered), sample stage, objective magnification lens, dielectric band-pass or 'barrier' filter, dichroic filter, final magnification lens, and the imaging detection plane. Traditionally, selecting wavelengths of interest has been accomplished using a wheel between the excitation and barrier filters. Switching times for filter wheels are relatively slow for many time-dependent phenomena.

Temporal resolution in filter-based spectral imaging microscopy is limited by the speed of wavelength switching, which is on the order of 40ms for filter wheels, 100ms for liquid crystal tunable filters, and $<100\mu s$ for conventional acousto-optical tunable filters (AOTFs). Grating or prism spectrometers acquire information on entire continuous spectrum profiles but require mechanical scanning of the sample to capture an area of interest, one line section at a time. They thus also collect much data of no interest, requiring significant storage and post-processing capacity. Any gains in speed are compromised by the need to physically sweep the image.

A wide variety of broadband sources, typically xenon, mercury, or halide lamps, are available for most clinical and research applications, delivered as part of an integrated system or as stand-alone enhancements. Requirements for high-precision

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spectral lines or variability have been addressed in the past by high-throughput monochromators, and more recently by individual and combined laser sources, typically helium-neon and increasingly diode-pumped versions. Both have limitations and are restricted to single spectral band passes.

Laser-based systems typically depend on the combined output of multiple diode laser sources that are coupled via high-speed switching mirrors and sophisticated, costly fiber-optic coupling systems. They offer narrow bandwidth emissions at significant cost increases for each additional line. Monochromators provide a broad selection of spectral emission lines and band passes but tend to have limited output and switching rates. Multiline lamp sources, such as mercury, have restricted stability and lifetime, and spectral distributions often require shaping with filters to block out strong UV components. However, potential light sources for fluorescence microscopy have recently been introduced, based on developments in digital micromirror devices (DMDs) that allow for a programmable generation of modulated, single, or multiple lines and even entire spectral profiles that can be coordinated with the variable imaging filter.

New photonic tools

AOTFs are crystals whose optical properties can be controlled by sound waves generated by applying a radio-frequency electrical signal to a specially designed transducer bonded to the crystal. All filter parameters, including center wavelength, bandwidth, and throughput, are under electronic control. The technology is solid-state in that there are no moving parts or maintenance, and an indefinite lifetime of use. AOTFs also permit wavelength, bandwidth, and intensity to be changed at any time and are extremely fast, with switching times on the order of 100μs.

Although AOTFs were introduced more than 30 years ago, their use has been restricted due to poor image quality arising from the type of acoustic wave pattern propagating in the crystal. This issue was recently addressed through the use of proprietary technology by ChromoDynamics that offers innovative transducer designs and long interaction-length crystals (see Figure 1). The diffraction-limited image quality has variable bandwidth resolution down to 1.5nm (see Figure 2). Wavelength switching time, including computer control overhead, is <100ms. The hyperspectral imaging system features an integrated electron-multiplying charge-coupled device with scientific camera, AOTF-based spectral filter system, instrument driver and control module, and a sofware suite for image capture and analysis.

Figure 2. Variable bandwidth capability of AOTFs.

Figure 4. Schematic of a digital micromirror-based spectral source, the Optronic Laboratories OL 490.

Similarly, DMDs have found their way into spectral light sources.[4] These products of microelectromechanical systems technology, developed by Texas Instruments, constitute the central component in digital light projection displays (DLP technology). When coupled to a broadband source illuminating a dispersive element, such as a spectral grating, the DMD can select specific band passes or entire spectral profiles while varying their intensity. These can be modulated, then combined and transmitted via liquid light guide or other output to the sample in an imaging platform. Some firms have recently packaged and commercialized these sources.

The OL 490 Agile Light Source (Optronic Laboratories) offers programmable and variable high-intensity and high-resolution spectral output. It can produce emissions at a single wavelength or broad spectrum, steady-state or varying with time, providing flexibility and speed for a wide range of scientific and technical applications. The unit's stable lamp sources offer output intensities exceeding those of conventional monochromator sources, more than 250mW across a spectral range of 380–780nm. By varying the slit width, the user can trade off between resolution and output power. Control software enables operators to set multiple bands, sweeps, and trigger modes to produce the desired spectrum, combination of spectral lines, modulation, or sequence with a switching rate of 12,500 spectra per second and a range of more than 49,000 intensity levels output to a 3mm liquid light guide (see Figure 4).

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Figure 3. *Three-color tissue sample.*

Figure 3 shows an AOTF-acquired image of a three-color tissue sample, exhibiting substantial colocalization of the probes. The trio of images in the center shows the results of using linear pixel unmixing to separate out the blue, green, and red components, respectively. The image at the right, a pseudo-colored classified image based on these results, contains quantitative information about the amount of each component present at every pixel. For illustrative purposes, the AOTF-acquired spectrum of the red fluorescence signal is shown in the inset.

Using conventional interference filters, four to five different probes can be detected in a single cell. More than this is quite difficult due to overlap of probe emission spectra. If an AOTF is used in conjunction with a properly designed interference filter set, however, it is possible to separate closely overlapping emission spectra, with the potential for distinguishing 10 or more probes in a single cell. This approach can be used for in situ imaging of fluorescent hybridization (FISH)-labeled cells, in which specific DNA sequences are used to detect the distribution of specific genes. High-speed hyperspectral modalities such as AOTF imaging enable rapid discrimination of multiple probes and are ideal for use in high-throughput systems for clinical diagnostics.

Potential applications

High-speed hyperspectral imaging provides fluorescence microscopy opportunities not only for unconventional observations but also as a means to address current limitations. For example, spectral overlap between fluorescent dyes can be separated by a computational process known as linear unmixing. Typically a reference spectrum is measured for optimal results, but certain unmixing techniques benefit from data supplied by the full profile. To fully exploit the multilabel samples with closely overlapping or dense spectral emissions, 'unmixing' algorithms are applied. These linear programs employ a priori knowledge of the fluorophores' spectral emissions to interpolate their individual profiles in such instances.

Time-dependent techniques such as fluorescence recovering after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) could also benefit from simultaneous multispectral imaging. FRET relies on proximity-dependent transfer of energy from excited molecules and can be used to study the interactions of subunits of macromolecular structures such as microtubules and ribosomes. With FRAP the gradual recovery of fluorescence from molecules migrating into a zone previously exposed to high levels of excitation is used to measure diffusion of lipids and proteins in biological membranes.

In such applications, pixel-based wavelength information coupled with fast processing speed is critical. Widespread adoption of multi- and hyperspectral imaging will also drive implementation and development of new classes of fluorescent dyes that, for example, change under certain conditions or interactions. Quantum dots, with their uniform excitation profile and well-defined, customizable emission spectra, are ideal labels for hyperspectral imaging platforms.

Hyperspectral technologies are now beginning to be integrated with other imaging technologies in the life sciences, used not only in wide-field but also confocal microscopes. The resulting images provide a dynamic 3D view, not of cellular systems but of whole live organisms. Hyperspectral imaging also permits simultaneous use of various fluorescence techniques in 'multimode' and multiparameter microscopy, where a broad selection of labels may be combined. Automated imaging platforms incorporating conventional optical microscopes with hyperspectral imaging systems and intelligent software have the potential to transform diagnostic medicine through high-content analysis

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(HCA). Clinical diagnostics involves high volumes of 'test tube' measurements now done with microplates and readers. HCA can process such measurements faster and more accurately. This will in turn help implement personalized medicine, the novel therapeutic approach that selects drugs from a library of potential candidates based on the patient's genomic profile. Drug development also stands to benefit from in vivo application of such techniques.

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