FRET, FRAP, FISH - Technology & Techniques

FRET, FRAP, FISH - Technology & Techniques. The use of fluorescence microscopy in the life sciences runs the gamut from basic photodocumentation to dynamic single-molecule fluorescence (SMF) studies. Recent advances in digital imaging technology are helping to expand the utility and popularity of many fluorescence microscopy techniques, including Förster resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), and fluorescence in situ hybridization (FISH). The newly created Microimaging Applications Group, which comprises Photometrics, Media Cybernetics, QImaging, Gatan, and MAG Biosystems, offers a broad range of innovative imaging solutions designed to enhance life science research capabilities.

FRET Imaging

Förster resonance energy transfer is a phenomenon in which nonradiative transfer of energy occurs between donor and acceptor molecules in close proximity (2-7 nm). Since FRET efficiency decays as a function of the inverse sixth power of the distance between the donor and acceptor, this phenomenon can be leveraged to provide solid evidence of an interaction between the donor and acceptor in a FRET pair.

In FRET, the donor molecule is returned to a ground state without fluorescence emission while the acceptor molecule is raised to an excited state. Upon decay of the acceptor's excited state, fluorescence emission may be witnessed. Thus, an increase in FRET between label molecules will result in a decrease in donor emission and a simultaneous increase in acceptor emission. Using FRET detection, interactions between molecules can be monitored in subcellular compartments and tracked as a function of time. FRET applications include evaluating the structure of proteins, determining the spatial distribution and assembly of protein complexes, monitoring receptor/ligand interactions, and sensing the presence of small molecules in living cells. FRET experiments are often performed using standard ratio imaging techniques. Depending on the application, FRET is used to qualitatively or quantitatively investigate experimental phenomena. While qualitative experiments focus on simply determining the presence or absence of FRET as an indicator of interaction, quantitative experiments require a more
To help ensure accurate results, next-generation Photometrics electron-multiplying CCD (EMCCD) cameras provide exceptionally high quantum efficiency, quantitative stability across 16 bits, and linear EM gain up to 1000x. Even with superior camera performance, sequential imaging techniques (e.g., using an emission filter wheel or switching microscope filter cubes) can make proper data calibration and correction very difficult, if not impossible, when dynamic samples are used. Therefore, many quantitative FRET applications require that the donor and acceptor emissions be simultaneously imaged. To meet this criterion, MAG Biosystems offers easy-to-use instrumentation that splits the incident beam from the microscope into independent beams. Each of the resultant emission channels is projected onto a region of a CCD or EMCCD array. A precision optical and mechanical design allows subpixel image registration and minimizes light loss for simultaneous multichannel acquisition.

**FRAP Imaging**

Fluorescence recovery after photobleaching is useful for examining intracellular molecular variables such as nuclear protein complex dynamics, diffusional mobility of membrane proteins, and cytoskeletal dynamics. FRAP is a powerful mode of fluorescence light microscopy in which a specialized illumination strategy is implemented in order to permit perturbation of the steady-state fluorescence distribution by bleaching fluorescence in selected regions of a sample. After the bleaching step, researchers can observe and analyze how the fluorescence distribution returns to the steady state. Because the photobleaching of fluorophores is permanent, changes in the fluorescence intensity in both the bleached and unbleached regions are attributable to the exchange of bleached and unbleached fluorescent molecules between those regions. FRAP microscopy is typically geared towards dynamic, lowlight endeavors.
Recently, MAG Biosystems introduced a widefield imaging system designed to study the intracellular dynamics of proteins and other macromolecular complexes via FRAP and iFRAP (inverse FRAP) experiments in 2D plus time and 3D plus time. Photoactivation and photo-conversion studies with fluorescent proteins such as PA-GFP, EOS, KFP, Kaede, and Dronpa can be performed. Several technological innovations, including Burst mode and a custom optical path, provide a combination of speed, sensitivity, and ease of use not found in other FRAP systems. When run in Burst mode, the delay between the end of the bleach pulse and the first recovery image is minimized, enabling fast dynamic analyses. The FRAP-3D system also lets researchers photobleach-on-the-fly by simply clicking within a live image display window to bleach the region appearing under the cursor. FRAP-3D includes a galvanometer-based FRAP head, an advanced laser launch module, high-speed I/O circuitry to control all system components, acquisition and analysis software with an intuitive graphical user interface (GUI), and a configured workstation. The head can be mounted to many inverted microscopes through the epi-illumination port in order to enable simultaneous laser and widefield illumination. A versatile optical design allows researchers to switch seamlessly between FRAP studies and standard widefield applications without reconfiguring the system's hardware. To meet user-specific quantum efficiency, spatial resolution, and frame rate requirements, the FRAP-3D system utilizes high-performance quantitative CCD and EMCCD cameras from Photometrics. To ensure the utmost instrumentation utility, FRAP-3D allows future upgrades for spinning-disk confocal microscopy and other imaging modalities.

FISH Imaging
Fluorescence in situ hybridization is a biochemical means of labeling specific nucleic acid sequences in cell preparations for the purposes of confirming the presence of certain genes and for spatial localization of sequences of interest within a cell or on chromosomes. Essentially, FISH provides a way to visualize and map genetic material in single cells. FISH has been instrumental in elucidating a variety of chromosomal abnormalities and genetic anomalies. The technique is used heavily in the basic research arena as well as the clinical arena. The use of FISH continues to grow quickly in such areas as genetics, cytogenetics, prenatal research, and tumor biology. The first step in FISH is the production of sequence-specific probes, which is accomplished by synthesizing antisense strands to sequences of interest and conjugating these antisense strands to fluorescent probes so that they can be detected using fluorescence microscopy. The power of FISH is greatly enhanced by the simultaneous use of multiple fluorescent probes. By using a multiplexing strategy, numerous nucleic acid sequences of interest can be detected and mapped.

There are three basic types of FISH probes: (1) locus-specific probes, (2) alphoid or centromeric repeat probes, and (3) whole-chromosome probes. Locus-specific probes, which bind to a particular region of a chromosome, are useful for determining which chromosome a gene is located on once a small sequence of a particular gene has been isolated. Centromeric repeat probes, which are generated from repetitive sequences found in the middle of each chromosome, are utilized to determine whether a cell has the correct number of chromosomes. Whole-chromosome probes, which are collections of genetic sequences common to a particular chromosome, can be used to map individual chromosomes as well as to identify different chromosomes in respect to one another.

Many researchers utilize two- to fourcolor FISH analysis on fixed samples. The main requirement for imaging of this kind is an ultra-high-resolution detector that allows all of the spatial information to be preserved under high magnification. Since the preparations most often contain fixed cells, more intense illumination can be used to produce stronger signals. A midrange-performance camera may be deemed adequate for fixedsample, moderate-light FISH studies, provided it offers sufficiently high spatial resolution.

With the maturation of techniques such as spectral imaging, many researchers are now enhancing their FISH experiment capabilities by using a far greater number of fluorescent probes at one time. Spectral imaging enables the identification of probes based on their spectral curves, allowing differentiation of closely overlapping fluorophores. When a camera is utilized in conjunction with a spectral imaging system, a specimen's fluorescent emission can be split into component wavelengths prior to reaching the detector. Thus, excellent camera performance
and sensitivity become critical. To facilitate the use of FISH, QImaging has engineered a wide selection of easy-to-operate CCD cameras that address myriad resolution, speed, and sensitivity requirements. QImaging also offers several color options, including solutions that combine the use of Bayer mask CCDs and innovative data interpolation methods to deliver high-fidelity color rendition.

**Software Considerations**

Fluorescence microscopy techniques are evolving at a rapid pace. New fluorescent probes, camera technologies, and optics offer researchers an expansive set of investigative capabilities. The importance of using intelligent software for image acquisition, analysis, and management cannot be overstated. Powerful packages designed specifically for life science experiments, such as the Media Cybernetics line of software solutions, provide researchers the latest image analysis tools for object tracking, 3D rendering, image deconvolution, and a broad diversity of fluorescence-based techniques. These programs integrate extensive hardware automation support and flexible image acquisition and processing into an easy-to-use GUI. Media Cybernetics also offers an image-asset management solution that lets life science researchers store, query, and share a large number of images (as well as data) via a client-server database application or the internet. Simple click-and-choose tools streamline archiving, searching, displaying, customizing, and reporting.

**Future Trends**

As imaging instrumentation simultaneously becomes more sophisticated yet simpler to use, the list of applications for FRET, FRAP, and FISH gets longer every day. Other fluorescence-based techniques benefiting from these technological advances include total internal reflection fluorescence (TIRF), fluorescence lifetime imaging microscopy (FLIM), and fluorescence anisotropy and polarization. Instrumentation providers such as the Microimaging Applications Group will continue to introduce high-performance cameras, versatile software, and complete imaging systems that offer life science researchers increasingly powerful and efficient capabilities for fluorescence microscopy.

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