SPECTRAL IMAGING SYSTEMS
FOR FLUORESCENCE MICROSCOPY

MAG BIOSYSTEMS
Multichannel Imaging

Over the last decade there has been a substantial increase in the number of life science applications that require fluorescence microscopy to investigate dynamic phenomena in cells and living tissue. Due to the spatial, spectral, and temporal complexities associated with acquiring quantitative emission ratios, it is often necessary to use two or more channels simultaneously to track changes in fluorescence signal.

MAG Biosystems™ multichannel systems project multiple images onto the detector(s) at the same time, thus removing electromechanical positioning of filter optics as a rate-determining factor. With these systems, investigators can take full advantage of newer, low-light, high-speed imaging sensors like EMCCDs.

Utilizing FRET

Förster, or fluorescence, resonance energy transfer (FRET) is a phenomenon utilized in microscopy to observe interactions between biological molecules. For this technique, two biological molecules of interest are labeled with different fluorescent dyes, one with a donor fluorophore and the other with an acceptor fluorophore. When the donor and acceptor molecules are within close proximity of each other, the excitation energy of the donor fluorophore will transfer nonradiatively to the acceptor. This results in quenching the donor’s fluorescence emission and stimulating the fluorescence emission of the acceptor.

The ability to acquire the donor and acceptor emissions simultaneously is critical to measuring FRET accurately. The Dual-View™, Quad-View™, and Dual-Cam™ designs are optimized for one-shot imaging of the donor and acceptor emissions, giving researchers more quantitative information about molecular separation and interaction distances.

Measuring Polarization

Fluorescence polarization is another technique that is being used to investigate the biochemical properties of samples, such as protein denaturation, protein-ligand interactions, protein-protein interactions, protein-DNA interactions, and the rotational rates of proteins. MAG Biosystems multichannel imaging systems are well suited for this type of investigation because they provide the ability to image simultaneously the parallel and perpendicular components of a fluorescence emission.

Multicolor Imaging

The capability to acquire up to four separate spectral channels (e.g., blue, green, red, far red) simultaneously provides flexibility in multicolor bio-investigation. Not only does such multicolor imaging make it possible to colocalize specific time-critical molecular phenomena, it also speeds up investigations by capturing more fluorophores in each exposure.

For example, with the Dual-View, a living cell can be imaged for both CFP fluorescence and YFP fluorescence in one shot, with perfect registration. With the Quad-View, DIC and three fluorescent probes can be imaged simultaneously.

Ratiometric Mapping

Ratiometric indicator dyes, such as indo-1, SNARF, JC-1, and di-4-ANEPPS, have significant advantages over single-emission probes where changes in dye concentration can cause changes in the emission intensity that are independent of changes in the item being studied. Ratiometric dyes eliminate potential artifacts resulting from uneven dye loading, photobleaching, dye leakage, and varying cell thickness.

Coupling emission ratio dyes with simultaneous multichannel-emission acquisition allows even the most rapid cellular events to be studied. With sequential imaging techniques, the concentration of dye molecules at any given pixel may change between acquisitions, resulting in an artificially altered emission ratio and inaccurate quantitation of the response being investigated. Thus, the precise temporal registration of the Dual-View, Quad-View, and Dual-Cam systems offers more accurate data and better experiment repeatability.
Fluorescence microscopy is now a standard technology in investigating dynamic phenomena in cells and living tissue. It has given rise to advances in molecular manipulation, a growing variety of fluorescent dyes, and the incorporation of multiple fluorophores into single imaging experiments.

Many fluorescent proteins, however, have overlapping emission spectra that make them difficult to separate with traditional filtering methods. Furthermore, cellular microenvironments like molecular interactions, pH, and viscosity behave unpredictably to create additional emission-fluorescence signatures that can confuse experiment results. The intrinsic autofluorescence present (to varying levels) in almost all biological samples also serves as a formidable hurdle to performing quantitative fluorescence imaging.

With the development of the MAG Biosystems SpecEM™ system, full spectral information is acquired for each pixel in an image, and the emission of multiple fluorophores is obtained quickly, quantitatively, and reliably. A two-channel filter cube converts the MAG Biosystems SpecEM into a dual-band imaging system that acquires two fluorescence channels in a single shot, enabling real-time multispectral investigations.

**Quantitative Spectral Unmixing**

Many factors can hamper fluorescent signal measurements in molecular investigations. For example, it is often hard to separate a fluorescent signal of interest from the autofluorescence of the background. The SpecEM system is able to make this distinction absolute by quantifying all the spectra in the sample via spectral imaging and subsequent spectral unmixing.

First, the SpecEM system scans across the sample, collecting slice after slice of spectra until the entire sample is imaged. After the spectra are acquired, the system’s Mélange™ software assembles a three-dimensional image cube of the sample spectra.

The software then utilizes both linear and blind unmixing algorithms to determine the absolute contribution of each fluorophore. The blind unmixing algorithms determine the contribution of unknown spectra, such as those associated with autofluorescence or when probes shift spectra due to intracellular environments.

**Application Flexibility**

The ultimate advantage of the SpecEM system is that it can use both full-spectrum imaging and multispectral (two-channel) collection modes. The system’s Mélange software can then take the fluorescence profiles obtained and deliver true spectral unmixing of the data, thus providing unprecedented quantitative analysis for critical fluorescence techniques such as FRET and the removal of autofluorescence (among many others).

These SpecEM-enabled fluorescence techniques facilitate a broad range of biological applications, from monitoring molecular interactions to sequencing DNA and sensing membrane potential. (See Zimmermann et al., “Spectral Imaging and Its Applications in Live Cell Microscopy,” *FEBS Letters* 546 [2001]: 87–92.)

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**[a]** A longpass (500-nm) image of a liver section* with actin filaments stained with Alexa Fluor® 488 (Molecular Probes) phalloidin. In this image, it is difficult to determine which structures have been stained with the Alexa Fluor 488.

**[b]** Unmixed autofluorescence and Alexa Fluor 488 **[c]** images produced using Mélange software after the SpecEM system acquired a spectral stack of images. The unmixed Alexa Fluor image reveals the exact location of the actin filaments.

**[d]** A color-overlay image showing the unmixed actin filaments and autofluorescence much more clearly than the original longpass image.

*Liver sections courtesy of Simon Watkins, University of Pittsburgh.*
**MULTICHANNEL APPLICATIONS**

- Real-time multicolor imaging
- FRET imaging
- Calcium imaging with fluo-3/Fura Red™ (Molecular Probes) or dual-emission indo-1 imaging
- Fluorescence polarization/anisotropy imaging
- Simultaneous fluorescence/DIC imaging
- Drug discovery with Cy3/Cy5
- Single-molecule fluorescence (SMF)
- pH imaging with SNARF
- Multiwavelength TIRF
- Multiwavelength FLIM
- Voltage sensing with di-4-ANEPPS
- Other emission ratio analyses
- Fluorescence in situ hybridization (FISH)
- cAMP imaging with FICHR
- Multichannel confocal microscopy when used in conjunction with a spinning-disk confocal
- Two-color polarization/anisotropy studies
- Simultaneous calcium and pH studies with indo-1 and SNARF
- Three-color fluorescence and DIC
- Polarized FRET analysis

**FULL SPECTRAL APPLICATIONS**

- Separation of specific fluorescence and autofluorescence
- Quantitative separation of true FRET signal from bleed-through signal of donor
- Colocalization of multiple spectrally overlapping fluorophores
- Spectral karyotyping (SKY)
- Multicolor FISH (mFISH)
- Monitoring changes in fluorescence emission as function of subcellular localization
- Multiple quantum dot imaging
- Fluorochrome development and characterization