

Imaging Solutions for Biological Applications 2009



Performance, Quality, Support

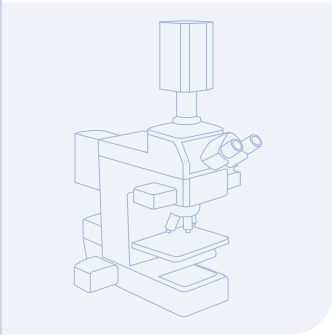
Proven Performance for the Life Sciences

Founded in 1978, Photometrics® is the world's leading designer and manufacturer of high-performance CCD and EMCCD camera systems for the life sciences.

By working closely with our global network of representatives, which includes subsidiaries in Benelux, France, Germany, Japan, and the United Kingdom, we're able to distribute and support high-performance digital imaging hardware and software across the world.

We can provide state-of-the-art CCD and EMCCD camera systems that come with everything you'll need for your application.

Contact your local Photometrics representative today!



Quality You Can Count On

We take pride in creating intelligent, reliable solutions to meet the needs of researchers and original equipment manufacturers (OEMs) alike.

Statistical process control, rigorous custom testing, and individual camera calibration and certification are integral parts of our manufacturing procedures.



Responsive Support

Superior customer service is a Photometrics hallmark.

Our PhD application scientists and camera technicians are dedicated professionals. Whether rendering assistance via phone, email, internet (WebEx™ session), or in person, these imaging specialists treat each issue with the utmost respect and urgency.

To help protect your valuable investment, Photometrics also offers an extensive selection of service contracts and product warranties.



Digital Camera Systems for Microscopy

We engineer our camera systems specifically for microscopy, taking many important considerations into account:

- › **Resolution** — match camera resolution to microscope resolution
- › **Speed** — keep pace with transient phenomena while preserving requisite sensitivity
- › **Sensitivity** — optimize detection of wavelengths of interest, minimize live samples' exposure to bleaching effects and toxicity of illumination energy
- › **Automation** — streamline operation of camera system
- › **Triggering** — synchronize experiment

Our design and implementation of low-noise electronics and CCD cooling have long set industry standards for quantitative imaging. We utilize front- and back-illuminated CCDs from many different manufacturers, as well as a number of original, proprietary CCDs and coatings that enhance spectral response.

Breakthroughs in Biological Imaging

Through the years, we've played a key role in the facilitation and improvement of numerous microscopy applications, including:

- › **FISH** — cameras with full-frame, front-illuminated CCDs for projects such as cataloging the human genome
- › **High-speed calcium imaging** — cameras with back-illuminated, frame-transfer CCDs for high quantum efficiency and fast frame rates
- › **GFP** — cameras with CCDs featuring indium tin oxide (ITO) gates for enhanced quantum efficiency in the blue-green region of the spectrum
- › **Single-molecule fluorescence** — EMCCD cameras with on-chip signal amplification for unprecedented low-light-level sensitivity at supravideo frame rates



Please visit www.photomet.com for more information on digital camera systems for microscopy.

The **Evolve 512** camera uses a back-illuminated, frame-transfer EMCCD to deliver incredible sensitivity, fast frame rates, and industry-leading quantitative stability for biological imaging.



- 512 x 512 imaging pixels
- 16 x 16- μ m pixels
- >90% quantum efficiency
- -85°C (regulated) cooling
- On-chip signal amplification (EM gain)
- Exceptional gain stability
- Quantitative bias stability
- Self-calibration feature (ensures predictable gain levels)
- EMCCD and slow-scan readout modes
- Multiple readout speeds (10, 5, or 1 MHz)
- Field-calibratable gain table

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Evolve™

Photometrics' highly popular Cascade® series was the world's first line of electron-multiplying CCD (EMCCD) cameras engineered specifically for use by microscopists. The introduction of the Evolve imaging platform now ushers in another new era in EMCCD camera technology. Our Evolve 512 is the first and only camera to offer EM gain with true quantitative stability across 16 bits.

The Evolve provides:

- Smallest, most powerful scientific EMCCD camera on the market
- Most advanced feature set available for low-light applications
- Lowest dark current available for an EMCCD camera
- Lowest read noise available for an EMCCD camera
- Ideal for sophisticated researcher and multi-user labs
- Superb electron multiplication (EM) gain and bias stability
- Most accurate EM calibration technique in the industry
- Backed by Photometrics' worldwide support team

EMCCD Basics

EMCCD technology enables multiplication of charge (i.e., electrons) collected in each pixel of the CCD's active array. Secondary electrons are generated via an impact-ionization process that is initiated and sustained when higher-than-typical clock voltages are applied to a special "extended" portion of the CCD's serial register.

The level of EM gain can be controlled by either increasing or decreasing the voltage applied to the pixels. Multiplying the signal above the read noise of the output amplifier enables ultra-low-light detection at high operation speeds.

Advanced EMCCD Quantification

The Photometrics Evolve:512 is the ultimate deep-cooled, back-thinned EMCCD camera. Years of engineering expertise have enabled Photometrics to perfect every element of the Evolve:512. The Evolve:512 offers life science researchers the world's first advanced feature set designed specifically for EMCCD cameras and for low-light-level bio-imaging applications. Each of the Evolve camera's revolutionary features can easily be enabled or disabled by the camera user via software control. This sophisticated functionality enhances the quantitative nature of the camera while simultaneously allowing researchers to concentrate on acquiring image data relevant to their work.

Outstanding Versatility

The Evolve:512 camera offers multiple speed settings, giving you the ability to tailor operation for either the fastest image visualization or the most precise photometry. Flexible binning options permit optimization of experiments both for desired spatial resolution and required detection efficiency.

For enhanced flexibility, the Evolve:512 utilizes dual readout amplifiers in order to deliver optimized performance not only for applications that demand tremendous low-light-level sensitivity but also for those requiring wide dynamic range.

Application Benefits

The quantitative stability of the Evolve:512SC camera lets you conduct accurate ratiometric analysis in time-course experiments, acquire reproducible data during long-term studies, and capture streaming data for multidimensional time-lapse investigations — all with single-molecule sensitivity.

The Evolve:512SC features a back-illuminated, frame-transfer EMCCD for >90% peak quantum efficiency and high-speed operation. Owing to the camera's exceptional sensitivity, very short exposure times are possible, thereby permitting more rapid data acquisition and allowing greater temporal resolution in experiments.

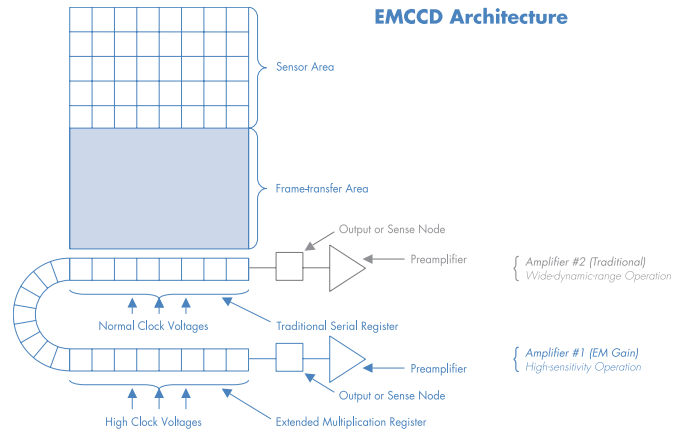
Applications such as intracellular calcium or pH ratio imaging, fluorescence recovery after photobleaching (FRAP), total internal reflection fluorescence (TIRF), fluorescence resonance energy transfer (FRET), widefield confocal microscopy, and spectral imaging benefit greatly from the impressive set of capabilities offered by the Evolve:512SC.

Widefield Confocal Microscopy

Spinning-disk and swept-field confocal detection technologies permit high-resolution imaging of thin optical sections of a sample that are free from out-of-focus blur in near real-time. This capability permits high-speed 3D spatial resolution of probe localizations in a cell and increases the contrast of 2D images. Such technologies operate under low-light-level conditions and rapid frame-readout rates. The ideal sensor for these systems is the electron-multiplying CCD. On-chip electron multiplication is vital to permitting fast camera readout with a high signal-to-noise ratio. Photometrics offers a full line of industry-leading EMCCD cameras for spinning-disk and swept-field microscopy. Photometrics' 16-bit EMCCD cameras provide very wide dynamic range along with peak quantum efficiencies exceeding 90% in the visible range on back-thinned models.

Spectral Imaging

An exciting development in microscopy is the increasing availability of signal-detection systems capable of recording emission spectra at each pixel in an image. Evidence of the ability of these systems to solve problems such as distinguishing the identity and relative concentration of fluorochromes with overlapping emission profiles is well established. There are numerous strategies for implementing a spectral-detection device. The advantages of a particular system will depend on the scope of applications the instrument is expected to handle; nevertheless, all systems display reduced photon flux at each individual pixel on the detection array. In order to obtain the best signal-to-noise ratio possible, it is critical to employ a CCD camera capable of high-quantum-efficiency detection. Meanwhile, in order to reduce the amount of time necessary to produce such spectral datasets, it is beneficial to be able to read out the camera exposures as quickly as possible. These requirements qualify EMCCDs as the ideal detection technology for widefield spectral imaging. The Evolve EMCCD platform is the ideal choice for spectral imaging, providing quantitative detection capabilities at extremely low light levels and fast readout speeds.



Please visit www.photomet.com to learn more about high-performance EMCCD cameras from Photometrics.

photometrics® evolve™

512 x 512 imaging array
16 x 16- μ m pixels

- ▶ Smallest, most powerful scientific EMCCD camera on the market
- ▶ Most advanced feature set available for low-light applications
- ▶ Lowest dark current available for an EMCCD camera
- ▶ Lowest read noise available for an EMCCD camera
- ▶ Ideal for sophisticated researcher and multi-user labs
- ▶ Superb electron multiplication (EM) gain and bias stability
- ▶ Most accurate EM calibration technique in the industry
- ▶ Backed by Photometrics' worldwide support team



Primary applications:

- Quantitative FRET
- Multiprobe experiments
- Ratiometric ion imaging
- Confocal microscopy
- Live-cell fluorescence imaging

Features	Benefits
EM gain	Very high sensitivity Low-noise, impact-ionization process
Back-illuminated EMCCD	Highest available quantum efficiency (>90% peak QE)
512 x 512 imaging array 16 x 16- μ m pixels	Optimized field of view and highest sensitivity
Intelligent FPGA design	Precise linearization of EM gain Self-calibrating linearization ensures truly quantitative data consistently, time and again Ensures bias stability to guarantee a stable background
PAR feedback system (Photometrics Active Regulation)	Delivers unsurpassed EM gain stability for outstanding signal fidelity across 16 bits
ACE technology (Advanced Clocking Enhancement)	Pixel-clock timing resolution 12x better than other EMCCD cameras Provides lowest noise floor and minimizes generation of spurious charge and background events
10-MHz readout	Excellent for high-speed image visualization
5- and 1-MHz readout	Perfect for high-precision photometry
Dual amplifiers	Select readout mode via software for optimized (1) high-speed / high-sensitivity performance (EM) or (2) wide-dynamic-range performance (traditional CCD mode)
16-bit digitization	Wide dynamic range allows detection of bright and dim signals in the same image
Frame-transfer EMCCD	100% duty cycle to collect continuous data No mechanical shutter required
C- mount	Easily attaches to microscopes, standard lenses, or optical equipment
Turbo-1394™ interface (IEEE-1394a)	High-bandwidth, uninterrupted data transfer with no dropped frames Windows® XP/Vista 32 and Mac OS X compatibility
PVCAM® Circular buffers Device sequencing	Supported by numerous third-party software packages Real-time focus Precise integration with shutters, filter wheels, etc.

Photometrics Advanced Features

Features	Benefits
Quant-View™	Allows camera to read out pixel values in terms of electrons measured, thereby enabling user to calculate actual photon flux
Electrons-per-gray-level selector	Allows user to select how many electrons will cause a single gray-level increase in the image data, thus permitting utilization of Quant-View while maintaining full dynamic range of EMCCD
Rapid-Cal™	3-minute EM calibration process is most accurate in industry; camera does not need to be detached from microscope; no special attachments required; user-initiated
Background Event Reduction Technology™	Identifies pixels that are likely to contain spurious event data and then makes corrections, if desired
Black-Lock™ / Top-Lock™	Intensity-filtering tool narrows visualization to the intensity range of the image features in which the user is most interested
Vari-Bit™ selectable bit depth (8-16 bits)	Improves image quality by matching digitization bit depth to actual intrascene dynamic range

The Photometrics Evolve:512 is the ultimate deep-cooled, back-thinned EMCCD camera. Years of engineering expertise have enabled Photometrics to perfect every element of the Evolve:512. The Evolve:512 offers life science researchers the world's first advanced feature set designed specifically for EMCCD cameras and for low-light-level bio-imaging applications. Each of the Evolve camera's revolutionary features can easily be enabled or disabled by the camera user via software control. This sophisticated functionality enhances the quantitative nature of the camera while simultaneously allowing researchers to concentrate on acquiring image data relevant to their work.

Specifications

Read noise (e- rms @ Gain State 3)		
10 MHz EM Port	45e-	
5 MHz EM Port	32e-	
5 MHz non-EM Port	12e-	
1.25 MHz non-EM Port	6e-	
Bias stability		
A measurement of the camera stability when no light hits the sensor. A slope of zero would be ideal. See footnote #1.	≤0.0006	
Gain stability		
A measurement of the stability of the electron multiplication applied to images. Ideal value would be 0. The stability of the EM gain applied can be quantified by measuring the slope of a sequence of images with known amount of light and EM Gain applied. See footnote #2.	0.0012 (@ 10MHz, 350X, Gain State 3, 20K ADU)	
Field uniformity		
The image quality of the EMCCD is assessed for gradients. A complete lack of any gradient (i.e a flat image) would provide a numerical value of 1.00 See footnote #3.	10 MHz EM	1.04
	5 MHz EM	1.10
	5 MHz STD	1.06
	1 MHz STD	1.02
Baseline bias value		
	500ADU but can be varied	
Cooling temperature		
	Air cooled (@ ambient air 20°C)	-85°C
	Chilled liquid (@ 10°C)	-100°C
Dark current		
	0.001 e-/pixel/sec (See footnote #4.)	
Background events		
events/pixel/sec (10 MHz, 1000X EM gain) Standard operation	0.0045	
events/pixel/sec (10 MHz, 1000X EM gain) BERT operation	not detectable (See footnote #5.)	
Relative charge transfer efficiency		
Photometrics is able to measure this on each camera and optimize this parameter.	Optimized on each camera (See footnote #6.)	
Charge transfer efficiency		
	As specified by CCD manufacturer's data sheet (See footnote #7.)	
Dark signal non-uniformity (DSNU)		
	As specified by CCD manufacturer's data sheet (See footnote #7.)	
Photoresponse non-uniformity (PRNU)		
	As specified by CCD manufacturer's data sheet (See footnote #7.)	
Parallel shift rate		
	0.7 μsec. This is optimized with Charge Transfer Efficiency (CTE) and CIC. Test results demonstrate that increasing parallel shift rate further can decrease CTE and increase CIC adversely affecting image quality and sensitivity.	

Note: Specifications are subject to change.

Ultra-Low-Light Solutions

Single-Molecule Fluorescence (SMF)

In order to minimize any effects that fluorophores may themselves make on the system being studied, many bio-researchers are seeking to reduce the concentration of fluorescent molecules used to label samples. Some researchers have chosen to track events at the single-molecule level. In order to perform dynamic imaging experiments at such low light levels, superior camera technology is required. Several years ago, Photometrics broke exciting new ground with the world's first commercially available EMCCD camera designed for microscopy. Today, Photometrics continues to lead the way in the evolution of this technology. Current offerings in the Evolve line of EMCCD cameras boast greater than 90% peak quantum efficiency, 16-bit digitization, and the lowest noise levels in the industry.

Deep-Cooling EMCCD Cameras

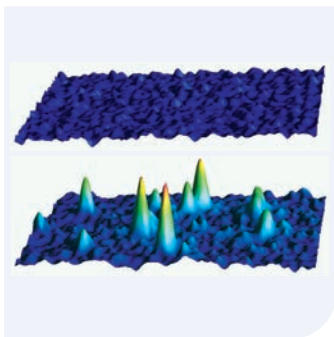
The amount of EM gain obtained from a set voltage can be enhanced by cooling the detection device. While this gain enhancement can also be achieved by increasing the amount of voltage applied, such as with the Photometrics QuantEM imaging platform, the generation of dark current is nonetheless still dependent on temperature. Thus, for applications in which electron multiplication is necessary and dark current is an issue (generally when longer acquisition times are required) it can be beneficial to deeply cool the EMCCD. Our deep-cooling Evolve:512 camera is well suited for this class of applications.

TIRFM

Total internal reflection fluorescence microscopy (TIRFM) is a form of near-field illumination in which the difference of refractive index between the sample and coverslip is used to generate an evanescent wave of illumination that penetrates to a very shallow depth (on the order of 200 nm). This illumination strategy is ideal for imaging events at or very near the cell membrane without contributions from out-of-focus blur. Many researchers have utilized this technique to image endocytic and exocytic events as well as protein interactions at the cell membrane. EMCCD technology is well suited to TIRFM owing to the superb temporal resolution and sensitivity such cameras provide in the context of dynamic imaging studies. The Cascade series of EMCCD cameras has been used to extremely good effect under highly demanding TIRF imaging conditions.

Fluorescent Speckle Microscopy

Visualizing fluorescent proteins, whether injected or expressed, in living cells continues to yield much information. Fluorescent speckle microscopy is a labeling strategy that uses low levels of fluorochrome such that the label is incorporated into the structure of interest in a non-homogeneous manner (i.e., the structure of interest is seen to be “speckled” with fluorochrome). This approach has utility for studies exploring dynamic assembly, movement of structures, and turnover of proteins at the limits of optical resolution. The strategy also reduces out-of-focus fluorescence and greatly improves the visibility of fluorescently labeled structures in thicker regions of living cells. Due to the low amount of fluorescent signal and the fast dynamic events usually being imaged in speckle microscopy, selecting a high-sensitivity, low-noise camera is of paramount importance. Evolve EMCCD cameras are engineered with the industry’s most innovative electronics to enable excellent detection and quantitation capabilities.



Single molecules of perylene diimide in polymethylmethacrylate polymer. Fluorescence emission acquired using a Cascade camera with EM gain off (top) and on (bottom). Images courtesy of Kallie Willets and Stefanie Nishimura, W.E. Moerner Lab, Department of Chemistry, Stanford University.

The **Evolve:512** camera uses a back-illuminated, frame-transfer EMCCD and deep thermoelectric cooling to provide truly spectacular low-light-level sensitivity at fast frame rates.



- 512 x 512 imaging pixels
- 16 x 16- μm pixels
- >90% quantum efficiency
- Detector thermoelectrically cooled to -80°C
- EM gain
- Multiple-speed operation (10, 5, or 1 MHz)
- Back-illuminated, frame-transfer EMCCD
- Lifetime vacuum guarantee
- 100% duty cycle
- 16-bit images
- Dual readout modes permit superb low-noise slow-scan readout as well as high-speed EMCCD operation

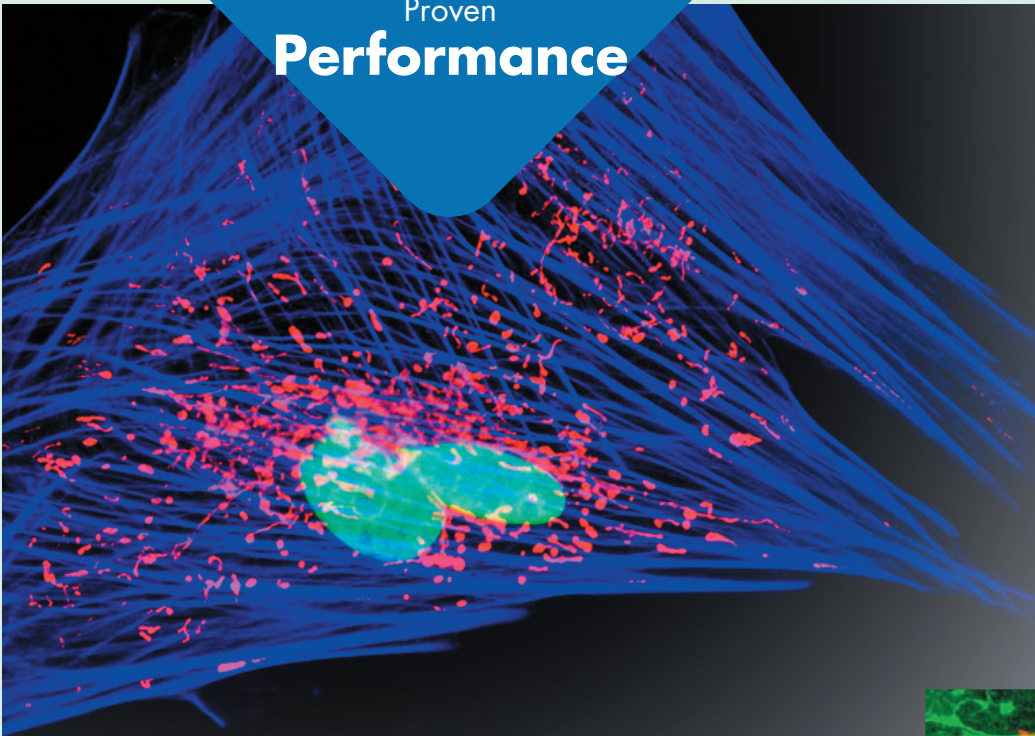
The **Cascade:128+** EMCCD camera features a small imaging array that delivers ultrafast frame rates for low-light-level, live-cell microscopy applications.



- 128 x 128 imaging pixels
- 24 x 24- μm pixels
- >90% quantum efficiency
- Detector cooled to -30°C
- EM gain
- Greater than 500 full frames per second
- 12-MHz operation
- Back-illuminated, frame-transfer EMCCD
- 100% duty cycle
- 16-bit images

Please visit www.photomet.com to learn more about high-performance EMCCD cameras from Photometrics.

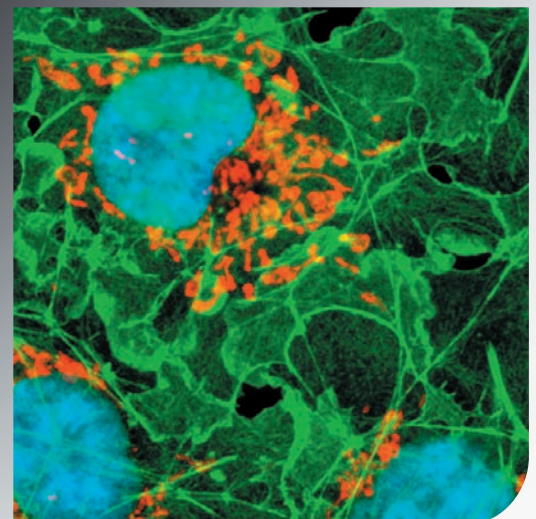
Proven Performance



The **CoolSNAP_{HQ}²** offers optimal low-light-level sensitivity across the entire visible spectrum — and even into the near-infrared region. This interline camera is an ideal choice for a broad range of high-speed, high-resolution microscopy applications.



- 1392 x 1040 imaging pixels
- 6.45 x 6.45- μ m pixels
- >60% quantum efficiency
- Detector cooled to -30°C
- Dual-speed readout modes (10 MHz or 20 MHz) permit ultra-low-noise readout or rapid readout
- User-friendly gain slider for higher-speed qualitative imaging as well as discrete quantitative gain modes
- 14-bit images
- Turbo 1394 interface (FireWire)
- Extreme Eclipse Technology™



GFP Imaging

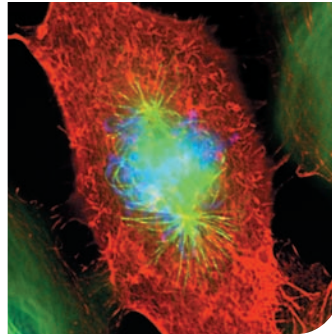
The ability to conduct meaningful studies in the context of live cells has been fortified in large part due to the introduction of genetically encoded fluorescent proteins. This technology has revolutionized cell biology. Today's researchers can investigate gene expression, protein interactions, chemical environments in discrete cellular compartments, protein localization, and more in real-time using living cells. An extraordinary signal-to-noise ratio coupled with industry-leading quantitative digitization makes the CoolSNAP_{HQ}² a top choice for GFP imaging. For lower GFP-expression levels and higher temporal-resolution requirements, where photons are typically in short supply, a high-resolution EMCCD camera such as the Cascade:1K can provide an ideal solution.

FRET Imaging

Förster, or fluorescence, resonance energy transfer (FRET) is a powerful tool that is being increasingly used in biological studies to determine the close proximity (on the order of 10 nm) of molecules labeled with different fluorophores. This method transcends the optical resolution limit of light microscopes and thus permits the imaging of dynamic events at the molecular level. FRET imaging can be very difficult to conduct due to the fact that usually only a small fraction of the total energy being emitted from the sample is attributable to FRET and that this energy can be lost in the imaging system noise. The CoolSNAP^{HQ2} delivers the industry's best low-noise specifications (as well as advanced features like noise-reducing Extreme Eclipse Technology†) to provide a perfect platform for FRET imaging under low-light, slow-scan conditions. Furthermore, the wide dynamic range of the CoolSNAP^{HQ2} extends application flexibility by permitting a greater span of intensity values to be quantified in the same image. For higher-speed acquisition under the low-light conditions typical of FRET imaging, Photometrics EMCCD cameras offer a number of exceptional solutions.

Ion Imaging

Intracellular ion flux is of major concern to biologists. Bio-researchers often wish to quantify both absolute concentrations and spatial variations of ion concentrations within cell and tissue samples. Fluorescent dyes have been designed to have specificity for particular ions and exhibit predictable changes in absorption or emission profiles due to changes in ionic concentration. Such changes in absorption or emission profile are measured to reveal behavior of ion regulation in cells. These dynamic events may unfold very rapidly, or longer-term trends may be of interest. Photometrics is pleased to offer a broad selection of both slow-scan CCD and rapid-imaging EMCCD cameras for ion-imaging studies.



High-speed operation and good QE performance are important for low-light, time-resolved imaging applications.*
Image courtesy of Woods Hole AQLM.

The **Cascade:1K** EMCCD camera features a 2/3" imaging format that offers megapixel resolution for low-light-level, live-cell microscopy applications.



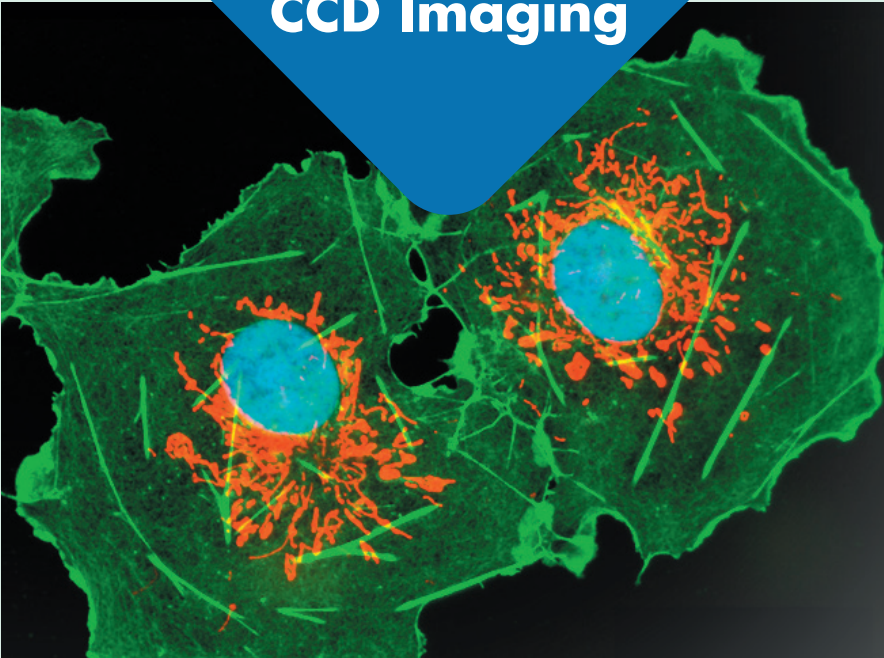
- 1004 x 1002 imaging pixels
- 8 x 8- μ m pixels
- >60% quantum efficiency
- Detector cooled to -30°C
- EM gain
- 10-MHz operation
- Front-illuminated, frame-transfer EMCCD
- 100% duty cycle
- 16-bit images

Please visit www.photomet.com to learn more about high-performance CCD and EMCCD cameras from Photometrics.

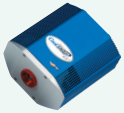
* Monochrome images have been pseudocolored after acquisition for display purposes.

† The CCD used in the CoolSNAP^{HQ2} has inherent electronic noise. Extreme Eclipse Technology (EET) from Photometrics is a detector mode that totally eliminates this noise.

CCD Imaging

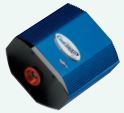


The **CoolSNAP_{EZ}** interline camera has been optimized for moderate- to low-light-level fluorescence microscopy applications that demand both moderate speed and high resolution.



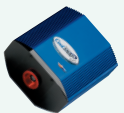
- > 1392 x 1040 imaging pixels
- > 6.45 x 6.45- μ m pixels
- > >60% quantum efficiency
- > Detector cooled to 0°C
- > 20-MHz operation
- > Subcompact design
- > 12-bit images
- > Turbo 1394 interface (FireWire)
- > Extreme Eclipse Technology

The **CoolSNAP_{EZ}** interline camera is an excellent choice for moderate-light-level fluorescence microscopy applications that demand both moderate speed and high resolution.

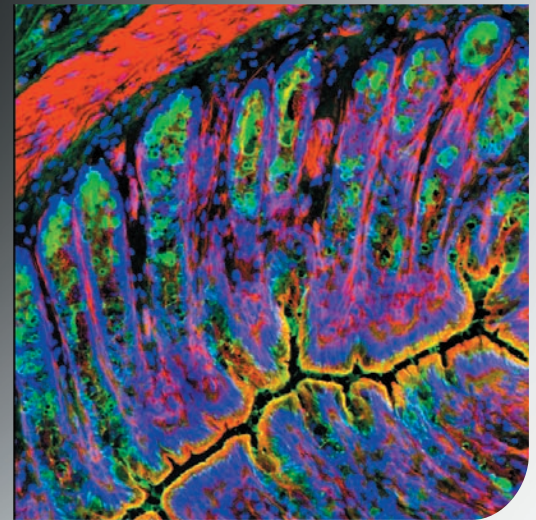


- > 1392 x 1040 imaging pixels
- > 6.45 x 6.45- μ m pixels
- > >60% quantum efficiency
- > Detector cooled to 5°C below ambient
- > 20-MHz operation
- > Subcompact, fanless design
- > 12-bit images
- > Turbo 1394 interface (FireWire)

The **CoolSNAP_{cf}** interline camera has been tailored specifically for moderate-speed microscopy applications that require the highest spatial resolution.



- > 1392 x 1040 imaging pixels
- > 4.65 x 4.65- μ m pixels
- > >40% quantum efficiency
- > Detector cooled to 5°C below ambient
- > 20-MHz operation
- > Subcompact, fanless design
- > Monochrome model (12-bit images)
- > Color model (36-bit color images)
- > Turbo 1394 interface (FireWire)



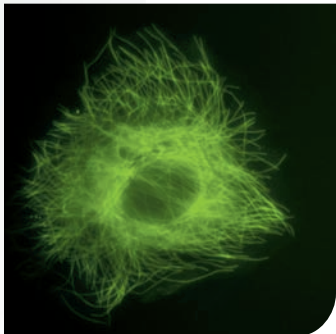
Pathology

Photodocumentation in pathology is not typically a photon-limited application, rather pixel density is of primary concern.

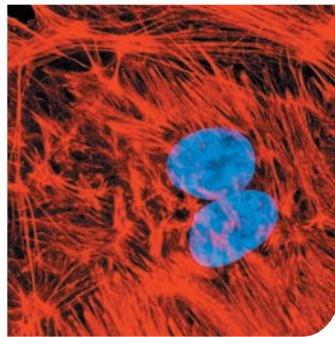
The CoolSNAP_{K4} CCD camera offers maximum field of view along with high resolution and low noise for pathology documentation. For situations that do not require maximized area capture, the CoolSNAP_{ES} and CoolSNAP_{EZ} cameras represent excellent imaging solutions with low-noise characteristics. The CoolSNAP_{cf} camera, meanwhile, is offered in a 36-bit color model and makes an ideal choice for photodocumentation of multicolor histological sections in brightfield.

Photodocumentation

Applications for photodocumentation extend beyond clinical pathology. Researchers often wish to document general morphology of live cells in culture using phase-contrast illumination. More specialized applications of qualitative imaging include differential interference contrast, darkfield illumination, and broadband polarized-light illumination. Photometrics offers the world's highest-performance and most reliable scientific-grade CCD cameras for morphometric photodocumentation.



Low noise and high resolution produce detailed images under fluorescence.*



Viewing Cellular Organelles and Compartments Using Stains

In order to identify specific cytological structures and observe localization of such structures to subcellular compartments, specialized vital stains can be employed in living cells. These probes are usually specific for a particular organelle or chemical micro-environment and utilized in the context of fluorescence microscopy to provide high contrast. Such imaging studies are generally mildly photon-limited, thus the low-noise performance of either the cooled CoolSNAP^{ES2} or CoolSNAP^{EZ} camera is a perfect fit.

The CoolSNAP^{K4} interline camera enables full field-of-view fluorescence microscopy without any intermediate demagnifying optics.

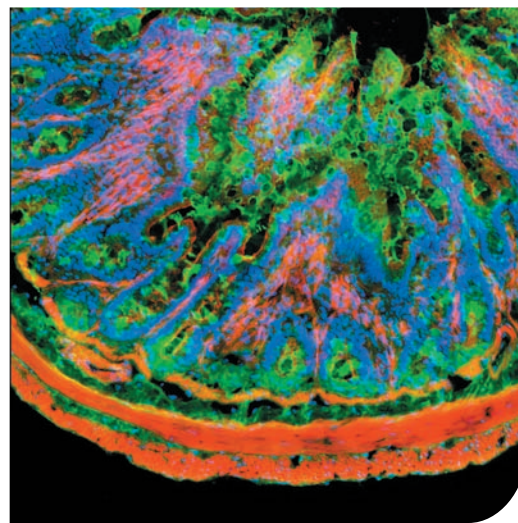


- 2048 x 2048 imaging pixels
- 7.4 x 7.4- μm pixels
- >50% quantum efficiency
- Detector cooled to -25°C
- 20-MHz operation
- 12-bit images

Please visit www.photomet.com to learn more about high-performance CCD cameras from Photometrics.

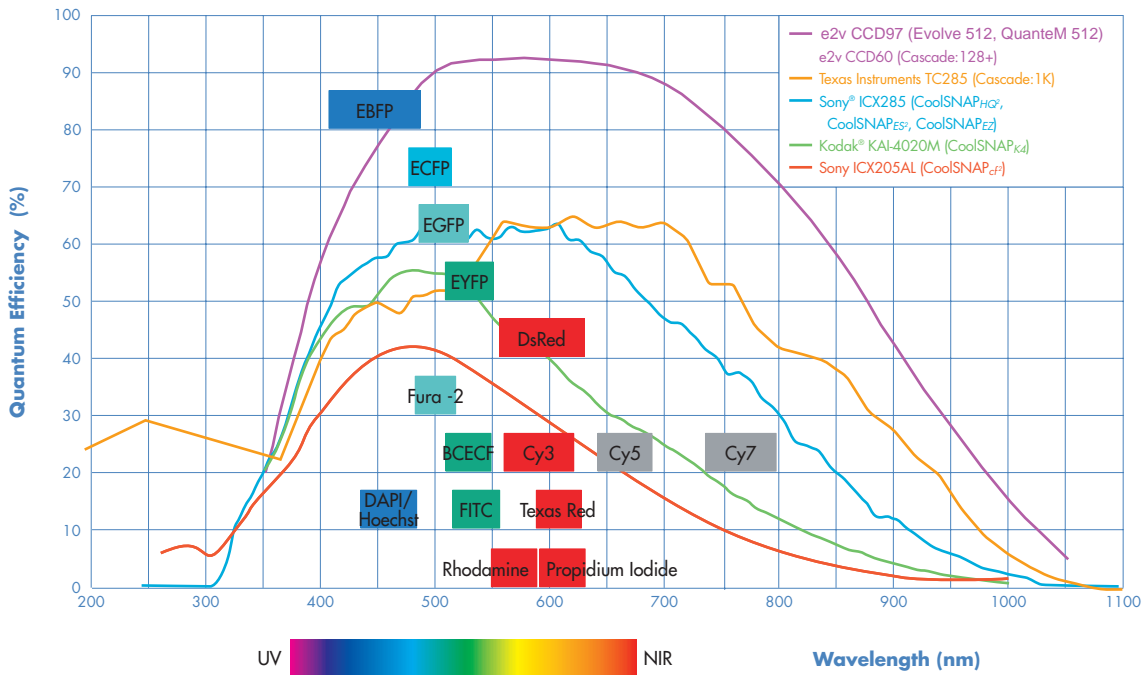
Immunostaining

Immunolabeling technology permits the presence and accurate localization of proteins to be determined. Immunolabeling studies may involve colorimetric reporters observed under brightfield conditions or may utilize fluorescent labeling. The CoolSNAPTM camera series offers appropriate solutions for either of these approaches to signal detection. The CoolSNAP^{cf2} color camera model is well suited for documentation of colorimetric reporters under brightfield conditions, while the low-noise, cooled CoolSNAP^{ES2} and CoolSNAP^{EZ} cameras are ideal for fluorescence studies, where photon flux is necessarily much lower.



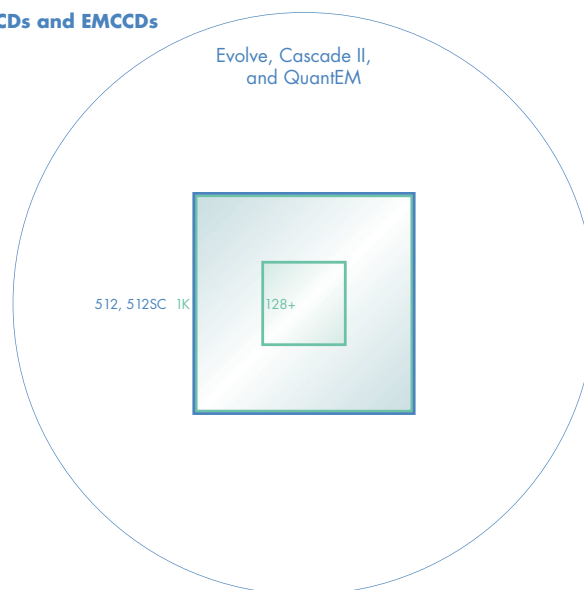
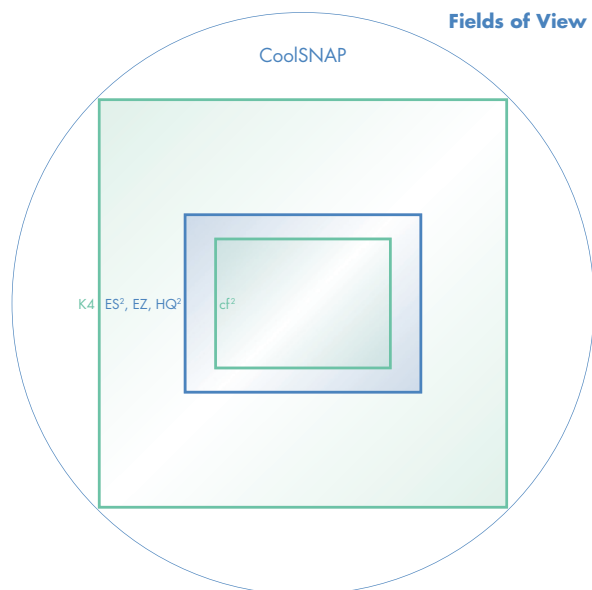
* Monochrome images have been pseudocolored after acquisition for display purposes.

		histology	DIC microscopy	immuno- fluorescence	FISH	fixed-cell GFP imaging	live-cell GFP imaging
	QUANTEM: 512SC				✓		✓
	evolve™				✓		✓
	Cascade 1K						✓
	Cascade 128+						✓
	CoolSNAP ² HQ ²			✓	✓	✓	✓
	CoolSNAP ² ES ²	✓	✓	✓	✓	✓	✓
	CoolSNAP ² EZ	✓	✓	✓	✓	✓	✓
	CoolSNAP ² cf ²	✓	✓				
	CoolSNAP ² K4	✓	✓	✓	✓	✓	



high-speed calcium imaging	3D deconvolution	4D / 5D / 6D imaging	single-molecule fluorescence	FRAP	FRET	FLIM	widefield confocal
✓	✓	✓	✓	✓	✓		✓
✓	✓	✓	✓	✓	✓		✓
	✓	✓		✓	✓		✓
✓		✓		✓	✓		
	✓	✓		✓	✓	✓	
	✓	✓			✓		
	✓				✓		

Fields of View for CCDs and EMCCDs



drawn to scale

Please visit www.photomet.com to learn more about high-performance CCD and EMCCD cameras from Photometrics.

Software

PVCAM®

Photometrics Virtual Camera Access Method

The exclusive PVCAM application programming interface for high-performance digital cameras is a set of software library routines that implements a camera's operations in a hardware-independent, platform-independent ("virtual") suite of function calls.

Once an application has been written to control one PVCAM-enabled camera, every PVCAM-enabled camera is then compatible with the application.

The transparent PVCAM interface supports a wide variety of popular third-party imaging packages. All Photometrics cameras are PVCAM-enabled.

Interface features include:

- Multiple-port access for PVCAM-enabled cameras
- Circular buffers and event streaming that improve high-frame-rate performance
- Software Developers' Kit that helps expert users efficiently access and utilize PVCAM's full range of capabilities
- Compatibility with Windows® 2000/XP/7, Mac OS X, and SUSE® Linux® 9.2 (kernel version 2.6)

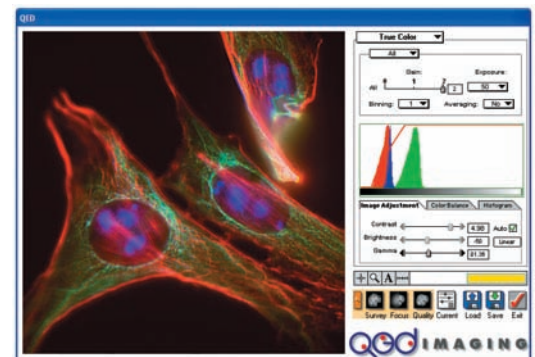
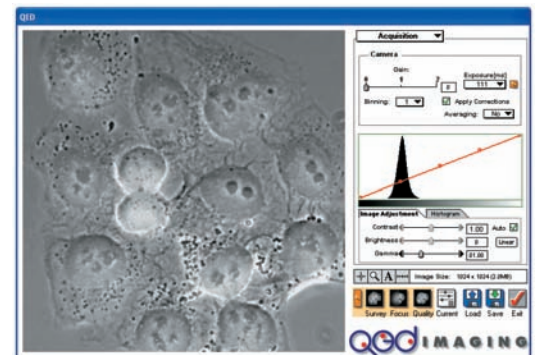
Please visit www.photomet.com for more information about software.

Third-Party Software

Utilizing the right software with your Photometrics CCD or EMCCD camera can improve both the efficacy and efficiency of your digital imaging application.

Dozens of popular third-party software packages are available that fully support the many features built into Photometrics' quantitative camera systems. The analysis and presentation functions provided within these packages are specifically designed to generate precise and accurate data.

In order to facilitate truly optimal camera performance, users are encouraged to learn more about appropriate third-party software options by contacting Photometrics.



OEM Capabilities

Our OEM Programs and Services Group has its own account managers, application engineers, and technical support specialists dedicated solely to serving OEM customers. This structure allows us to anticipate and respond quickly to your changing needs.

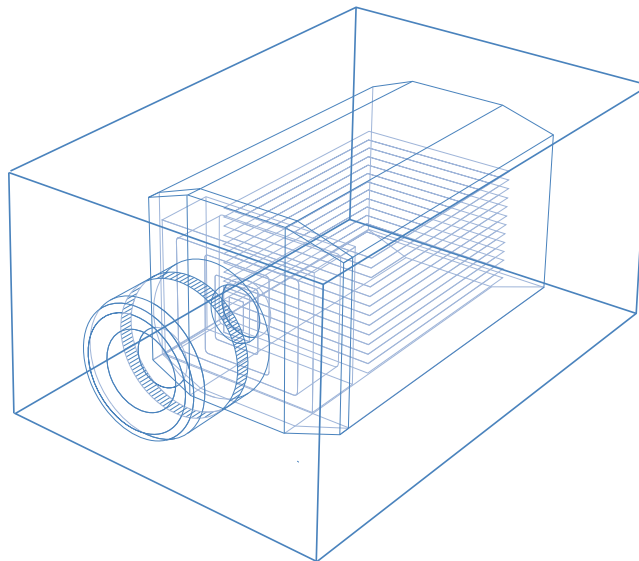
We provide high-performance CCD and EMCCD cameras in a wide variety of configurations, from complete, ready-to-run camera systems to component-level, single-board cameras. Our engineering team can help you rapidly integrate Photometrics products with your software, optical, electrical, and mechanical elements.

In addition, our extensive experience with numerous imaging applications makes us better able to support you. No matter what the scenario, we work closely with you to deliver the best solutions for your unique requirements.

The list of our areas of proven expertise is always growing:

- › Drug discovery
- › Electrophoresis
- › Film digitization
- › Genomics/proteomics
- › Cancer detection
- › Medical imaging
- › Light microscopy

Please visit www.photomet.com to learn more about our OEM capabilities.



Multichannel Imaging Systems

The **Dual-View™** utilizes a single beamsplitter that splits the incident beam from the microscope into two independent beams. One beam contains all the emission reflected off of the beamsplitter; the other contains all the emission transmitted through the beamsplitter. Each of these emission channels is projected onto half of the CCD array.



- › Simultaneous acquisition of two emission channels
- › Easily mounts to most microscopes
- › Uses standard 25-mm-diameter emission and polarization filters
- › Bypass mode permits no-hassle, full-FOV imaging
- › Removable filter cube makes configuring different experiments a snap
- › Precision optical and mechanical design allows subpixel image registration and minimizes light loss
- › Anodized aluminum finish on all parts
- › Compatible with many Photometrics cameras*

The **Quad-View™** allows simultaneous acquisition of up to four emission channels. The operation of the Quad-View system is similar to the Dual-View system except that there are four images formed on the detector, one in each quadrant.



- › Simultaneous acquisition of four images
- › Easily mounts to most microscopes
- › Uses standard 25-mm-diameter emission and polarization filters
- › Bypass mode permits no-hassle, full-FOV imaging
- › Removable filter cube makes configuring different experiments a snap
- › Precision optical and mechanical design allows subpixel image registration and minimizes light loss
- › Anodized aluminum finish on all parts
- › Compatible with many Photometrics cameras*

Over the last decade there has been an explosion of life sciences applications that require fluorescence microscopy to investigate dynamic phenomena in cells and living tissue. One of the biggest challenges has continued to be the imaging of time-critical cellular events. Much of the most important data at the cellular level changes in seconds or less. The only way to accurately capture these events is for two or more channels to be imaged *simultaneously* to track changes in fluorescence signal.

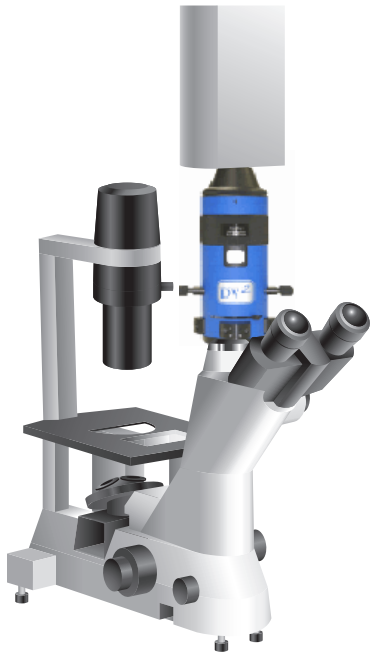
The Multichannel Advantage

Multichannel imaging systems from Optical Insights project multiple images onto the detector(s) at exactly the same time. This alleviates problems associated with temporal resolution. Investigators can finally take full advantage of newer, low-light, high-speed cameras in multilabel experiments.

Multicolor Imaging

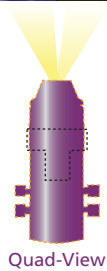
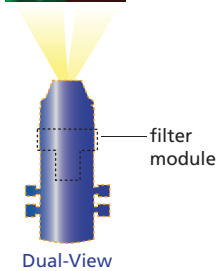
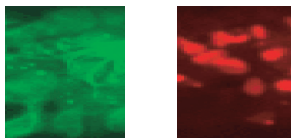
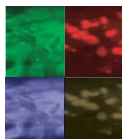
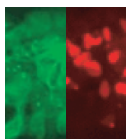
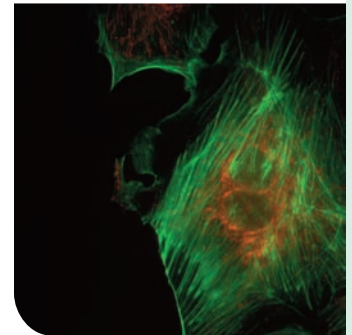
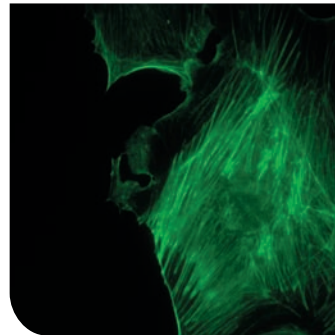
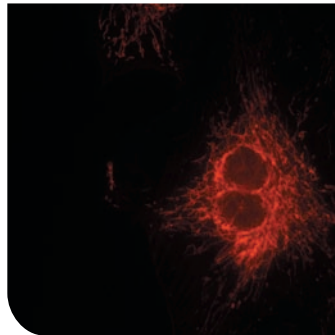
The capability to simultaneously acquire up to four separate spectral channels (e.g., blue, green, red, far red) provides incredible flexibility in multicolor bio-investigation. Such multicolor imaging makes it possible to correlate specific time-critical molecular phenomena.

*Please contact your local Photometrics sales manager to verify compatibility.



The **Dual-Cam™** system works in essentially the same manner as the Dual-View, except that it permits each channel to take up a full detector array. This is crucial for applications where the full field of view for each emission channel is required. The Dual-Cam system is particularly ideal for high-content screening.

- Simultaneous acquisition of two full-FOV emission channels in a single snapshot
- Increases acquisition by factor of two without sacrificing resolution
- Easily mounts to most microscopes
- Removable filter slider makes configuring different experiments easy
- Bypass mode allows user to send all of the emission light to a single detector
- Precision optomechanical design allows subpixel image-to-image registration and minimizes light loss
- Anodized aluminum finish on all parts
- Compatible with many Photometrics cameras*



Please visit www.photometrics.de/multichannel.html to learn more about multichannel imaging systems.

How Does It Work?

Incident light from the microscope is split by one or more dichroic filters, polarizing beamsplitters, or amplitude beamsplitters into multiple (two or four depending on model), independent light paths. Each of these light paths, or channels, is projected onto the image sensor(s) at precisely the same point in time.

The **Xtender XT²** is a simple-to-install collimated emission-port adapter that enables correct emission imaging by eliminating bandpass shift and preserving parafocality between the detector and the microscope eyepiece.

- Two-piece optical adapter creates collimated (infinity) space at emission port of microscope, thus allowing addition of other optical devices without sacrificing performance or introducing optical aberrations
- Provides "extra space" at microscope emission port for installation of instrumentation such as filter wheels and liquid-crystal tunable filters (LCTFs)
- Flexible enough for use with a variety of optical devices with C-mounts
- Fits in between virtually any microscope and CCD



GFP Imaging in Living Cells

APPLICATION BRIEF

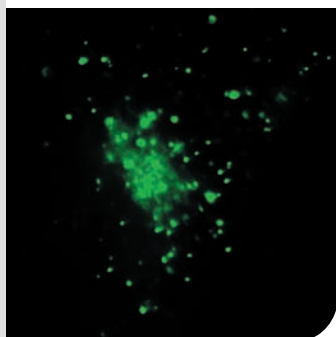


Figure 1. GFP wild-type Rab4. Image courtesy of Photometrics.

The Green Fluorescent Protein (GFP) is a 27-kilodalton protein isolated from the jellyfish *Aequorea victoria* that fluoresces in the green upon illumination with UV light. The cDNA has been cloned and placed into high-level expression vectors optimized for use in organisms ranging from plants to mammalian cells. With the availability of this cDNA, GFP can be introduced easily into virtually any cell type using standard transfection and selection methods, producing a stable cell lineage with maintained GFP expression.

The fusion of GFP with other proteins generates labeled proteins that can be constitutively expressed *in situ* for long-term studies. These chimeric proteins often maintain normal function when GFP is added to the NH₂ or COOH terminus of the fusion partner. Hence, GFP is an ideal tool for labeling cellular proteins to follow their spatial and temporal localization in live-cell preparations (see **Figures 1** and **2**).

In addition, red-shifted spectral variants of GFP have been produced using mutagenesis to alter sequences in the region of the chromophore. With multiple spectral variants of GFP available, it is possible to perform simultaneous monitoring of multiple labels within a single cell/tissue sample. By using optical-filtering devices such as the Optical Insights Dual-View or Quad-View (which can be easily coupled to any Photometrics camera), multiple emission channels can be observed *simultaneously* in order to reveal the movement and/or interactions of different proteins in living cells.

Imaging GFP Chimeras

Illumination of living cells with light in the UV range is highly detrimental to the cells (the amount of damage is dependent on cumulative exposure time). Even with 488-nm laser line illumination of GFP there can be a disruption of normal cellular events such as mitosis. Thus, to obtain physiologically relevant data, the excitation light must be kept to an absolute minimum.

The primary consideration when selecting a camera to image GFP chimeras in living cells, therefore, is sensitivity. It is important to choose a low-noise camera that maximizes signal-to-noise ratio. Additionally, to perform precise localization of the labeled structures within the cell, a medium- to high-resolution detector is preferred.

Both quantum efficiency (QE) and readout noise are factors in determining the sensitivity limit of a detector. For instance, a typical back-illuminated CCD has a QE of 92% at 520 nm, which is more than three times higher than the QE of a standard front-illuminated CCD. When a back-illuminated CCD is read out at a slow rate, the noise of readout can reach levels that are three times lower than CCDs running at higher speeds.

The major limitations of older-style back-illuminated CCDs are large pixel size (typically 24 x 24 microns for the SiTe 502B) and high readout noise. Large pixel size lowers the spatial resolution of the detector for a given magnification and high readout noise limits the speed at which the detector can be operated.

Photometrics has addressed these problems by working with leading CCD manufacturers to produce a series of the industry's lowest-noise CCDs with QEs optimized for the visible region. These CCDs also have smaller pixels, allowing biologists to maintain maximal spatial resolution while imaging their fluorescent proteins. They can be run at speeds up to 20 megapixels per second and are available in a variety of sizes in four distinct camera formats (CoolSNAP, Evolve, Cascade II, and QuantEM).

Evolve Camera System

Highest Sensitivity, High Speed

The Evolve:512 monochrome camera system is optimized for high-speed imaging experiments in live cells. The camera combines the sensitivity of a back-illuminated electron-multiplying CCD (EMCCD) with the high-speed imaging capability of a frame-transfer device. Its EMCCD is cooled to -80°C via a Peltier device, minimizing noise attributable to dark current.

The combination of speed and sensitivity afforded by the Evolve:512 is ideal for 3D imaging of live cells. Note that when collecting 4D (3D over time) data sets, the camera's high sensitivity becomes even more important.

Please visit www.photometrics.de to learn more about high-performance CCD and EMCCD cameras from Photometrics.

GFP can be used to monitor a huge variety of processes in cells, tissues, or whole organisms. For example, in cell-trafficking experiments, fluorescent proteins can be tagged to proteins of interest and then monitored for intracellular localization and itinerary in living cells using real-time data acquisition. Detection of low-light-level fluorescent protein expression is vital for such studies, as it reduces the chances of artifacts due to over-expression. A camera like the Evolve 512 is an excellent choice for this type of application. The detector's 16-micron square pixels enable subcellular structures labeled with GFP to be resolved quite easily.

In order to synchronize image collection with a fast wavelength-switching device or a piezo-driven objective, the Evolve:512 provides various TTL triggers that reflect the exact status of the exposure or image readout. Additionally, the Evolve:512 can acquire data continuously (the light-sensitive portion of the EMCCD array 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 acquire data at rates ranging from 29 frames per second (fps) at full resolution up to >300 fps on binned subregions of the EMCCD.

For applications where fast frame rates are not as critical, the Evolve:512 offers an additional software-selectable readout speed of 5 MHz. The lower-noise readout performance at this slower speed enables higher signal-to-noise data collection.

QuantEM:512SC Camera System

Quantitative Stability Over Time

The QuantEM:512SC monochrome camera system utilizes an intelligent FPGA design and patent-pending technologies to achieve true quantitative stability across 16 bits — unprecedented for an EMCCD imaging platform. The QuantEM:512SC is engineered specifically for use in ultra-low-light, high-speed applications requiring calibrated, linearized EM gain as well as both bias offset and EM gain that are exceptionally stable over time. To provide high quantum efficiency and fast frame rates, the camera features a back-illuminated, frame-transfer EMCCD. The outstanding signal-to-noise ratio offered by the QuantEM:512SC is especially beneficial for low-light, live-cell imaging. The camera's use of Photometrics' industry-leading Turbo 1394 interface facilitates quick, easy connectivity. This unique IEEE 1394 (FireWire) implementation permits the fastest parameter-switching image acquisition on the market.

CoolSNAP_{HQ}² Camera System

Highest Resolution

The CoolSNAP_{HQ}² monochrome camera system is a premier high-spatial-resolution GFP imaging device whose interline-transfer CCD delivers good QE across the full visible spectrum (>60% at 520 nm). The detector's 1392 x 1040 format and 6.45-micron square pixels provide ultra-high-resolution images. The camera also boasts an industry-leading read-noise specification, typically around 4.0 e⁻ (at 10 MHz in gain state 3). Thermoelectric cooling to -30°C minimizes dark current, enabling longer exposure times.

For greater application flexibility, the CoolSNAP_{HQ}² employs 14-bit digitization as well as both high-speed (20 MHz) and high-sensitivity (10 MHz) readout modes. The "live feel" of the camera's video output makes setting up and focusing extremely easy — without sacrificing low-noise performance. Camera vibration is eliminated via electronic shuttering, which also facilitates fast triggering.

The CoolSNAP_{HQ}² is best suited for GFP imaging that requires the absolute highest resolution possible at high frame rates. Under experimental conditions where resolution requirements are not as high, the camera can be run in binned mode to increase signal-to-noise ratio and further shorten readout times. The camera also allows programmable subregion readout, which reduces the digital data load while increasing frame rates for kinetic imaging applications.

With many of the same features and only a slight decrease in noise performance, another Photometrics camera, the CoolSNAP_{ES}², represents an economical, streamlined alternative to the CoolSNAP_{HQ}².

For assistance comparing the performance characteristics of the Cascade II:512, QuantEM:512SC, and CoolSNAP_{HQ}², consult **Table 1**.

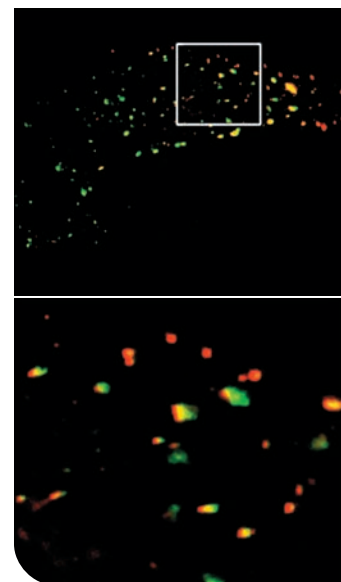


Figure 2. Peripheral region of a HeLa cell transiently transfected with GFP wild-type Rab5 and labeled with Alexa Fluor® 594 (Molecular Probes) cholera toxin. The GFP compartments label the early endosomes, in which can be seen some of the cholera toxin. Image courtesy of Photometrics.

	Evolve:512	QuantEM:512SC	CoolSNAP _{HQ} ²
Format	512 x 512 pixels	512 x 512 pixels	1392 x 1040 pixels
Pixel size	16 x 16 µm	16 x 16 µm	6.45 x 6.45 µm
Diagonal	11.6 mm	11.6 mm	11.2 mm
Peak QE	92%	92%	62%
A/D converter	10, 5 MHz (8 to 16 bits)	10, 5 MHz (16 bits)	20, 10 MHz (14 bits)
Read noise (fast/slow)	<1 e ⁻ with EM gain	<1 e ⁻ with EM gain	5.5 e ⁻ / 4.5 e ⁻
Frame rates (1x1 binning)	29 fps	31 fps	11 fps
Regulated cooling	-85C, liquid -100C	-30°C	-30°C
Summary	High-speed, live-cell, nonshuttered imaging	High-speed, live-cell, nonshuttered imaging with quantitative stability over time	Fixed/live-cell, nonshuttered imaging

Table 1. Feature comparisons for Photometrics cameras.

Multicolor Live-Cell Imaging

APPLICATION NOTE

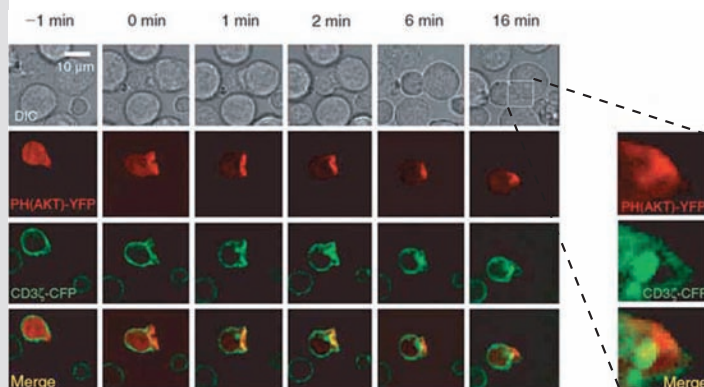


Figure 1. CD3 ζ and PKB localization. Despite rapid internalization of CD3 ζ , PKB [PH(AKT)-YFP] activity remains localized proximal to the T cell / B cell contact interface.¹

In order to image molecular interactions and signaling processes in living cells both temporally and spatially, it is frequently necessary to multiplex fluorescent markers. Today, there is a rich variety of organic dyes and genetically encoded fluorescent proteins available that can be combined in studies of intra- and intercellular function. This type of high-resolution, multiple-probe, time-lapse imaging in living cells places a number of appreciable demands on imaging systems; advanced CCD imaging solutions from Photometrics have been demonstrated to produce significant and impressive results in this context.

Multiple-Probe Imaging

Exploring the Role of T Cell Receptor Signaling in T Cell / B Cell Communication and T Cell Activation

In 2003, Johannes Huppa, Michael Gleimer, Cenk Sumen, and Mark Davis published a study that examined the involvement of T cell receptor (TCR) signaling at the synapse between helper T cells and B cells¹. In this study, multicolor 3D time-lapse microscopy was used to monitor and correlate the redistribution of CD3 ζ on the cell membrane with localized TCR-dependent signal-cascade activity. Levels of intracellular calcium, a marker of T cell activation, were monitored using ratiometric imaging of a calcium-indicator dye. TCR-dependent maintenance of the T cell / B cell synapse was assayed by imaging the distribution of receptors on cell surfaces at the synaptic junction. A third aspect of the study monitored T cell proliferation using a persistent cell-proliferation-tracking dye.

To examine the relationship between the TCR-CD3 ζ complex and early signaling at the synapse between T cells and antigen-presenting B cells, genetically encoded fluorescent proteins were used as fluorescent markers. A CD3 ζ coupled to cyan fluorescent protein (CFP) was utilized to monitor CD3 ζ localization. Yellow fluorescent protein (YFP) fused to the pleckstrin homology domain of protein kinase B (PKB) was utilized to image localization of kinase activity. Using this labeling approach, the researchers were able to image the dynamics of both fluorophores in three-dimensional space by taking rapid exposures at multiple focus positions.

Low-noise images were acquired with a Photometrics CoolSNAP_{HQ} CCD camera and subsequently post-processed using deconvolution software to re-assign out-of-focus blur to the point of origin. Not only was the camera's ability to minimize noise critical to successful deconvolution, but the exceptional low-noise performance provided by the CoolSNAP_{HQ} was ideal for imaging under low-light conditions. In addition, the pixel size (6.45 x 6.45 microns) and 1392 x 1040 array of the CoolSNAP_{HQ} permitted high-resolution imaging over a large field of view.

Using this approach, Huppa *et al.* were able to image antigen-dependent recruitment of PKB from the cytoplasm to the areas of CD3 ζ clustering at the cell membrane. The researchers also witnessed the rapid internalization of CD3 ζ at the T cell / B cell interface (see **Figure 1**). Despite this rapid internalization of CD3 ζ from the cell surface, PKB activity proximal to the cell membrane remained high until TCR stimulation was blocked using monoclonal antibodies (see **Figure 2**).

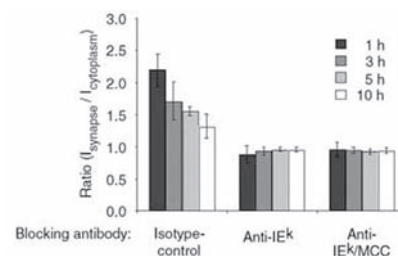


Figure 2. PKB localization proximal to the T cell / B cell contact interface is blocked by the addition of an antibody reagent that blocks antigen-dependent TCR signaling.¹

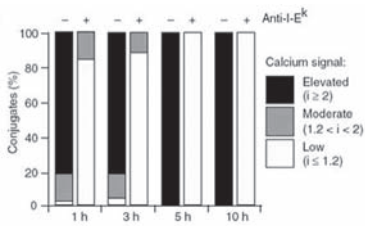


Figure 3. Raised intracellular Ca²⁺ levels (an indicator of T cell activation) are observed to decrease rapidly with the addition of an antibody reagent that blocks antigen-dependent TCR signaling.¹

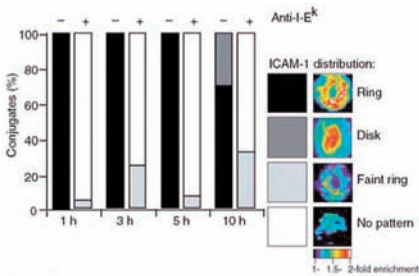


Figure 4. ICAM distribution on B cells involved in synapse with T cells. The characteristic ring-shaped distribution of the immune synapse is observed to dissolve with the addition of an antibody reagent that blocks antigen-dependent TCR signaling.¹

The calcium-sensitive dye fura-2 was used to monitor intracellular calcium levels as well as the dependence of these levels on continued TCR stimulation. Ratiometric analysis of fura-2 fluorescence at excitation wavelengths of 340 and 380 nm was utilized to quantify the Ca²⁺ levels. The low noise, superb linearity, and 12-bit dynamic range of the CoolSNAP_{HQ} enabled excellent quantitation of the fluorescent signals.

The ratiometric analysis showed that the Ca²⁺ signal associated with TCR stimulation persisted for many hours after conjugate formation; however, the elevated Ca²⁺ levels were found to be dependent on continuous TCR signaling. This dependence on TCR stimulation was confirmed by imaging the suppression of Ca²⁺ levels using an antibody blockade (see **Figure 3**).

Maintenance of the T cell / B cell synapse was also confirmed to deteriorate upon blocking TCR stimulation, which was accomplished by monitoring the characteristic distribution of green fluorescent protein-labeled ICAM receptors on B lymphocytes involved in synapse with T lymphocytes (see **Figure 4**). This finding points to a requirement for continuous TCR-mediated signaling to maintain functional cell adhesion between T cells and B cells in the immune response.

To explore the importance of continuous T cell antigen recognition in cell proliferation of helper T cells, interleukin-2 production and thymidine uptake were assayed using enzyme-linked immunosorbant assay (ELISA). Flow cytometry was utilized to monitor cell division in T cells marked with carboxyfluorescein diacetate succinimidyl ester (CFSE), a fluorescent live-cell marker that is distributed equally to daughter cells. Blocking of TCR signaling was found to down-regulate antigen-mediated T cell proliferation.

New Technology

Like the original CoolSNAP_{HQ} used by Huppa *et al.*, Photometrics' new CoolSNAP_{HQ}² has been designed for quantitative fluorescence microscopy and employs a state-of-the-art implementation of interline CCD technology that delivers good quantum efficiency across the visible spectrum. Electronic shuttering eliminates camera vibration and facilitates fast triggering, while thermoelectric cooling to -30°C minimizes the noise attributable to dark current. For even greater sensitivity, adjacent 6.45-micron square pixels in the detector's 1392 x 1040 array can be binned to increase the signal registered per pixel. Dual readout modes, optimized for high-speed or high-sensitivity imaging, provide maximum camera versatility.

The CoolSNAP_{HQ}² employs up to 14-bit digitization, thus offering wider dynamic range than the original CoolSNAP_{HQ}. Furthermore, the recently introduced CoolSNAP_{HQ}² Turbo 1394 camera, a powerful addition to the high-performance CoolSNAP product line, features an innovative implementation of the IEEE-1394 (FireWire) interface in order to deliver the fastest parameter-switching image acquisition available for demanding life sciences applications.

Additional Information

To learn more about Dr. Mark Davis' research, please visit: cmgm.stanford.edu/micro/fac/davis.html

Citations

- Huppa, J.B., Gleimer, M., Sumen, C., and Davis, M.M. (2003). Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nature Immunol.* **4**, 8: 749-755.

Please visit www.photomet.com to learn more about high-performance CCD cameras from Photometrics.

TIRF Microscopy

APPLICATION BRIEF

Total internal reflection fluorescence (TIRF) microscopy is a highly useful mode of fluorescence-light microscopy that employs a carefully refined illumination scheme to allow direct, time-resolved visualization of various intracellular events, including endocytosis¹, exocytosis², cytoskeletal dynamics³, and dynamic single-molecule imaging⁴.

A relatively recent development in biological fluorescence microscopy, TIRF permits ultrahigh axial resolution — on the order of 200 nm — at the sample/coverglass interface⁵. The benefit of such high optical-sectioning resolution at this interface is that the signal-to-noise ratio for studies involving the direct observation and recording of cell membrane function, vesicle fusion, and membrane proteins will likewise be very high.

TIRF is based on the principles of refraction and reflection of light. When light strikes an interface between two materials with differing refractive indices, the light rays are bent (refracted). The degree to which the light path is refracted depends on the degree of difference between the refractive index of the two materials. Note that the refractive index may vary as a function of the wavelength of the light itself. When the angle of the light incident on the interface between refractive indices becomes sufficiently great, however, then the light will begin to reflect rather than penetrate and refract (see **Figure 1**). The angle at which this phenomenon begins to occur is known as the critical angle. The critical angle depends on the ratio of the two media's refractive indices per the relationship below:

$$\Theta_c = \sin^{-1} (n_2/n_1)$$

In this equation, Θ_c is the critical angle, n_1 is the first refractive index, and n_2 is the second refractive index at the wavelength of light being used. For total internal reflection to occur, n_1 must be greater than n_2 , while the angle of incidence must be greater than Θ_c .

The phenomenon of total internal reflection creates an electromagnetic field at the distal side of the interface. The electromagnetic field, known as an evanescent wave, is of the same frequency (or wavelength) of the light being internally reflected. The energy of this field drops exponentially with penetration depth into the sample (see **Figure 1**). The practical implication of this decrease in energy is that only the portion of the sample within the first couple hundred nanometers is illuminated with enough intensity to provide a robust signal. Therefore, background signal and/or scattered illumination will not contribute to the field of interest such as is typical when using a conventional, far-field illumination setup.

There are two basic approaches to designing a TIRF microscopy workstation. The first, and original, method is to make use of a high-refractive-index prism to guide an incident laser beam to the coverglass media interface distal to the microscope objective⁵ (see **Figure 2**). The laser beam is directed by the prism to impinge on the coverslip/media interface at an angle greater than or equal to the critical angle, thereby setting up an evanescent wave for excitation of fluorescent probes within about the first 200 nm of the coverslip/cell interface. The advantages of the prism approach are that the cost of implementation is relatively low and that very high signal-to-noise ratios can be achieved. Disadvantages include the technical expertise required to align the laser illumination optics and the lack of access to the specimen. Another disadvantage is that the signal from the specimen must traverse the entire sample thickness before it can be collected by the objective. In theory, this can introduce artifacts attributable to spherical aberration and absorption.

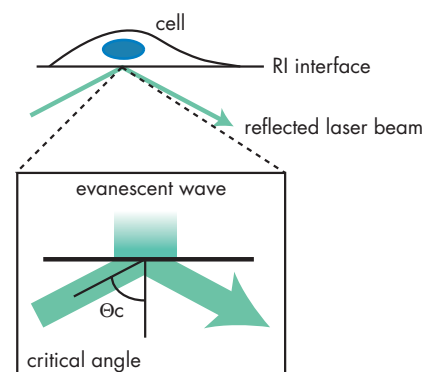


Figure 1. Total internal reflection fluorescence. A laser beam incident on an interface between high- and low-refractive-index materials is reflected at 100% efficiency at or above the critical angle, thus setting up an electromagnetic field of the same frequency called an evanescent wave. The evanescent wave excites the specimen only within a couple hundred nanometers of the refractive-index interface.

Please visit www.photomet.com to learn more about high-performance EMCCD cameras from Photometrics.

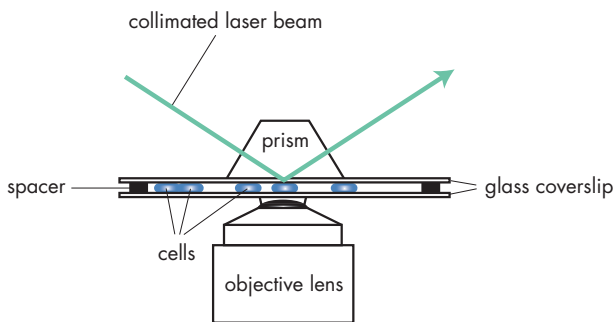


Figure 2. TIRF illumination using a prism. The laser illumination must be directly coupled; alignment is facilitated using beam-steering mirrors on an optical bench. The interface located across the sample from the objective lens generates the evanescent wave.

More recent proprietary systems utilize high-numerical-aperture objectives to set up the conditions necessary for TIRF⁶. This approach permits illumination from the same surface of the sample that is closest to the objective. Thus, sample manipulations can be performed from above (on an inverted microscope base) and there are potentially fewer distorting moieties between the fluorescent markers and the collection lens (see **Figure 3**). An additional advantage is ease of use and encapsulation of the technology in a turnkey system; users do not need an optical bench with beam-steering mirrors to directly couple and adjust the laser input.

Systems have been developed that use white light as an alternative to laser illumination as well. White-light-based systems offer lower-intensity illumination and lower signal-to-noise ratios than laser-based systems, but are more affordable. Furthermore, because they use a broadband illumination source, white-light-based systems are more versatile in terms of the variety of fluorochromes that can be accommodated.

TIRF microscopy is usually used for dynamic, low-light endeavors. In order to optimize the available signal under such conditions, it is important to employ high-quantum-efficiency detectors. The quickly occurring events investigated in these studies often dictate that frame rates be maximized and readout noise be minimized. Photometrics offers an industry-leading line of advanced electron-multiplying CCD (EMCCD) cameras to meet this demanding requirement. The Cascade II:512 camera features a back-thinned

EMCCD with greater than 90% peak quantum efficiency in the visible spectrum as well as a 16-bit digitizer to permit a wide range of brightness values to be recorded. For very low dark noise, the Cascade II:512 is cooled to -80°C. Photometrics EMCCD cameras also provide convenient LVDS connectivity to researchers' TIRF workstations for rapid data transit and fast, low-light imaging.

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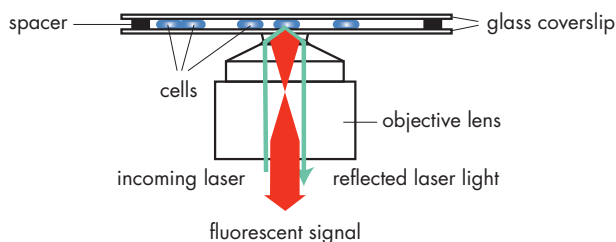


Figure 3. Objective lens TIRF strategy. The illumination source is directed at the outside regions of the objective lens so that the light is directed at the sample at the critical angle. The portion of the lens with a numerical aperture corresponding to less than the critical angle is used to collect the fluorescent signal coming back from the sample.

Live-Cell TIRF Microscopy

APPLICATION NOTE

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 event¹.

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 events².

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 process¹.

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.

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 complex³. Recently, it has also demonstrated the role of cortactin in the scission process during endocytosis⁴. 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.

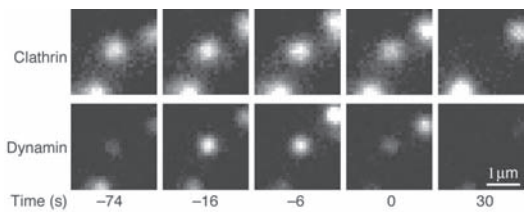


Figure 1. Dynamin is recruited to the clathrin-coated pit and leaves the plasma membrane with the vesicle. These time sequences show images of a clathrin-coated structure (top row) and dynamin fluorescence (bottom row) under evanescent illumination. The dimming of the clathrin-coated structure is preceded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

Enabling Technologies

The researchers utilized a high-performance Cascade:512B electron-multiplying CCD (EMCCD) camera from Photometrics and a beamsplitting Optical Insights Dual-View 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.

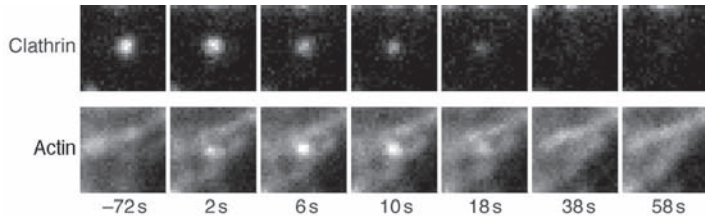


Figure 2. Actin is recruited to the clathrin-coated pit and leaves the plasma membrane with the vesicle. The top row is a time sequence of clathrin–DsRed fluorescence imaged under evanescent illumination. The bottom row displays green fluorescence images of EGFP–actin taken under evanescent illumination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

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

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Please visit www.photomet.com to learn more about high-performance EMCCD cameras from Photometrics.

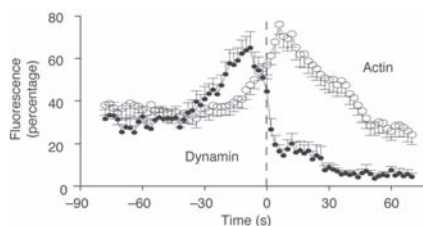


Figure 3. Due to the high temporal and spatial resolution afforded by this experimental setup, it was found that dynamin recruitment to the site of endocytosis peaks just prior to the endocytic event, whilst actin is recruited just after the endocytic event.¹

Applications

FISH

FISH

APPLICATION NOTE

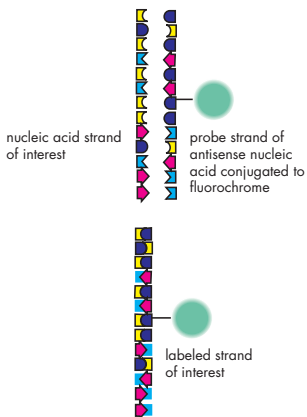


Figure 1. The FISH probe concept. An antisense strand is generated to a nucleic acid sequence of interest. This antisense strand is labeled with a fluorochrome, introduced into a cell preparation, and permitted to anneal. Excess unbound probe strands are washed away.

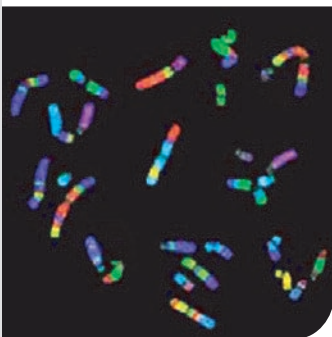


Figure 2. Chromosomes painted with a variety of FISH probes directed at different DNA sequences. Such technology can be used to map the locations of genes on chromosomes and to determine whether translocation events have occurred.

Fluorescence *in situ* hybridization (FISH) 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 chromosomes¹. In other words, 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 rapidly 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 may be detected using fluorescence microscopy (see **Figure 1**). The power of FISH is greatly enhanced by the simultaneous use of multiple fluorescent probes². By using a multiplexing strategy, numerous nucleic acid sequences of interest can be detected and mapped (see **Figure 2**).

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 bind to a particular region of a chromosome. This kind of probe is useful for determining which chromosome a gene is located on once a small sequence of a particular gene has been isolated. Centromeric repeat probes are generated from repetitive sequences found in the middle of each chromosome. Researchers utilize these probes to determine whether a cell has the correct number of chromosomes. Centromeric repeat probes can be used in concert with locus-specific probes to determine if an individual cell is missing genetic material on a particular chromosome.

Whole-chromosome probes, or whole-chromosome paints, are collections of various genetic sequences common to a particular chromosome; each individual probe is labeled with a different color fluorescent probe. These multicolor collections of probes can be utilized to map individual chromosomes as well as to identify different chromosomes in respect to one another. Whole-chromosome probes are very useful for determining if translocation of genetic material from one chromosome to another has occurred. Chromosome painting is also utilized to make a comparison between two species or individuals by using DNA from one species or individual as a probe on another. This method can help identify chromosomal abnormalities and evolutionary relationships.

The FISH concept has been extended to visualize the movement of RNA in living cells³. More recently, fluorescence *in vivo* hybridization (FIVH) has been enhanced with the use of genetically encoded fluorescent proteins, such as GFP⁴. In this approach, termed the MS2-GFP method, a phage sequence containing the stem-loop binding sites for the phage capsid protein MS2 is added to the RNA sequence of interest and incorporated into a plasmid. A second plasmid that codes for the MS2 capsid protein that binds single-stranded RNA, fused to a fluorescent protein, is developed. Together, the two plasmids are transfected into living cells and subsequently expressed. The binding of MS2 to the stem-loop binding site is highly specific and stable, thus providing a powerful system for following the localization and movement of specific RNA sequences in living cells.

Many researchers utilize two- to four-color FISH analysis on fixed samples. The main requirement for imaging of this kind is an extremely 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. In some cases, a midrange-performance CCD camera may be adequate for FISH imaging.

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 cameras are utilized for spectral imaging with purpose-built systems, a specimen's fluorescent emission can be split into component wavelengths. As a result, excellent camera performance and detector sensitivity become critical.

CoolSNAP_{HQ}² Camera

The CoolSNAP_{HQ}² camera system utilizes a CCD that has greater than 1.44 million 6.45-micron square pixels, allowing collection of images with very high spatial resolution (see **Figure 3**). In fact, this pixel format helped make the original CoolSNAP_{HQ} the most highly published camera for this application area.

The new CoolSNAP_{HQ}² camera system comes in a compact housing and provides the industry's lowest-noise readout, software-selectable gains, and a comprehensive set of subregion readout and binning controls. It also has an additional visual gain setting to enable optimal visual display and a controllable fan to minimize vibration. The quantum efficiency (QE) of the CCD is best for wavelengths ranging from 500 to 800 nm. This detector is particularly well suited for "very long wavelength" probes (near IR).

Cascade II:512 Camera

The Photometrics Cascade II:512 camera system utilizes a back-illuminated electron-multiplying CCD (EMCCD) that offers unparalleled sensitivity for bio-imaging. Back-thinning the device results in >92% QE, making it one of the most efficient light-gathering detectors available to bio-researchers. This Cascade camera's electron-multiplying capability, coupled with deep cooling (-80°C), facilitates extremely low-light imaging.

The performance of the Cascade II:512 lends itself to spectral imaging for FISH by minimizing integration times and noise factors while maximizing sensitivity. Additionally, more than 262,000 16-micron square pixels allow the camera to collect images with good spatial resolution.

The ultrahigh QE and electron-multiplying capability of the Cascade II:512 allow unsurpassed spectral-imaging data collection. For the utmost in application versatility, the camera system can even be operated in a traditional CCD mode (in which electrons are not multiplied) that delivers high-QE, low-read-noise performance when photons are not as scarce.

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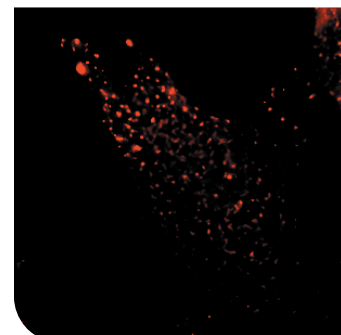
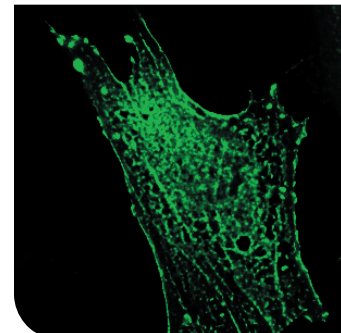


Figure 3. GFP actin and β -actinFISH images taken at specific wavelengths with a CoolSNAP_{HQ} camera to show expression of actin in a mouse myoblast(C2C12) cell. Image courtesy of Alex Rodriguez, Robert Singer Lab, Albert Einstein College of Medicine, New York.

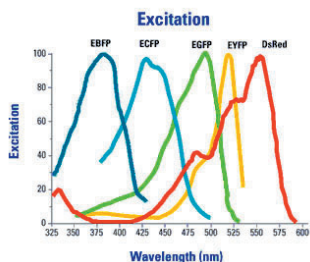
Please visit www.photomet.com to learn more about high-performance CCD and EMCCD cameras from Photometrics.

FRET Imaging

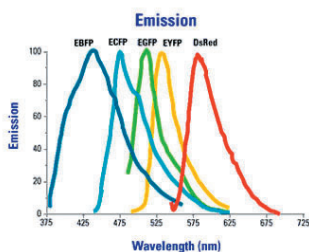
APPLICATION BRIEF

Introduction

Förster resonance energy transfer (FRET) is a phenomenon utilized in microscopy to observe distance-dependent interactions between biological molecules. In this approach, two biological molecules of interest are labeled with different dyes: one with a donor fluorophore, another with an acceptor fluorophore. Typically, the donor fluorophore is excited with narrowband illumination, which will stimulate fluorescence of the donor-labeled molecule. As the acceptor-labeled molecule comes into close proximity of the donor-labeled molecule, the donor transfers its energy without the emission of a photon (i.e., nonradiatively) to the acceptor. As a result of this energy transfer, the donor fluorescence is quenched and the acceptor fluorescence is stimulated. Since the efficiency of the energy transfer process is dependent on the inverse of the sixth power of the distance separating the donor- and acceptor-labeled molecules, the FRET process can provide very sensitive measurements on the separation distance of these two molecules. Typically, interaction distances of 10 to 100 Å can be measured using this approach.



Excitation spectra for different fluorescent proteins (BD Biosciences Living Colors® probes).



Emission spectra for different fluorescent proteins (BD Biosciences Living Colors® probes).

FRET Experiments

FRET experiments are 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 determining simply the presence or absence of FRET as an indicator of interaction, quantitative experiments require a more methodical strategy.

Applications

- › Determining interactions of single molecules
- › Evaluating the structure of proteins
- › Spatial distribution and assembly of protein complexes
- › Monitoring receptor/ligand interactions
- › DNA sequencing operations
- › Sensing presence of small molecules in living cells

A number of experimental conditions need to be satisfied (see the “conditions” list) for successful FRET. In addition, there are certain effects inherently present in any FRET measurement (see the “potential sources” list) that need to be carefully accounted for and may need to be calibrated out of the data. The degree of calibration and correction depends on the accuracy required for the experiment. A variety of algorithms exist in the current literature that can be used for this calibration and correction.¹

Sequential imaging techniques (e.g., using an emission filter wheel or switching microscope filter cubes) can make this calibration and correction very difficult, if not impossible. Therefore, many quantitative FRET applications require that the donor and acceptor emissions be simultaneously imaged using an image-splitting device like the Optical Insights Dual-View.

Conditions for Successful FRET

- › Concentrations of donor and acceptor tightly controlled
- › Photobleaching minimized
- › Large overlap between donor emission and acceptor excitation spectra
- › Direct excitation of acceptor at the excitation maximum of donor is minimized
- › Donor must have a sufficiently long fluorescence lifetime

Potential Sources of Error in FRET

- › Dependence of FRET on donor and acceptor concentrations
- › Bleedthrough of donor fluorescence into acceptor emission channel
- › Direct excitation of acceptor fluorescence by donor excitation

Citations

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FRET Determination

APPLICATION NOTE

Dynamic FRET Imaging

Förster resonance energy transfer¹ (FRET) 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 the proximity between a 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 methods of FRET detection, interactions between molecules can be monitored in subcellular compartments and tracked as a function of time.

FRET Microscopy

Revealing the Interaction between Cytochrome c and Inositol (1,4,5) Triphosphate Receptors in Apoptosis

In 2003, Darren Boehning *et al.* published a study investigating the role of cytochrome c binding to inositol (1,4,5) triphosphate receptors in the regulation of calcium-dependent programmed cell death². The data obtained indicate that cytochrome c binding to inositol (1,4,5) triphosphate (InsP3R) receptors, above certain concentrations, abolishes calcium-mediated inhibition of InsP3R-mediated calcium release. It was determined that cytochrome c translocates from the mitochondria to endoplasmic-reticulum-associated InsP3R, and that binding of cytochrome c to InsP3R is a specific interaction.

Microscopic imaging studies using a low-noise CoolSNAP_{HQ} CCD camera from Photometrics were conducted in order to clarify the temporal evolution of calcium release and the roles played by cytochrome c and InsP3R. These studies revealed that large, sustained increases in early apoptosis are concomitant with the association of cytochrome c and InsP3R. The intimate interaction between cytochrome c and InsP3R was revealed in live cells using FRET microscopy.

The first topic of the study conducted by Boehning *et al.* sought to identify proteins that interact with InsP3R. Through these efforts, it was determined that cytochrome c binds to a specific amino acid sequence on the InsP3R protein. The influence of cytochrome c binding to InsP3R on InsP3R-mediated calcium release was evaluated and it was discovered that above 1-nM concentration, cytochrome c abolishes the calcium-dependent inhibition of InsP3R-mediated calcium release activity.

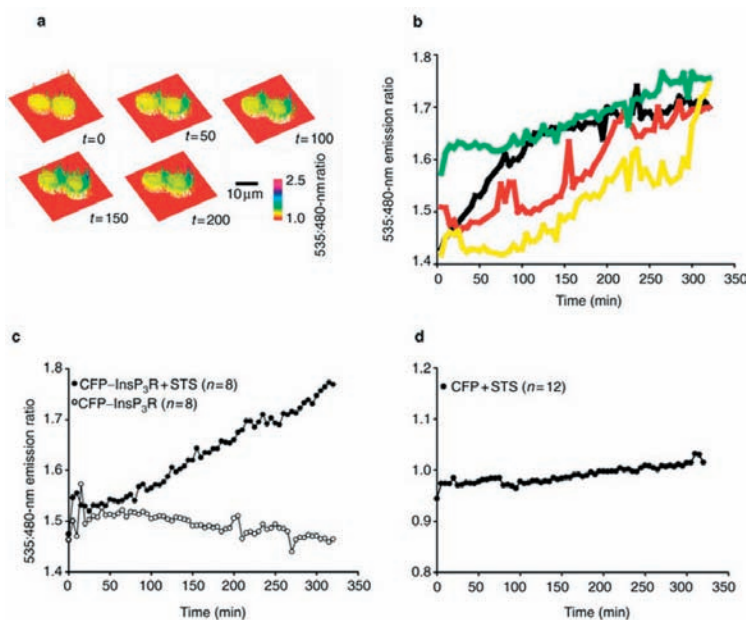


Figure 1. Cytochrome c-InsP3R interaction during apoptosis demonstrated by FRET. **(a)** Surface plot display of the 535:480-nm ratio in two PC12 cells stably expressing YFP-cytochrome c and co-expressing CFP-InsP3R after induction of apoptosis. **(b)** 535:480-nm ratio of four PC12 cells stably expressing YFP-cytochrome c and cotransfected with CFP-InsP3R. This plot demonstrates the heterogeneity of cytochrome c-InsP3R interactions observed in response to induction of apoptosis. **(c)** 535:480-nm ratio of YFP-CytC PC12 cells co-expressing CFP-InsP3R in response to induction of apoptosis or vehicle. Only cells in which apoptosis had been induced are positive for FRET activity. **(d)** 535:480-nm ratio of YFP-cytochrome c PC12 cells cotransfected with cytosolic CFP in response to induction of apoptosis. No significant change in the 535:480-nm ratio was observed. Data courtesy of Prof. Solomon Snyder, Department of Neuroscience, Johns Hopkins School of Medicine.

Next, the investigators explored the possibility that the cytochrome *c* / InsP3R interaction plays a role in apoptosis. InsP3R in cells is usually associated with the endoplasmic reticulum (ER), whereas cytochrome *c* is generally found in the mitochondria. The hypothesis was formed that cytochrome *c* translocates from the mitochondria to the InsP3R in the ER during apoptosis. To test this hypothesis, groups of cultured cells were induced to undergo apoptosis and then fractionated at different time points. The subcellular fractions were then run on Western blots and probed for InsP3R and cytochrome *c*. It was found that cytochrome *c* immunoreactivity decreased in the mitochondrial fraction and increased in the endoplasmic reticulum fraction several hours after induction of apoptosis. Cytochrome *c* and InsP3R were co-immunoprecipitated from the ER fraction, indicating an association between the two proteins.

These results suggest that cytochrome *c* is released from the mitochondria during the early stages of apoptosis to bind with the InsP3R located in the closely adjacent ER. The end result of this interaction is diminished feedback regulation of InsP3R-mediated calcium release and subsequent mitochondrial and cytosolic Ca⁺⁺ overload. The role of InsP3R in translocation of cytochrome *c* to the ER was further investigated using cells lacking InsP3R. It was found that cytochrome *c* fails to translocate to the ER in such cells, but rather appears in the cytoplasm.

Cell lines cotransfected with EYFP-labeled cytochrome *c* and ECFP-labeled InsP3R were used to evaluate interactions between the two proteins in intact cells. Close association between the two proteins in the living cells results in a FRET interaction between the linked fluorescent protein probes. When apoptosis was induced in the transfected cells, the researchers witnessed a gradual increase in the FRET signal (see **Figure 1**). No FRET signal increase was observed in cells that were not induced to undergo apoptosis or in cells in which CFP not linked to InsP3R was expressed.

Further imaging studies were conducted to ascertain whether there was a link between cytochrome *c* binding to InsP3R. In these investigations, YFP-linked cytochrome *c* and calcium levels were imaged in cells induced to undergo apoptosis. Calcium spiking and cytochrome *c* release were observed to be temporally linked. A peptide competition of cytochrome *c* binding to InsP3R demonstrated that *in vivo* release of cytochrome *c* and altered Ca⁺⁺ regulation is dependent on the cytochrome *c*-InsP3R interaction (see **Figure 2**). Expression of the cytochrome-*c* binding sequence of InsP3R was found to attenuate the calcium release due to induction of apoptosis in living cells. This blocking effect was not witnessed for cytochrome *c*-mediated caspase activation, and caspase inhibitors did not block translocation of cytochrome *c* to the InsP3R. Taken together, these results indicate that cytochrome *c* translocation to the ER and the changes in Ca⁺⁺ regulation leading to cell-wide cytochrome *c* release occur upstream of cytochrome *c*-mediated caspase activation.

Additional Information

To learn more about Prof. Solomon Snyder's research, please visit: neuroscience.jhu.edu/people/detail.asp?ID=1

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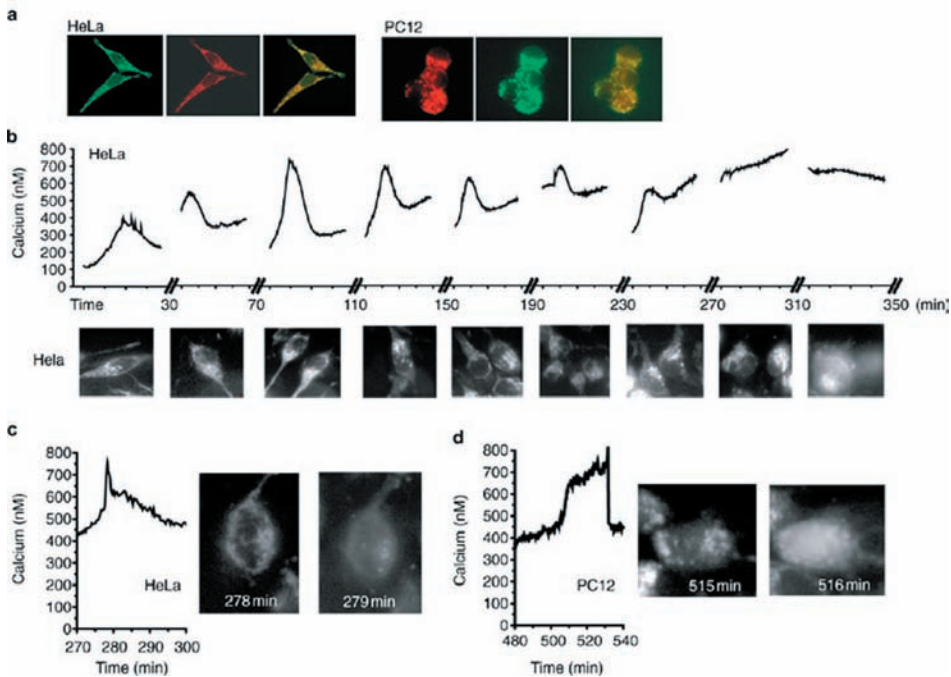


Figure 2. (a) YFP-cytochrome c colocalizes precisely with MitoTracker[®] Red (Molecular Probes), demonstrating that the stably expressed YFP-cytochrome c is contained within the mitochondria. (b) Fura-2 intracellular calcium measurements in response to induction of apoptosis in HeLa cells stably expressing YFP-cytochrome c. Marked oscillations are observed as early as 10 min and continue until approximately 270 min. Cells displayed beneath each 30-min time course manifest the representative distribution of YFP-cytochrome c during that time course. (c) Fura-2 intracellular calcium measurements of a single HeLa cell in response to induction of apoptosis over the 270-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome c into the cytosol, as depicted in the two adjoining images. (d) Fura-2 intracellular calcium measurements of a single PC12 cell in response to induction of apoptosis over the 480-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome c into the cytosol, as depicted in the two adjoining images. Data courtesy of Prof. Solomon Snyder, Department of Neuroscience, Johns Hopkins School of Medicine.

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Multicolor Imaging

APPLICATION BRIEF

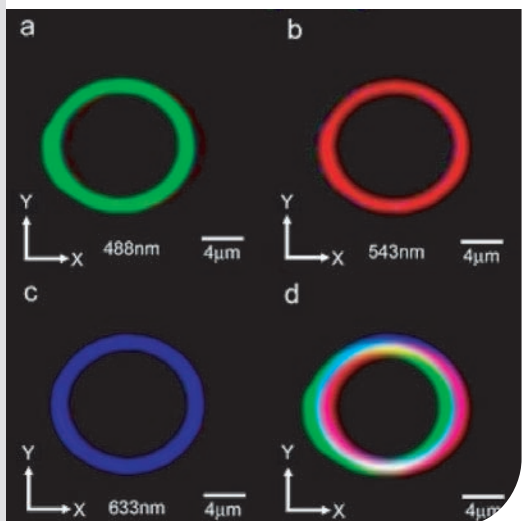


Figure 1. Pixel shift is evident in a multichannel image of an optical section taken through a fluorescent-coated bead. When the images corresponding to the (a) green, (b) red, and (c) blue channels are presented as an overlay (d), misregistration of the wavelength channels is evident in respect to the x-y image plane.

The ability to label multiple targets of interest in a single sample greatly extends the flexibility of fluorescence imaging. The traditional method utilized to acquire images of multiple probes is to capture multiple images in sequence while switching wavelength bandpass filters between exposures. After acquisition, the images are combined electronically to yield a multicolor image.

Unfortunately, this approach presents concerns regarding temporal resolution, spatial registration, and vibration of the images. Many dynamic biological processes take place on time scales that are simply too short to permit electromechanical switching of filter wheels and serial exposures. Another common issue related to sequential acquisition is the pixel shift that can occur when filters and dichroic beamsplitters are physically moved to permit excitation and detection of a different wavelength range (see **Figure 1**).

These drawbacks can make it impossible to use electromechanical filter wheels in a wide variety of quantitative experiments. One effective way to circumvent the aforementioned limitations is the use of stationary optical splitters to split various wavelength bands and project them onto different areas of a CCD simultaneously (see **Figure 2**).

Because the individual images taken at different wavelengths are precisely aligned prior to image capture, stationary beamsplitters such as the Optical Insights Dual-View do not exhibit pixel shift. The images are integrated and read off of the CCD at the exact same point in time, thus solving the temporal problems associated with sequential exposures using a filter wheel.

This powerful approach is utilized successfully for many demanding applications, including ratiometric ion probe imaging (calcium, pH, etc.), polarization anisotropy measurement, FRET determination, and simultaneous fluorescence and DIC imaging. Furthermore, the lack of vibration from the use of stationary precision beamsplitting optics lends itself well to ultra-high-resolution imaging modalities such as multicolor TIRF microscopy and combined atomic force microscopy and fluorescence imaging.

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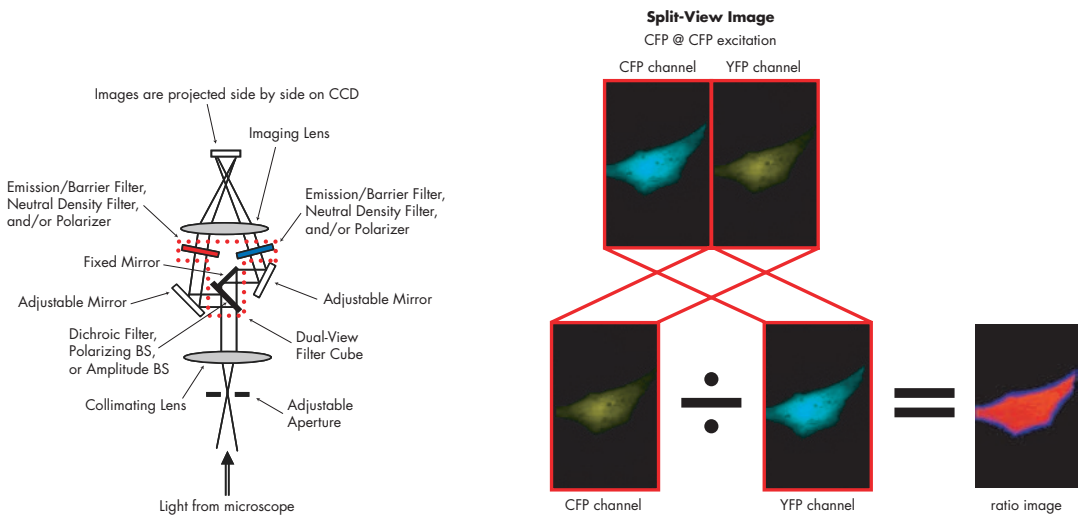


Figure 2. Principle of a multispectral imaging beamsplitter. **(a)** The image output of the microscope is split with a dichroic optic and the images at different wavelengths are projected onto two halves of a single CCD. **(b)** The two side-by-side images can be separated in software and processed. In this case, the signal from the YFP detection channel is divided by the signal from the CFP channel to create a ratio image. For certain types of FRET studies, changes in the ratio of intensity values in these two images are proportionate to FRET interaction.

Ratiometric Calcium Imaging

APPLICATION BRIEF

Indo-1 Imaging

Indo-1 is a UV-excitable ratiometric indicator that is used to study the role of calcium in cellular regulation. Ratiometric indicators have significant advantages over single-emission probes (i.e., probes for which changes in ion concentration cause changes in emission intensity only at a single wavelength). Measurements of ion concentrations using fluorescence microscopy are sensitive to the effects of uneven dye loading, photobleaching, leakage of dye, and unequal cell thickness. By using ratioing techniques, the measurements are less sensitive to these effects.

The Optical Insights Dual-View system streamlines the emission ratioing approach by enabling simultaneous acquisition of two emission images on a single CCD. This simplification has led to a renewed interest in emission ratioing techniques, especially since these techniques do away with rotating filter wheels and sequential imaging as well as their associated problems. Since indo-1 dye exhibits a shift in emission wavelength with changes in calcium concentration, it is a perfect candidate for ratio imaging.

Simultaneous Calcium and pH

While indo-1 is effective at targeting calcium ions, it is also effective at targeting other ions with similar properties, such as hydrogen ions¹. As a result, the pH of the environment has an impact on the measurement and needs to be monitored at the same time. Previous research monitored two wavelengths for calcium and two wavelengths for SNARF, a pH indicator dye, using a four-camera video microscope.

Figure 1 shows an image of rat pituitary intermediate lobe melanotropes obtained with this system².

The Optical Insights Quad-View simplifies the instrumentation needed for this application. By acquiring four separate spectral images simultaneously on a single CCD, the Quad-View can monitor two wavelengths of indo-1 for calcium and two wavelengths for a pH indicator like SNARF-1.

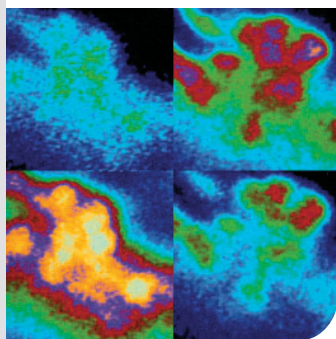


Figure 1. Rat pituitary intermediate lobe melanotropes labeled with indo-1 AM and carboxy SNARF-1, AM, acetate indicators. Pseudocolored fluorescence from indo-1 is shown at 405 and 475 nm (left panels). Pseudocolored fluorescence from carboxy SNARF-1 is shown at 575 and 640 nm (right panels).

Fura-2 / DIC Imaging

In the past, excitation ratioing has been more practical than emission ratioing. Thus, the excitation ratio indicator fura-2 has been the indicator of choice for calcium concentration measurements. When fura-2 is utilized, the Dual-View system is useful for monitoring cell morphology and calcium concentration at the same time. When configured for simultaneous fluorescence/DIC imaging, the Dual-View allows researchers to visualize the location and concentration of calcium in the fluorescence channel, as well as visualize changes in cell morphology in the DIC channel. The fluorescence image can be overlaid on the DIC image and the whole sequence of images can be played in real time to provide a multi-dimensional view of the cellular environment.

Fluo-3 / Fura Red™ Imaging

Due to the need for UV optics and objectives in the context of indo-1 and fura-2 ratiometric imaging, the experimental setup can be somewhat complex. As an alternative in the visible excitation range, dyes such as fluo-3 and Fura Red can be used. Unfortunately, neither of these dyes is an emission ratiometric indicator when used by itself; however, because the two dyes have reciprocal shifts in intensity due to calcium binding, they can be used together in a ratiometric probe strategy³. By doing so, the benefits of a ratiometric approach to ion-concentration determination can be realized.

Cameleon Probes

Förster resonance energy transfer⁴ (FRET) 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 the proximity between a 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 methods of FRET detection, interactions between molecules can be monitored in subcellular compartments and tracked as a function of time.

The development of GFP-derived FRET biosensors has enabled researchers to study many dynamic cellular processes, including cellular transport, signaling, and development. FRET-based GFP biosensors are genetically encoded proteins that incorporate a fluorescent protein FRET pair, typically CFP and YFP, into strategic locations on the macromolecule. In some GFP biosensors, changes in molecular conformation due to biochemical events will alter the distance between donor and acceptor on the biosensor macromolecule. In turn, this will alter the apparent FRET efficiency as recorded with fluorescence microscopy.

Genetically encoded Ca^{2+} indicators, known as cameleons⁵, were derived by sandwiching calmodulin, a peptide linker, and the calmodulin peptide-binding protein M13 between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Binding of Ca^{2+} makes calmodulin wrap around the M13 domain, closing the distance between the CFP donor and YFP acceptor (the FRET pair). This results in a large increase in measurable FRET signal from the biosensor. Because cameleons are genetically encoded, they can be targeted to specific intracellular locations as well.

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Dual-View Configurations for Calcium Imaging

Indo-1 Imaging

Filter Sets	Part Number
Microscope Exciter Filter Set	12-EX
Dual-View Emission Filter Set	12-EM
Comments: This filter set is designed to excite indo-1 at 365 nm and monitor the emission at 405 nm (saturated calcium) and 485 nm (free calcium). The emission filter set uses a 440-nm dichroic to split the two emission images.	

Fura-2 / Brightfield Imaging

Filter Sets	Part Number
Microscope Exciter Filter Set	400DCLP
Dual-View Emission Filter Set	19-EM
Comments: The excitation system will need a monochromator or filter wheel to allow dual excitation of the fura-2 at 340 nm and 380 nm. The emission filter set uses a 565-nm dichroic to split the fluorescence at 510 nm and brightfield channel into separate images.	

Fluo-3 / Fura Red Imaging

Filter Sets	Part Number
Microscope Exciter Filter Set	10-EX
Dual-View Emission Filter Set	10-EM
Comments: This filter set is designed to excite fluo-3 and Fura Red, which are visible dyes used for ratiometric imaging of calcium. The emission filter set records images at 535 nm (saturated Ca^{2+}) and 660 nm (free Ca^{2+}). The emission filter set uses a 565-nm dichroic to split the two images.	

Cameleon Imaging

Filter Sets	Part Number
Microscope Exciter Filter Set	05-EX
Dual-View Emission Filter Set	05-EM
Comments: This combination of excitation and emission filters is specifically designed for real-time ratio imaging of CFP and YFP emission to determine dynamic changes in FRET signal from cameleon probes. This set uses a single excitation band centered around 436 nm to excite the CFP donor component of the cameleon molecule and detects bands centered around 480 nm (CFP emission) and 535 nm (YFP emission). Changes in the ratio of CFP:YFP emission indicate changes in FRET efficiency linked to Ca^{2+} concentration.	

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Dynamic Ratio Imaging of Intracellular Calcium

APPLICATION NOTE

Cytosolic and organellar free calcium concentrations show dramatic spatial and temporal fluctuations associated with intracellular messaging; therefore, the ability to monitor intracellular Ca^{2+} flux has wide-ranging utility in the life sciences. Today, a significant number of *in vitro* and *in vivo* investigations involve the monitoring of calcium.

Mapping Thermoresponsive Neurons with Ratiometric FRET

Researchers at the Michael Welsh laboratory (Howard Hughes Medical Institute and the University of Iowa College of Medicine) employed a FRET-based optical approach using an engineered Ca^{2+} -sensitive fluorescent protein biosensor, yellow cameleon 2.1, to identify neurons responding to temperature changes.¹

The researchers utilized genetically encoded Ca^{2+} indicators, known as cameleons,² by sandwiching calmodulin, a peptide linker, and the calmodulin peptide-binding protein M13 between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Binding of Ca^{2+} makes calmodulin wrap around the M13 domain, thereby closing the distance between the CFP donor and YFP acceptor (the FRET pair). Consequently, there is a large increase in measurable FRET signal from the biosensor.

When the stoichiometry of the donor and acceptor is fixed, as it is when they are fused in a single polypeptide chain, the ratio of acceptor fluorescence to donor fluorescence signal can be used as a convenient means to measure FRET efficiency (as well as changes in FRET efficiency) with superb temporal resolution. For CFP-YFP biosensors such as cameleons, numerator and denominator images can be taken using a single excitation wavelength (that of the donor). In this scenario, the ratio would be $(\text{FYFP})/(\text{FCFP})$, where FYFP is the fluorescent image from the YFP channel using the CFP excitation wavelength, and FCFP is the fluorescent image from the CFP channel using the CFP excitation wavelength.

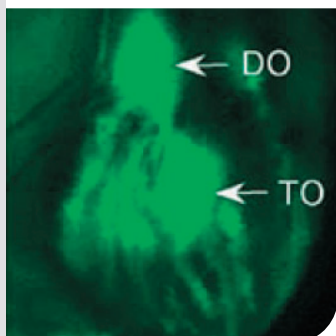


Figure 1. Cameleon fluorescence (YFP) in larval terminal (TO) and dorsal (DO) organs.¹

In their study, Welsh *et al.* used transgenic *Drosophila* larvae that express cameleon in their neurons to identify thermosensitive cells (see **Figure 1**). For microscopy, the larvae were submerged in water and immobilized using a glass cover. An Optical Insights Dual-View was used to separate and project two wavelength channels simultaneously on a single CCD camera sensor. This strategy permitted the perfect spatial and temporal registration needed for unambiguous, dynamic, dual-channel FRET detection.

Results

Neuronal activity was assayed in the peripheral neurons as the temperature was changed. The results of the study indicated that the terminal organ is a thermosensitive structure that responds to cool temperatures (see **Figure 2**). Its response profile was found to resemble the behavior documented for commonly observed mammalian cold receptors. Some body-wall neurons also showed a FRET response correlated to temperature shifts; however, they responded to warm temperatures (see **Figure 3**).

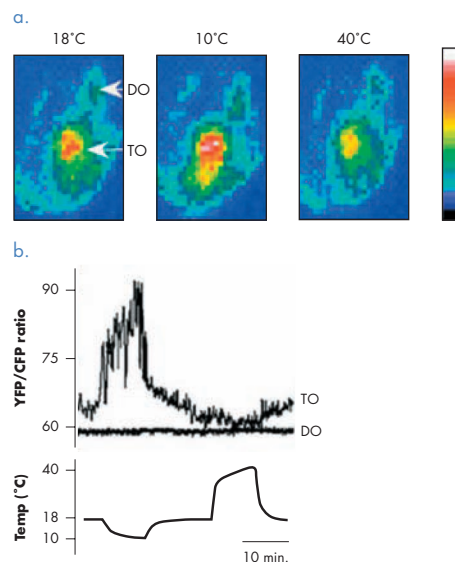


Figure 2. (a) Representative images of cameleon YFP/CFP ratios at the three temperatures indicated in terminal and dorsal organs; white represents a high fluorescence ratio, whereas purple is a low fluorescence ratio. (b) Effect (top) of temperature (bottom) on the YFP/CFP fluorescence intensity ratio from the terminal and dorsal organs.¹

Enabling Equipment for Dynamic FRET Imaging

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. Toward this end, the advent of GFP-derived FRET biosensors has enabled researchers to study many dynamic cellular processes, including cellular transport, signaling, and development.

The Optical Insights Dual-View system enables simplified, automated capture of ratio images for biosensor imaging. The design of the beam path permits careful pre-alignment of the individual channels and incorporates a multiwavelength dichroic so that the images recorded at different wavelengths remain in perfect register throughout an experiment. Such precision is extremely difficult to accomplish using conventional automated filter wheels and dichroics, or manually positioned filter blocks.

Additionally, the absence of electromechanical instrumentation required for multiple-image capture with the Dual-View reduces the complexity of software automation, minimizes the number of potential sources of vibration and electrical noise, and increases the overall dependability of the system.

It is important to note that when studying rapid biological processes, images of the CFP and YFP channels must be acquired simultaneously. If this is not done, the signal molecules may shift to a different pixel location and the donor and acceptor images will be misaligned in the resultant ratio images. In practice, this artifact is often misinterpreted as a loss of FRET signal.

The Dual-View directly addresses this issue by projecting both images onto the imaging sensor at precisely the same moment. Thus, the system not only eliminates problems associated with poor temporal registration, it also removes electromechanical positioning of filter optics as the rate-determining factor with respect to the speed at which multichannel images can be recorded. In regard to the temporal resolution of data recording, the digital camera becomes the primary limiting factor; therefore, investigators can make use of the full potential of newer, low-light, high-speed imaging sensors such as EMCCD technology.

Additional Information

To learn more about Dr. Michael Welsh's research, please visit www.medicine.uiowa.edu/welshlab.

Citations

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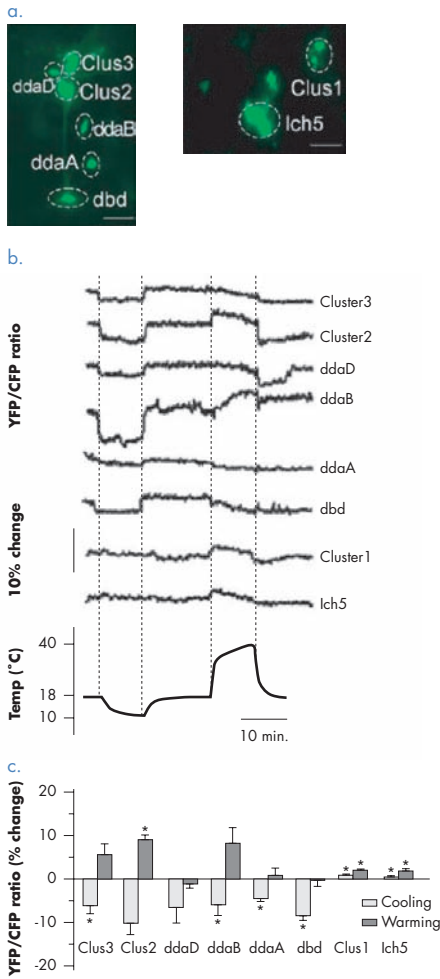


Figure 3. (a) Optical recording of cameleon fluorescence in the dorsal, lateral body wall segment identifying neurons. (b) Representative traces of YFP/CFP ratios during temperature changes (bottom) for specific neurons and clusters (top). (c) Changes in YFP/CFP ratio in response to heating and cooling for the indicated neurons and clusters.¹

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Live-Cell Ratiometric Imaging of Intracellular pH

APPLICATION NOTE

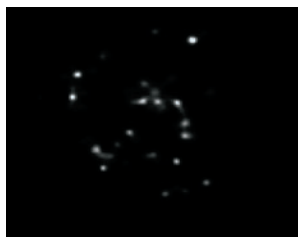


Figure 1. Acridine-orange-labeled secretory lysosomes in NK cell after porcine endothelial cell activation.¹

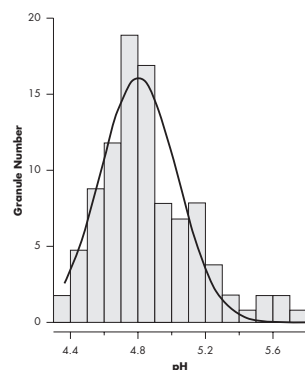


Figure 2. The pH values of individual granules showing a single peak.¹

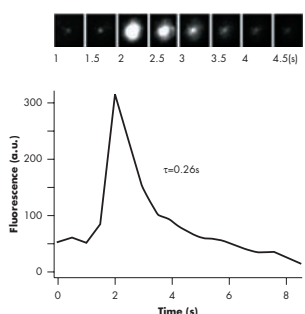


Figure 3. Fusion of an acridine-orange-labeled vesicle revealed through TIRFM.¹

Intracellular ion fluxes can be spatially and temporally mapped via fluorescence imaging. There are fluorescent indicators available for most biologically important ions, including calcium, hydrogen (pH), magnesium, sodium, potassium, and chloride. This application note describes the use of a fluorescent indicator to study the formation and sensitization of secretory lysosomes that serve a degradative and secretory function for natural killer (NK) cells.

Secretory Lysosome Formation in NK Cells

NK cells play a key role in cell-mediated immunity against tumors, viral infections, and foreign-tissue implants (xenograft rejection). These killer cells store their secretory proteins in membrane-bound cell organelles that recently have been recognized as secretory lysosomes¹. Though many studies have investigated the exocytosis of secretory lysosomes from endocrine and neuronal cells, little is known about how they behave in NK cells². In 2004, researchers in China successfully used ratiometric fluorescence imaging techniques to monitor secretory lysosome production and release in a human NK cell line (NK92)¹.

In order to gain an understanding of the origin of secretory lysosomes in sensitized NK cells, acridine orange was used as a ratiometric indicator of these acidic vesicles in conjunction with live-cell imaging. For microscopy, the secretory lysosomes were labeled by incubating the cells in a 3- μ M solution of acridine orange in physiological buffer for 10 minutes (see **Figure 1**).

Acridine orange displays a pH-dependent spectral shift in its emission spectra, where higher pH environments result in a red-shifted fluorescence signature. By simultaneously collecting the green- and red-shifted intensity for each pixel in the image, it was possible to monitor the ratio and determine the pH distribution of vesicles in living cells. An Optical Insights Dual-View was used to separate and project two wavelength channels onto the detector at the same time. This strategy permitted the exact spatial and temporal registration necessary for unambiguous, dynamic, dual-channel ratiometric imaging.

The pH at each pixel in the image was estimated by calculating the fluorescence ratio of red emission to green emission. Calibration of the observed fluorescence ratio to pH values was conducted *in situ* using solutions of defined pH (see **Figure 2**). Total internal reflection fluorescence microscopy (TIRFM) was used to image acridine-orange-labeled vesicles as they underwent fusion with the plasma membrane (see **Figure 3**).

Results

The results of this study indicate that there is a rapid *de novo* formation of secretory lysosomes after target-cell recognition (see **Figure 4**). The number of secretory vesicles was observed to increase from 61 ± 4 in resting NK cells to 202 ± 12 upon activation of the cells through contact with porcine endothelial cells. Furthermore, it was observed that rapid synthesis of low-pH secretory vesicles was abolished via pre-incubation with Gö6983, a protein kinase C inhibitor.

In contrast, pre-incubation with the fungal metabolite brefeldin A, an inhibitor of trans-Golgi network-derived vesicle budding, did not block the formation of secretory vesicles upon contact between NK cells and porcine endothelial cells. Phorbol 12-myristate 13-acetate was employed as an alternative means of protein kinase C activation.

The researchers suggest that target-cell recognition triggers rapid biogenesis and sensitization of secretory lysosomes in NK cells through activation of protein kinase C. They note that the fact that brefeldin A did not block the rapid *de novo* formation of secretory lysosomes suggests that the nascent secretory lysosomes are not derived from the trans-Golgi network. Their research methods and equipment also provide a general means of study.

Enabling Equipment for Multichannel Acquisition of Ratiometric Indicators

Response times of some ion-sensitive probes occur on the order of 10 milliseconds or less. When studying such rapid biological processes, ratio images of the emission channels must be acquired simultaneously. If this is not done, the signal molecules may shift to a different pixel location and the two images will be misaligned in the resultant ratio images. Such artifacts will confound accurate determination of ion concentrations.

The Optical Insights Dual-View system directly addresses this issue by projecting both images onto the imaging sensor at precisely the same moment. The design of the beam path permits careful pre-alignment of the individual channels and incorporates a multiwavelength dichroic so that the images recorded at different wavelengths remain in perfect register throughout an experiment. Such precision is extremely difficult to accomplish using conventional automated filter wheels and dichroics, or manually positioned filter blocks.

The Dual-View system not only eliminates problems associated with poor temporal registration, it also removes electromechanical positioning of filter optics as the rate-determining factor with respect to the speed at which multichannel images can be recorded. With regard to the temporal resolution of data recording, the digital camera becomes the primary limiting factor; therefore, investigators can make use of the full potential of newer, low-light, high-speed imaging sensors such as EMCCD technology.

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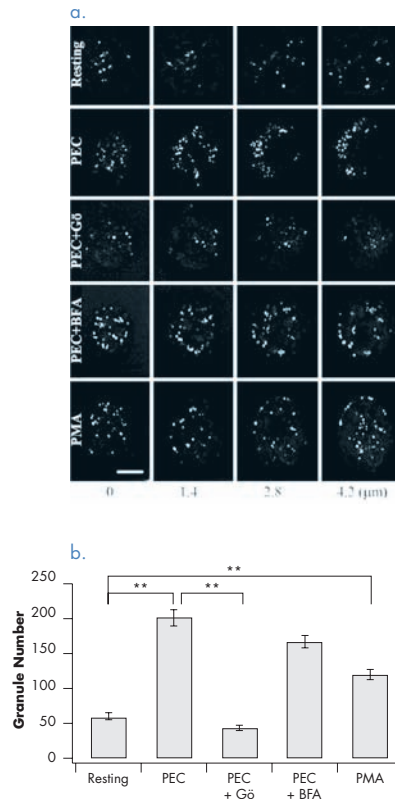


Figure 4. Images showing the increase in secretory lysosomes of acridine-orange-stained NK cells: (a) porcine endothelial cell (PEC)-activated, porcine endothelial cell plus Gö6983, Gö6983 plus brefeldin A (BFA), and phorbol 12-myristate 13-acetate (PMA)-treated; (b) graphical comparison of the total number of secretory lysosomes under different conditions than in 4a.¹

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Fluorescence Polarization

APPLICATION BRIEF

Introduction

While the majority of fluorescence microscopists investigate cell structure and function using only the intensity information from spectral emission, an increasing number of scientists are turning their attention to the technique of fluorescence polarization to probe biophysical properties of fluorescent samples. The degree of depolarization in the emission can be used to infer physical properties of the sample. Radiationless energy transfer among fluorophores (e.g., FRET) may also lead to a rapid depolarization of the emission signal. When such effects occur on the order of the timescale of the rate of fluorescent emission, the depolarization can be significant.

If a fluorescent molecule is excited with polarized light, it will emit light of the same polarization assuming the molecule does not rotate during its emission lifetime. Depolarization, or a decrease in light being emitted in the same polarization direction, occurs when the molecule rotates during its emission lifetime. Polarization and anisotropy measurements are often used to estimate the rate and extent of rotational diffusion during the lifetime of the excited state. These, in turn, have been used to quantify biochemical properties such as protein denaturation, protein-ligand association reactions, and the rotational rates of proteins. The anisotropies of fluorophores bound to membranes have been measured in order to estimate the internal viscosities of membranes and the dependency of membrane phase transitions on membrane composition¹.

Example Application: Anisotropy/FLIM

Recent research has shown that the Optical Insights Dual-View system can be used to help simultaneously acquire two-dimensional polarization and lifetime images, which can then be transformed to yield maps of rotational correlation time and fluorescence lifetime². This technique has been labeled time-resolved fluorescence anisotropy imaging (TR-FAIM), an extension of time-domain FLIM. It utilizes linearly polarized laser pulses to excite a sample. The parallel and perpendicular components of the fluorescence emission of the sample are then imaged simultaneously using the Dual-View.

Figure 1 shows the result of applying this technique to the images of several wells of a standard multiple-well plate with rhodamine 6G in methanol, ethylene glycol, trimethylene glycol, and glycerol. The acquired images are processed to recover the rotational correlation times and unperturbed fluorescence lifetimes of the sample. These results are used to determine the local viscosity and refractive index of the probe environment.

System Configuration

The Dual-View has been used with tremendous success in various fluorescence imaging applications for microscopy. In most applications, the Dual-View is fitted with dichroic filters and spectral emission filters for simultaneous imaging of multiple fluorescent labels; however, the Dual-View can also be fitted with a high-efficiency polarization beamsplitter and high-efficiency broadband polarization filters for polarization imaging of fluorescent samples. This configuration allows simultaneous acquisition of two polarization images: one parallel to the polarization of the source and the other perpendicular to the polarization of the source. These images are used to calculate the two-dimensional polarization/anisotropy of the sample. The operation of this system is shown conceptually in **Figure 2**.

Technique Summary

For anisotropy measurements, a fluorescent sample is excited with polarized light, yielding fluorescent emission from the sample. This simple property of fluorescent samples can be used to probe different biochemical properties of cellular structures. When implemented on a microscope, the technique requires excitation of the sample with vertically polarized light. On the emission side, two separate images need to be acquired. One image is taken with a vertically polarized filter in one emission channel; the second is taken with a horizontally polarized filter in the other emission channel. The first image is $I_{||}$ and the second image is I_{\perp} . The two images can then be used to determine the polarization (P) and anisotropy (r) at each pixel in the image.

If both images have been acquired simultaneously, then using the ratio of the images has significant advantages because it normalizes for fluctuations in source intensity, photobleaching, and uneven dye loading.

Citations

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2. Siegel, J., Suhling, K., Leveque-Fort, S., Webb, S.E.D., Davis, D.M., Phillips, D., Sabharwal, Y., French, P.M.W. (2003) "Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): Imaging the rotational mobility of a fluorophore," *Review of Scientific Instruments* **74**, 1, 182-192.

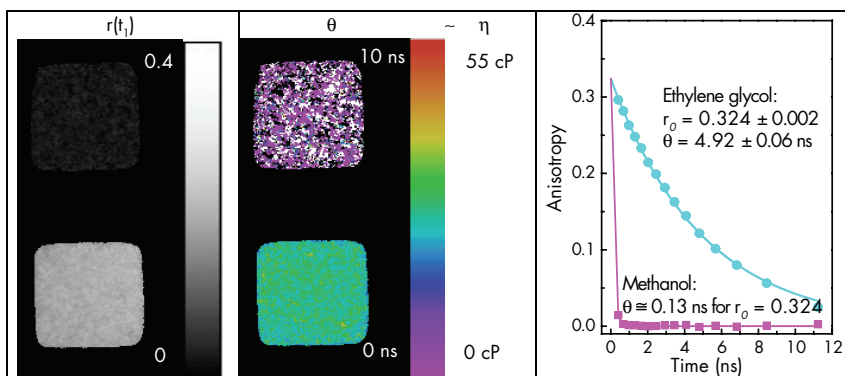


Figure 1. TR-FAIM results of two wells filled with rhodamine 6G in methanol (top) and ethylene glycol (bottom). Left panel: Transient anisotropy 400 ps after the excitation pulse, showing strong anisotropy in ethylene glycol and apparently weak anisotropy in the sample in methanol since it has already decayed almost entirely. Middle panel: Map of the rotational correlation time θ in a pseudocolor scale from 0 ns to 10 ns. The timescale has also been converted directly into a viscosity scale ranging from 0 cP to 55 cP. Right panel: The anisotropy decays averaged over the individual well areas of the (middle panel). Images courtesy of Siegel *et al.*

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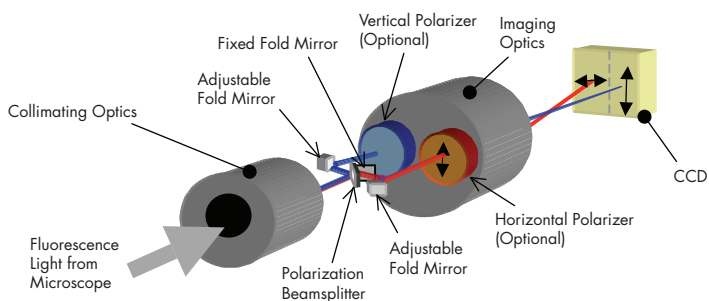


Figure 2. Operation of Dual-View fluorescence polarization imaging system.

Matching Resolution

Researchers using a CCD camera in conjunction with a microscope desire to work at the maximum possible spatial resolution allowed by their system. In order to accomplish this, it is necessary to properly match the magnification of the microscope to the CCD.

The first step in this process is to determine the resolving power of the microscope. The ultimate limit on the spatial resolution of any optical system is set by light diffraction; an optical system that performs to this level is termed “diffraction limited.” In this case, the spatial resolution is given by:

$$d = 0.61 \times \lambda / \text{N.A.}$$

where d is the smallest resolvable distance, λ is the wavelength of light being imaged, and N.A. is the numerical aperture of the microscope objective. This is derived by assuming that two point sources can be resolved as being separate when the center of the airy disc from one overlaps the first dark ring in the diffraction pattern of the second (the Rayleigh criterion).

It should be further noted that, for microscope systems, the N.A. to be used in this formula is the average of the objective’s numerical aperture and the condenser’s numerical aperture. Thus, if the condenser is significantly underfilling the objective with light, as is sometimes done to improve image contrast, then spatial resolution is sacrificed. Any aberrations in the optical system, or other factors that adversely affect performance, can only degrade the spatial resolution past this point. However, most microscope systems do perform at, or very near, the diffraction limit.

The formula above represents the spatial resolution in object space. At the detector, the resolution is the smallest resolvable distance multiplied by the magnification of the microscope optical system. It is this value that must be matched with the CCD.

The most obvious approach to matching resolution might seem to be simply setting this diffraction-limited resolution to the size of a single pixel. In practice, what is really required of the imaging system is that it be able to distinguish adjacent features. If optical resolution is set equal to single-pixel size, then it is possible that two adjacent features of like intensity could each be imaged onto adjacent pixels on the CCD. In this case, there would be no way of discerning them as two separate features.

Separating adjacent features requires the presence of at least one intervening pixel of disparate intensity value. For this reason, the best spatial resolution that can be achieved occurs by matching the diffraction-limited resolution of the optical system to two pixels on the CCD in each linear dimension. This is called the Nyquist limit.

Expressing this mathematically we get:

$$(0.61 \times \lambda / \text{N.A.}) \times \text{Magnification} = 2.0 \times (\text{pixel size})$$

Let’s use this result to work through some practical examples.

Example 1: Given a camera with a Kodak KAF1401E CCD (pixel size $6.8 \mu\text{m}$), visible light ($\lambda = 0.5 \mu\text{m}$), and a 1.3 N.A. microscope objective, we can compute the magnification that will yield maximum spatial resolution.

$$M = (2 \times 6.8) / (0.61 \times 0.5 / 1.3) = 58$$

Thus, a 60x, 1.3 N.A. microscope objective provides a diffraction-limited image for the KAF1401E CCD camera without any additional magnification. Keep in mind, however, that this assumes that the condensing system also operates at an N.A. of 1.3. This high N.A. means the condenser must be operated in an oil-immersion mode, as well as the objective.

Example 2: Given a camera with an e2v CCD37 (pixel size $15.0 \mu\text{m}$), visible light ($\lambda = 0.5 \mu\text{m}$), and a 100x microscope objective with an N.A. of 1.3, we can compute the magnification that will yield maximum spatial resolution.

$$M = (2 \times 15.0) / (0.61 \times 0.5 / 1.3) = 128$$

Since the microscope objective is designed to operate at 100x, we would need to use an additional projection optic of approximately 1.25x in order to provide the optimum magnification.

It should be kept in mind that as magnification is increased and spatial resolution is improved, field of view is decreased. Applications that require both good spatial resolution and a large field of view will need CCDs with larger numbers of pixels. It should also be noted that increasing magnification lowers image brightness on the CCD. This lengthens exposure times and can limit the ability to monitor real-time events.

Readout vs. Frame Rate

Readout rate is defined as the inverse of the serial conversion time, that is, the time required to digitize a single pixel. Readout rates are usually given in pixels/second (e.g., 500 kilopixels/second).

Frame rate is the inverse of the time needed for the CCD to acquire an image and then completely read that image out. Frame rate is typically expressed in frames per second (fps). Often, the frame rate can be approximately calculated from the total number of pixels and the readout rate, combined with the total exposure time. Specifically,

simplified frame rate = $1 / (\# \text{ of pixels/digitizer rate} + \text{frame acquisition time})$ in frames per second.

However, there are a number of other factors that can have a significant effect on frame rate, depending on operating conditions.

To better understand frame rate, we'll define two other quantities called **frame acquisition time (FAT)** and **frame read time (FRT)**, which will take into account all these factors. Frame rate is then defined as:

true frame rate = $1 / (\text{frame acquisition time} + \text{frame read time})$

frame acquisition time = (clear count x parallel clear time) + shutter open + close delay + exposure time

frame read time = serial clear time + (parallel shift time x parallel size) + (serial discard time x pixels pre & post subarray) + (serial conversion time x pixels being read)

Let's now examine what some of these parameters mean.

Clear count and parallel clear time: Depending upon the application conditions, it may be necessary to clear the CCD array of accumulated charge prior to acquiring an image. Sources of this accumulated charge can be dark current and even cosmic ray events. The array may have to be cleared several times to completely rid it of charge (in newer CCDs, this is typically done 1 to 3 times). Clear count is defined as the number of times this charge clearing is done, and parallel clear time is the time it takes to accomplish each clear. Since charge only needs to be cleared, and not digitized, parallel clear time takes less time than a normal readout.

Serial clear time: As in the parallel register case, it may sometimes be necessary to flush the serial register of accumulated charge prior to transferring charge from the parallel register. This is serial clear time.

Parallel shift time and parallel size: Parallel shift time is the time required to shift one row of pixels into the serial register during image readout. If binning is being performed, this must then be multiplied by the parallel size of the superpixel to obtain the total time needed to shift charge.

Serial discard time: When reading a subarray from the CCD, it may be necessary to discard pixels both before and after the region of interest. Serial discard time is the time taken to accomplish this. Also, the serial registers on most CCDs have a number of pixels (typically 20 to 50) placed between the data portion of the serial array and the output amplifier. These pixels must be discarded prior to reading data.

The significance of these factors depends highly upon the particular conditions under which the CCD is being used. There are sometimes large differences in the results, especially when reading subarrays, or reading the CCD at very high speeds.

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Signal-to-Noise Ratio

Signal-to-noise ratio (SNR) describes the quality of a measurement. In CCD imaging, SNR refers to the relative magnitude of the signal compared to the uncertainty in that signal on a per-pixel basis. Specifically, it is the ratio of the measured signal to the overall measured noise (frame-to-frame) at that pixel. High SNR is particularly important in applications requiring precise light measurement.

Photons incident on the CCD convert to photoelectrons within the silicon layer. These photoelectrons comprise the signal but also carry a statistical variation of fluctuations in the photon arrival rate at a given point. This phenomenon is known as photon noise and follows Poisson statistics. Additionally, inherent CCD noise sources create electrons that are indistinguishable from the photoelectrons. When calculating overall SNR, all noise sources need to be taken into consideration:

Photon noise refers to the inherent natural variation of the incident photon flux. Photoelectrons collected by a CCD exhibit a Poisson distribution and have a square root relationship between signal and noise.

$$(\text{noise} = \sqrt{\text{signal}})$$

Read noise refers to the uncertainty introduced during the process of quantifying the electronic signal on the CCD. The major component of readout noise arises from the on-chip preamplifier.

Dark noise arises from the statistical variation of thermally generated electrons within the silicon layers comprising the CCD. Dark current describes the rate of generation of thermal electrons at a given CCD temperature. Dark noise, which also follows a Poisson relationship, is the square root of the number of thermal electrons generated within a given exposure. Cooling the CCD from room temperature to -25°C will reduce dark current by more than 100 times. In addition, many scientific-grade CCDs employ multi-pinned-phase (MPP) technology to even further reduce dark current.

Taken together, the SNR for a CCD camera can be calculated from the following equation:

$$\frac{IQEt}{\sqrt{(IQEt + Ndt + Nr^2)}}$$

where:

I = Photon flux (photons/pixel/second)

QE = Quantum efficiency

t = Integration time (seconds)

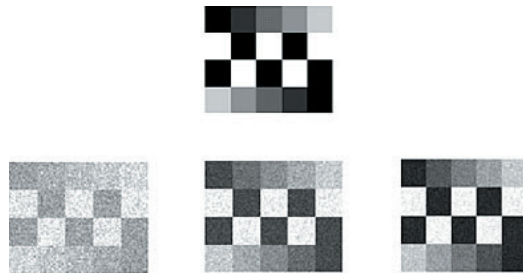
Nd = Dark current (electrons/pixel/sec)

Nr = Read noise (electrons)

Under low-light-level conditions, read noise exceeds photon noise and the image data is said to be “read-noise limited.” The integration time can be increased until photon noise exceeds both read noise and dark noise. At this point, the image data is said to be “photon limited.”

An alternative means of raising the SNR is to use a technique known as binning. Binning is the process of combining charge from adjacent pixels in a CCD during readout into a single “superpixel.” Binning neighboring pixels on the CCD array may allow one to reach a photon-limited signal more quickly at the expense of spatial resolution.

Once you have determined acceptable values for SNR, integration time, and the degree to which you are prepared to bin pixels, the above equation can be solved for the minimum photon flux required. This is, therefore, the lowest light level that can be measured for given experimental conditions and camera specifications.



Visual impact of increasing SNR of a typical test pattern.

Linearity

The fundamental process that occurs in CCD imaging is the conversion of photonic input to electronic output. Photons incident on the CCD will be converted to electron/hole pairs and the electrons will be captured under the gate electrodes of the CCD. These electrons are then transferred in a “bucket brigade” fashion to the output amplifier where the charge is converted to a voltage output signal. An analog processing chain further amplifies this signal and finally it is digitized before being transferred to a host computer for display, image processing, and/or storage. The transfer function between the incident photonic signal and the final digitized output should vary linearly with the amount of light incident on the CCD. Hence, nonlinearity is a measure of the deviation from the following relationship:

$$\text{Digital Signal} = \text{Constant} \times \text{Amount of Incident Light}$$

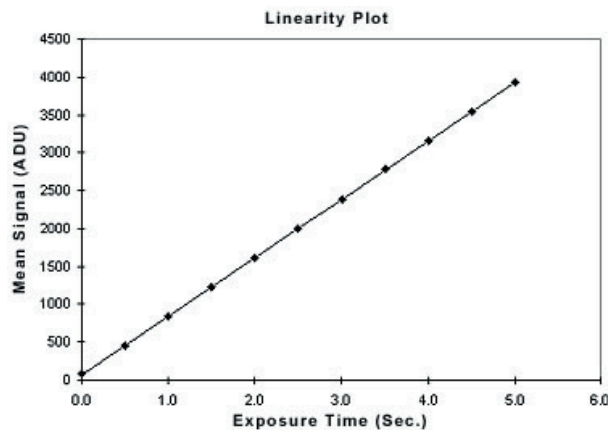
High-performance CCD imagers have extremely good linearity. Deviations from linearity are often less than a few tenths of a percent for over five orders of magnitude. This is far superior to video CCDs and other solid-state imagers, which can exhibit nonlinearity of several percent or more. For quantitative imaging, linearity is a stringent requirement. CCDs must be linear in order to perform image analysis such as arithmetic ratios, shading correction, flat fielding, linear transforms, etc.

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There is no standard method for measuring or reporting linearity values. Typically the numbers are reported as percent deviations from linearity (it may be specified as linearity or nonlinearity, however).

One method that can be used is to plot the mean signal value versus the exposure time over the full linear range (linear full well) of the CCD. A linear least-squares regression can then be fit to the data. The deviation of each point from the calculated line gives a measure of the nonlinearity of the system. The nonlinearity can be reported as the sum of the maximum and minimum deviation divided by the maximum signal as a percentage:

$$\text{nonlinearity (\%)} = \frac{(\text{MaxPositiveDeviation} + \text{MaxNegative Deviation})}{\text{MaximumSignal}} \times 100$$



Dark Current

Dark Current Noise

Dark current arises from thermal energy within the silicon lattice comprising the CCD. Electrons are created over time that are independent of the light falling on the detector. These electrons are captured by the CCD's potential wells and counted as signal. Additionally, this increase in signal also carries a statistical fluctuation known as dark current noise. CCDs can be cooled either with thermoelectric coolers (TECs) or liquid nitrogen to reduce this effect. Ideally, the dark current noise should be reduced to a point where its contribution is negligible over a typical exposure time.

MPP Operation

Some CCDs operate in multi-pinned-phase (MPP) mode. MPP devices are fabricated and operated in such a way as to significantly reduce thermal charge generation (dark current). The largest contribution to dark current results from the interface between the silicon dioxide and epitaxial silicon layer within the CCD. Boron implantation into the epitaxial silicon layer and proper biasing of the various clock phases drive the dark current electrons away from the potential wells that comprise a pixel, thus reducing the number of electrons per pixel per second (e/p/s) collected due to dark current.

Dark Current vs. Dark Current Noise

Each high-performance CCD camera carries a dark current specification. Dark current noise is the statistical variation of this specification. For instance, a given camera might have a dark current specification of 1.0 e/p/s. For a 4-second exposure, a total of 4 electrons/pixel are generated (1.0 e/p/s x 4 sec). Since dark current noise follows Poisson statistics, the rms dark current noise is the square root of the dark current or, in this case, 2 e/p.

Dark Current Noise Contributions

Noise sources in CCD cameras add in quadrature (the square root of the sum of the squares). In the low-light regime, the significant noise sources are read noise and dark current noise. Again, using the previously mentioned camera as an example, we can easily compare the relative sizes of these noise sources. Using 13 electrons/pixel as the read noise and the dark current noise calculated above (2 e/p) for a 4-second exposure, the total camera noise is calculated as follows:

$$\begin{aligned}\text{Total Noise} &= \sqrt{(\text{read noise}^2 + \text{dark noise}^2)} \\ &= \sqrt{13^2 + 2^2} \\ &= 13.15 \text{ electrons (for a 4-second exposure)}\end{aligned}$$

Thus, the dark current noise generated in a 4-second exposure has virtually no effect on the total camera system noise. Similarly, for a 30-second exposure we find that the total system noise equals 14.1 electrons. Again, even at a 30-second exposure, dark current noise barely contributes to the total camera system noise.

Hot Pixels

Occasionally, an individual pixel may have a different dark current generation rate than the rest of the CCD array. Remember, the dark current specification is an ensemble average of the entire array. Those pixels that have a higher-than-average dark current are known as hot pixels. These pixels will repeatedly have higher backgrounds than the vast majority of pixels. Since this is an effect that arises from the CCD manufacturing process, each hot-pixel location will remain fixed and can therefore be corrected.

Dynamic Range

Dynamic range refers to intrascene performance (i.e., the ability to quantitatively detect very dim and very bright parts of a single image). Because the smallest measurable intensity varies between applications and experimental conditions, CCD manufacturers have adopted a definition for specifying dynamic range that is independent of how the camera is used. This definition is defined mathematically as:

$$\text{linear full well (electrons)}/\text{read noise (electrons)}$$

and is thus a dimensionless number. The linear full well is a specific measure of pixel well capacity. With a high-performance, cooled camera, the read noise (the noise associated with a single readout event) is therefore minimized to yield the largest dynamic range possible.

As a specific example, consider a Kodak 1401E CCD, which has a full well capacity of 45,000 electrons. At a typical readout rate of 1 MHz, the read noise is 11 e⁻. The dynamic range of this sensor is therefore 45,000:11, or 4,091:1. In order to take full advantage of this dynamic range, cameras incorporating Kodak 1401E chips usually utilize a 12-bit A/D converter (4096 gray levels). It is important that the camera's readout and signal-processing electronics be optimized so that low read noise is maintained, otherwise the dynamic range will be compromised.

To extend dynamic range beyond the 12 bits given in the previous example, a camera with a lower read noise or a CCD with a larger full well capacity is required. Full well capacity is related to pixel size. For instance, the Thomson 7895 has a capacity of 375,000 e⁻ and a read noise of 5 e⁻ rms at 40 kHz. The dynamic range is thus 75,000:1. In commercial cameras, this is usually coupled to a 16-bit A/D converter (65,536 gray levels).

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