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Leica MICROSYSTEMS

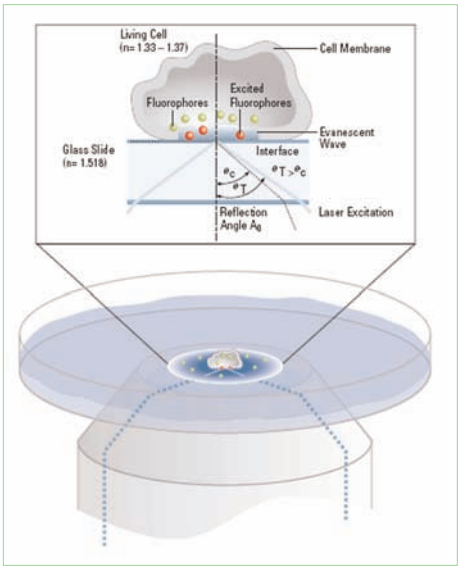
Reveal Additional Information Using TIRF

By Nicola Bettesworth, *Leica Product Manager*

Live cell imaging is always a challenge – achieving the highest signal, with the blackest background while maintaining cell viability is one of the most difficult tasks in microscopy. Eliminating background noise in an image that originates from outside of the focal plane is one way to dramatically increase the signal-to-noise ratio. TIRF – Total Internal Reflection Fluorescence microscopy is one method to increase this ratio and reveal additional specimen information.

Many molecular events, including cell adhesion, cell binding, vesicle transport, membrane research, and single molecule detection, are now studied using TIRF imaging systems. With widefield fluorescence, the fluorophores of interest are often overwhelmed by intensity and/or not visible due to a high degree of background fluorescence. TIRF, on the other hand, produces evanescent waves that selectively illuminate and excite a small area. Instead of illuminating an entire specimen with excitation light, as in widefield fluorescence microscopy, the evanescent wave only penetrates the specimen to a depth of 100-300nm from the cover slip and decays exponentially with distance. This method enables a substantially improved signal-to-noise ratio, as no additional auto-fluorescence is generated through the excitation of fluorophores outside of the focal plane.

According to Snell's Law of Refraction, an incident light beam is totally reflected, rather than passed through material, when it strikes



Total internal reflection fluorescence

the refractive index interface (glass: water interface) at an angle greater than the critical angle (see illustration). This reflection generates an evanescent wave in the sample media that has identical wavelength properties to the original incident light.

The resulting evanescent wave excites the fluorophores within up to 300nm of the cover slip. Varying the angle at which the light, and resultant evanescent wave, is introduced can control the depth of penetration, and therefore fluorophore excitation. In order to initially achieve the critical angle, it is necessary to use a very high numerical aperture objective. The Leica HCX Plan Apo 100x objective, for example, has a numerical aperture of 1.46, and in conjunction with the precise positioning of a laser beam via a scanning mirror, allows complete control of the penetration depth (70-300nm) and direction of the evanescent wave propagation within the specimen. These features allow the exact location of structures to be determined and enable additional structural information to be easily revealed and easily reproduced.

In the past, determination of the initial critical angle and positioning of the laser were arduous and time-consuming tasks. The new Leica AM TIRF system incorporates a sensor that detects total reflection and provides complete confirmation that you are actually in TIRF. Further, the integrated refractometer function automatically determines the refractive indices of the specimen under examination. The exact penetration depth of the TIRF field is then calculated using the specific excitation wavelength, the refractive index of the sample

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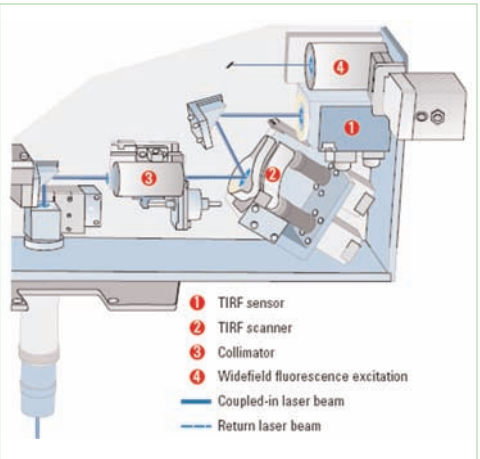
and the incident angle of the laser. All of this information is presented on screen thereby allowing the user to know how far from the cover slip molecular events are occurring.



Light path of the Leica TIRF module. The laser beam is positioned in the objective with the aid of a scanner. The returning beam of the total reflection is measured on a special sensor. This feedback allows precise, fully automatic, and reproducible setting of the penetration depth of the evanescent wave.

TIRF has several advantages over widefield fluorescence microscopy. The ability of the evanescent wave to excite only the fluorophores within this narrow range not only provides an exceptionally high signal-to-noise ratio, revealing structures that would normally be hidden, but the wave is also much less toxic to the sample itself. Sample viability is greatly increased when it is not continuously exposed to the high power excitation of regular widefield fluorescence. This increased viability helps enable the highest quality studies to be performed over longer periods of time.

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Fly and larvae sorting and screening

Leica Supports NASA's "Flies in Space"

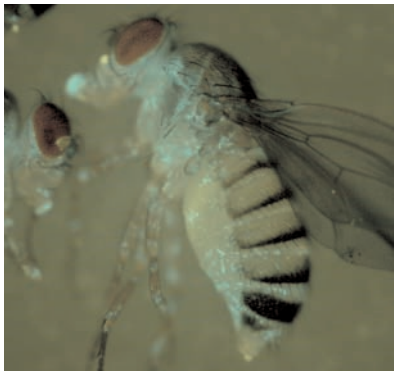
by Craig Wollschlager, *Leica Marketing Manager*

On July 14, 2006, NASA launched fruit flies into space aboard the Space Shuttle Columbia, destined for the International Space Station (ISS). The FIT (Fungal Pathogenesis, Tumorigenesis, and Effects of Host Immunity in Space) experiment is a comprehensive study to investigate susceptibility to fungal infection, progression of radiation-induced tumors, and changes in immune function in sensitized *Drosophila.* Leica Microsystems supported the study by providing top-of-the-line imaging systems to the NASA Kennedy Space Center's Life Sciences Laboratory for pre- and post-flight processing and analysis of the specimens.

Headed by Dr. Sharmila Bhattacharya (Principle Investigator; NASA Ames Research Center, Moffett Field, CA) and Dr. Deborah Kimbrell (Co-Investigator; University of California, Davis), the FIT experiment is primarily an examination of how immune system function is affected by the space flight environment. Other studies were coupled with this experiment as well, such as insect pathogen virulence in space.

Drosophila melanogaster was selected as the model organism due to its well-known genetic makeup, molecular pathways that control immune functions (similar to humans), and phagocytic immune cell functions (also similar to humans).

Drosophila eggs arrived on the ISS in larval form and hatched while in orbit. The flies were housed in habitats equipped with video cameras to allow researchers to monitor their behavior – courtship rituals, running speed, how they fly – all clues to genetic activity. The flies grew and bred to produce the foundation of approximately nine generations of flies.



Drosophila melanogaster

Both stereo (dissection) and compound microscope systems were used during laboratory analysis. Leica provided MZ16 F and S8 APO stereomicroscopes with fluorescence and brightfield cameras for high-resolution imaging. These systems were used to conduct fly and larvae sorting; determination of sex; physiological examination; dissection of flies, eggs, embryos, and larvae; and examination of whole flies and organs for GFP fluorescence of target proteins or cells. High-resolution images were used to archive the data for later study. The compound microscope examinations involved immunohistochemistry and fungal sporulation processes. For more information on this an other space research, including results, visit: **http://spaceresearch.nasa.gov**.

The Objective

Analyzing Dust-sized Particles Generated by Bolide Impacts on Earth

by Joe W. Fandrich, *Westwater Group Geological Research Facility and Adjunct Professor, Mesa State College, Grand Junction, CO*

A large bolide (asteroid or comet) impact on Earth creates objects ranging from greater-than-a-house sized blocks to particles less than 63mm in diameter. The impact results in the vaporization of the bolide and much of the target area. The intense heat generated by this event creates a Plinian cloud or massive fireball, which lifts most of the vaporized materials vertically into the atmosphere where cooling and condensation take place. Some of the smaller condensed particles rise to the stratosphere where they are dispersed throughout the Earth. In time, many of these scattered particles return to the Earth's surface where they are deposited with commonly generated sediments.

My research involves the study of these dust-sized, impact-generated microspherules that were created approximately two hundred and fifty million years ago. These particles are now present within solid rock, which must be cut with a diamond saw, sized, cemented to a glass slide, and polished for microscopic inspection. Thin sections for plain and polarized transmitted light are polished to 3mm in thickness. Rock sections, to be observed under reflected light, are cut to a maximum 1.27mm thickness.

The microspherules involved in this study are mostly spherical and smaller than most dust particles; they range in size from less than 45mm to as small as 250 nanometers. I locate these particles with a Leica DM LP light microscope with a CLEMEX digital imaging system. With the DM LP I am able to locate the particles in question and collect finely detailed color images using several options that enhance the visual impression of each particle in the horizontal plane. The DMLP allows measurement of the thickness of each particle as well as various associated objects and fragments of rock material. By employing multi-layer image capture, full focus of relatively uneven rock surfaces is easily obtained. Entire slides of rock samples are then imaged in mosaic format using the CLEMEX software. The resulting photomicrographs provide essential maps that indicate the location of pertinent particles to be further studied using a scanning electron microscope (SEM).

The value of the Leica DM LP/CLEMEX system for this research lies in the ability to collect a three-dimensional image of the particles being studied. Previously, I only had the use of an SEM to determine particle morphology. Now, more than 95% of the particle information can be obtained with the Leica light microscope system. The difference in operating expense of the SEM versus the DM LP/CLEMEX system is substantial. The SEM must undergo intensive and expensive maintenance every month, but the DM LP/CLEMEX is virtually maintenance-free (only a dust cover and good housekeeping techniques are employed). Hourly operation of the SEM is approximately $300/hour whereas the hourly operation of the Leica DM LP/CLEMEX system is negligible. Preparation of particles for the SEM by using the DM LP/CLEMEX has cut cost to a minimum and freed up the SEM for other projects.

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The Private Eye

Why Should I Use Deconvolution?

By, Chris Vega, *Leica Product Manager*

Deconvolution algorithms are commonly used to improve the quality of digitally acquired, fluorescence images. Why should one use deconvolution? This question is best addressed by asking "what is convolution?" and "why does it happen?"

Convolution is, primarily, a phenomenological property of light passing through the objective lens of a microscope. The resolution of an image acquired through a microscope is limited due to the diffraction of light that occurs at the objective lens; the resolution of a system is therefore referred to as diffraction-limited. The visual effect of this phenomenon is a blur that is independent of other forms of image degradation such as noise, glare, or scatter.

The blur caused by the lens is characterized by a point-spread function (PSF). The PSF is the representation of a three-dimensional diffraction pattern generated from an idealized point, e.g., a sub-resolution fluorescent bead. A single convolved digital image is therefore a collection of PSFs generated by the specimen through the objective lens. Since the optical properties of light passing through the objective lens can be mathematically described as a convolution function, algorithms have been defined that deconvolve, or reverse the blurring function of the objective lens if full PSFs are collected in a 3D image set (x, y, and z data).

The algorithms for performing deconvolution functions fall into two classes, deblurring and image restoration:

- Deblurring algorithms subtract blurred information from the images collected and are based on a relatively simple set of calculations that can be done quickly; but the results provide only qualitative improvement in image quality.
- Image restoration algorithms reassign the blurred light of PSFs into single points. These deconvolution algorithms better estimate the true object and can be used for quantitative analysis of image intensities.

Therefore, deconvolution should be used to "clean up" image sets with x, y, z components either qualitatively or quantitatively.

Leica Microsystems' AF6000 software allows users to collect 3D image sets and perform deconvolution algorithms for true image restoration.

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Your Educational Resource

Marine Biological Laboratory
The MBL offers advanced, graduate-level courses in embryology, physiology, neurobiology, microbiology, and parasitology for six to eight weeks each summer. Download the complete 2007 course schedule: **http://www.mbl.edu/education/courses/summer/index.html**

Cold Spring Harbor Laboratory
CSHL is a private, non-profit institution with research programs in cancer, neuroscience, plant genetics, genomics, and bioinformatics and a broad educational mission. Download the complete 2007 course schedule: **http://meetings.cshl.edu/courses.html**

Jackson Laboratory
The Jackson Laboratory's mission is to improve the quality of human life through discoveries arising from our own genetic research and by enabling the research and education of others. Download current and upcoming events: **http://www.jax.org/courses/events/current.do**

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Upcoming Events

Visit Leica Microsystems at the following exhibitions:

- Biophysical Society 51st Annual Meeting, Baltimore, MD, **March 3-7, 2007**, booth#726.
- Annual Drosophila Research Conference, Philadelphia, PA, **March 8-10, 2007**, booth#401.

For more events: **http://www.leica-microsystems.us**
(click on Company, then Events)

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Editorial Staff

Editors-in-Chief:	Molly Lundberg
Managing Editors:	Pam Jandura, Lon Nelson
Graphic Design:	M.N. Kennedy
Contributors:	Nicola Bettesworth, Chris Vega Craig Wollschlager
Special Thanks:	Joe W. Fandrich

Note: *We are interested in your comments and thoughts about the newsletter. Please feel free to email your comments to:* ***microscience.imaging@leica-microsystems.com***