Application Note

Live-Cell TIRF Microscopy

One of the goals of modern microscopy is to correlate the spatial and temporal data-gathering ability of fluorescence microscopy to the functional activity of biochemical events. When imaging molecular interactions and signaling processes in space and time, camera sensitivity and the ability to acquire images at a high rate of speed can have an appreciable impact on the quality of results obtained. Advanced CCD imaging solutions from Photometrics have been demonstrated to produce significant and impressive results in this context.

Endocytosis is a phenomenon by which a cell takes up molecular matter via invaginations on the cell surface that pinch from the plasma membrane and then move inside the cell. Understanding the intricacies of endocytosis more completely is thus of crucial importance to cell biologists; related applications range from cell invasion of pathogens to uptake of hormones and growth factors into cells.

This application note describes the use of simultaneous, multiple-wavelength acquisition coupled with total internal reflection fluorescence microscopy (TIRFM) to successfully image actin and dynamin recruitment during the final steps of clathrin-mediated endocytosis, thereby revealing the sequence in which dynamin and actin proteins are recruited to clathrin-coated pits during the endocytic event[1].

Since most of the key information about endocytosis occurs at the membrane surface of cells, an optical technique that can visualize these areas without fluorescence interference from the underlying cellular structure is favorable. In TIRFM, the illumination source imparts on the coverslip at a large angle (critical angle), resulting in the formation of an evanescent wave that illuminates the sample. The strength of the evanescent wave drops off exponentially with increasing distance from the coverslip interface, only exciting fluorochromes within 200 nm of the sample surface. This makes TIRFM a particularly powerful technique for studying endocytic events[2].

Dynamic Multicolor Imaging of Clathrin-Mediated Endocytosis

Working with several colleagues in 2002, Dr. Wolfhard Almers, at Oregon Health and Sciences University's Vollum Institute, used TIRFM and simultaneous multicolor imaging to visualize clathrin-mediated endocytosis and determine the order in which various proteins play a role in this process[1].

The study temporally resolved the involvement of certain proteins in clathrin-mediated endocytosis. The timeline of the appearance of dynamin (a protein believed to be involved in severing the clathrin-coated pit from the plasma membrane) and actin (a common structural protein) during the internalization of a clathrin vesicle from the plasma membrane was measured. Since the events associated with endocytosis occur within microseconds, high quantum efficiency (QE), low noise, and fast readout times were critical considerations when choosing a CCD camera for this study.

Periodic frames from time-lapse acquisition show the presence of clathrin-DsRed molecules (in regions believed to be clathrin-coated pits) at the plasma membrane (see **Figure 1**). Internalization of the clathrin-coated pit was determined by a decrease in the intensity of the clathrin-DsRed signal (top row). Simultaneously, the appearance of dynamin1-EGFP (bottom row) was measured and found to localize at the clathrin-coated pit just prior to internalization.

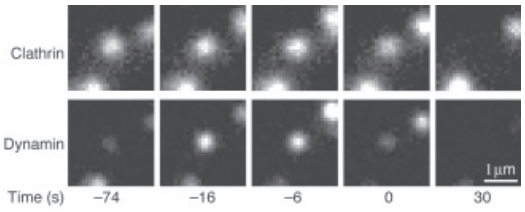

Figure 1. Dynamin is recruited to the clathrin-coated pit and leaves the plasma membrane with the vesicle. These time sequences show images of a clathrin-coated structure (top row) and dynamin fluorescence (bottom row) under evanescent illumination. The dimming of the clathrin-coated structure is preceded by transient recruitment of dynamin.[1]

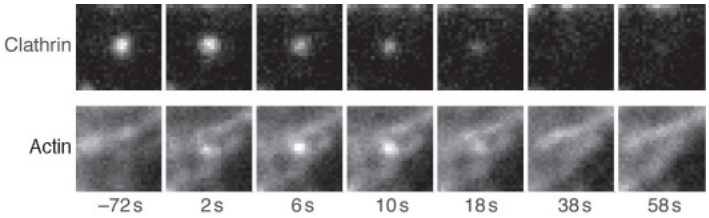

Figure 2. Actin is recruited to the clathrin-coated pit and leaves the plasma membrane with the vesicle. The top row is a time sequence of clathrin–DsRed fluorescence imaged under evanescent illumination. The bottom row displays green fluorescence images of EGFP–actin taken under evanescent illumination at the same times as the images in the top row.[1]

The authors then simultaneously observed clathrin-DsRed (first row of panels) over time and found that the appearance of EGFP-actin peaked at the clathrin pit after the clathrin signal started to decrease (see **Figure 2**).

When both results are plotted on a graph relative to the scission point of the endocytic event, it is revealed that the appearance of dynamin peaks prior to the scission event, while the actin appearance peaks after the event (see **Figure 3**). This is consistent with dynamin1 playing a role in the pinching of clathrin-coated pits from the plasma membrane. The fact that actin peaks after this event suggests that its role is likely post-internalization. The authors theorize that the actin may actually help provide the force for movement into the cytosol.

This work has since been extended to encompass N-WASP and the Arp2/3 complex[3]. Recently, it has also demonstrated the role of cortactin in the scission process during endocytosis[4]. Using this equipment setup and TIRFM, the researchers plan to investigate and further detail other events and protein interactions near the plasma membrane, including exocytosis, the function of caveolae, and signal transduction in lipid rafts.

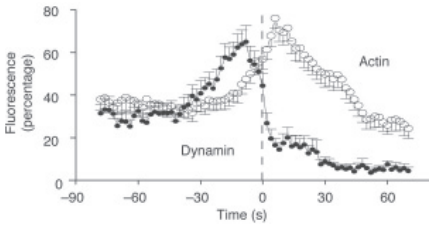

Figure 3. Due to the high temporal and spatial resolution afforded by this experimental setup, it was found that dynamin recruitment to the site of endocytosis peaks just prior to the endocytic event, whilst actin is recruited just after the endocytic event.[1]

Enabling Technologies

The researchers utilized a high-performance Cascade:512B electron-multiplying CCD (EMCCD) camera from Photometrics® and an Optical Insights Dual-View (currently know as the MAG Biosystems™ DV2™) for simultaneous, dual-channel, fluorescence image acquisition. Cascade® cameras employ state-of-the-art detector technology that provides excellent QE across the visible spectrum as well as on-chip EM gain in order to boost signal levels for high-speed imaging.

The Cascade:512B combines the sensitivity of a back-illuminated EMCCD with the high-speed imaging capability of a frame-transfer device. With the detector's 16-micron square pixels, subcellular structures labeled with GFP can be resolved quite easily. In addition, the camera can collect data continuously, since the photosensitive side of the EMCCD collects light while the stored image is being read out from underneath the permanent mask. When run in standard-mode operation at 10-MHz readout speed, the camera can collect data from 29 frames per second (fps) at full resolution to 300 fps and higher on binned subregions of the EMCCD.

The Cascade:512B also has an additional software-selectable readout speed (5 MHz) for use under conditions where fast frame readout is not as critical. The lower-noise readout performance at this slower speed enables higher signal-to-noise data collection. To minimize the dark noise that can accumulate during longer exposures, the camera is cooled to -30˚C.

The newest member of the Cascade family of EMCCD cameras is the Cascade II:512. The Cascade II:512 combines the sensitivity of a back-illuminated, deeply cooled (-80˚C) EMCCD with the speed of a frame-transfer device. This camera offers up to 92% QE, wide dynamic range (16-bit digitization), low dark noise, and high-speed readout in a single, versatile instrument. In standard-mode operation at 10 MHz, the Cascade II:512 can collect full-resolution images at 29 fps; adjacent pixels can be binned for even greater sensitivity and speed.

For applications that require longer exposures and exceptionally low noise, the Cascade II:512 provides an additional software-selectable readout speed of 5 MHz. While this slower speed reduces the camera's readout noise, cooling the EMCCD to -80˚C minimizes the dark noise that can accumulate during longer exposures.

Additional Information

To learn more about Dr. Wolfhard Almers' research, please visit:
www.ohsu.edu/vollum/faculty/almers

To learn more about the Cascade II:512, please visit:
www.photomet.com/pm_products/cascade_2_512.php

To learn more about the MAG Biosystems DV2, please visit:
www.magbiosystems.com/products/DV2.php

Citations

1. Merrifield, C.J., Feldman, M.E., Wan, L., and Almers, W. (2002). Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nature Cell Biology* **4**, 691-698.

2. Lambert, A. (2005). Microscopy is moving on. *American Biotechnology Laboratory* **23**, 8-10.

3. Merrifield, C.J., Qualmann, B., Kessels, M.M., and Almers, W. (2004). Neural-Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. *European Journal of Cell Biology* **83**, 13-18.

4. Merrifield, C.J., Perrais, D., and Zenisek, D. (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell* **121**, 593-606.

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