# PHOTOMETRICS®

## Scientific CMOS, EMCCD and CCD Cameras

# PRIME 95B™

## Customer Applications Handbook

# PHOTOMETRICS®
## Scientific CMOS, EMCCD and CCD Cameras

# Contents

## Camera Test Protocol

# PHOTOMETRICS

photometrics®

## PRIME 95B™

# Back Illuminated Scientific CMOS

## Discovery depends on every photon

Prime 95B is the Scientific CMOS with extreme sensitivity using high Quantum Efficiency (QE) Backside Illumination (BSI), a first for Scientific CMOS cameras. The 95B's sensor converts up to 95% of incident photons into a measurable signal. Unlike microlens approaches to increasing QE, which lose effectiveness as objective magnification is increased, Prime 95B's BSI sensor brings light into the pixel photodiode from behind, avoiding structures that reflect or absorb light. When combined with large 11µm pixels, Prime 95B can deliver over 300% more signal than other sCMOS cameras at 100X magnification.

More importantly, Prime 95B outperforms EMCCD cameras—with no excess noise that negates the benefit of using a high QE sensor, and additional limitations from EM gain calibration, stability, expense, and sensor lifetime With a true 16-bit dynamic range, Prime 95B easily accomplishes what EMCCD can not—detect weak and bright signals within the same image with photon-noise limited performance.

The extreme sensitivity not only allows fainter signals to be detected, it provides the flexibility to increase frame rates, or turn down the excitation intensity to reduce cellular photo-damage. Yet Prime 95B maintains the same high frame rates, field-of-view and extremely low read noise that has made sCMOS so popular for live-cell imaging.

**Primary applications:**

**Super-Resolution Microscopy**

**Confocal Microscopy**

**Single Molecule Fluorescence**

**Light Sheet Microscopy**

- ▸ **95% Quantum Efficiency**
- ▸ **11µm x 11µm Pixel Area**
- ▸ **1.6e- Read Noise (median)**
- ▸ **41fps @ 16-bit / 82fps @ 12-bit**
- ▸ **PrimeEnhance increases SNR 3-5X**

| Features | Advantages |
|---|---|
| High Quantum Efficiency 95% Peak QE | Maximizes ability to detect weak signals, enables short exposure times for high frame rates, minimizes phototoxicity across a wide range of wavelengths |
| Large 11µm Pixel Size | Maximize light collection while maintaining proper spatial sampling |
| Extremely Low Read Noise | Maximize your ability to detect faint fluorescence |
| Fast Frame Rates | Capture highly dynamic events with high temporal resolution |
| Large Field of View | Maximize the number of cells that can be tracked and monitored per frame |
| PrimeEnhance | Real-time quantitative denoising algorithm that improves image clarity by reducing photon-shot (Poisson) noise. Delivers an increase in Peak Signal to Noise Ratio of 3X to 5X |
| PrimeLocate | Dynamically evaluates and acquires only the relevant data for localization based super-resolution applications |
| Enhanced Dynamic Range | Measure both bright and dim signal levels within the same image 50,000:1 Dynamic Range (94 dB) |
| Multiple Expose Out Triggering | Control up to four light sources for multi-wavelength acquisitions |
| SMART Streaming | Faster acquisition rates with variable exposures, ideal for multi-probed live cell imaging Compatible with Multiple Expose Out Triggering |
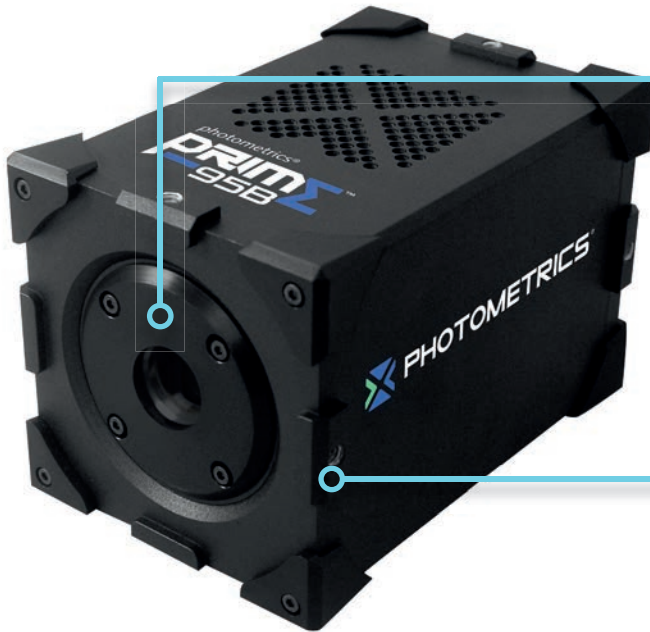
## 1.4 Megapixel BSI CMOS Sensor

Backside Illuminated Sensor
1.6e- Read Noise (Median)
>95% peak QE
80,000e- full well
11 x 11µm pixels
18.7mm diagonal

## Easily Mounted and Secured

C-mount
Two ¼"-20 mounting holes per side

## Convenient Interfaces

16-bit Data
  • 41fps
12-bit Data
  • 82fps

## Multiple Cooling Options

Forced Air Cooling
  • -20ºC Cooling
  • Selectable Fan Speed
Liquid Cooling
  • -25ºC Cooling
  • Leak-proof, quick-disconnect ports

## Advanced Application Triggers

Effective Global Shutter
Up to four selectable expose-out lines

# Real-time Application Optimization

## PrimeEnhance
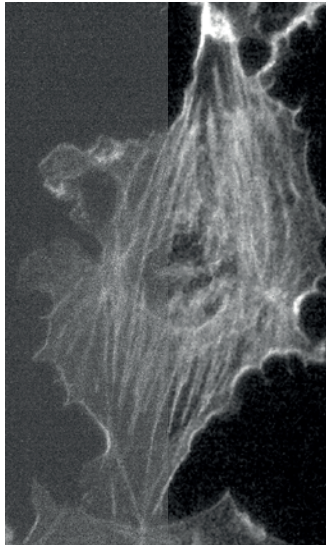


- ▸ Increase SNR 3x to 5x at low light levels by reducing photon shot-noise

- ▸ Preserve signal intensities ensuring quantitative measurements

- ▸ Extend cell lifetimes with reduced phototoxicity and photobleaching

- ▸ Extremely useful for low light imaging applications dominated by noise

With the near-perfect sensitivity of Backside Illuminated Scientific CMOS sensors, the latest generation of scientific cameras have enabled imaging using only a few photons per pixel. Unfortunately, these minute signals are dominated by the natural Poisson variation in light levels preventing useful quantitation.

PrimeEnhance uses a quantitative SNR enhancement algorithm used in Life Science imaging to reduce the impact of photon shot-noise present in acquired images, leading to an increase in Signal to Noise Ratio (SNR) by 3x to 5x with equivalent exposure times.

With PrimeEnhance, the exposure times can be reduced by a factor of 8-10X while maintaining the Signal to Noise ratio. This reduces the effects of cellular photo-damage and extends cell lifetimes.

Invented at INRIA and further optimized for fluorescence microscopy at the Institut Curie, the denoising algorithm used in PrimeEnhance uses a patch based evaluation of image data and knowledge of the each individual camera's performance parameters to reduce the effects of photon shot-noise. The patches of image intensities and their noise characteristics are processed and evaluated with increasing neighborhood sizes during which weighted intensity averages are taken. This iterative process preserves not only the quantitative nature of the measured intensities, but also the maintains the finer features present in biological samples.

Detailed performance and methodology of the algorithm is available in the following publication:
**Patch-based nonlocal functional for denoising fluorescence microscopy image sequences.**
Boulanger J, Kervrann C, Bouthemy P, Elbau P, Sibarita JB, Salamero J. IEEE Trans. *Med Imaging* 2010 Feb.
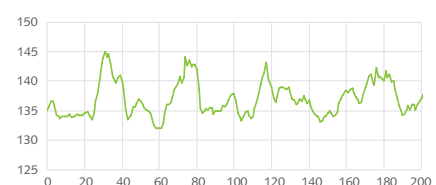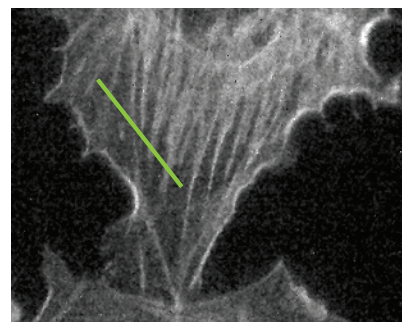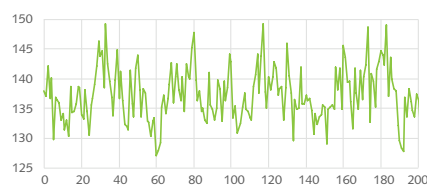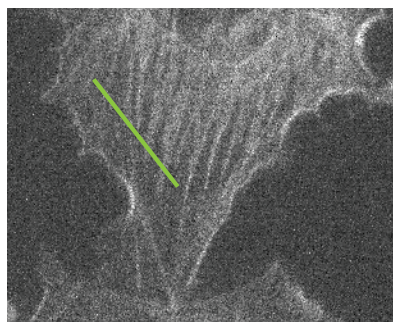
# Real-time Application Optimization

## PrimeLocate

Localization based super-resolution microscopy requires a sparsity of data to ensure proper localization of emitting molecules. Even with this sparsity, the full image frame is transferred to the host to be analyzed, creating a large amount data to be processed without adding useful information.

PrimeLocate dynamically evaluates image data and locates 500 regions per frame containing single molecule data relevant for super-resolution localization. Only these 500 regions are transferred to the host computer, drastically reducing the amount of data and time required for analysis.

By transferring only the relevant raw data, users have the freedom to use their preferred localization algorithm to generate super-resolution images.
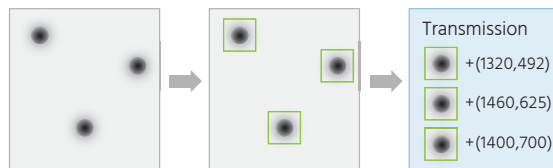


- Only the data within the patches is transferred to the host computer
- Processing time and storage requirements are easier to manage with the acquisition of only relevant data
- Ability to transfer 500 regions per frame
- Allows freedom to select preferred super-resolution localization algorithm

## Multi-ROI

The surplus of data generated by sCMOS devices is challenging to acquire, analyze, and store, requiring special interfaces and expensive SSDs. While a large Field of View (FOV) is convenient for imaging, at times, only certain areas contain the desired information.

Multi-ROI allows users to select up to 15 unique ROIs within the FOV, and only these selected regions are transferred to the host computer. This allows for a large reduction in the amount of data acquired but ensures that the critical information is obtained.
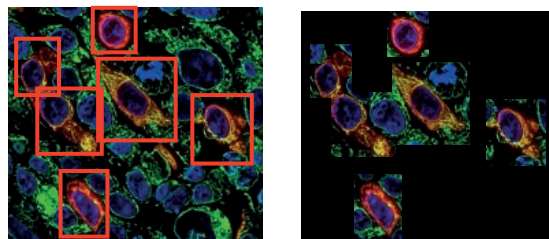


- Only the data within the user-defined ROIs is transferred to the host computer
- Select up to 15 unique regions
- Significantly reduce the amount of data being acquired

# Real-time Application Optimization

## Live Particle Tracking

Single molecule tracking is a technique often used to observe molecular interactions and behaviours at the single molecule level with high spatial and temporal resolution.

Photometrics Live Particle Tracking performs this process live on the camera with live statistics.

The Live Particle Tracking algorithm works by identifying individual single molecule particles and tracking them across the field of view by adapting a published algorithm[1] tuned for two-dimensional tracking.

Firstly, the camera determines only the dynamic portions of the image and disqualifies anything static from detection. The data is then run through a restoration step (Figure 1) which reduces both the high frequency and low-frequency noise, and allows the correction of any noise variation on a pixel-to-pixel basis as well as any background intensity modulations due to uneven illumination.

The points are then processed to determine the local-maxima and go through a refinement process to ensure a high efficiency in particle detection based on a threshold to reduce the susceptibility to false positives. Any remaining artifacts are filtered out during the non-particle discrimination step, aimed at hot pixels and cosmic events.

The particles are tracked and linked through the acquired frame stack. The metadata included with all images is updated to include the particle data within each frame, providing particle IDs as well as the ability to display particle path traces as well as boxes to outline each detected particle.

Live Particle Tracking can be used to determine whether the particles are behaving as expected before time consuming data acquisition for post-processing tracking analysis.
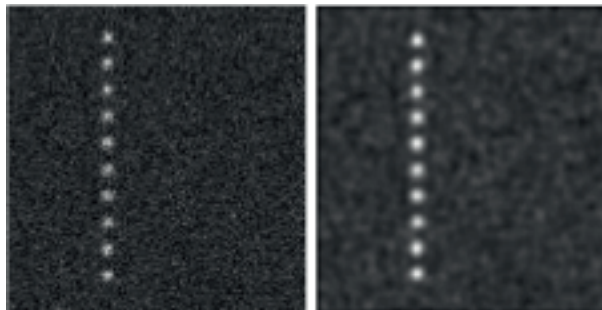


**Figure 1:** The input image of simulated single-particle data and the output of the image-restoration step to reduce image noise and pixel-to-pixel variation
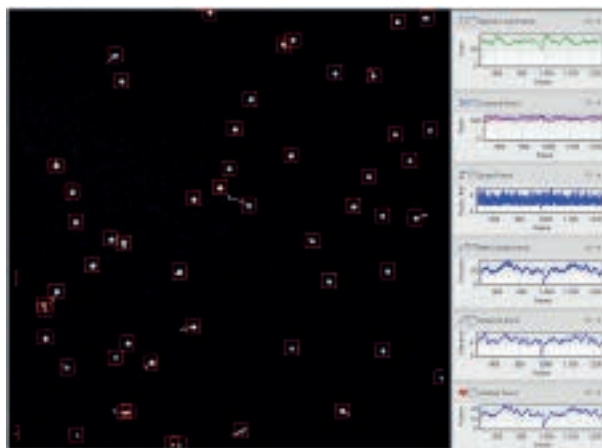


**Figure 2:** Live Particle Tracking running in Ocular software. Movement information of each particle is recorded, allowing tracking statistics to be displayed

[1]  I.F.Sbalzarini & P. Koumoutsakos. (2005) *Feature point tracking and trajectory analysis for video imaging in cell biology. J Struct Bio.* Aug;151(2):182-95.

## Three Field of View Options

Most modern microscope camera ports have a maximum field of view of 19 mm, 22 mm or, more recently, 25 mm. The Prime 95B Series is uniquely positioned to match each of these ports to deliver the largest obtainable field of view for imaging.

The Prime 95B and Prime 95B 22mm connect via the standard microscope C-mount and the Prime 95B 25mm connects via the larger format F-mount.

▶ Match the Prime 95B to the largest available microscope port

▶ Maximize field of view

▶ Increase throughput and maximize sample imaging area

### Prime 95B
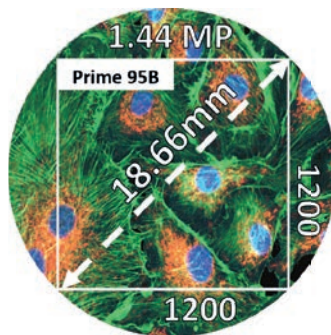
### Prime 95B 22mm
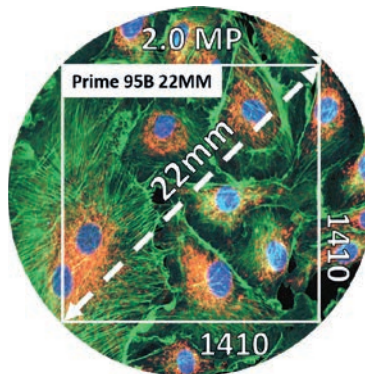
### Prime 95B 25mm
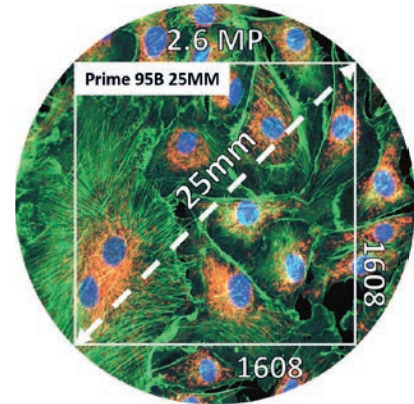
## Blue and UV Sensor Variants

Maximize sensitivity in the Blue and UV with the Prime 95B sensor variants, Prime 95B Blue (310 – 450 nm) and Prime 95B UV (250-310 nm). Capture more photons than before at these difficult wavelengths to reduce exposure times and increase speeds.

— Prime 95B

— Prime 95B UV

— Prime 95B Blue

**Prime 95B Blue**
Optimized for wavelengths between 310nm and 450nm

**Prime 95B UV**
Optimized for wavelengths between 250nm and 310nm
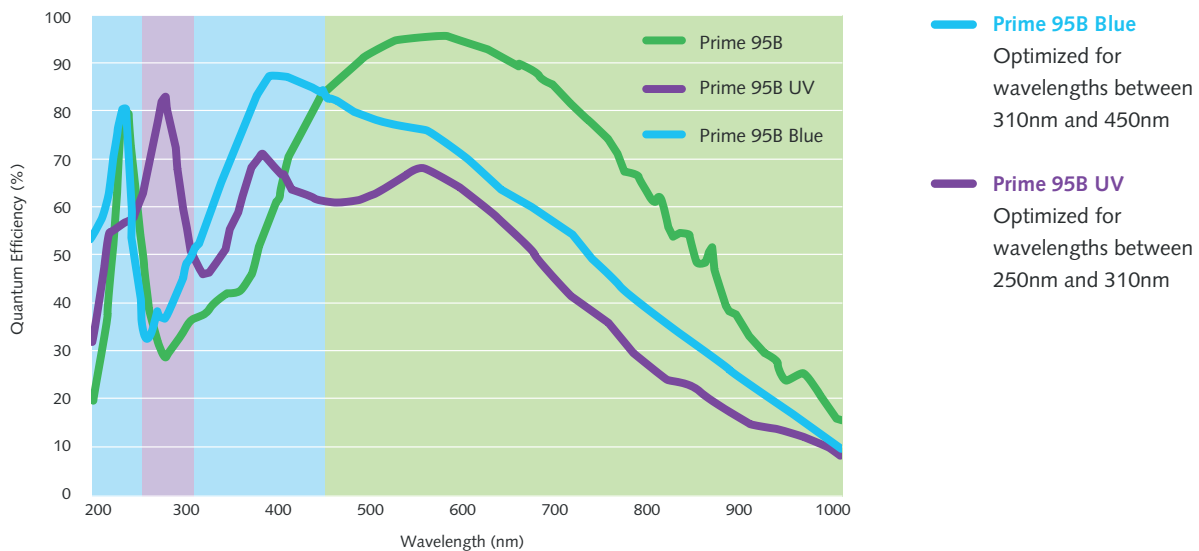
| Specifications | Camera Performance |
|---|---|
| Sensor | GPixel GSense 144 BSI CMOS Gen IV, Grade 1 in imaging area |
| Active Array Size | 1200 x 1200 pixels (1.44 Megapixel) |
| Pixel Area | 11µm x 11µm (121µm²) |
| Sensor Area | 13.2mm x 13.2mm<br>18.7mm diagonal |
| Peak QE% | >95% |
| Read Noise | 1.6e- (Median)<br>1.8e- (RMS) |
| Full-Well Capacity | 80,000e- (Combined Gain)<br>10,000e- (High Gain) |
| Dynamic Range | 50,000:1 (Combined Gain) |
| Bit Depth | 16-bit (Combined Gain)<br>12-bit (High Gain) |
| Readout Mode | Rolling Shutter<br>Effective Global Shutter |
| Binning | 2x2 (on FPGA) |

| Cooling Performance | Sensor Temperature | Dark Current |
|---|---|---|
| Air Cooled | -20ºC @ 25ºC Ambient | 0.55e-/pixel/second |
| Liquid Cooled | -25ºC @ 25ºC Ambient | 0.3e-/pixel/second |

| Specifications | Camera Interface |
|---|---|
| Digital Interface | PCI-E, USB 3.0 |
| Lens Interface | C-Mount |
| Mounting Points | 2 x ¼ 20" mounting points per side to prevent rotation |
| Liquid Cooling | Quick Disconnect Ports |

| Triggering Mode | Function |
|---|---|
| Input Trigger Modes | Trigger-First: Sequence triggered on first rising edge<br>Edge: Each frame triggered on rising edge<br>SMART Streaming: Fast iteration through multiple exposure times |
| Output Trigger Modes | First Row: Expose signal is high while first row is acquiring data<br>Any Row: Expose signal is high while any row is acquiring data<br>All Rows: Effective Global Shutter – Expose signal is high when all rows are acquiring data<br>        Signal is high for set Exposure time<br>Rolling Shutter: Effective Global Shutter – Expose signal is high when all rows are acquiring data<br>        Signal is High for set Exposure time – Readout Time |
| Output Trigger Signals | Expose Out (up to four signals), Read Out, Trigger Ready |

| Frame Rate (PCIe interface) | | |
| --- | --- | --- |
| **Array Size** | **16-bit** | **12-bit** |
| 1200 x 1200 | 41 | 82 |
| 1200 x 512 | 96 | 192 |
| 1200 x 256 | 192 | 384 |
| 1200 x 128 | 384 | 736 |
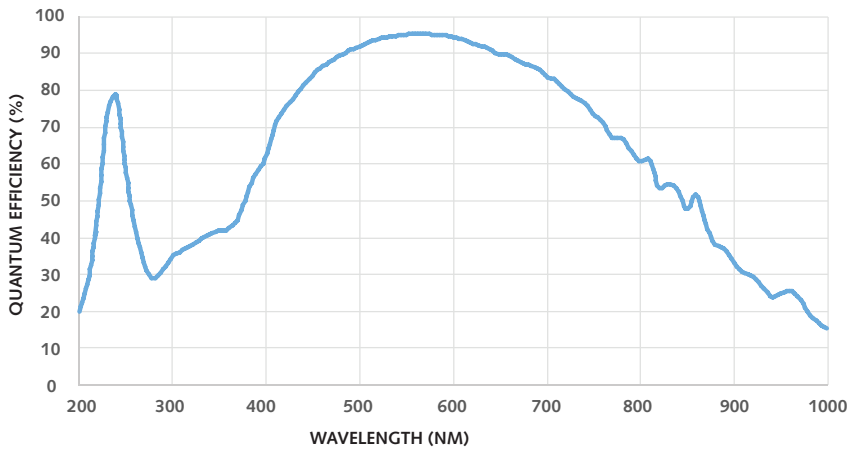
### Accessories (Included)

PCIe Card/Cable          Power Supply
USB 3.0 Cable            Manuals and QuickStart Guide
Trigger Cable            Performance and Gain Calibration Test Data

### Accessories (Additional)

Liquid Circulator
Liquid Cooling Tubes

## Distance from C-mount to sensor
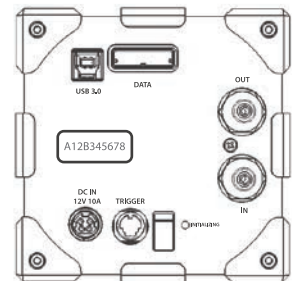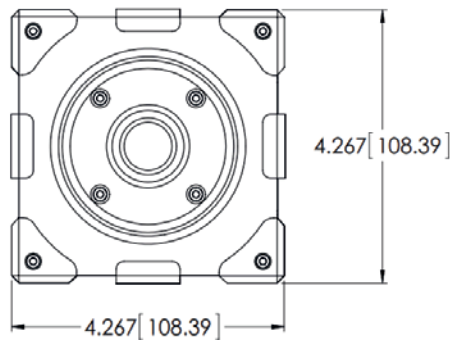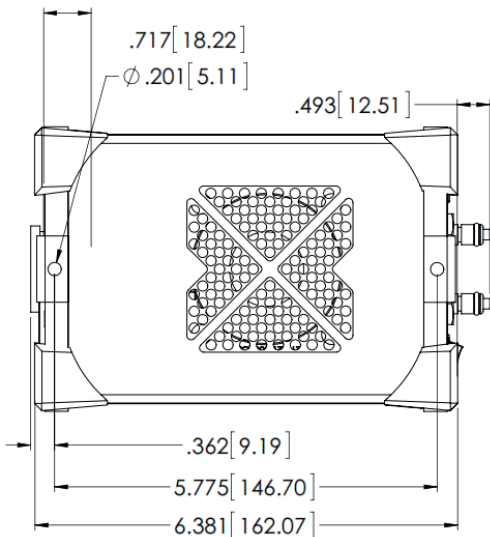
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Prime 95B™ Scientific CMOS Camera

# Photonics and Super-Resolution

**Paul French, Professor**

Photonics Group, Physics Department, Imperial College London

**BACKGROUND**

The Photonics Group in the Physics Department at Imperial College London develops instrumentation for multidimensional fluorescence imaging - spanning a wide range of applications, from super-resolved microscopy through automated fluorescence lifetime imaging for high content assays to endoscopy and optical tomography.

The availability of Scientific CMOS cameras has been transformative for their research because the technology provides unprecedented imaging performance with high resolution and high frame rates. The team particularly uses Scientific CMOS cameras for localization and light sheet microscopy.

**CHALLENGE**

STORM [i, ii] for super-resolved microscopy is a particular interest and the team recently published an approach using low-cost diode lasers [iii]. "Our goal is to develop instruments that provide state-of-the-art performance while reducing the cost where possible so that more users are able to access such advanced imaging capabilities," shared Professor Paul French.

**SOLUTION**

Scientific CMOS cameras are relatively cost effective compared to EMCCD cameras and they had already been incorporated into the team's STORM system. The group has since started using the Prime 95B.

*(continued...)*

"The Prime 95B camera is specified to provide >95% quantum efficiency, giving us the advantages of Scientific CMOS with fantastic sensitivity."

Learn more about the Photonics Group at Imperial College London:

http://www.imperial.ac.uk/photonics

## SOLUTION

French explained, "Since the achievable resolution is a function of the number of photons detected, we were excited to learn about the back-illuminated Scientific CMOS camera from Photometrics. The Prime 95B camera is specified to provide >95% quantum efficiency, giving us the advantages of Scientific CMOS with fantastic sensitivity."

When the group made a comparison of images taken with the Prime 95B and a standard Scientific CMOS camera, they were immediately impressed with the increase in signal to noise.

French concluded, "We look forward to implementing the Prime 95B camera in the new STORM microscopy platform that we are currently developing."



| dSTORM | dSTORM/wide-field overlay | wide-field |
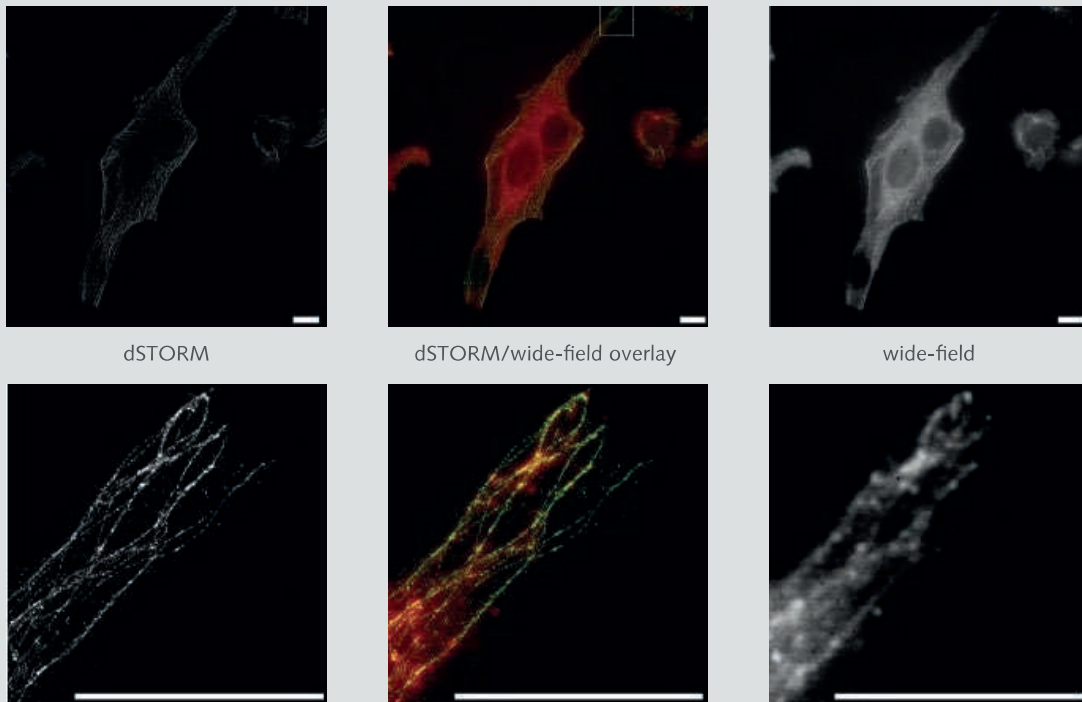
NIH3T3 mouse embryonic fibroblast, starved overnight and treated with 1µM Trichostatin A for 4h prior to fixation. Cell is stained by anti-acetylated tubulin with an Alexa Fluor 647 secondary antibody. Using a Cairn OptoTIRF system, 5000 image frames were taken with a 30ms exposure time which composed 2768763 individual localisations. Mean uncertainty is 11.54 nm. (Scale bars are all 10um)

### REFERENCES

[i]  M. J. Rust, M. Bates, and X. Zhuang, *Nat. Methods* 3, 793–796 (2006).

[ii]  M. Heilemann, S. van de Linde, M. Schuttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, and M. Sauer, *Angew. Chem. Int. Ed.* 47, 6172–6176 (2008).

[iii]  K. Kwakwa, A. Savell, T. Davies, I. Munro1 S. Parrinello, M.A. Purbhoo, C. Dunsby, M.A.A. Neil and P.M.W. French, *J. Biophotonics* 9 (2016) 948–95, DOI 10.1002/jbio.201500324

# Super-Resolution Fluorescence Microscopy

**Sang-Hee Shim, Principal Investigator and Assistant Professor of Chemistry**
Shim Group, Center for Molecular Spectroscopy and Dynamics,
Institute for Basic Science, Korea University

**BACKGROUND**

The Shim Group at Korea University is an interdisciplinary lab covering physical chemistry, biophysics and cell biology. Sang-Hee Shim, principal investigator and assistant professor of chemistry, leads a team composed of postdoctoral researchers and graduate students to develop new microscopic methods and apply them to answer complex biophysical questions.

Their core focus is to better understand life at the molecular scale by visualizing cell dynamics and the interactions of intracellular molecules. To do so, they explore the frontiers of optical microscopy with super-resolution fluorescence imaging.

**CHALLENGE**

The Shim Group previously used EMCCD technology for localization-based super-resolution fluorescence microscopy. However, although EMCCD offers better sensitivity than sCMOS technology, it suffers from excess noise generated by the process of electron multiplication. The precision and resolution of their experiments are highly dependent on the sensitivity and noise level of the camera so this presents an issue.

The group investigated potential solutions and found the Prime 95B Scientific CMOS camera from Photometrics - the first and only 95 percent quantum efficient CMOS device. The camera affords comparable sensitivity to EMCCD, yet offers far higher imaging speed and a larger field of view. After testing the camera, the group also found some cases in which it produced even better spatial resolution when compared to their existing EMCCD.

> " For single-molecule images like in DNA-PAINT, the Prime 95B combined with PrimeEnhance allows us to conduct super-resolution imaging with higher spatial resolution than that of EMCCD technology."

Additional information about the Shim Research Lab and their work is available at:
http://sodaus.wixsite.com/shimku

**SOLUTION**

The team compared the Prime 95B to their EMCCD camera using single-molecule DNA-PAINT imaging and found that the camera gave improved localization precision. This is suggested to be because the Prime 95B does not rely on electron multiplication to increase sensitivity. By removing the excess noise factor generated by the electron multiplication process, the Prime 95B Scientific CMOS can achieve a higher signal to noise ratio than an EMCCD.

The team also investigated PrimeEnhance™, the active denoising algorithm that accompanies the Prime 95B camera. They found that PrimeEnhance can amplify some noise and produced false localizations. When the localization software was optimized for PrimeEnhance, the localization precision was further improved and gave the best results among all tested conditions.

Shim explains, "For single-molecule images like in DNA-PAINT, the Prime 95B combined with PrimeEnhance allows us to conduct super-resolution imaging with higher spatial resolution than that of EMCCD technology." Shim adds, "Plus, the Prime 95B offers the additional benefits of higher frame rate and a larger field of view."
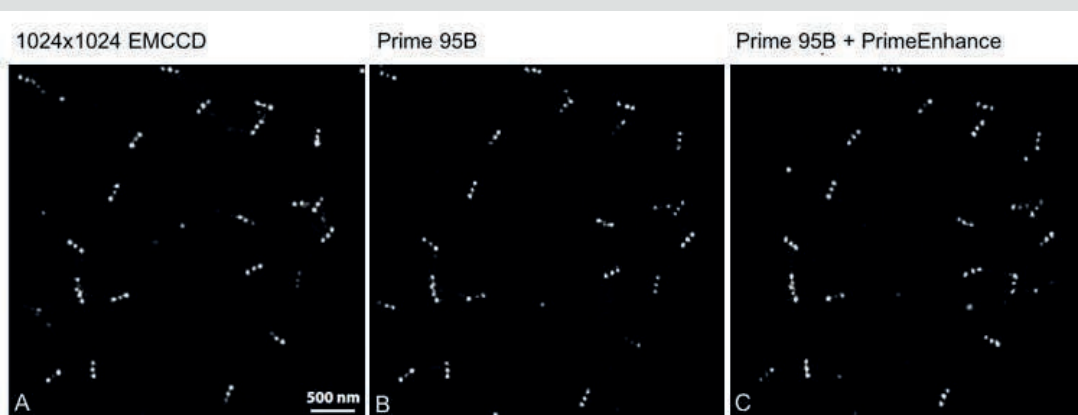


**Surface-immobilized DNA origami with 3 docking DNA strands with 80 nm gaps, imager DNA oligo transiently binding to the docking DNA strand, labelled with Atto 655**

**A.** 1024x1024 EMCCD camera with a 130 nm effective pixel size 32x32 field. Acquired at 47 fps with 20 ms exposure, 30x EM gain.  **B.** Prime 95B with a 110 nm effective pixel size. Acquired at 50 fps with 20 ms exposure time. **C.** Prime 95B plus PrimeEnhance with a 110 nm effective pixel size. Acquired at 28 fps with 20 ms exposure time.
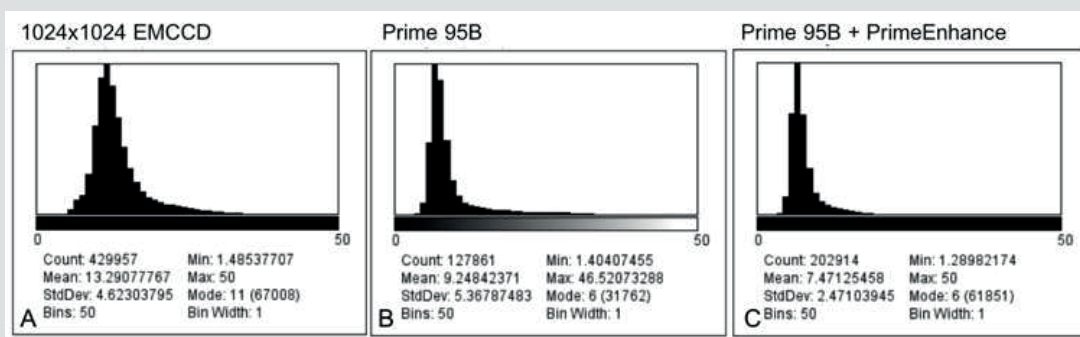


**Localization accuracy analysis of the DNA origami**

**A.** The 1024x1024 EMCCD camera shows a mean localization accuracy of 13.29 nm.  **B.** The Prime 95B shows a mean localization accuracy of 9.25 nm.  **C.** The Prime 95B plus PrimeEnhance shows a mean localization accuracy of 7.47 nm.

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# STORM Super-Resolution Microscopy

**Yandong Yin, PhD, Postdoctoral Fellow**
**Eli Rothenberg, PhD, Associate Professor**

New York University, School of Medicine

**BACKGROUND**

The laboratory of Dr. Rothenberg at the New York University School of Medicine focuses on new optical methods to study biological molecules and processes at real time and nanometer scale. The Rothenberg research team studies the mechanisms of enzymes and proteins that participate in repair of DNA damage leading to cancer, and develops new imaging methods that will enable them to visualize the behavior of individual biological molecules. STORM Microscopy is used to localize and track DNA as it replicates in the cell. "We try to look at the nucleus of a cancer cells as they are replicating the DNA. The DNA and proteins involved in DNA replication are labelled so we can understand what is going on when replication happens," Yandong Yin, PhD. Postdoctoral fellow states.

**CHALLENGE**

One of the challenges of imaging replicating DNA is that inside the nucleus of the cell there are many labeled components crowded together, as well as very small components that need to be clearly resolved. To determine how each component is organized spatially, the lab often performs STORM imaging using three or four colors sequentially, which makes resolution, sensitivity, and localization accuracy a great concern. "The DNA replication fork is very small. We can't image it without super resolution," says Yin. The laboratory calibrates their STORM post-processing conditions based on the variances of each pixel in the chip of the camera, correcting for any major variations, in order to better fit the point spread function of each fluorophore. Because of this, pixel to pixel variations, like those seen in patterned noise on CMOS cameras becomes a major problem.

> " If you have a shorter exposure time, you can **track faster** kinetics. More sensitivity and shorter exposure times with the **Prime 95B** allow you to image faster **and track** kinetics better. "

**SOLUTION**

The move away from EMCCD technology to the Prime 95B back-thinned CMOS was made easier because of the improved sensitivity in the detection of low-emission fluorophores, and the reduction in pattern noise when compared to other CMOS cameras. "For single-molecule localization imaging, the most important thing is the reconstruction process in how we fit each single-molecule point spread function into its centroid coordinate," Yin noted on his use of the Prime 95B. The post-processing of images collected with the Prime 95B are made significantly easier because of the reduction in pixel to pixel pattern noise, which makes the localization and reconstruction of fluorophores of multiple colors easier to do. Yin continued, "The variance for each pixel is much smaller than what is reported on other sCMOS cameras. We have found that more than 90% of the pixels fall within a very tight noise distribution."

Additionally, the large field of view coupled with the improved sensitivity allows the team to get an image that contains useful information more often. Since they often work with samples that are dark at the start of acquisition, it was an issue that they couldn't guarantee seeing something with the smaller field of view of an EMCCD. Yin says "Previously we used an EMCCD, but the EMCCD has a smaller chip. With a bigger chip we can see multiple cells simultaneously."
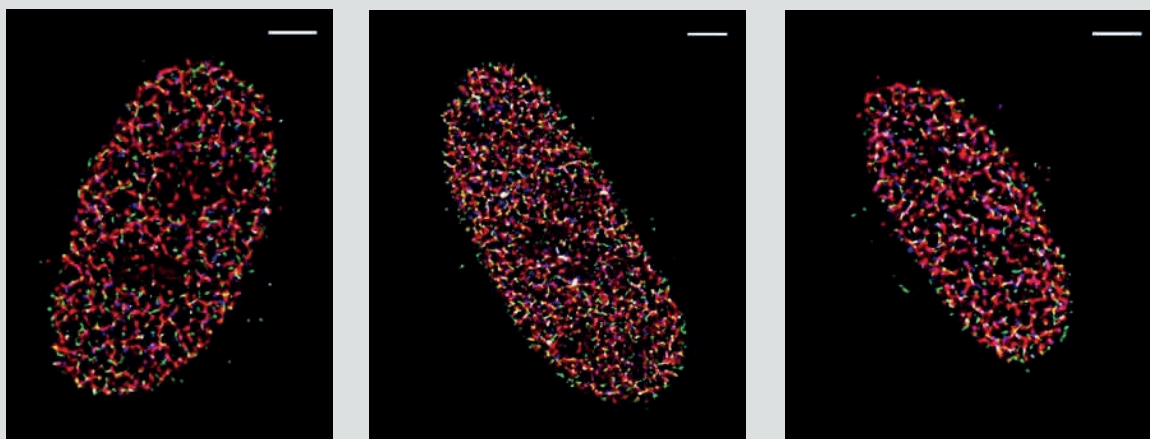


Cell Type: U2OS cells
Exposure time: 30 ms
Magnification: 150 times (the configured pixel size is ~ 73 nm)
Reconstruction Algorithm Used: Maximum Likelihood Estimation (MLE) method for single PSF fitting

Learn more about the Rothenberg Group at New York University:
https://med.nyu.edu/biomolpharm/research/biochemistry-macromolecules/eli-rothenberg

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# CUSTOMER REFERENCE

# Super-Resolution Fluorescence Microscopy

**Ke Xu, Principal Investigator and Assistant Professor of Chemistry**

Ke Xu Group, University of California Berkeley, College of Chemistry

**BACKGROUND**

The Ke Xu Group at Berkeley is an interdisciplinary lab that combines biophysics, physical chemistry and cell biology. Their goal is to understand how orders emerge in biological systems at the nano-meter scale from the interaction between biomolecules. They achieve this goal experimentally through the development and synergistic application of innovative quantitative methods such as super-resolution fluorescence microscopy.

Ke Xu, principal investigator and assistant professor of chemistry, successfully opened his lab in 2013 and today, leads a team that includes post-doctoral researchers and graduate students. The team recognizes and respects how living systems achieve versatile structural organizations at the nanoscale. Their dedication to gaining a greater understanding of this phenomenon has led to their ability to consistently achieve publication of their research findings.

**CHALLENGE**

Previously, EMCCD technology was the primary imaging solution in the Ke Xu Group. However, STORM experiments presented increasing demands on the existing imaging setup. The team decided to look at other technologies, specifically sCMOS solutions due to new advancements in CMOS sensors and more advanced capabilities becoming available.

Having reviewed available products, the team found the Prime 95B Scientific CMOS camera from Photometrics. The company was touting the first and only 95 percent quantum efficiency camera, which piqued their interest. Having the opportunity to fully test the camera, they discovered it offered comparable, if not better, results when compared to their existing EMCCD.

"[Prime 95B] allows us to conduct our STORM experiments with higher frame rates... 95 percent quantum efficiency allows for super-resolution imaging that's not achievable with conventional sCMOS cameras."

**SOLUTION**

When comparing to the EMCCD camera that was used previously, the Prime 95B provides many more benefits; faster imaging, comparable spatial resolution and a larger field of view. "The Prime 95B Scientific CMOS camera allows us to conduct our STORM experiments with higher frame rates and a larger field of view than with EMCCD technology," Xu shares. "Plus, the 95 percent quantum efficiency allows for super-resolution imaging that's not achievable with conventional sCMOS cameras," he adds.

**Prime 95B Scientific CMOS Camera Test**

β-tubulin-AF647
160 Hz
50k frames
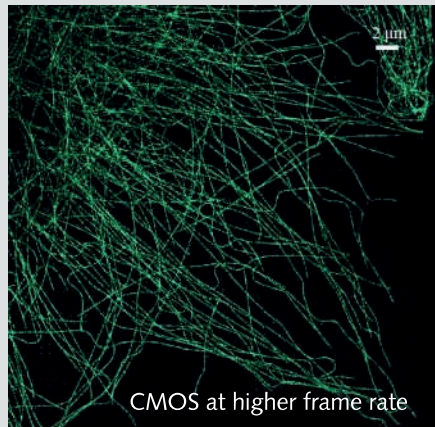


**Prime 95B Scientific CMOS vs EMCCD Camera Test**

β-tubulin-AF647
110 Hz
50k frames
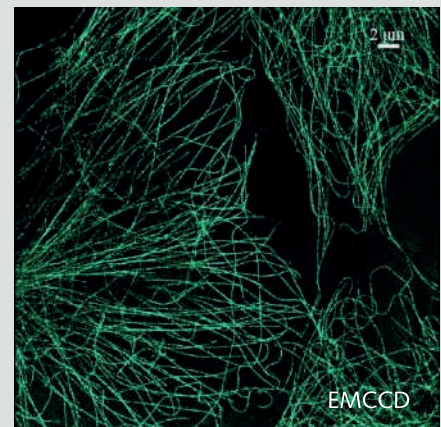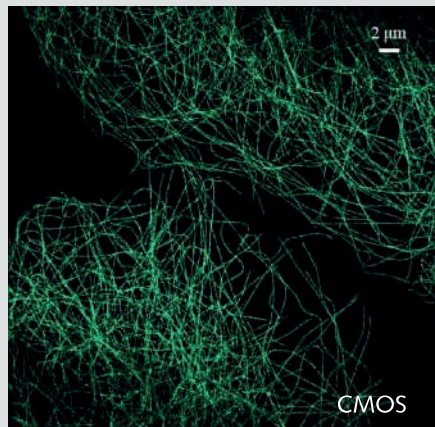


Learn more about the Ke Xu Group at the University of California Berkeley:

http://www.cchem.berkeley.edu/xuklab/

*Images courtesy of Rui Yan and Ke Xu, UC Berkeley*

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# CUSTOMER REFERENCE

## Super-Resolution Fluorescence Microscopy

### Dr. Kyle M. Douglass, Post-Doctoral Researcher

The Laboratory of Experimental Biophysics EPFL
Suliana Manley Lab, Lausanne, Switzerland

**BACKGROUND**

Dr. Kyle M. Douglass, a research scientist at the EPFL, has spent the past several years developing high-throughput and automation methods for super-resolution fluorescence microscopy. The Laboratory of Experimental Biophysics, which is led by Prof. Suliana Manley, uses these techniques to study the structural biology of multi-protein complexes such as chromatin foci, the bacterial division machinery, and the centrosome.

From the perspective of the technology, these structures share a common theme in that they require large datasets of high quality images to computationally combine into a structural model which can possibly consist of one or more disordered components. It is therefore imperative to acquire as much data as possible and to ensure that it meets the exacting standards required by the computational reconstruction pipelines.

**CHALLENGE**

These multiprotein complexes are well-suited to super-resolution approaches like STORM and PAINT because they are too small to see with traditional light microscopy. Furthermore, their rich and often heterogeneous composition precludes a complete study with electron microscopy , but this problem is easily overcome with multi-color super-resolution. Unfortunately, not every available dye is bright and stable. The quality of the measurements depends critically on how many photons can be recorded from each dye molecule, which means that the protein maps that are reconstructed from weak dyes will suffer from a loss of resolution and quality.

"We can now more precisely locate each fluorescent dye that is targeting a protein within a complex. This has the effect of improving the resolution of our structural models, allowing us to see details inside these complexes that we could not before."

**SOLUTION**

Because every photon counts, Douglass and colleagues upgraded their cameras to the Photometrics Prime 95B. The high sensitivity and large field of view allows the researchers to simultaneously image numerous structures at the same time while capturing even more photons than before. The increased throughput and quality of the data is paying dividends.
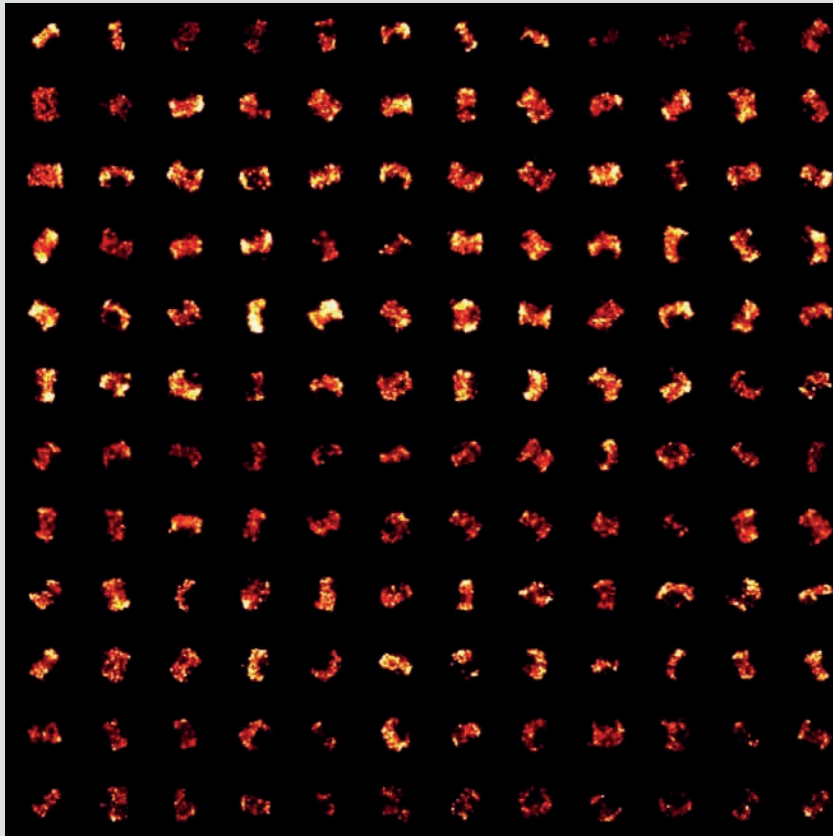


Image shows a montage taken from a particle library showing images of centriolar protein Cep152. Each particle is from a single centriole. The raw library typically consists of over 1,000 particles.

Learn more about the work of Dr. Douglass at EPFL:
http://kmdouglass.github.io

Visit the Laboratory of Experimental Biophysics at EPFL:
http://leb.epfl.ch/

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# SRRF and Super-Resolution Microscopy

**Dr. Ricardo Henriques, LMCB Group Leader, UCL Senior Lecturer, Experimental Optics Leader**
Medical Research Council Laboratory for Molecular Cell Biology (LMCB), University College London (UCL)

**BACKGROUND**

The Henriques group use various super resolution microscopy techniques to investigate cell signalling and host-pathogen interactions as well as creating and developing technology for cell biology research.

A big challenge in super-resolution microscopy is the requirement for intense illumination but this is usually phototoxic and incompatible with live-cell imaging. To tackle this problem the group developed a new approach – Super Resolution Radial Fluctuations (SRRF) – which enables super-resolution imaging using any fluorophore with far lower illumination intensities than conventional super resolution techniques.

**CHALLENGE**

The Henriques group recently started using the Prime 95B Scientific CMOS camera for some of their work. Dr. Henriques told us, "We've been actively using the Prime 95B as one of our main cameras for low-signal and super-resolution imaging at UCL. The Prime 95B Scientific CMOS is an outstanding camera, particularly due to its low-noise, high-sensitivity and large field-of-view."

The group is also using the Prime 95B as they adapt the SRRF algorithm for use with CMOS cameras. Dr Henriques explains, "Super-resolution microscopy using our SRRF method was designed for EMCCD cameras but we are currently updating the SRRF algorithm for improved quality and performance when using data from modern CMOS devices to take advantage of the large field of view and higher speeds available."

The accuracy of SRRF is directly related to the speed of acquisition so a faster camera would be advantageous.

"The Prime 95B Scientific CMOS is an outstanding camera, particularly due to its low-noise, high-sensitivity and large field-of-view."

**SOLUTION**

The updated algorithm is expected to be released soon. Below are images obtained with the Prime 95B to show how SRRF is evolving to work with Scientific CMOS sensors:
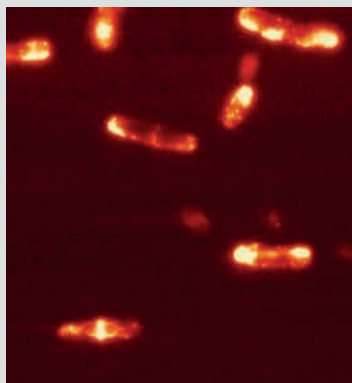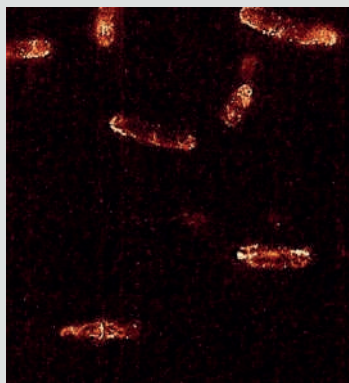


| Widefield | NanoJ-SRRF | SRRF4CMOS |
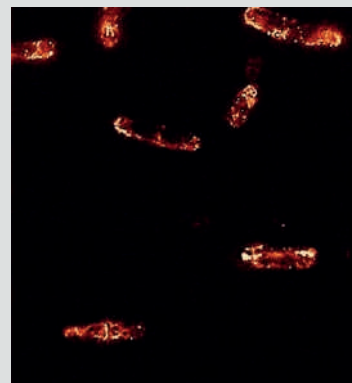
**LifeAct-GFP labelled fission yeast**

*Left:* Widefield image
*Middle:* Image after applying the conventional NanoJ-SRRF algorithm
*Right:* Image after applying the in-development novel version NanoJ-SRRF algorithm optimized for CMOS devices (to be named SRRF4CMOS).

All images acquired with the Prime 95B scientific CMOS camera with a 10 ms exposure time (yielding 1 super-resolution frame per second).

# Confocal Microscopy and STORM

**Uri Manor, Biophotonics Core Director**

**Salk Institute for Biological Studies, Biophotonics Core Facility**

**BACKGROUND**

The Salk Institute is home to a highly collaborative cadre of scientists who delve into a broad range of research areas, from aging, cancer and immunology to diabetes, brain science and plant biology. The group is supported by on-campus research centers and core facilities that are equipped with cutting-edge technology.

The Institute embodies Jonas Salk's mission to dare to make dreams into reality by exploring the very foundations of life, seeking new realities in neuroscience, genetics, immunology and more. The team lives to discover, be it cancer or Alzheimer's, aging or diabetes, they understand that every cure has a starting point. Salk is where cures begin.

Uri Manor, biophotonics core director works with the Salk researchers to provide collaborative support for a wide variety of research projects that require scientific imaging. Manor also works with the faculty steering committee to incorporate new and advanced imaging technologies into the repertoire of resources offered through the Biophotonics Core Facility.

**CHALLENGE**

The Facility provides technical and logistical access to Salk faculty, enabling the integration of imaging tools into a variety of biological research programs. To maintain its ability to advance science, the Facility must maintain the latest, cutting-edge commercial imaging and data analysis technologies available. This is especially important given that most projects involve sophisticated and complex research techniques.

The primary instrumentation that core researchers must have access to include technologies that support confocal microscopy (both fixed and live cell), TIRF microscopy, two-photon microscopy, electron microscopy and super-resolution microscopy as well as in-vivo imaging modalities.

> "The Prime 95B provides the speed, field of view and resolution of a CMOS camera, with the added sensitivity of an EMCCD camera for our more demanding experiments."

**SOLUTION**

To continue supporting the diverse work of the Salk researchers, Manor must stay abreast of the advancements being made in scientific imaging. It is important that the team always have access to the newest and most advanced solutions.

Manor learned of the Prime 95B Scientific CMOS camera from Photometrics and was interested in learning more about the claim for 95 percent quantum efficiency. Of greater importance was how the camera was being touted as the most sensitive in the industry – sensitivity is always in high demand among Salk researchers.

After seeing the camera and testing it with his own samples, Manor realized the camera did stand up to the hype and would integrate well into the Facility's imaging tools. The back illuminated technology and high QE make the camera exceptionally versatile. Manor shares, "The Prime 95B provides the speed, field of view and resolution of a CMOS camera, with the added sensitivity of an EMCCD camera for our more demanding experiments."

The Prime 95B easily supports multiple scientific applications, this flexibility also makes it a very good investment for the Salk Institute.
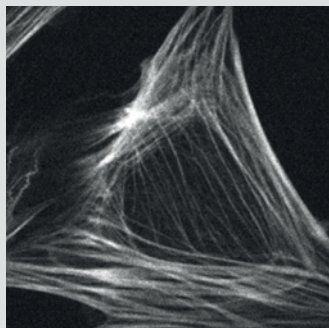


25ms
Scientific CMOS
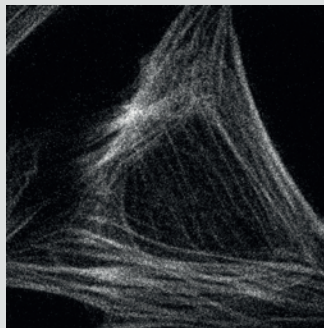
25ms, 400x EM gain
EMCCD
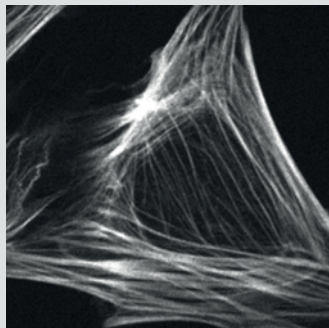
50ms
Scientific CMOS
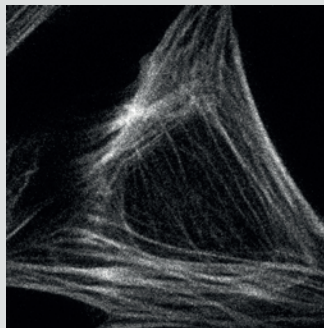
50ms, 400x EM gain
EMCCD

Images depict the actin cytoskeleton as stained by AlexaFluor488-Phalloidin. EMCCD used was the Photometrics Evolve 512 and the Scientific CMOS was the Photometrics Prime 95B. Images were captured using Micro-Manager on a Zeiss Confocal microscope with a Yokogawa spinning disk scan head. 25ms and 50ms exposures were acquired with laser power set to the minimum of 5% with the EM Gain of the EMCCD set to 400.

Learn more about the Salk Institute for Biological Studies:
http://umanor@salk.edu

View Uri Manor's professional profile at Salk Biophotonics Core:
http://www.salk.edu/science/core-facilities/advanced-biophotonics/faculty-staff/

# Spinning Disk Confocal

**Peter March, Senior Experimental Officer**

University of Manchester, Bioimaging Facility

**BACKGROUND**

The research being performed at the University of Manchester has a real-world impact beyond the lab. The team is at the forefront of the search for solutions to some of the most pressing issues in biology, medicine and health. The Bioimaging Facility delivers a broad range of state-of-the-art imaging solutions to the University, Faculty of Biology, and Medicine and Health. A key technology used in biological imaging of live cells is Spinning Disk Confocal Microscopy. Spinning Disk allows for long-term, high-speed, three-dimensional imaging of live samples with multiple channels of illumination.

**CHALLENGE**

One of the primary reasons for using Spinning Disk Microscopy is to generate confocal images without photobleaching or damaging live samples. "Bright cells are not necessarily healthy cells," warns Peter March, senior experimental officer at the university. "Using less GFP in cells matches their natural behavior more closely," he adds.

Correspondingly, sensitivity is among the most important features of a camera. Until recently, and due to their ability to achieve >90% quantum efficiency, EMCCD cameras were the preferred imaging device for Spinning Disk Microscopy. March and his team typically use a 60x objective, resulting in the need to address the large pixels of an EMCCD, which require extensive optical adjustments to reach acceptable sampling levels. This severely limits field of view, making samples harder to find and capture. Additionally, EMCCD cameras cause excessive, visible noise across the sample, even at high exposures.

> "The Prime 95B is the **perfect** camera for **Spinning Disk** - the image quality **is a big** improvement **over our EMCCDs, and the field of** view makes **samples** much easier **to find.**"

28

**SOLUTION**

The Prime 95B back-illuminated Scientific CMOS is the perfect match for Spinning Disk Microscopy because it delivers much greater image quality at a higher resolution than possible with a EMCCD camera. In addition, its sensitivity is much higher than other CMOS cameras currently on the market. March is most impressed with the increase of field of view as he shares "With EMCCD cameras, finding the sample was often an issue. Field of view is all-important, and the Prime 95B is a big improvement here."

Without the excess noise factor of EMCCDs or the pattern noise seen in 2x2 binned front-illuminated sCMOS, March says, "The difference in image quality is huge". The Prime 95B provides the ability to capture more of a sample at equal exposure times compared to EMCCD cameras, and it produces more impressive images as a result.
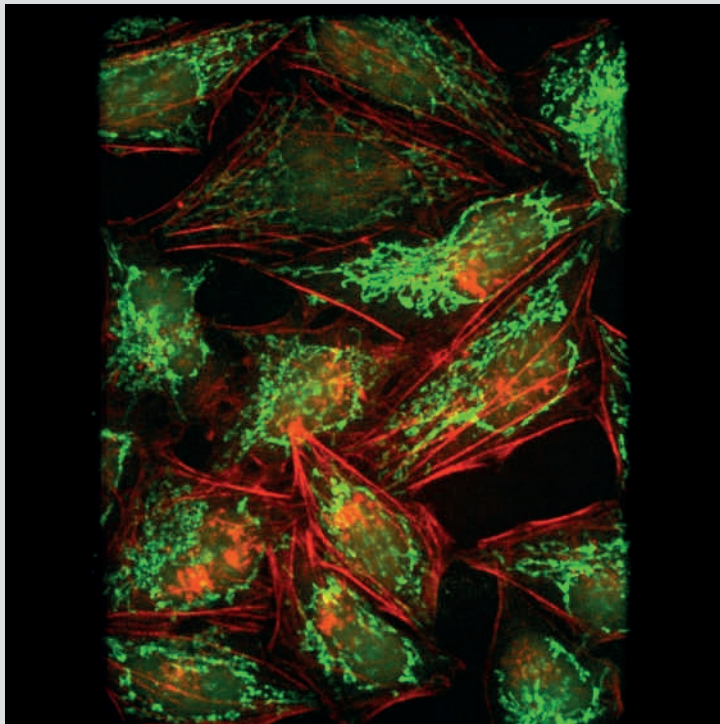
Mitochondria (green, 488nm) and Actin filaments (red, 566nm).

Sample prepared by Vicki Allen, imaged using a Yokogawa CSU-X1 with a 63x oil, 1.4NA objective.

Additional information about the research being performed in the Bioimaging Facility at the University of Manchester is available at:
https://www.bmh.manchester.ac.uk/research/

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# Live Cell Spinning Disk Microscopy of Endogenously Expressed, Low Abundance Proteins

**Dr. Jan Felix Evers**

Centre for Organismal Studies at University of Heidelberg, Germany

> " **Photometrics cameras are r e l i a b l e with great performance. We can now v i s u a l i z e things that we could not see before.** "

**BACKGROUND**

The Center for Organismal Studies (COS) Heidelberg has set the goal of researching organismal biology beyond the boundaries of the biological organizational stages. Research and teaching at the COS are devoted to the biology of organisms from the molecular basis to cell biology, developmental biology and physiology to evolution and biodiversity as well as system biology and biotechnology in plant and animal systems. Dr. Jan Felix Evers and his team's primary interest is in how neuronal circuits form in the central nervous system, both on the cellular and molecular level. Their focus lies on investigating these issues with spinning disk live cell imaging, which requires very high sensitivity and resolution.

**CHALLENGE**

Synaptogenesis is very sensitive to overexpression artifacts. To study synaptogenesis with minimal interference, the research team devised a system to visualize endogenous gene expression in single neurons. Their challenge lies in working with a low copy number of fluorophores down to the single molecule level and therefore low fluorescent yield.

Learn more about Dr. Evers and his team's work at the Centre for Organismal Studies at University Heidelberg: www.cos.uni-heidelberg.de

**SOLUTION**

The team wanted the best solution to cover all of their applications. The Prime 95B Scientific CMOS offers the largest usable field of view with the highest sensitivity of all available Scientific CMOS cameras, and an optimal sampling density with a 60x magnification. In addition, the Evolve 512 EMCCD provides the best sensitivity and signal-to-noise for detecting low endogenous gene expression at the single molecule level. "After carefully testing a larger range of cameras, we selected equipment from Photometrics. The Evolve provides the lowest noise floor and highest sensitivity for our most demanding samples while the Prime 95B provides a higher sampling density with signal-to-noise that is almost as good as an EMCCD" shares Dr. Evers. "Photometrics cameras are reliable with great performance," Dr. Evers concludes. "We can now visualize things that we could not see before."
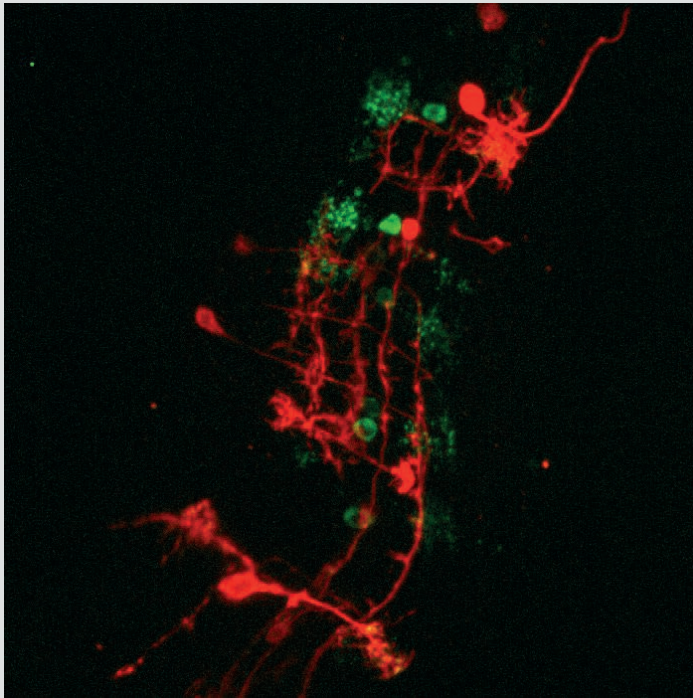


Image captured with the Prime 95B Scientific CMOS camera from Photometrics.

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**CUSTOMER REFERENCE**  Prime 95B™ Scientific CMOS Camera

## Structured Illumination Microscopy (SIM)

**Guy Hagen, PhD, Research Associate**

University of Colorado, Colorado Springs

### BACKGROUND

Dr. Guy Hagen, Research Associate from the University of Colorado, Colorado Springs creates high performance image reconstruction methods and open-source software to process super-resolution microscopy data. In 2014 Dr. Hagen released ThunderSTORM, an ImageJ plug-in for automated processing of photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) data.[1] In 2016, he introduced SIMToolbox, a MATLAB toolbox for processing SIM data. It has the flexibility to process 2D and 3D images, including both optical sectioning and super-resolution applications, and can be used on data acquired from commercial systems.[2] SIMToolbox also includes maximum a posteriori probability estimation (MAP-SIM), a super-resolution restoration method that suppresses out of focus light, improves spatial resolution, and reduces reconstruction artifacts.[3] Dr. Hagen is currently developing live cell imaging using SIM, and is continuing to develop data analysis methods for super-resolution microscopy.

### CHALLENGE

SIM is a widefield fluorescence technique that uses illumination patterns with high spatial frequency to illuminate samples. Algorithms applied to a combination of images taken with different phases and orientation of the illumination pattern are used to reconstruct a high-resolution image. Reconstruction of images routinely requires many images per focal plane. Fluorescent signals need to withstand photobleaching and the acquisition rate must be fast enough to observe live cell dynamics.

"Imaging twice as fast with the Prime 95B is a great advantage. The reduction of pixel-to-pixel variance and the reduction of visible column structure in the camera greatly improves our results."

**SOLUTION**

Because multiple images are required for the reconstruction of SIM data, a sensitive camera will allow for shorter exposure times and faster acquisitions, reducing phototoxic and photobleaching effects on samples.

The back-illuminated Prime 95B Scientific CMOS camera has a near perfect 95% quantum efficiency and large 11 µm pixels, making it extremely sensitive. The Prime 95B allowed Dr. Hagen to reduce exposure times to acquire a set of SIM images in about half the time required with typical sCMOS cameras.

Pixel-to-pixel and column-to-column gain variations which are present in typical sCMOS cameras can degrade SIM images during the reconstruction process. The Prime 95B reduces image artifacts by implementing several pixel noise filters to detect and correct dynamic fluctuations, and the static variation in gain and offset is calibrated for every pixel. Because of this, raw SIM images have a reduction in noisy pixels and visible readout lines, resulting in higher quality processed SIM images.
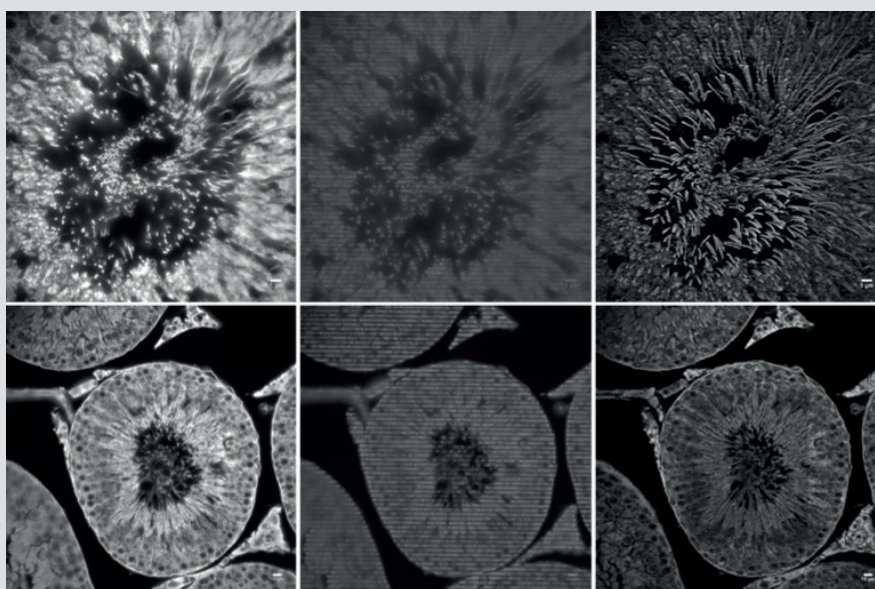


Cross section of a rabbit seminiferous tubule acquired using Dr. Hagen's structured illumination microscopy system and a Prime 95B camera with a 100X/1.47NA objective (top row) or 40x/1.3NA objective (bottom row). Images were reconstructed using SIMToolbox [2]. Conventional widefield fluorescence images (left) display out of focus light, raw SIM images (center) display the illumination pattern, and the 3D SIM reconstruction (maximum intensity projection, right) shows high quality, optically sectioned images.

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# Single Molecule Fluorescence Imaging

**Madhavi Krishnan, Professor**
Krishnan Group, University of Zürich

**BACKGROUND**

The electrostatic properties of macromolecules—specifically, their electrical charge and interior dielectric characteristics— are a vital component of their function as they contribute to the physical basis of mechanisms that range from molecular recognition, signalling and enzymatic catalysis to protein folding and aggregation, and are of fundamental relevance in experiment and theory.

The Krishnan group at the university of Zurich are pioneering the use of the "electrostatic fluidic trap" to perform novel experiments in the spatial control, manipulation, and measurement of nanoscale matter in solution. Their primary focus is on biological molecules such as proteins and nucleic acids but some experiments also involve inorganic entities displaying interesting photonic properties.

The unique "field-free" trap offers high-precision measurement of the effective electrical charge of a single molecule in solution. They are able to measure a macromolecule's electric charge with the precision of a single charge and below (<1e-). One of their goals is to use this approach to read out three-dimensional conformational changes or fluctuations in single macromolecules in real time.[1, 2]

**CHALLENGE**

Professor Krishnan shared, "Imaging single molecules labeled with a single fluorophore can be challenging as single fluorophores generally emit relatively weak signals. We also need to work at high speeds to visualize the motion of the molecules in the electrostatic fluidic trap."

This means that the group is using very low exposure times so they are constantly working in a low signal to noise environment.

The group had previously been using an EMCCD camera for this work but to reach the required speed they could only use a small field of view which only allowed them to visualize a few molecules at a time.

"The high speed and large field of view of the Prime 95B are a massive advantage for our work."

Additional information about the Krishnan Lab and their work is available at:
http://krishnanlab.uzh.ch/

**SOLUTION**

The near-perfect 95% quantum efficiency of the Prime 95B Scientific CMOS camera provides sensitivity that is equivalent to an EMCCD but with the high speed and large field of view expected of a CMOS device.

Having the ability to go 82 frames per second with high sensitivity on the large 1200x1200 sensor with an 18.66 mm diagonal increased the number of single molecules that could be detected in a single frame by a large margin. Professor Krishnan told us, "The high speed and large field of view of the Prime 95B are a massive advantage for our work."
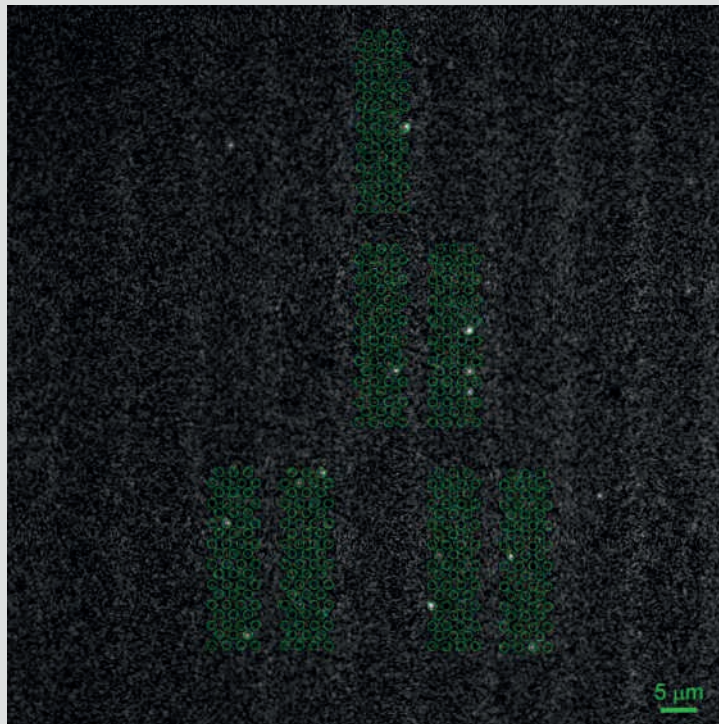


**Figure 1: Atto 532 labelled DNA fragments in electrostatic fluidic traps (indicated by green circles) arranged in rectangular lattices.**

Image acquired with the Prime 95B under 40x magnification with an 8 ms exposure time using the full frame (1200x1200 pixel) field of view.

**REFERENCES**

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# Single Molecule Fluorescence Imaging

## Anders Kyrsting, Post-doc

The Linke Group, University of Lund, Sweden

**BACKGROUND**

The Linke Group at the University of Lund, Sweden, creates artificial molecular motors and nanowires to better understand the role of biological motors in cellular processes such as cargo transportation, muscle contraction and cell division. The group tags the motors with quantum dots and images them using single molecule TIRF. They have further plans to expand their investigation with optical trapping and STORM super-resolution microscopy.

**CHALLENGE**

Anders Kyrsting, post-doc with the Linke Group, explained, "Molecular motors move very fast so to track them, we need a camera with a very high frame rate." The group was previously using an EMCCD camera but the slower frame rate of its architecture meant that they had very limited temporal resolution.

Kyrsting continued, "Investigating single molecules means working with very low fluorescence signal. So, a camera with high sensitivity is equally as important as our need for a fast camera." For this reason, the group couldn't afford to sacrifice sensitivity for speed.

"I don't think I'd use an EMCCD again, I don't know why I'd use it with the performance we get out of the Prime 95B."

**SOLUTION**

The near-perfect 95% quantum efficiency and high speed of the Prime 95B Scientific CMOS camera made it a clear fit for their work and the group was excited to implement the camera into their system.

Kyrsting told Photometrics, "The Prime 95B enabled us to reach the 200 fps speed that we needed with a far larger field of view than would be possible on any EMCCD camera."

Additionally, with the large 11 µm pixel of the Prime 95B, the group could achieve perfect diffraction limited resolution with a 100x objective, without using any additional optics.

Kyrsting continued, "The speed and sensitivity were exactly what we were looking for and the bonus of having such a large field of view has really helped our data throughput." In conclusion, Kyrsting added, "I don't think I'd use an EMCCD again, I don't know why I'd use it with the performance we get out of the Prime 95B."

Alumina (Al2O3) coated gallium phosphide (GaP) nanowires functionalized with biotinylated BSA (bovine serum albumin). Streptavidin labelled with three fluorescent dyes (FITC, TRITC and Cy5), imaged under 100x magnification.

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# Total Internal Reflection Fluorescence (TIRF)

**Shiaulou Yuan, Ph.D.**
**Postdoctoral Associate in Pediatrics (Cardiology)**
Yale University

**BACKGROUND**

The Martina Brueckner lab at Yale University School of Medicine studies the genes of children with congenital heart disease. "We take a human genetics approach combined with animal models, such as mouse and zebrafish, and in vivo imaging. In particular, we do a lot of live imaging of cilia, hair-like structures on the cell surface that function as cellular antennas," Shiaulou Yuan, a postdoctoral researcher explained. "The cilium is a tiny organelle that is packed with hundreds of distinct signaling molecules. We know these must be important because mutations in genes that are important for cilia biogenesis or signaling can cause congenital heart disease. The challenge is that for many of these genes, we simply don't know how they can cause congenital heart disease. To understand how these genes are functioning requires us to create a whole-animal, as well as apply high-resolution live imaging approaches, that are guided by human genetics. It's exciting because it's the type of science that can only be done nowadays rather than twenty years ago, because of the remarkable advances in genomic and imaging technologies. It all comes together to enable us to understand the problem from new angles."

" The quantum efficiency of the camera is a really important factor for us. If we can use less excitation power, we can increase the length of our imaging and minimize photodamage to the animal. The sensitivity of the Prime 95B is truly transformative for our type of work. "

**CHALLENGE**

Image collection for Dr. Yuan relies not only on sensitivity and resolution, but also fast speeds. "Much of work depends on genetically encoded biosensors or GFP-knock-ins, which are endogenously tagged with a single copy of GFP. They are not that bright, but at the same time, we are also doing live imaging of mouse or zebrafish embryos that require rapid image acquisitions over several hours as the animals happily develop. We have a limited photon budget, yet we must capture a lot of images very rapidly and over a long period of time. We also need high resolution because we're looking at tiny cilia, but also speed, because they move very fast. Finally, on top of all this, we must keep the laser excitation power low because the animal has to stay alive during all of this – the imaging needs to be gentle."

**SOLUTION**

"The quantum efficiency of the camera is a really important factor for us. If we can use less excitation power, we can increase the length of our imaging and minimize photodamage to the animal. The sensitivity of the Prime 95B is truly transformative for our type of work. Besides the sensitivity, the combination of speed and resolution of the Prime 95B, which is superior to an EMCCD, makes it killer for our experiments. In fact, we have been limited in the past due to insufficient camera technologies. With the Prime 95B on hand, we are now able to attack these questions."



A live image of a cilium from a mouse cell expressing a genetically encoded calcium biosensor (green) and a membrane localized fluorophore (red).

Recording by Shiaulou Yuan and Mohammed Mahamdeh using a Zeiss 63X 1.2NA water immersion objective.

Learn more about the Martina Brueckner lab at Yale University School of Medicine:

https://medicine.yale.edu/pediatrics/cardiology/

**CUSTOMER REFERENCE**          Prime 95B™ Scientific CMOS Camera

# TIRF Microscopy

**Gerry Hammond, Ph.D.**
*Assistant Professor*

**Simon Watkins, PhD.**
*Founder and Director of the Center for Biologic Imaging*

**University of Pittsburgh**

**BACKGROUND**

Gerry Hammond studies membrane trafficking and intracellular signaling and regulation in the Center for Biologic Imaging at University of Pittsburgh. Working with Simon Watkins, Director of the Center, they use gene editing technology to fuse GFP to endogenous alleles. They then use a combination of Total Internal Reflection Fluorescence Microscopy (TIRF) and other techniques to study the mechanisms of how inositol lipids effect membrane function and transport of nutrients in disease. Dr. Hammond shared, "We use TIRF because we are interested in what's happening at the interface between the plasma membrane and the endoplasmic reticulum which sits very close to it."

**CHALLENGE**

When tagging genes at their endogenous loci, low expression levels are often a problem. This results in low levels of fluorescence and produces images with very low signal to noise. "The amount of fluorophore that you've got [in the sample] is much less than you'd normally have, but of course that's the advantage because we get to see how the endogenous protein is behaving in a living cell," Dr. Hammond explains.

> " I think the Prime 95B is a very innovative product. Partly because of the back thinning but also because the pixel size is more appropriate than conventional scientific CMOS cameras."

**SOLUTION**

Dr. Hammond told us, "The advantage of the Prime 95B for me was the improved sensitivity over other CMOS cameras but with all the advantages that come with CMOS technology like acquisition speed and a larger chip size."

Dr. Watkins added, "The 11 micron pixel size with the 100x objective that Gerry and I are using gets us much closer to Nyquist sampling in XY... If you combine the back-thinning and the more appropriate pixel size you have much better resolution at higher sensitivity."
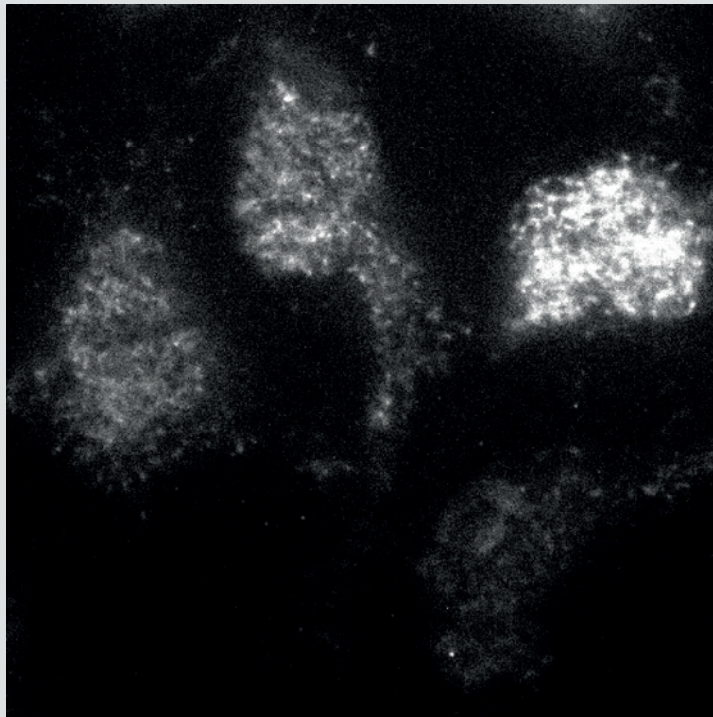


**HEK 293 cells with GFP fused to Sec 61 at its endogenous allele.**

Camera: Prime 95B

Magnification: 100x TIRF

Binning: 2x2

Additional Information:
Learn more about the Center for Biologic Image at the University of Pittsburgh, Pennsylvania:
http://www.cbi.pitt.edu/

Learn more about Simon Watkins PhD at the University of Pittsburgh, Pennsylvania:
http://www.cbp.pitt.edu/faculty/watkins.html

To learn about the Hammond Group at the University of Pittsburgh, Pennsylvania:
http://www.cbp.pitt.edu/faculty/hammond.html

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# Single Molecule TIRF Microscopy

**Aleks Ponjavic, Postdoctoral Researcher**

Klenerman Group, University of Cambridge

**BACKGROUND**

The Klenerman group investigates intracellular signalling in T-cells, a vital component of the human adaptive immune response. They are particularly interested in the kinetic-segregation model of T-cell signalling which proposes that signalling is only possible when CD45 molecules on the T-cell surface are sterically excluded from the T-cell receptor site.

The group observes these cell-surface molecules using single molecule TIRF microscopy to add further structural support for the kinetic-segregation theory.

**CHALLENGE**

The group needs to ensure reliable detection of single molecules so high signal to noise is of great importance. To achieve this, they have been using the Photometrics Evolve® 512 EMCCD camera but made the decision to purchase a Prime 95B because of its high sensitivity combined with the multiple benefits of CMOS architecture. Aleks Ponjavic, postdoctoral researcher with the Klenerman group, told us, "I was very interested to compare the high sensitivity of the Prime 95B to an EMCCD for single molecule imaging."

**SOLUTION**

When asked how the Prime 95B compared to an EMCCD camera, Aleks told us, "I find the performance of the Prime 95B to be comparable to state-of-the art EMCCDs but at lower cost and higher speed." He went on to say, "I would definitely choose the Prime 95B over an EMCCD for any high sensitivity application that would benefit from the high speed offered by a CMOS camera."

> "I find the performance of the Prime 95B to be comparable to state-of-the-art EMCCDs."

Additional information about Klenerman Group and their research is available at:
http://www.klenermangroup.co.uk/
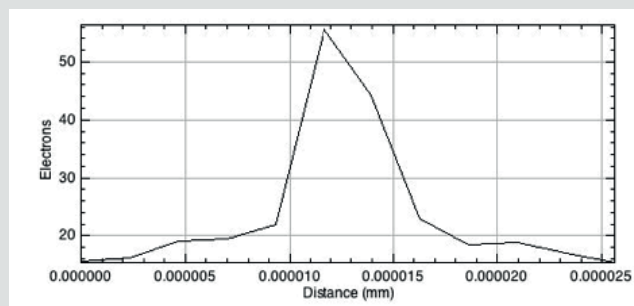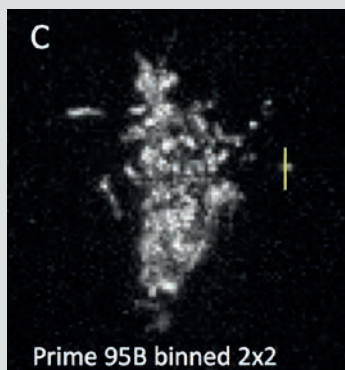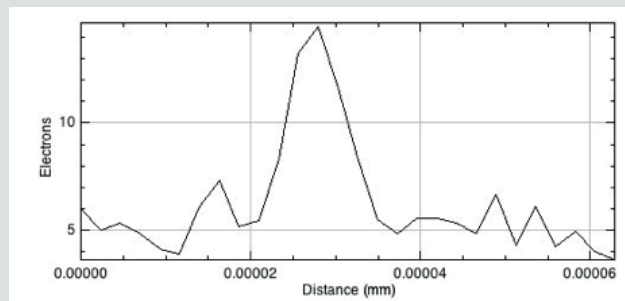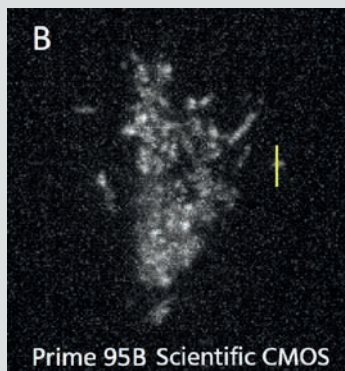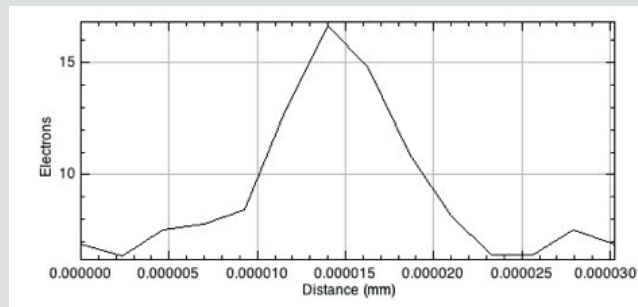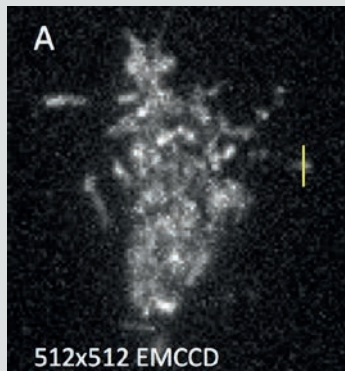
**Jurkat T-cells bound to fibronectin. CD45 molecules on the cell surface labelled with Alexa 488.**

**A.** *Left:* Maximum intensity projection of 500 frame 33 ms exposures taken with the Evolve Delta EMCCD camera using 250x EM gain. *Right:* Line profile taken through the marked area of the image (yellow line).

**B.** *Left:* Maximum intensity projection of 500 frame 33 ms exposures taken with the Prime 95B.
*Right:* Line profile taken through the marked area of the image (yellow line).

**C.** *Left:* Maximum intensity projection of 500 frame 33 ms exposures taken with the Prime 95B binned 2x2.
*Right:* Line profile taken through the marked area of the image (yellow line).

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# Single Molecule Microscopy

**Prof. Mark Leake**

**Professor Anniversary Chair of Biological Physics**

University of York

**BACKGROUND**

Prof. Leake founded and leads the Biological Physical Sciences Institute at the University of York, which brings together scientists researching the biomolecular interactions, biological modelling imaging and quantitation of complex data. His work targets a broad range of fundamental processes and open questions in biology, and has provided insight into topics such as the behavioural mechanics of the flagellar motor of bacteria, protein transport, DNA replication, repair and remodelling, signal transduction, gene regulation and oxidiative phosphorylation.

**CHALLENGE**

Prof. Leake's lab is at the forefront of developing new biophysical instrumentation to probe cells and biological specimens on a single-molecule level. Single-molecule imaging is a hugely powerful cutting-edge technique for examining fundamental processes and interactions in biological systems. However, the technique is also among the most demanding low-light imaging applications, as the fluorescent response from a single fluorophore is very low, and photobleaching must be avoided. Determining the precise quantity and localisation of single-molecule signals requires an excellent signal to noise ratio for the weakest of signals, while simultaneously delivering high spatial and temporal resolution.

"Altogether, there is no **better** camera for cutting-edge **single** molecule microscopy than the Prime 95B Scientific CMOS."

**SOLUTION**

The Prime 95B Scientific CMOS camera uses back illumination to reach near-perfect quantum efficiency, which combined with low readout noise and large pixels, provides the sensitivity required for single-molecule fluorescence. "The Prime 95B combines the speed of a CMOS camera with sensitivity at or better than an EMCCD, allowing us to push single-molecule microscopy into larger specimens, imaging even faster phenomena than before," Prof. Leake tells us.

"The camera is extremely easy to set up, with very few settings so you can get straight to imaging. It even includes its own programmable logic outputs which allow the 95B to control and synchronise with other components on the microscope. Altogether, there is no better camera for cutting-edge single-molecule microscopy than the Prime 95B Scientific CMOS."
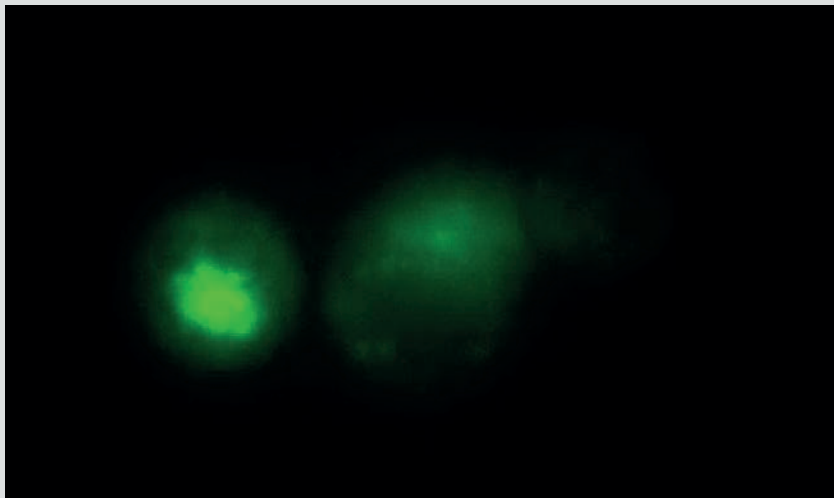


Live yeast cells displaying GFP labeled Mig1, an essential transcription factor, at millisecond sampling and single-molecule sensitivity.

Additional information is available at: http://single-molecule-biophysics.org/

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# Single Molecule Biophysics and DNA Mechanics

**Dr. Christoph Baumann**
**Lecturer in Molecular Biophysics**
Department of Biology, University of York

## BACKGROUND

Dr. Christoph Baumann, Lecturer at the University of York, Department of Biology and his group work with advanced imaging techniques to push forward our understanding of spatio-temporal dynamics in the bacterial cell envelope. Using a Photometrics camera, the group was the first to observe that, contrary to expectations, proteins in the outer cell membrane don't diffuse significantly when tracked, and that new proteins are inserted predominantly at mid-cell during growth.[1] This means that bacteria can very quickly turnover their outer membrane proteins to adapt to new environmental challenges during growth, and this work initiated a new investigation of inter-membrane crosstalk in the Gram negative bacterial cell envelope.[2]

## CHALLENGE

To pursue this research area, Dr. Baumann and his colleagues use TIRF microscopy alongside laser scanning confocal FRAP microscopy. When tracking single molecules, sensitivity and speed are all-important. "Better temporal and spatial resolution means better quality data," Dr. Baumann shares. Previously, the large pixels, slow speed, and the excess noise factor of their EMCCD camera limited both these aspects. Further, the small sensor size and the need to use a 1.6x magnification optic to match pixel size lead to a very small field of view, and using additional lenses lost them precious light.

"Better temporal and spatial resolution means better quality data. We'll definitely be using the Prime 95B Scientific CMOS camera for our upcoming experiments."

**SOLUTION**

The Photometrics Prime 95B Scientific CMOS camera was the perfect fit for the team's research, with the 11 µm pixels giving optimal resolution paired with the 100x objectives used for TIRF microscopy. The faster speed and higher signal to noise ratio of the back-illuminated CMOS also provided better temporal resolution. "This increased resolution is of great benefit for all the research we do, not just this experiment," Dr. Baumann told us. "Since the camera is USB3.0, it's very easy to move the camera to other setups for short periods."

The huge field of view of the Prime 95B is not only useful for increasing throughput, but allows the team to use single-camera optical splitting technology. Dr. Baumann concluded, "We're going to use a polarization splitter in an upcoming experiment, and the Prime 95B's chip is large enough to still have a great field of view with both images side by side."
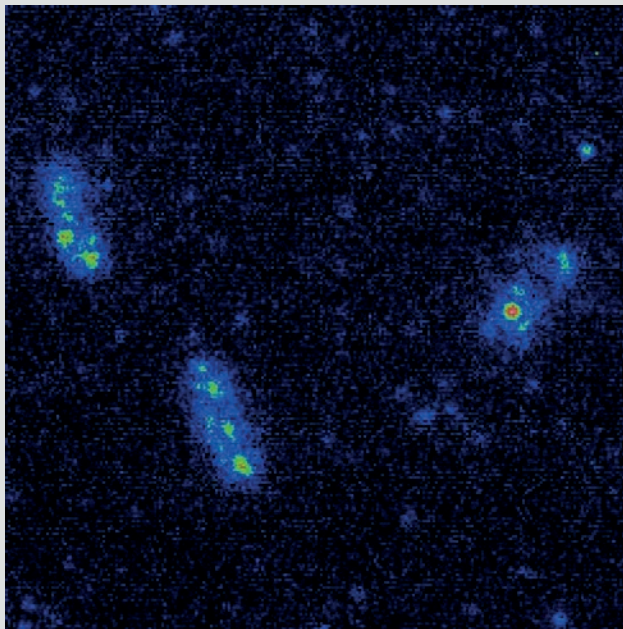


Alexa Fluor 488-labelled colicin E9 protein molecules bound to extracellular face of BtuB transmembrane receptors in the outer membrane of live *Escherichia coli* JM83 cells (256 x 256 pixels$^2$, 16.9 x 16.9 µm$^2$, 30 frame sum, video data collected with 33 ms exposure and global shuttering).

**REFERENCES**

1 Supramolecular assemblies underpin turnover of outer membrane proteins in bacteria, P. Rassam et al., 2015, *Nature* 523: 333-336.

2 Intermembrane crosstalk drives inner-membrane protein organization in *Escherichia coli,* P. Rassam et al., 2018, *Nature Commun* 9: 1082.

Additional information is available at:
https://www.york.ac.uk/biology/research/biochemistry-biophysics/

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# Light Sheet and Single Molecule Tracking

**Dr. Martin Lenz, Senior Research Associate**

Cambridge Advanced Imaging Centre (CAIC)

University of Cambridge

**BACKGROUND**

The Cambridge Advanced Imaging Centre (CAIC) at the University of Cambridge develops modern imaging techniques to answer some of the most pressing and challenging biological questions. Keeping in mind the needs and demands of biologists, one of the current developments is a localization based 3D super-resolution microscope. One of its applications include investigation of Notch pathway transcription factor dynamics in *Drosphila* salivary gland cells and mapping out the arrangement of chromatin inside *Drosphila* spermatocytes. Working in close collaboration with biologists requires CAIC to adapt and apply technological advancements in biomedical imaging to answer some of the most challenging questions in the field.

**CHALLENGE**

The research team uses single molecule tracking (SMT) to explore protein dynamics in living tissues of *Drosphila* and *Zebrafish*. A complete picture of different diffusing populations require images with high signal to noise ratio (SNR) at low excitation laser powers and short exposure times. One of the key points for achieving this is an efficient collection and detection of emitted photons.

To investigate the architecture of chromatin in *Drosphila* spermatocytes the team uses single molecule detection in combination with double-helix point spread function (DHPSF). This can give high-resolution in all three spatial dimensions. Losses in generating DHPSF and splitting the number of photons into two lobes of the DHPSF, requires highly efficient collection of single molecule emissions for this technique to be successful.

> "The high QE of the Prime 95B will allow us to improve our investigation of protein dynamics and extend single molecule tracking to more challenging samples."

**SOLUTION**

Dr. Lenz, Senior Research Associate at CAIC shares, "The Photometrics Prime 95B will help us to progress in both SMT as well as chromatin mapping projects. The high QE of the 95B compared to other sCMOS cameras currently used by us, will allow us to improve our investigation of protein dynamics and extend SMT to more challenging samples."

Single molecule light sheet microscopy is one of the key applications for future work that will benefit the most from the increased sensitivity. For this application, the larger than usual pixel size of 11µm together high quantum efficiency will be highly advantageous.



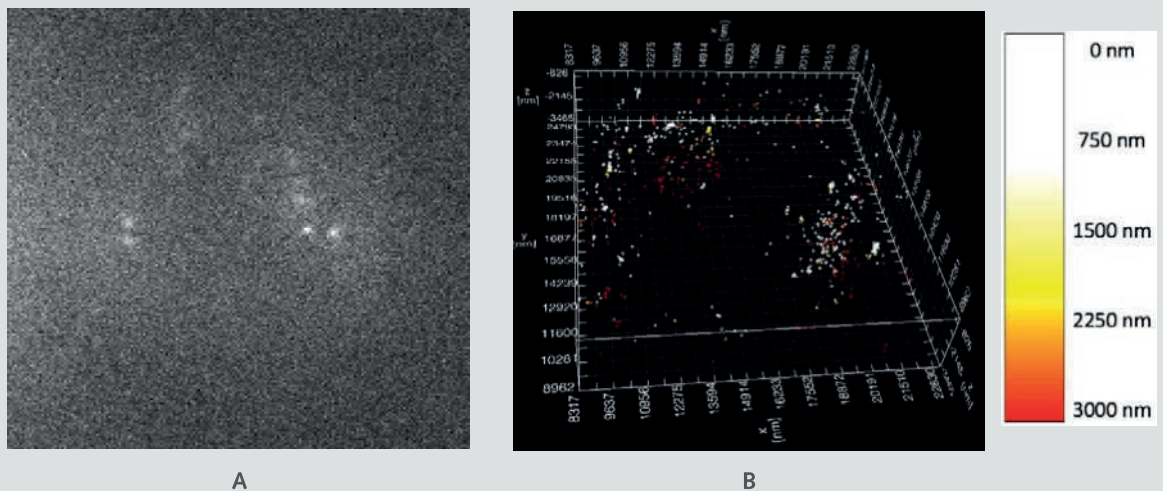A                                  B

**Figure 1: A) Raw image of spermatocytes using double-helix point spread function and B) 3D reconstruction from the raw data. Localizations in different colors represent their axial position.**

Additional information is available at: https://caic.bio.cam.ac.uk/

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**CUSTOMER REFERENCE**

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# Mizar TILT Light Sheet Fluorescence Microscopy

**Dr. Paul Maddox,** Assistant Professor
The University of North Carolina at Chapel Hill, Biology Department

### BACKGROUND

Conventional light sheet fluorescence microscopy (LSFM) is performed with two objectives oriented orthogonally to each other so that one objective introduces the light sheet and the other detects the fluorescence signal. However, this orientation requires the detection objective to be placed slightly away from the sample to prevent the two objectives colliding in space. Therefore, a long working distance detection objective is necessary which means that high NA, oil-immersion objectives are incompatible with the conventional LSFM design.

This presents a problem for the detection of cellular or subcellular structures which require a high NA detection objective and coverslip-based mounted samples for the superior resolution and light collection efficiency.

The Mizar TILT overcomes this problem by removing the illumination objective and introducing a tilted light sheet through a photomask and cylindrical lens which can be made to converge at the working distance of high NA objectives. In this way, high magnification and high NA (60x, 1.49), oil-immersion objectives can be used to image coverslip-based mounted samples.

### CHALLENGE

Like all light sheet systems, the Mizar TILT is designed to minimize photodamage and photobleaching to live samples by reducing the light source intensity and reducing exposure times. This allows for longer acquisitions to be made to monitor live processes over longer timescales.

One way to reduce exposure times is to use a more sensitive camera. CMOS devices are typically used in LSFM for the combination of a large field of view and fast speed but the sensitivity isn't that high. EMCCD cameras are more sensitive than CMOS devices but suffer from small fields of view and slow speeds which makes them unappealing for LSFM applications.

> "The Prime 95B Scientific CMOS camera is, right now, the best solution we have found for TILT imaging. Coupling the Prime 95B to the TILT generates an extremely **powerful** imaging system!"

**SOLUTION**

The back-illuminated Prime 95B Scientific CMOS camera with an almost perfect 95% quantum efficiency and large 11µm pixels is the perfect fit for the Mizar TILT.

The sensitivity of the Prime 95B is equivalent to an EMCCD but it retains the field of view and speed advantages of a CMOS camera. Furthermore, the larger pixels of the Prime 95B allow for high-resolution imaging with higher magnification objectives which the Mizar TILT was designed to use. This allows exposure times to be reduced and cells to be imaged for much longer with high detail.

Dr. Paul Maddox, assistant professor at the University of North Carolina at Chapel Hill is the creator of the Mizar TILT and founder of Mizar Imaging, shares with us, "The Prime 95B Scientific CMOS camera is, right now, the best solution we have found for TILT imaging. The outstanding quantum efficiency and pixel size allow imaging of a wide diversity of samples of varying brightness whilst enabling Nyquist sampling in space and time for even the most challenging samples. Coupling the Prime 95B to the TILT generates an extremely powerful imaging system!"
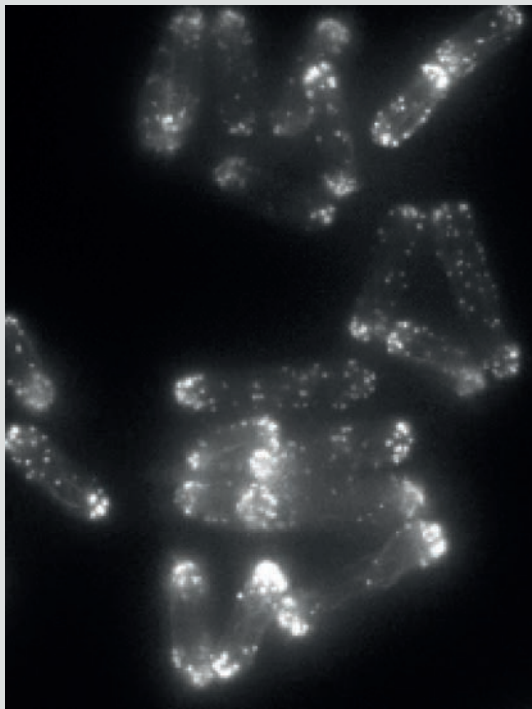


**Maximum intensity projection of fission yeast expressing LifeAct-mCherry**

Image acquired on the Mizar TILT under 150x magnification with a 1.49NA TIRF objective, 0.2 µm step size. 100 ms exposure using the Prime 95B scientific CMOS camera, cropped to 550x750 pixels.

Sample kindly provided by
Dr. Dan Mulvihill,
University of Kent
https://www.kent.ac.uk/bio/profiles/staff/mulvihill.html

Additional information is available at:
http://bio.unc.edu/people/faculty/maddox-paul/
https://mizarimaging.com/

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# Live Cell Fluorescence Microscopy

**Christof Osman, PhD, Professor**

Ludwig-Maximilian University, München, Germany

Department of Biology II, Faculty of Biology, Cell and Developmental Biology

**BACKGROUND**

The lab of Prof. Osman's at the Ludwig-Maximilian University in München, Germany is interested in understanding the power plants of cells – mitochondria – and the way their functionality and network activity is maintained during cell division. The underlying process of ensuring that the mitochondrial DNA is correctly distributed between cells undergoing cell division so that cells can produce the required proteins, is the lab's goal. The team developed a tool which allows them to track in yeast mitochondrial DNA, in the least-possible invasive manner, with high-speed live-cell fluorescence microscopy.

**CHALLENGE**

The lab's research organism is yeast, which is notoriously challenging to image as samples are small and low in fluorescence. Moreover, as the process of cell division and the function of mitochondria is very sensitive to exposure with light (in particular with wavelength in the UV and blue end of the spectrum), the research team would like to use a light dose, as low as possible, for excitation. This allows them the ability to image samples under physiological conditions. Additionally, the events to be observed can be relatively rare, and therefore it is important to image as many cells as possible in one attempt to achieve optimal statistics.

> " The Prime 95B c a m e r a provides the r e q u i r e d sensitivity that is needed to image our yeast cells with as little excitation light as possible."

**SOLUTION**

Prof. Osman is using two Prime 95B 25mm Scientific CMOS cameras with a new TwinCam which is suited for the 25mm diameter light path of the Nikon Ti2 microscope. "The Prime 95B camera provides the required sensitivity that is needed to image our yeast cells with as little excitation light as possible. This prevents phototoxic effects and allows us to maintain cells under physiological conditions throughout long imaging experiments," Prof. Osman shares. The TwinCam allows the ability to image two channels simultaneously without having the need to switch filters in-between which leads to losing temporal resolution.
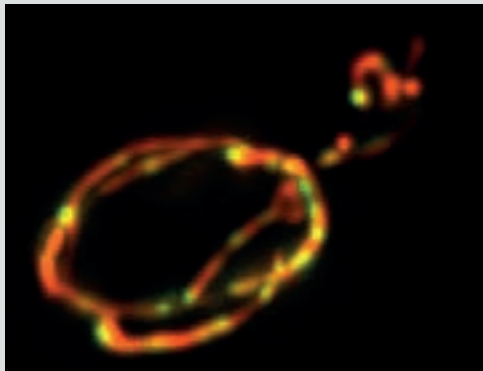


**Maximum intensity projection of 45 planes showing live yeast mitochondria labelled with mKate2 and mitochondrial DNA with tandem-mNeonGreen.** Image was taken with a Photometrics Prime 95B 25mm CMOS camera on a Nikon Ti2 microscope.

Learn more about the the lab of Prof. Osman's at the Ludwig-Maximilian University:

http://www.cellbiology.bio.lmu.de/research_groups/osman/index.html

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# Microfluidics and Live Cell Imaging

**Andrew deMello, Principal Investigator and Professor for Biochemical Engineering**
**Simon Berger, Doctoral Student**

deMello Group, ETH Zürich (Switzerland)
Department of Chemistry and Applied Biosciences

**BACKGROUND**

The deMello Group at ETH Zurich is engaged in a broad range of activities in the general area of microfluidics and nanoscale science. Primary specializations include the development of microfluidic devices for high-throughput biological and chemical analysis, ultra-sensitive optical detection techniques, nanofluidic reaction systems for chemical synthesis, novel methods for nanoparticle synthesis, the exploitation of semiconducting materials in diagnostic applications, the development of intelligent microfluidics and the processing of living organisms.

In recent years the deMello group has developed a range of microfluidic tools for the long-term imaging of living organisms, specifically the nematode *Caenorhabditis elegans*. Currently, work is focused on the creation of novel microfluidic devices for worm manipulation and the study of a wide range of developmental processes, previously inaccessible.

**CHALLENGE**

Live fluorescence imaging has seen a tremendous change over recent years. The development of sCMOS cameras has transformed image acquisition rates, fields of view and noise suppression, while also lowering unit costs. However, until recently the EMCCD has been the gold-standard for high sensitivity applications, significantly outperforming sCMOS devices with quantum efficiencies in excess of 95%, but lacking considerably with respect to sensor size and acquisition speed.

Simon Berger, doctoral student with the DeMello group, explains, "The primary challenge in live cell/organism imaging is the extraction of high quality images, both bright and with high contrast, while ensuring that phototoxicity and photobleaching are kept to a minimum. In this way, imaging does not affect sample viability and the biological processes under investigation."

The need to ensure low photobleaching/phototoxicity often limits attainable image quality, as well as the frequency and detail with which images can be acquired. While the higher sensitivity associated with EMCCDs can remedy the effects of photobleaching/phototoxicity, the acquisition rates, the small fields of view and limited dynamic range, severely limit their usefulness for *in vivo* imaging.

> "The Prime 95B allowed us to acquire high contrast fluorescence images using low **excitation** intensities, and subsequently allowed us to image over longer periods of time and at higher frequencies than **previously possible.**"

**SOLUTION**

Compared to its peers, the Prime 95B combines the features of sCMOS cameras (high acquisition rates, large sensor size, low noise and high dynamic range) with the exceptional sensitivity previously only available through EMCCDs.

Simon explains, "The Prime 95B allowed us to acquire high contrast fluorescence images using low excitation intensities, and subsequently allowed us to image over longer periods of time and at higher frequencies than previously possible. This allowed for intrusion-free study of many sensitive developmental processes."
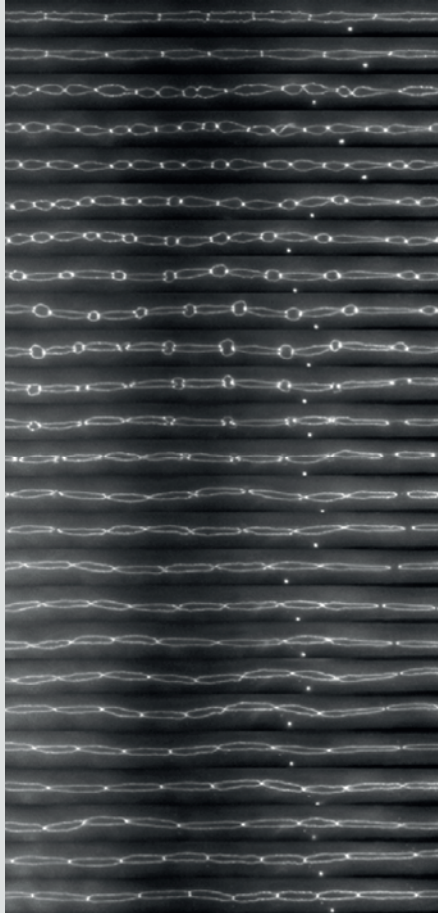


Expression of DLG-1::eGFP, apical junctional protein in the seam cell epithelium. Each image represents a 10 ms exposure taken every 20 minutes for a full sequence time of 4.5 hours.

C. elegans strain courtesy of Dr. H. Ribeiro Pires and Dr. M. Boxem, Utrecht University.

Additional information about the the deMello Group and their research is available at:
https://demellogroup.ethz.ch/en/index

# Microfluidic Chip Imaging of Live Yeast

**Marc Fouet, Postdoctoral Researcher**

University of California, Berkeley

**BACKGROUND**

The Rine lab at the University of California, Berkeley is working towards understanding mechanisms underlying establishment, maintenance, and epigenetic inheritance of gene silencing in yeast. The lab has developed a genetic strategy to capture transient losses of gene silencing of heterochromatin in *S. cerevisiae*, and translating these dynamic processes as a permanent modification of fluorescence expression. By using the CRASH (cre-reported altered states of heterochromatin) reporter, red fluorescent yeast cells become green once gene silencing by Sir proteins has been lost.[1] Monitoring fluorescence of yeast colonies can be used to describe dynamic epigenetic phenomena quantitatively. Recently, the Rine lab began using high-throughput yeast aging analysis (HYAA) chips[2] with high-resolution time-lapse microscopy to monitor the relative life span of yeast and understand better the relationship between gene silencing and aging.[3] Dr. Marc Fouet, a postdoctoral researcher in the Rine lab, is working on solutions to detect events leading to a lower signal of fluorescence and on the microfluidic process intensification to image an increasing number of single cells.

**CHALLENGE**

Rare transcription events within heterochromatin occur in approximately 1/1000 cell divisions and cells divide every 90 minutes. In order to capture these rare events, 40 images are taken of 5,000 yeast cell traps for each sample. Images are captured every 5 to 10 minutes to measure cell division and fluorescent protein levels. Phototoxicity and photobleaching should be minimized, and resolution is increasingly important when machine learning algorithms are used to segment fluorescence data.

"The bigger our field of view and the lower our exposure times are, the more cells we can image overall as less images are needed and the acquisition is faster."

**SOLUTION**

The 95B has a 95% quantum efficicency (QE) and can detect low levels of fluorescent proteins in yeast. The high sensitivity of the 95B allowed Marc to lower the exposure time to reduce phototoxicity and photobleaching.

The sensitivity and large field of view reduces acquisition time of the microfluidic chip. In addition to faster exposure times, the large field of view (18 mm diagonal) reduces the amount of images needed per chip and decreases acquisition times.

The 95B is a very versatile camera and is useful in multiple applications in the Rine group. In addition to time lapse imaging, the 95B can acquire full frame images at 82 fps in the 12-bit mode. This is fast enough to capture rapid events that occur frequently in microfluidic chips, for example, loading of a cell trap.
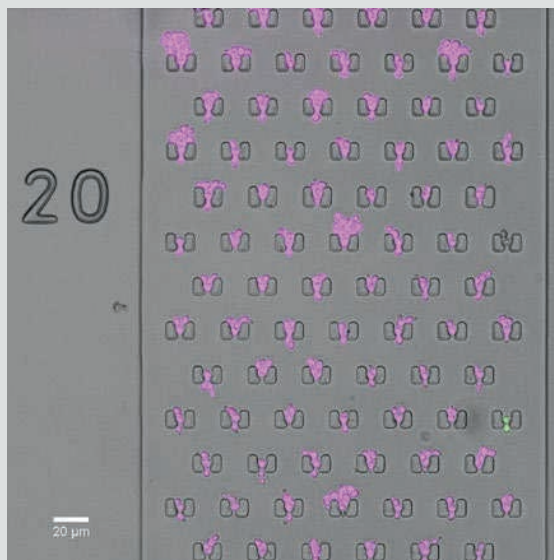


This microfluidic HYAA chip[2] contains single-cell traps and are used with time-lapse microscopy in aging assays of *S. cerevisiae.*

Figure 1 is an image from a chamber with cells carrying the CRASH (cre-reported altered states of heterochromatin) reporter that induces a permananet and hertibale switch from expressing red fluorescent protein (RFP) to expressing green fluorescent protein (GFP) when loss of silencing of the auxiliary mating-type locus *HMLα2* occurs.[1]
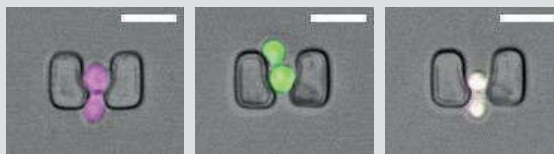


Figure 2 is a close up view of a single trap with single cells that are budding. They can be expressing either RFP (fig. 2A), GFP (fig. 2B), or expressing both colors when they are switching (fig. 2C), scale bar represents 10 µm.

**REFERENCES**

1  Dodson AE, Rine J. Heritable capture of heterochromatin dynamics in Saccharomyces cerevisiae. *Elife* (2015), doi: 10.7554/eLife.05007.

2  Jo MC, Liu W, Gu L, Dang W, Qin L. High-throughput analysis of yeast replicative aging using a microfluidic system. *Proc Natl Acad Sci U S A* (2015), doi: 10.1073/pnas.1510328112.

3  Schlissel G, Krzyzanowski MK, Caudron F, Barral Y, Rine J. Aggregation of the Whi3 protein, not loss of heterochromatin, causes sterility in old yeast cells. *Science* (2017), doi: 10.1126/science.aaj2103.

# Neuroscience and Calcium Imaging

**Geoffrey G. Murphy, Ph.D., Research Professor**

Molecular & Behavioral Neuroscience Institute
University of Michigan

**BACKGROUND**

Dr. Geoffrey Murphy, professor of physiology at the University of Michigan's Molecular & Behavioral Neuroscience Institute studies the how the mammalian brain encodes, stores and retrieves information. Dr. Murphy explains, "We do a lot of mouse molecular genetics, in vitro neurophysiology, and mouse behavior. We are interested in the dendritic architecture, and the calcium signaling within the dendritic structure versus the somatic structure."

**CHALLENGE**

For many neuroscience researchers, speed, sensitivity, and resolution are all critical to visualizing small changes in calcium signals in different regions of the neuron. Dr. Murphy told us, "Being able to image at 100 Hz is very important to us. That allows us to get high resolution, rapid calcium signals." Additionally, the samples to be imaged require a sensitive camera to visualize as the samples are often thick and can be troublesome to image through.

> " The Prime 95B allowed us to not only increase the frame rate we were using to acquire images, but we also achieved higher resolution. For us, that meant being able to look at subcellular structures in real time."

**SOLUTION**

For Dr. Murphy, the 11µm pixel size and speed of the Prime 95B provided a solution to the challenges of calcium imaging in neurons. Dr. Murphy told us, "Previously, we had been using cameras that had a lower frame rate and worse resolution, so using the Prime 95B allowed us to not only enhance the frame rate that we were using to acquire images, but get higher resolution too. For us, that meant being able to look at subcellular structures in real time so the speed is certainly something that we like. Also, it's a really easy camera to use and it interfaces well with our preferred software application [for further analysis]."
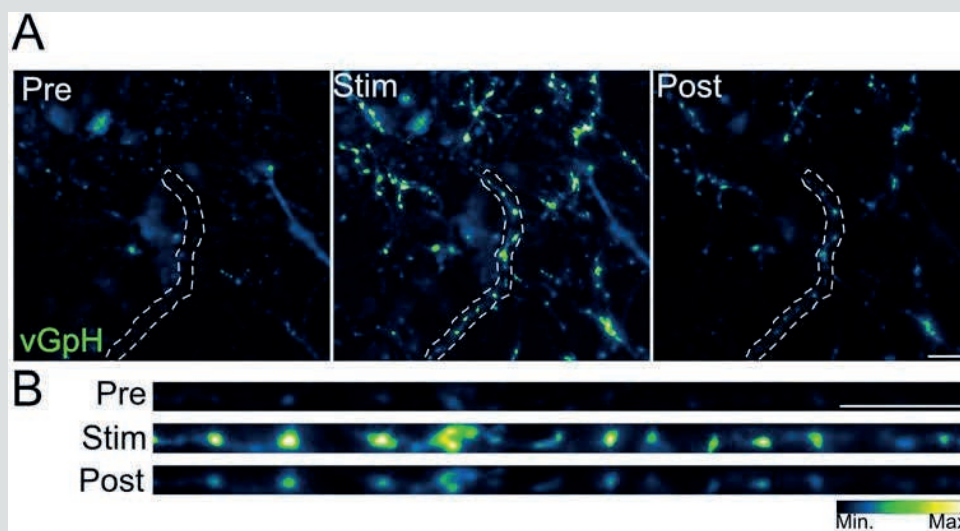


**Fluorescence imaging of the fluorescent reporter for vesicle cycling (VGLUT1-pHluorin, vGpH) in hippocampal neurons.**
**A:** Images of fluorescent intensity before (Pre), during (Stim) and after electrical stimulation (100 pulses, 10 Hz) of neurons.
**B:** Enlarged view of an axonal segment (from outlined region in A).

Learn more about the Murphy Lab at the University of Michigan:

https://sites.google.com/a/umich.edu/murphy-lab/home

# PHOTOMETRICS ®

# Fluorescence Correlation Spectroscopy

**Prof. Enrico Gratton, Founder and Principle Investigator**
Laboratory of Fluorescence Dynamics
University of California, Irvine

**BACKGROUND**

The lab of Prof. Enrico Gratton at the University of California, Irvine, is interested in the dynamics of the cell interior. The group investigates this using microscopy combined with mathematical approaches such as fluorescence correlation spectroscopy. As the founder and Principle Investigator of the Laboratory of Fluorescence Dynamics, Prof. Gratton's team has developed numerous fluorescence-based methods to measure diverse cell properties. This includes measurement of the absolute concentrations of molecules within cellular compartments, detection of aggregation of proteins and the detection of barriers to diffusion. Prof. Gratton also developed Globals for Images, a set of software packages to perform this analysis.

**CHALLENGE**

To reliably detect the incredibly small fluctuations in image signal required for fluorescence correlation spectroscopy – on the order of 10 electrons of signal - stable and predictable noise characteristics are necessary. Noise is an inherent feature of camera sensors and is corrected to reveal fluorescent fluctuations which are detected and measured. Typical experiments using EMCCD cameras require the noise characteristics, including correlated noise and light-independent pixel variance, to be mapped before every experiment to account for instability in the camera noise over time. After fast, time series fluorescence imaging, the previously mapped noise is removed to detect the variance due to fluorescent fluctuations in each pixel, and allowed mathematical analysis of the signal. With fluctuations in signal being less than 10 electrons in amplitude, high camera sensitivity and being able to accurately correct for the noise are crucial in providing the necessary signal to noise for this analysis.

> Prof. Gratton now uses the Prime 95B back illuminated Scientific CMOS camera to take advantage of the stable noise and uncorrelated pixel noise properties.

**SOLUTION**

Prof. Gratton now uses the Prime 95B back illuminated Scientific CMOS (sCMOS) camera, to take advantage of the stable noise and uncorrelated pixel noise properties. Light-independent pixel noise characteristics are stable over time which means the camera only requires calibration and mapping once, rather than before every experiment.

Prof. Gratton shared, "I didn't need to account for camera noise in the analyses, unlike when using EMCCDs and other CMOS cameras, which suffer from unstable noise. The reliability and stability of the pixel noise, makes the Prime 95B Scientific CMOS camera an ideal imaging solution for fluorescence correlation spectroscopy methods." Having predictable and easily modelled pixel to pixel variation produces the highest signal to noise ratio and results in detection of smaller fluctuations than possible when using an EMCCD.
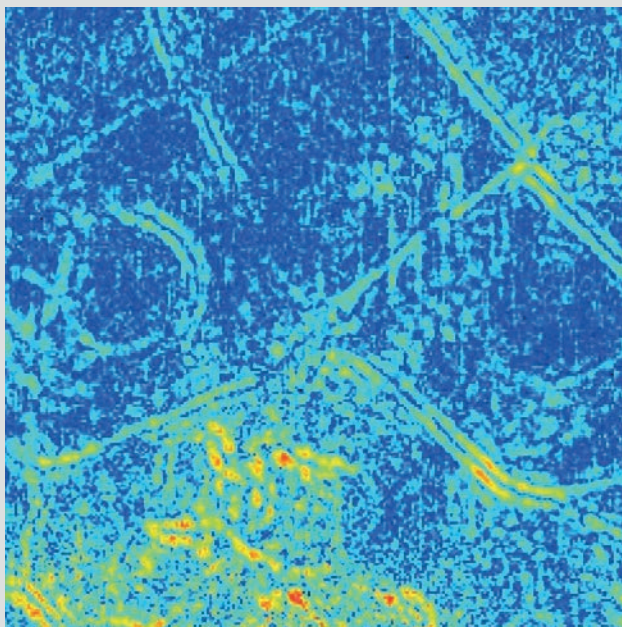


Arabidopsis in agar expressing GFP, imaged using SPIM with the Prime 95B uncorrected for camera noise. Pair correlation function showing obstacles to diffusion due to the cell walls in regions of strong anisotropic motion.

The parallel lines of signal clearly define the boundaries of diffusion at each side of the cell walls. Unlike super-resolution microscopy or other methods, fluctuation correlation spectroscopy images the diffusion itself, rather than the cellular structures which are often not visible.

Time series of 2100 frames over 10.5s, 256x256 pixels, 140nm effective pixel size.

**REFERENCES**

Introduction to Fluorescence Correlation Spectroscopy (PDF)

Fluctuation Correlation Spectroscopy in Cells, Determination of Molecular Aggregation (PDF)

Globals Software, G-SOFT Inc.

Additional information about the Laboratory of Fluorescence Dynamics is available at:
https://www.lfd.uci.edu/

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# PHOTOMETRICS ®

# Single Molecule Tracking PALM

**Dr Thomas Etheridge, Professor**
Antony Carr Group, Genome Damage and Stability Centre
University of Sussex

**BACKGROUND**

The Carr Lab at the Genome Damage and Stability Centre, University of Sussex, investigates DNA metabolism processes such as DNA replication and repair. They are interested in the challenges cells face during DNA replication and the cellular processes that help the cell overcome replication fork stalling or collapse. To study this, they focus on the behaviors and interactions of individual proteins involved in DNA replication in both fission yeast and human cells.

**CHALLENGE**

One approach used to examine the DNA association of proteins is to break open the cell, extract the proteins and perform western blotting. However, this approach reveals nothing of the dynamics of association, and destroys cells in the process.

A more demanding alternative is to use an imaging technique capable of single protein resolution. To this end, Dr. Etheridge uses Single Molecule Tracking PALM to observe the association of proteins with the DNA in real time, non-invasively, in both yeast cells and human cells. Single fluorescent molecules move quickly and give off very little light so this technique requires high speed and the highest sensitivity.

The fluorescent proteins used in the yeast cells are very dim and, until now, Dr. Etheridge has been using an EMCCD camera to observe them. However, the field of view given by his EMCCD camera is quite small. For human cells, brighter synthetic dyes can be used so dynamics can be observed with better temporal resolution. Unfortunately, the lower speed of his EMCCD camera doesn't allow for this.

> "Compared to an EMCCD, the Prime 95B gives us a field of view large enough for our most demanding experiments as well as faster frame rates."

**SOLUTION**

The back illuminated Prime 95B scientific CMOS camera has the speed and field of view advantages expected of CMOS cameras, but with the sensitivity to rival or beat EMCCDs. This gives researchers using single molecule techniques unprecedented low-light detection and temporal resolution.

Dr. Etheridge shared, "To image the demanding yeast cells with our EMCCD, we were having to do multiple experiments on different areas of the sample to generate enough data for it to be worth processing. With the field of view of the Prime 95B, we can gather enough cells in the field of view to process in a single shot. In the human cell system, using the brighter dyes, we can image our favorite proteins at much faster speeds."
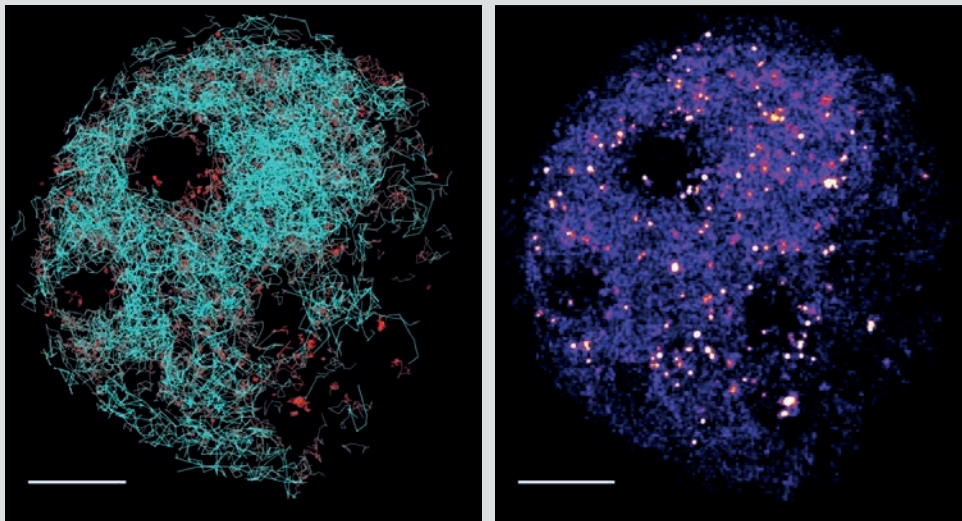


**Human cell nucleus with DNA repair complex labelled with HaloTag-PA-JF549**.
*Left:* Superimposed tracks of detected molecules. Red = DNA-bound, static molecule.
Cyan= freely diffusing complexes. *Right:* Localization map of complexes. Hot spots indicating locations of DNA bound molecules. Scale bar: 5μm.

Additional information about the Genome Damage and Stability Centre at the University of Sussex is available at: http://www.sussex.ac.uk/gdsc/

# Increasing CMOS Camera Sensitivity Through Back-Illumination

## Introduction

Scientific camera sensitivity is determined by three main factors; quantum efficiency (QE), pixel size and noise characteristics. Quantum efficiency is the measure of the effectiveness of the camera to produce electronic charge (electrons) from incident photons, where a higher QE results in the conversion of more photons to electrons of signal. Electrons go on to be converted into a digital signal that can be read by a computer and visualized. Pixel size relates to the physical area of the pixel, where a larger pixel can collect more photons and therefore deliver more electrons of signal. Noise characteristics, particularly read noise at low-light levels, determine how much the electron signal can fluctuate per pixel. The higher the signal over the noise, the higher the signal-to-noise ratio and therefore image quality. There will be no sample detection if noise exceeds signal.

This technical note will focus on quantum efficiency and how it was made possible to increase sensitivity on CMOS cameras by increasing QE to an almost perfect, 95% through the process of back-illumination.

## Quantum Efficiency

Quantum efficiency can be defined as the percentage of electrons produced from the number of incident photons. For example, if 100 photons hit a 95% quantum efficient sensor, 95 electrons would be theoretically generated. Likewise, if 100 photons hit a 65% quantum efficient sensor, 65 electrons would be theoretically generated.

This process is a property of the photovoltaic effect, where light energy (photons) incident on the silicon substrate of a pixel creates electron-hole pairs. These electrons are then read out by the device and converted into a digital signal that can be interpreted by a computer.

There are many conditions that affect the photovoltaic effect and thereby determine the number of electrons generated by a single photon. Of these, the two most important conditions are the absorption coefficient and the chemical and physical properties of the material on the sensor surface. As these conditions determine the number of electrons that can be generated by a single photon, they directly influence the quantum efficiency of the camera.

### Absorption Coefficient

The absorption of photons into the silicon substrate of the pixel is wavelength dependent. This is the reason why quantum efficiency is shown on camera datasheets as a curve, such as the curves shown in Figure 1.
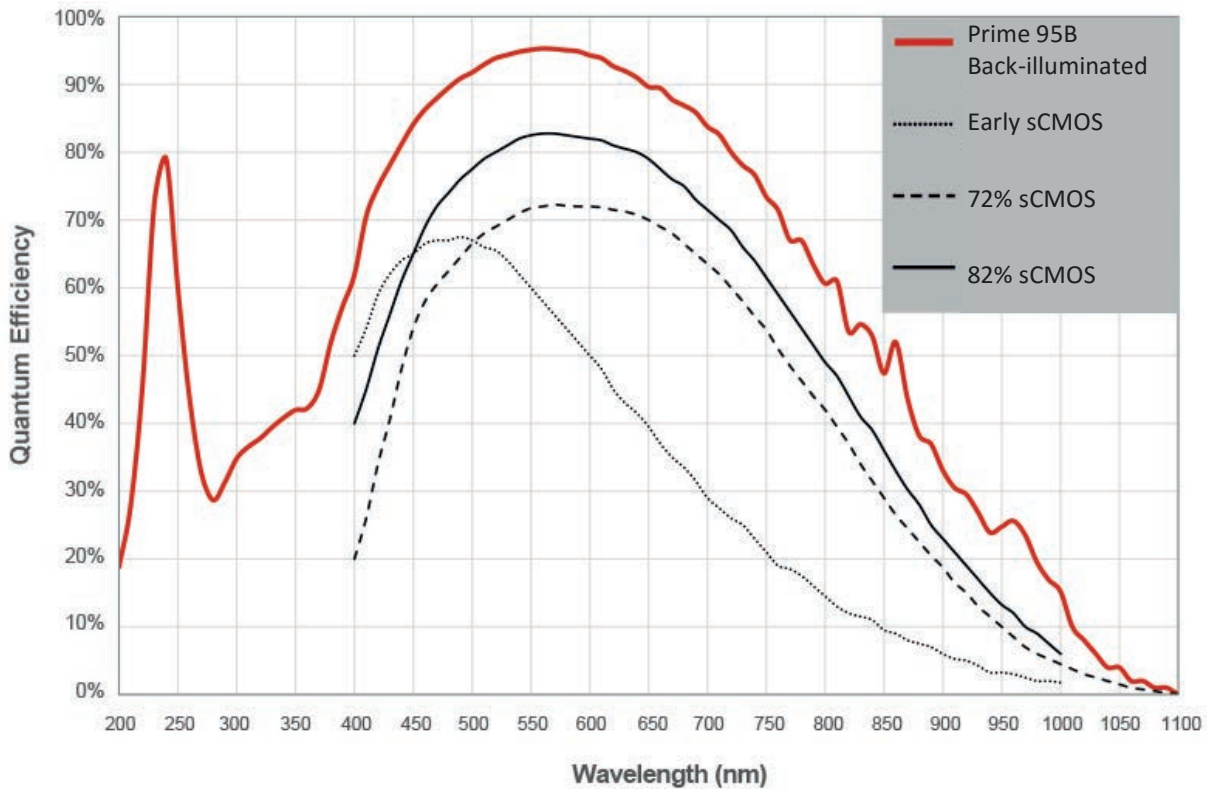
**Figure 1: Comparison of quantum efficiency curves of some typical CMOS cameras.**
*Adapted from Princeton Instruments, Kuro sCMOS*

Quantum efficiency is higher in the green and yellow region (500 nm – 600 nm) because these wavelengths penetrate well into the region of the silicon substrate of the pixel where the photovoltaic effect takes place (Figure 2).
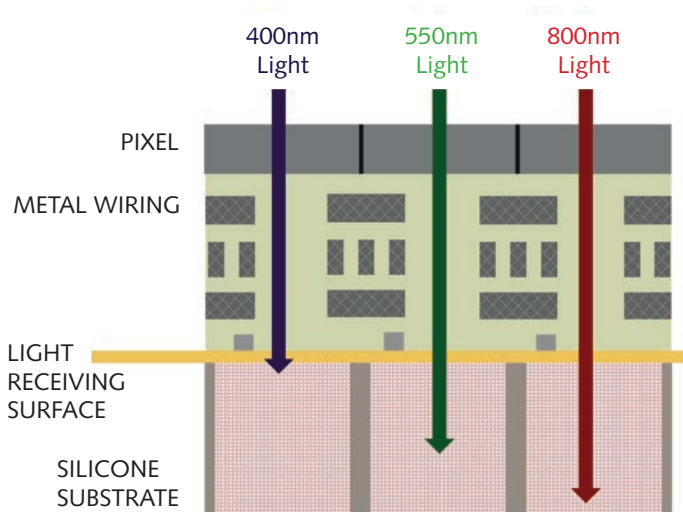


**Figure 2: Light wavelength and silicon penetration.**
*Adapted from Sony, back-illuminated CMOS image sensor*

Shorter wavelengths do not penetrate deep enough so many photons are lost before reaching the silicon substrate. At the other end of the spectrum, longer wavelengths penetrate too far so photons pass straight through the silicon substrate.

There is usually a quantum efficiency cut-off at around 400 nm where the majority of the photons are lost before they can reach the silicon substrate.

There is also a critical wavelength, usually at around 1100 nm, where incident photons have insufficient energy to produce an electron-hole pair so no signal can be generated.

## The Sensor Surface

On CMOS sensors, a certain fraction of the pixel surface is covered in the metal tracks, wiring and transistors (the circuitry) necessary to collect and transport charge (Figure 3). This has the unfortunate side effect of making that area completely light insensitive.



PIXEL

METAL WIRING

LIGHT RECEIVING SURFACE

SILICONE SUBSTRATE

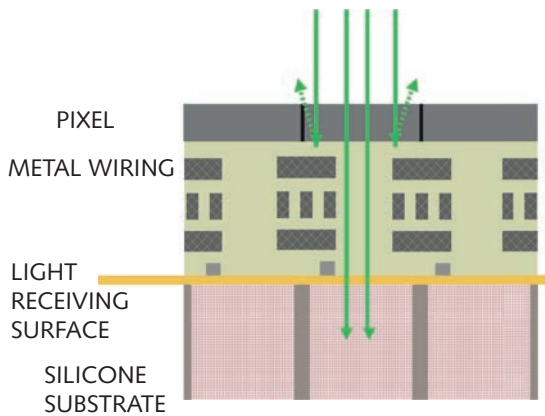**Figure 3: Photons unable to pass the metal tracks, wiring and transistors present in front of the CMOS sensor.**



PIXEL

METAL WIRING

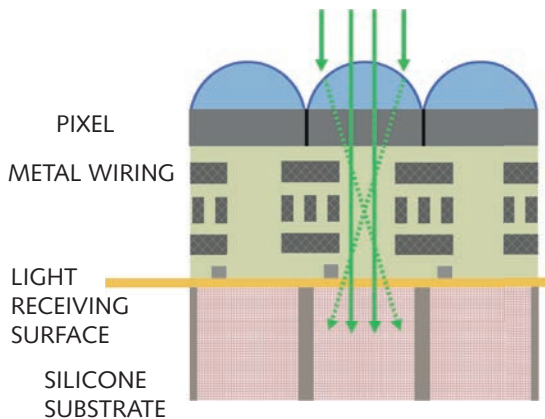LIGHT RECEIVING SURFACE

SILICONE SUBSTRATE

**Figure 4: The use of microlenses to increase quantum efficiency of CMOS sensors.**

The photons landing on this area can't reach the silicon substrate because they are physically impeded. These photons, therefore, won't be converted into electrons and so the quantum efficiency will be negatively affected. CMOS sensors using this architecture typically have a peak QE of 82%, so almost a fifth of the photons arriving at the pixel never make it to the silicon substrate.

The highest quantum efficiency sensors using this architecture was made possible through the addition of microlenses on the sensor surface (Figure 4). The microlenses are designed to focus the incident light away from the circuitry and onto the silicon substrate. This effectively increases the number of photons reaching the silicon substrate and therefore increases QE. CMOS Sensors using this architecture claim a QE of up to 82%.

A downside of microlenses, however, is that they are most effective when the incident angle of light is normal to the sensor surface. When light enters the sensor from any other angle, the effectiveness of the microlenses can become severely reduced. This means that the reported QE increase of a CMOS camera with microlenses may not accurately reflect the real QE increase.

Regardless of the issues with microlenses, the real problem to overcome is clearly the position of the circuitry. To address this, sensor manufacturers have recently started creating back-illuminated CMOS sensors. By inverting the sensor and bringing light in from the back, the circuitry can be avoided completely.

## Back-Illumination

A back-illuminated sensor is one that has essentially been flipped over so light enters directly into the silicon substrate rather than having to pass through the circuitry (Figure 5). Any light loss due to objects on the sensor surface is thereby eliminated.
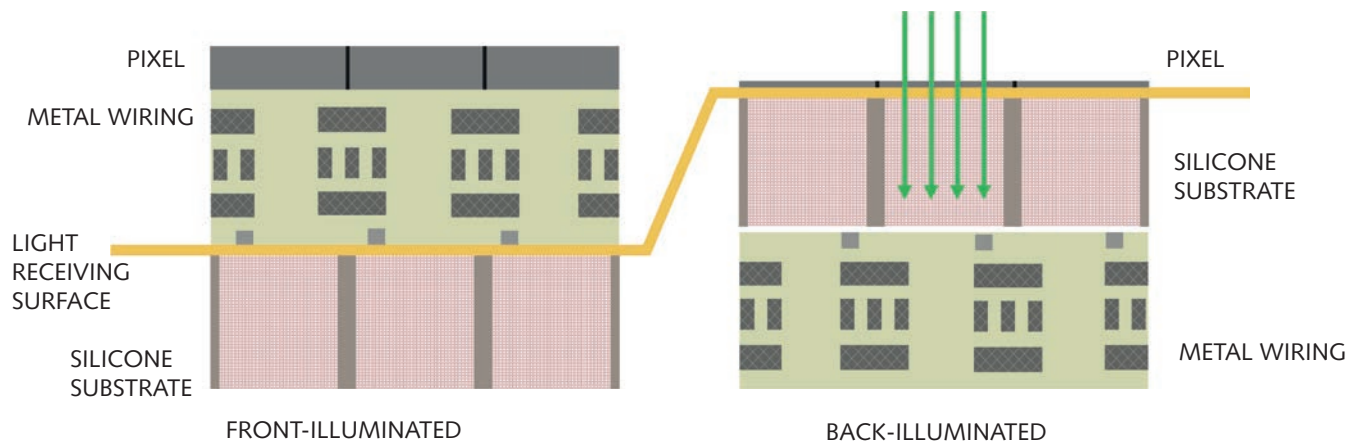


**Figure 5: Comparison of front and back illuminated sensors.**

To allow the photons to penetrate deep enough into the silicon substrate to be converted into electrons, the silicon must also be thinned at the back. For this reason, a back-illuminated sensor may also be referred to as a back-thinned sensor.

The result is a sensor with an almost perfect, 95% quantum efficiency at its optimum wavelength. This can be seen in Figure 1, which shows the QE curve of the Prime 95B back-illuminated CMOS compared to front-illuminated CMOS devices, such as the 82% CMOS camera with microlenses.

Another advantage of back-illumination, highlighted in Figure 1, is the ability to achieve a high QE with shorter, UV wavelengths of light. It's possible to achieve UV light detection on other CMOS devices but, as stated earlier, the fill-factor becomes limiting. This problem is completely overcome with a back-illuminated sensor. Moving the circuitry below the silicon substrate allows the fill-factor to reach 100%, granting both a high UV response and a high QE over a wide spectral range.

To test the theoretical increase in quantum efficiency of a back-illuminated sensor over a front-illuminated sensor, we performed a simple experiment designed to compare the sensitivity of multiple cameras.

# Control of Noise and Background in Scientific CMOS Technology

## Introduction

Scientific CMOS (Complementary metal–oxide–semiconductor) camera technology has enabled advancement in many areas of microscopy imaging. However, this technology also poses problems that camera manufacturers need to solve to produce a device capable of accurate quantitative imaging. To achieve this, several features of CMOS sensors have to be understood and then corrected. In this technical note, we'll briefly discuss the most important points to consider when producing a scientific CMOS camera.

The main consideration is correction of noise, which will always occur during the acquisition of an image. Noise is the uncertainty which accompanies the acquired signal and can be divided into two major groups: camera-related noise and sample-related noise. As a camera manufacturer, there's little we can do to improve the sample-related noise but our goal is to minimize all camera-related noise.

## Sample-Related Noise

### Photon Shot Noise

Photon shot noise is the inherent natural variation of the incident photon flux - there is always uncertainty associated with the process of emission of photons from a fluorescent structure upon excitation (quantum nature). Photon shot noise follows a Poisson distribution and has a square root relationship between signal and noise where:

$$Photon\ shot\ noise = \sqrt{Signal}$$

All values are displayed in electrons (e⁻).

This noise cannot be improved by advances in camera design as it is a physical phenomenon that can't be removed.

## Camera-Related Noise

One of the main differences between standard CCD/EMCCD cameras and scientific CMOS cameras is the signal readout structure. A CCD/EMCCD camera converts charge (electron signal) to a voltage at the preamplifier, the voltage is then digitized by the Analogue to Digital Converter (ADC) to produce a digital signal that can be read by a computer (figure 1). CCD/EMCCDs, therefore, have a single preamplifier and ADC which digitize every single pixel one by one.

CMOS sensors, on the other hand, have a capacitor and amplifier on every pixel to convert charge into a voltage. This voltage is then digitized by an ADC at the end of every column (figure 2). This greatly increases the readout speed of the sensor but introduces other complications.

## Read Noise

Read noise is the noise introduced as the signal is read out i.e. passed through the preamplifier and ADC.

The architecture of CCD/EMCCD sensors (Figure 1) typically consists of a single preamplifier to convert charge into voltage. This means that every single pixel is treated the same way and so read noise will follow a Gaussian distribution. All pixels will be affected and fluctuate by a similar degree.
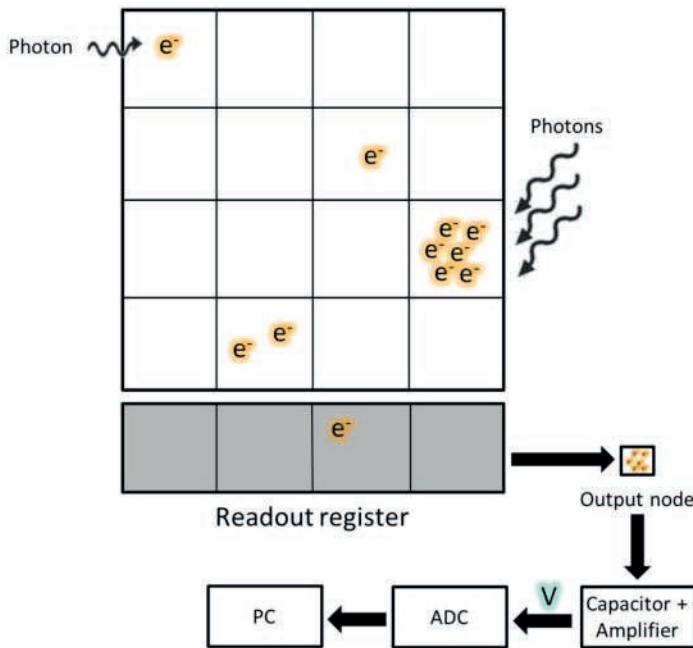


**Figure 1: CCD architecture.**
The number of electrons created is directly proportional to the number of photons hitting the pixel. After exposure, electrons are moved down, row by row, until they reach the readout register. The readout register shuttles the row of electrons one at a time into the output node which is connected to a capacitor and amplifier.

On CMOS sensors, each pixel has its own individual readout structure to convert charge into voltage. Furthermore, each column has its own ADC. Some scientific CMOS cameras also use a split sensor design where there are two ADCs instead, one for the top half of the chip and one for the bottom half. Although this results in a great increase in readout speed, a consequence of having multiple readout structures is that the read noise is now a distribution
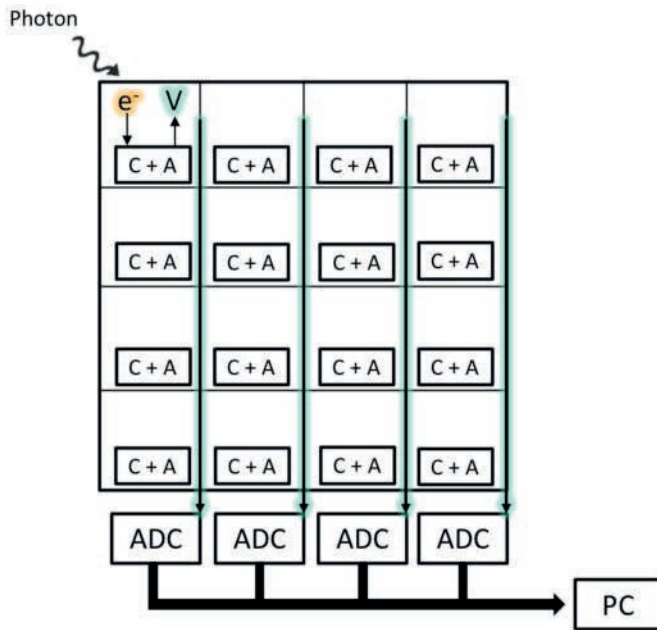
**Figure 2: Scientific CMOS architecture.**
Photons hit the pixels and create electrons, individual capacitors and amplifiers are on every pixel. The generated voltages are sent down the whole column to the analogue to digital convertor (ADC) and the digital signal is read by a computer. This makes CMOS cameras much faster because they have one amplifier per pixel.

The true distribution of read noise which can be measured on CMOS sensors is not Gaussian but more of a skewed histogram. For this reason, read noise will be quoted on datasheets both as root mean square (RMS) and median. If a datasheet reports a median read noise of 1 electron, this means half of the pixels will report less than 1 electron read noise and half will report more. However, within this group, some pixels may report very high read noises such as three electrons or more. For this reason, the datasheet will also report an RMS value which is a far more meaningful description of read noise. This value will represent a true mean read noise and will, therefore, be higher than the median.

## Pattern Noise

Pattern noise (Figure 3) is a noticeable pattern of 'hot' (bright) and 'cold' (dark) pixels in the background of the image and is produced regardless of illumination conditions. It goes hand-in-hand with CMOS read noise variation and a difference in the background offset (bias) value of individual columns, it's caused by small differences in the responsivity of individual pixels on the sensor.
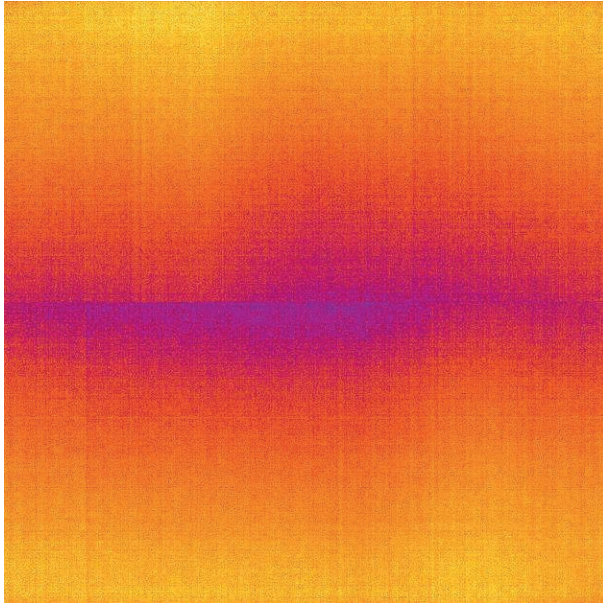
**Figure 3: Fixed pattern noise.**
Fixed pattern noise on a typical scientific CMOS camera with a split sensor. Variation across the background is around 6 e⁻.

## Roll Off

Roll off (Figure 4A) is a phenomenon which can often be observed on CMOS sensors making use of split sensor technology. The roll off highlights the seam between the two sensors and displays a bias dip across it. This variation in the bias and the consequent lack of linearization makes quantitative imaging very difficult.

Newer, single-read and back-illuminated sensors such as those used in newer Photometrics scientific CMOS cameras (Figure 4B) prevents this problem from occurring and provides a better environment for quantitative imaging.
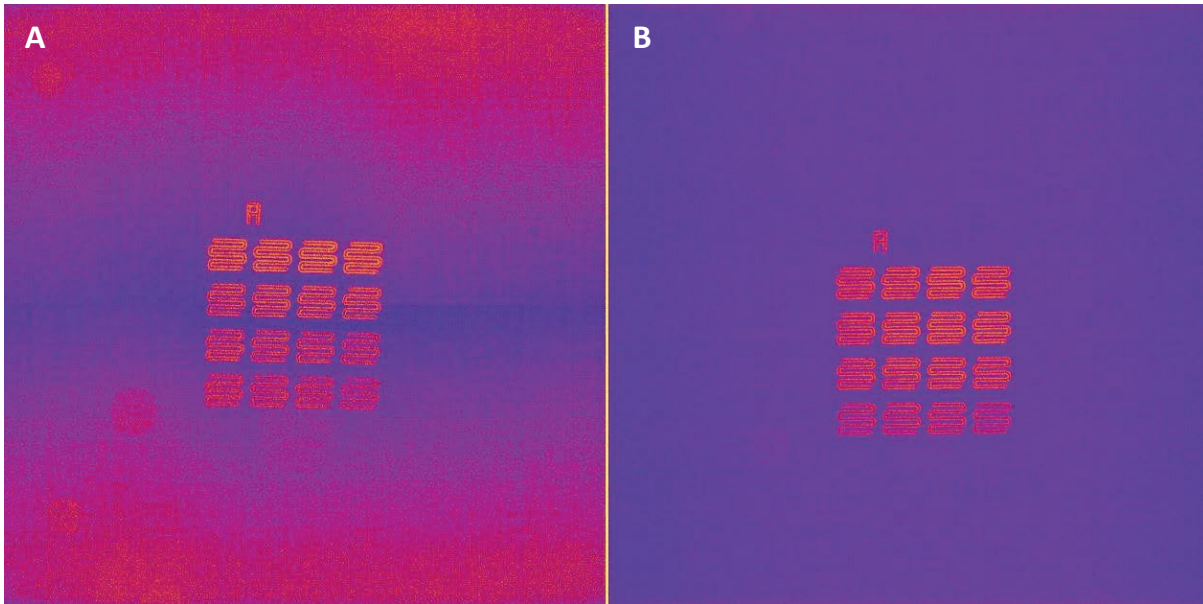
**Figure 4: Roll off comparison**
A) Current, standard, split sensor scientific CMOS camera and B) the GPixel GSENSE2020BSI sensor used in the Photometrics Prime BSI.

## Dark Current

Dark current arises from charge building up on the sensor caused by thermal energy. Crucially, this noise is light independent. Because dark current is a thermal effect, cooling (e.g. with a Peltier element) is used as a measure to counteract this problem. Typically, dark current can be halved for every 7°C of cooling.

All high-performance scientific CMOS cameras will come with a dark current specification on the data sheet. For instance, a Photometrics Prime BSI has a dark current specification of 0.5 e$^-$/p/s, resulting in 1 electron/pixel generated upon a 2 s exposure time.

## Clock Induced Noise & Random Telegraph Noise

It's possible to run EMCCD cameras at a very high clocking speed to achieve high frame rates but this introduces additional noise which can add up to 5 e$^-$ of noise per pixel. This is called clock induced charge (CIC) and occurs when an electron is inadvertently generated without being induced by an incident photon. CIC has to be corrected for by using a spurious noise filter to identify the affected pixels and replace the measured signal by taking a mean value from its neighbours (nearest-neighbor correction).

On a CMOS sensor, the increased speed reduces the need to run the pixel clock of the chip at or over the maximum. As a result, the effect of clock induced noise is also greatly reduced.

However, CMOS sensor architecture suffers instead from random telegraph noise (RTN) or 'salt'n'pepper' noise (Figure 5). This noise is caused by charge moving in and out of pixel defects. The output from a single pixel thereby fluctuates between bright, average, and dark states many times over the course of an acquisition. Similar correction as that used for CIC applies to RTN and it is corrected on the sensor in engineering.
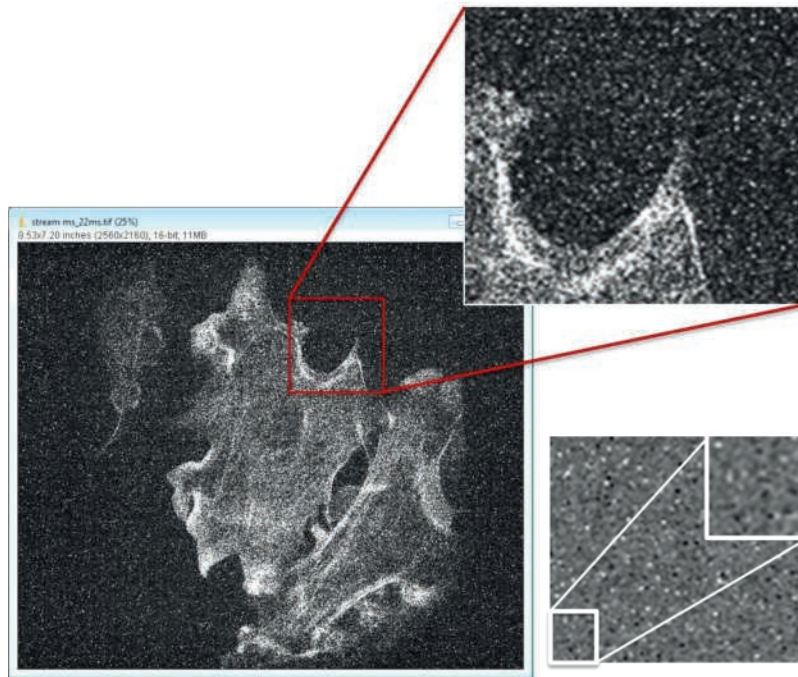


**Figure 5, Random Telegraph Noise.**
Example of random telegraph noise displaying the fluctuation between bright, average and dark pixel states.

### Correlated Noise

Correlated noise occurs due to capacitive coupling on the sensor. This is where energy is transferred between pixels by means of displacement current which causes neighboring pixels to share charge, effectively correlating the measured pixel signal. This results in an overestimation of the camera system gain which, by extension, overestimates the read noise, dark current and quantum efficiency of the camera.

This is also corrected in engineering until it is certain that all charge originates on the pixel and isn't being influenced by the charge of any surrounding pixel.

Further Considerations

Binning is a common method to increase speed and sensitivity on CCD/EMCCD cameras. When binning, the signal of a 2x2 or 4x4 square of neighbouring pixels is combined. This increases the signal by summing the charge of all these pixels and as a result, fewer effective pixels require digitization which decreases the speed bottleneck at the ADC. On CCD/EMCCD cameras, summing the charge of neighboring pixels occurs before the ADC so read noise is only applied once.

In contrast, the parallelization of the read out on scientific CMOS cameras will apply read noise to each column. The combination of neighbouring pixels' signal will occur afterwards. Pixels from the same column will combine their information before read noise is applied but combining the information from pixels originating from neighbouring columns will sum their according read noise. Effectively, binning on scientific CMOS technology is partially performed in software.

As a result, binning does not have the same speed and noise benefits on scientific CMOS as it does on CCD/EMCCD cameras. Furthermore, scientific CMOS fixed pattern noise will be substantially amplified by binning. On early scientific CMOS cameras, this was a problem which couldn't be solved. However, improved correction algorithms for CMOS sensors have minimized this effect at the current state of this technology.

## Photometrics Solution for Minimizing Camera Noise

Photometrics' solution to the problems of camera noise is to carefully characterise each individual sensor and fine-tune for all eventualities. Scientific CMOS technology is in this respect very challenging as pixel-to-pixel variations, but also column-to-column variations need to be taken into consideration. Equalling bias value, linearizing the response and gain of each individual pixel across the chip is key to achieve those targets. The aim is for all columns to be perfectly balanced.

Other levers to unify the behaviour across the chip is tuning the use of facilitated correlated double sampling. Where two signals per pixel are effectively measured during acquisition: (a) bias for the offset and (b) amount of light. The difference between the two will effectively give the real signal.

Background Event Reduction Technology (BERT) enables researchers to identify the pixels that are likely to contain spurious event data and then correct the data if desired. Originally designed for EMCCD cameras, this technology has also been optimized for and applied to Photometrics scientific CMOS cameras. BERT removes spurious events which could not have been generated via incident photons in real-time. It allows the image captured to more accurately represent what the sample is and remove artefacts.

Spuriously large pixel values which could not have come from the actual sample being imaged are replaced with a best approximation by taking the mean of the surrounding pixels and absolutely minimizes the influence of thermally-induced amplified events on acquired data.

# Maximizing Microscope Field of View

## Introduction

Microscope field of view (FOV) is the maximum area visible when looking through the microscope eyepiece (eyepiece FOV) or scientific camera (camera FOV), usually quoted as a diameter measurement
(Figure 1). Maximizing FOV is desirable for many applications because the increased throughput results in more data collected which gives a better statistical measurement for detecting subtle effects and also decreases time needed at the microscope.

The FOV of a microscope is ultimately limited by a number of factors, such as the objective lens, the tube-diameter of the microscope's internal optical-system, the eyepieces, the scientific camera sensor size and the camera mounting adaptor.

It's usually possible to find the maximum FOV of the microscope by referring to the field number (FN) displayed on the eyepieces and on some objective lenses. The field number is simply the maximum FOV of the objective or eyepiece in millimetres, so an objective lens with a field number of 18 would have a maximum FOV of 18 mm. However, the field number always assumes no magnification so to calculate the actual FOV, the field number should be divided by the objective magnification:



**Figure 1:** Microscope field of view measured as a diameter

$$FOV = \frac{Field\ Number}{Objective\ Magnification}$$

A 20x objective with a field number of 18 would actually have a FOV of 0.9 mm. Likewise, a 100x objective with a field number of 18 would have a FOV of 0.18 mm. The more an object is magnified, the smaller the field of view will be. Therefore, when looking to increase FOV, one of the first considerations should always be whether it's possible to decrease magnification (Figure 2).
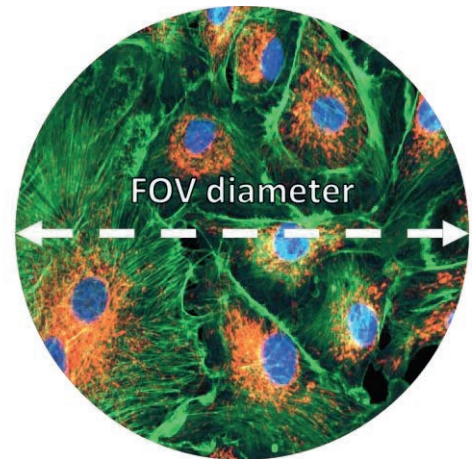
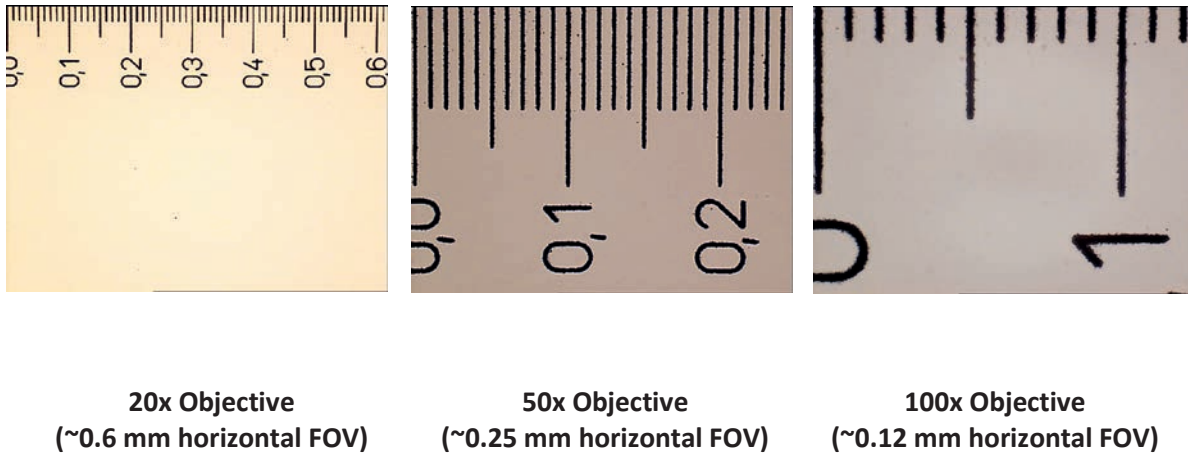| 20x Objective | 50x Objective | 100x Objective |
|---|---|---|
| (~0.6 mm horizontal FOV) | (~0.25 mm horizontal FOV) | (~0.12 mm horizontal FOV) |

**Figure 2:** Reduction in field of view with increasing magnification. The visible length of the graticule measures ~0.6 mm under 20x magnification but ~0.25 mm at 50x and only ~0.12 mm at 100x.

## Matching Scientific Camera FOV to Microscope FOV

Using the field number to calculate microscope FOV works well when imaging using the eyepieces but not when imaging using a scientific camera. Like most digital cameras, scientific cameras use square or rectangular sensors. This means that a scientific camera cannot capture the whole, circular FOV that the microscope is capable of. Instead, the camera FOV must fit inside the microscope FOV (Figure 3).

Camera specification sheets will display the camera FOV as a diagonal measurement (usually in millimeters). Ideally, the diagonal camera FOV should match the diameter of the microscope FOV to capture as much of the available image as possible. However, this does mean that the horizontal and vertical FOV of the camera will be less than the microscope diameter.

It's possible to use a camera with a larger diagonal FOV than the microscope to capture the entire microscope FOV (Figure 4). However, this is not optimal as there will be substantial vignetting at the corners of the image. Ideally, when choosing a scientific camera, it should have a diagonal FOV that matches the specifications of the microscope it will be used with.
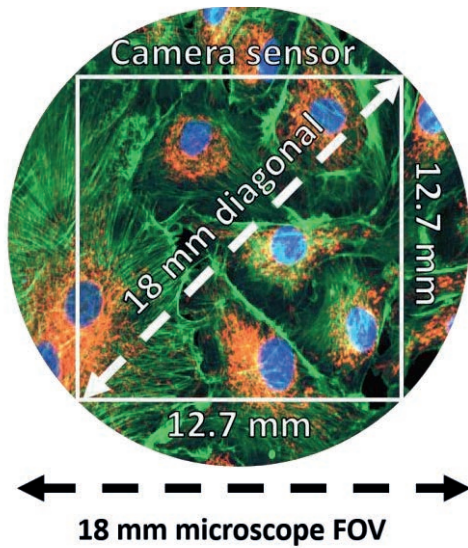
**Figure 3:** A scientific camera with an 18 mm diagonal FOV ideally fits an 18 mm microscope FOV. The x and y sides of the camera sensor measure 12.7 mm to allow for this diagonal FOV.
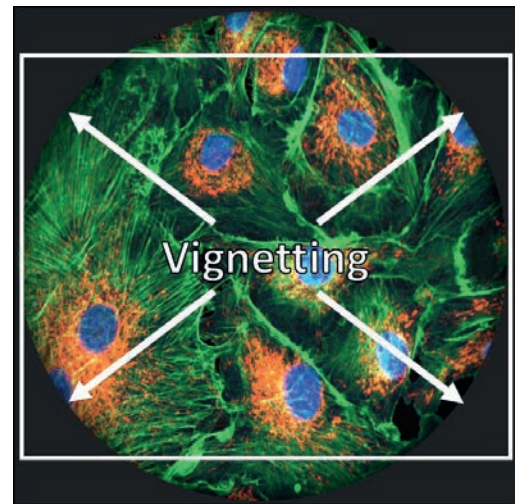


**Figure 4:** A camera sensor with a larger diagonal FOV than the microscope FOV would show considerable vignetting in the corners of the image.

## Matching Adaptor FOV to Camera and Microscope FOV

A microscope C-mount or F-mount adaptor is needed to connect a scientific camera to the microscope camera port. The mount threading is standardized which means that a C-mount adaptor will connect to all scientific cameras that connect via C-mount. However, the adaptors are microscope specific which means that although any C-mount camera will connect to a C-mount adaptor, the adaptor will only fit microscopes of the matching brand.

Adapters can have lenses in them to magnify or demagnify the image before it reaches the camera. This can be used to better match the camera FOV to the microscope FOV. For example, if the camera has an 11 mm diagonal FOV but the microscope is capable of an 18 mm FOV, a 0.67x adaptor would demagnify the image and allow it to be displayed on the 11 mm camera. However, this increase in FOV comes at the cost of reduced resolution.
If the goal is simply to attach the camera to the microscope, a 1x adaptor contains no additional lenses and provides no additional magnification or demagnification. This is often the preferred method as it introduces no additional lenses into the system. Every extra lens reduces the number of photons reaching the camera by 3-4% so many researchers will try to avoid this.

Adaptors can also affect the microscope and camera FOV depending on the type of adaptor used. A C-mount adaptor is the most popular microscope camera adaptor and is restricted to a maximum 22 mm FOV. The F-mount adaptor is a larger format adaptor capable of reaching >30 mm FOV.

The development of larger FOV microscopes and scientific cameras that can take advantage of the F-mount is relatively recent - at the time of writing only one commercially available 25 mm microscope exists. Most modern microscopes have a 19 mm or 22 mm FOV and are therefore still able to use the C-mount. The largest format spinning disk confocal systems are also limited to a 22 mm FOV.

## Choosing a Camera to Maximize Microscope FOV

At Photometrics, we aim to create cameras that can optimally match the FOV of all modern microscopes (Table 1). For this reason, the Prime 95B Series comprises a 19 mm camera, a 22 mm camera and a 25 mm camera. Additionally, the Prime BSI and Iris 9 both fit a 19 mm microscope FOV and the Iris 15 fits a 25 mm microscope FOV.

| Camera | Microscope FOV | | |
|---|---|---|---|
| | **19 mm** | | |
| **Prime 95B™** | ✔ | | |
| **Prime 95B 22mm™** | ✔ | ✔ | |
| **Prime 95B 25mm™** | | | ✔ |
| **Prime BSI™** | ✔ | | |
| **Iris 9™** | ✔ | | |
| **Iris 15™** | | | ✔ |

**Table 1:** Photometrics cameras are optimized to match any microscope with a FOV of 19 mm, 22 mm or 25 mm.

By recognizing that FOV requirements can be highly variable, we are able to better serve the needs of our customers and offer a broad range of camera FOV options.

## Conclusion

The maximum field of view of the microscope is affected by the objective lens, the tube-diameter of the microscope's internal optical-system, the eyepieces, the scientific camera sensor size and the camera mounting adaptor. For optimal imaging performance, it's best to match the microscope FOV to the scientific camera FOV to capture as much information as possible and avoid vignetting. Typical microscopes have a 19 mm, 22 mm or 25 mm FOV which is why Photometrics cameras are designed to match these specifications to offer the maximum field of view possible.

# PrimeEnhance™

## 2D Active Image Denoising

There are several sources of noise when imaging faint signal levels which can affect the Signal-to-Noise Ratio (SNR) of your measurement, the main types being dark noise, read noise and shot noise. Camera manufacturers make design choices to minimize the presence of noise in the image and to maximize the quality and SNR of the collected images. Dark Noise is reduced by cooling the sensor, and read noise is minimized through sensor performance and electronic design.

Photon shot noise however, is an inherent property of light. There is always a statistical variation in the number of photons (or photoelectrons) detected in a given time period. This uncertainty is dependent on the amount of signal photoelectrons being measured and has the statistical property of a Poisson distribution. This relationship is expressed as:

$$Shot\ Noise = \sqrt{Signal}$$

While shot noise increases with signal, it increases more slowly (as the square root). This results in SNR improving with light levels. At low light levels, SNR is low even with a perfectly acquired image.

| Signal Level (e-) | Shot Noise (e- RMS) | Percent of Signal |
|---|---|---|
| 5 | 2.23 | 44.8% |
| 10 | 3.16 | 31.6% |
| 50 | 7.07 | 14.1% |
| 100 | 10 | 10% |
| 500 | 22.36 | 4.5% |
| 1000 | 31.62 | 3.2% |

Table 1

At these lower signal levels, there have been only a few ways to improve SNR, each with a tradeoff.

- **Increase the exposure time and collect signal for a longer time**
  This allows for a higher signal level, reducing the impact of shot noise. The ability to image at a desired frame rate may be sacrificed, and the cell is illuminated for a longer time, increasing phototoxicity and photobleaching. Finally, if the exposure time is long enough, the noise from dark current can become a larger portion of the signal.

- **Average frames to reduce noise**
  This allows for a reduction in total image noise as a square root of the number of frames averaged. The ability to image at adequate frame rates will again be sacrificed, and is generally less productive than simply increasing exposure time.

- **Increase the excitation intensity**
  This allows for a higher signal level without trading off temporal resolution. The rate at which phototoxicity and photobleaching occurs is also increased, reducing cell viability.

A remaining technique for the reduction of noise is the use of a "denoising algorithm" that dynamically examines the image collected in order to separate and remove noise. The Prime™ family of cameras from Photometrics introduces a new real-time method for dynamic noise reduction called PrimeEnhance.

## PrimeEnhance

There are  many challenges when  processing data to  reduce noise such as preserving the quantitative nature of the recorded  pixel intensities, as well as  preserving key features like edges, textures and details with low contrast. Further, processing has to be accomplished without introducing new image artifacts like  ringing, aliasing or blurring.  Many algorithms are inflexible with different image types,  resulting in  these intrusive artifacts. Additionally, because noise tends to vary with the level of signal,  it is  difficult for many denoising algorithms to distinguish signal from  noise, and as  a consequence, small details tend to be removed.

Using an  algorithm invented at INRIA and  optimzed for fluorescence microscopy in  collaboration with the  Institute Curie, PrimeEnhance implements a 2D denoising process which evaluates and  processes incoming images to reduce the effects of photon shot noise at low signal levels. The algorithm also  preserves the finer details and features of biological samples, and  does not introduce image artifacts. One key facet of PrimeEnhance is  the quantitative nature of the algorithm, ensuring that intensity values remain  unchanged.

> *Patch-based nonlocal functional for denoising fluorescence microscopy image sequences.*
>
> Boulanger J, Kervrann C, Bouthemy P, Elbau P, Sibarita JB, Salamera J, IEEE Trans. *Med Imaging* 2010 Feb.

PrimeEnhance works by  being aware of each  camera's characteristics and  specifications. It  uses this knowledge to first evaluate the  image data and  perform a  variance stabilization transform, which  removes the dependency between the mean intensities and  their noise characteristics. Then a small  patch of pixels is  compared to similar sized patches in  iteratively increasing areas of surrounding pixels (neighborhoods). The pixels within the  neighborhood are selectively weighted based on  their similarity to the intensity values of the original patch,  and using these weighted corrections, the original patch  is updated. This process is  repeated through the entire  image, updating each  patch and  reducing the impact of shot noise.  Once this process has been  completed, the inverse variance stabilizing transform is applied to ensure that the quantitative nature of the  pixel  values is maintained.

## PrimeEnhance   Evaluation

Fluorescence  images were  acquired  with  (Fig  1a)  and  without (Fig 1 b)  PrimeEnhance  enabled,  to  demonstrate  its  functionality  and give  a proper  comparison.  The  following  image  was  acquired  with a  100ms exposure time. The image statistics are  avaiable in  Table 2.
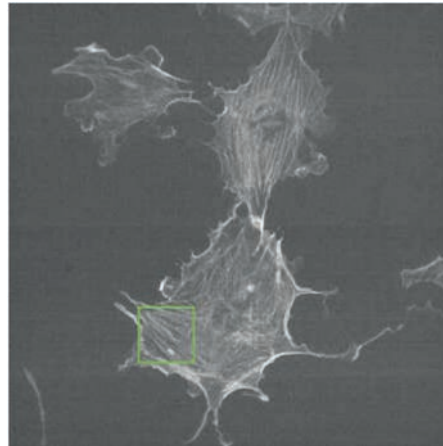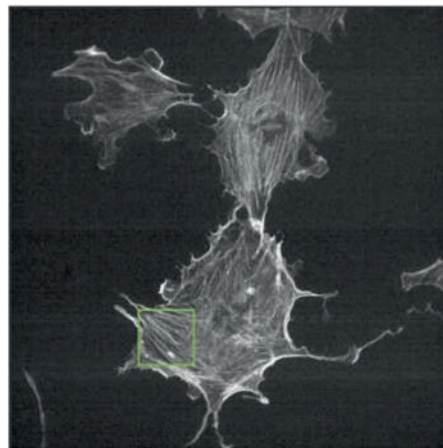


Figure  1a.
Original Image



Figure  1b.
PrimeEnhance

| | | Original   Image | PrimeEnhance |
|---|---|---|---|
| **Full Image** | Average  Intensity | 131.7 | 131.1 |
| | St  Dev | 6.68 | 4.78 |
| | Min Value | 72 | 71 |
| | Max Value | 363 | 362 |
| **Region  of Interest** | Average  Intensity | 138.8 | 138.2 |
| | St  Dev | 7.98 | 5.02 |
| | Min  Value | 87 | **88** |
| | Max Value | 241 | 241 |

Table  2

As shown  by  the intensities, the mean  intensity values, minimum intensity value,  and  maximum intensity value remain essentially unchanged  between the original
noisy image and  the denoised  image - ensuring that all measurements made remain quantitative and  are  relatable to each  other. The standard  deviation has  been  reduced, indicating the removal of noise.

A difference image (Fig 1c) between the original and PrimeEnhance image shows that only noise has been removed by PrimeEnhance, with the brighter regions showing higher noise levels in keeping with the relationship discussed in the introduction.

Figure 2a and 2b provide an increased zoom level on the structures within the cell, and show that features are preserved while no artifacts have been generated. The line profiles demonstrate PrimeEnhance's ability to reduce the shot noise present in the image, extracting features that were previously undistinguishable from the noise.
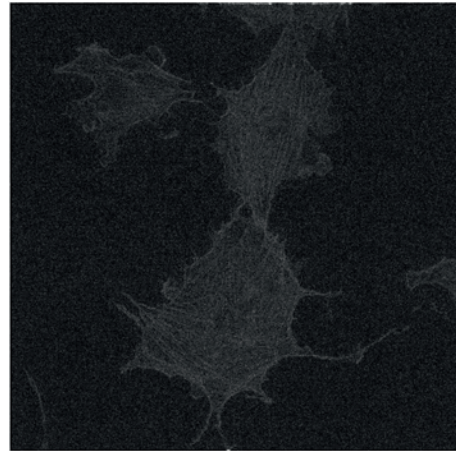


Figure 1c. Difference Image
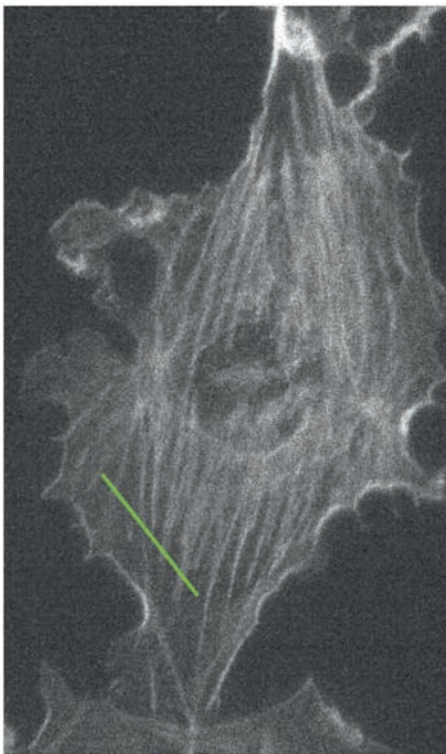


Figure 2a. Original Image
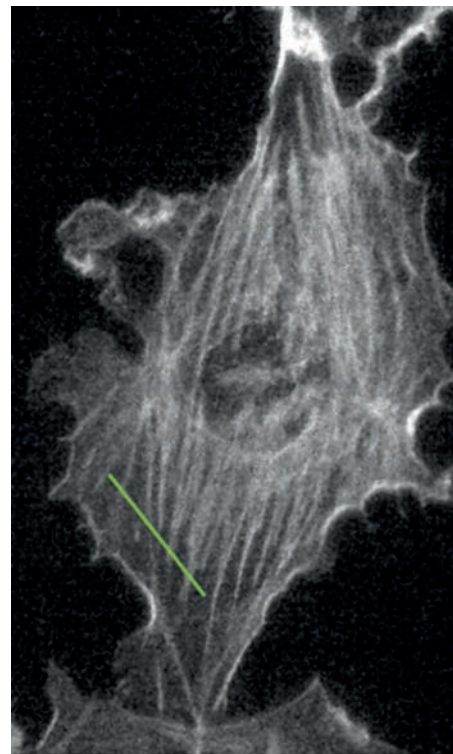


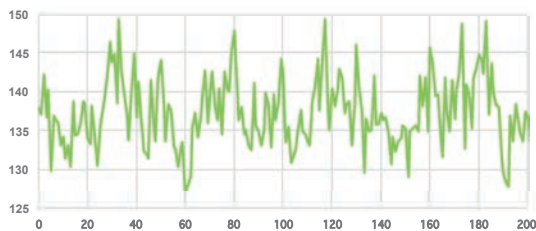Figure 2b. PrimeEnhance



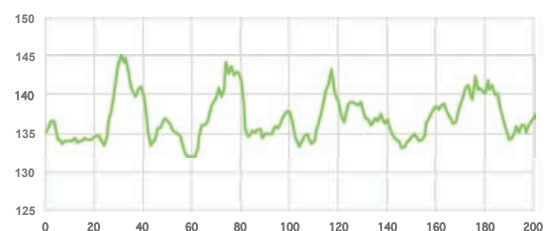Figure 2c. Line Profile for Original Image



Figure 2d. Line Profile for PrimeEnhance

## PrimeEnhance  Experimental  Impact

By  increasing  the  effective  signal  to  noise  in  each  frame,  it  is  possible  to  acquire  high  quality  images  at  lower exposure  times,  reducing  the  effects  of  phototoxicity  and  photobleaching  on  samples.  The  following  are  images  acquired  of a faint samples with a 100ms exposure compared to images acquired with an  800ms exposure.

The comparison  between the 800ms exposure and the 100ms PrimeEnhance exposure, as  evidenced  by the line-profiles, demonstrates the increase of image and  data quality possible with  PrimeEnhance at 8X lower exposure times.
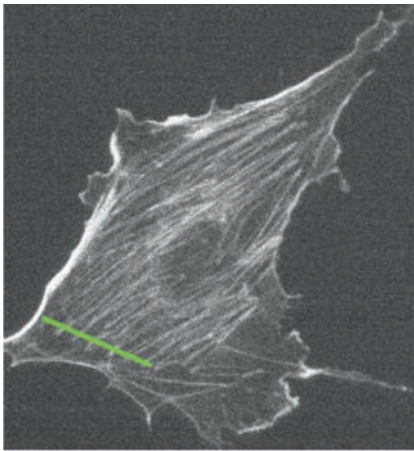


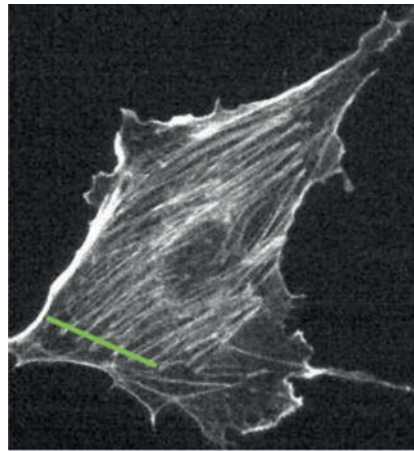Figure   3a.  Raw Image at 100ms exposure



Figure   3b.  PrimeEnhance at 100ms exposure
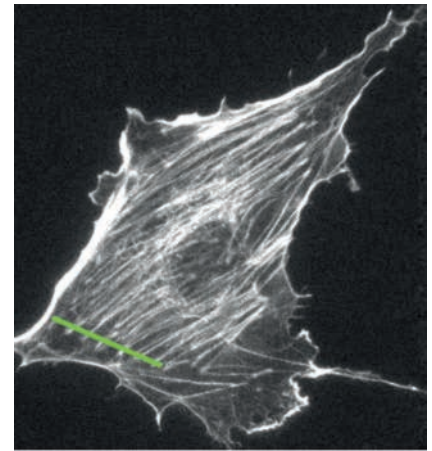


Figure   3c.  Raw Image at 800ms exposure
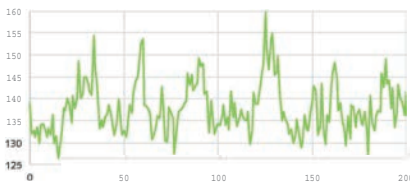


Figure   3d.  Line  Profile of Raw Image at 100ms
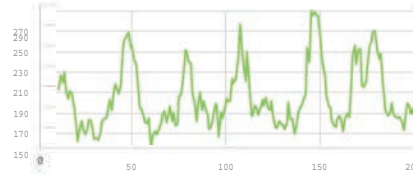


Figure   3e.  Line  Profile of PrimeEnhance Image at 100ms
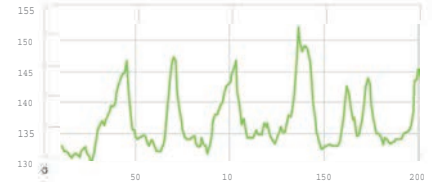


Figure   3f.   Line  Profile of Raw Image at 800ms

## Conclusion

PrimeEnhance provides a real-time quantitative increase in  signal to noise ratio by  reducing the effects of photon shot noise at low light levels,  which improves the quality of images and  data. The finer features within images are preserved and  no  unwanted processing artifacts are generated. A comparison  between a 100ms denoised image and  a 800ms standard fluorescence image shows equivalent results in  image quality, indicating the ability to significantly reduce  exposure times while maintaining the quality of captured  data.

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PHOTOMETRICS®

# PrimeLocate™

An excess of image data may seem like an abundance of riches, except when the data does not contain useful information. Then it becomes data that must be stored, processed and ultimately discarded. The Prime™ family of cameras from Photometrics solves this problem at its source with PrimeLocate, one if its advanced, real-time processing features. When enabled, PrimeLocate automatically finds and transfers regions of interest to the host computer, either over PCIe or USB3. In this way massive amounts of data containing no useful information is eliminated from consideration. *PrimeLocate can identify up to to 512 of the brightest 3x3 patches within each image and transmit only that portion of the image, reducing the data and processing requirements from 6.7Gbps down to 100Mbps.*

The PrimeLocate algorithm is most appropriate for localization microscopy methods such as STORM and PALM. The hallmark feature of localization microscopy is that sparse images of individual point emitters blink at random times during an image sequence. By finding the centroid of each emitter's diffraction limited spot in a given frame, and combining the localization results from each frame, a super-resolution image of the original fluorescence can be reconstructed.
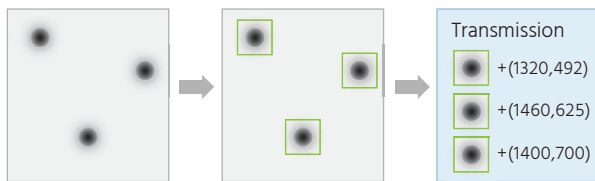


**Figure 1.** The PrimeLocate process in the camera. From left, an image containing sparse events is analyzed. The regions containing these events are identified, and only those regions are transmitted. Coordinates of the regions are also transmitted as metadata attached to each region.

The PrimeLocate algorithm scans each full image (or single ROI, if enabled) for the brightest local maxima within a 3x3 window. Each identified bright local maxima forms the center of a transmitted patch. The transmitted patch size is under user control and can vary from a radius of "1" to transmit a patch of 3x3 pixels, to a radius of "15" for transmitting a patch of 31x31 pixels. The number of transmitted patches can vary from one to a maximum of 512 patches per frame.
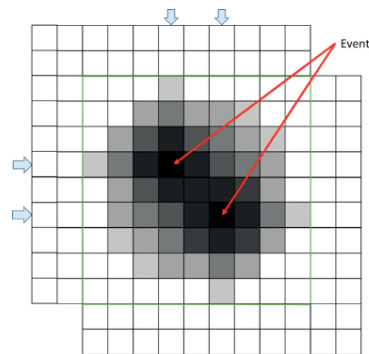


**Figure 2.** Example of PrimeLocate for two partially overlapping fluorescence events. The patch maxima are separated by more than two pixels, so two patches are transmitted with partially redundant pixel data. This ensures all data needed for correct localization is included. The blue arrows mark the center rows and columns for the patches.
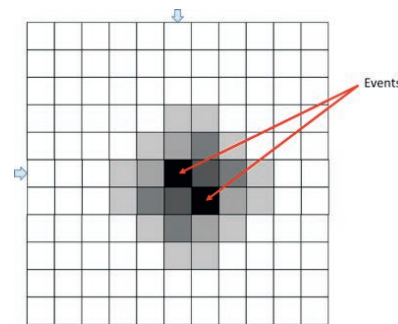


**Figure 3.** Example of 11x11 patch data for two small, overlapping fluorescence events. The event maxima are close enough together that a single 11x11 patch captures both. The blue arrays mark the center row and column for the patch.

Using a small 3x3 search window ensures that when two fluorescence events happen in close proximity, two or more patches are transmitted to capture both events (Figure 2). The 3x3 exclusion region discounts two or more events occurring within a radius of two pixels as being independent, as events this close together can be captured within a single, transmitted patch (Figure 3).

PrimeLocate always transmits the predetermined number of patches per frame, regardless of the number of fluorescence events present. For example, when using the maximum 512 regions, after patches containing true fluorescence events are transmitted, remaining patches will simply contain the brightest

noise peaks from the image. In this way, a consistent amount of data is transmitted per frame. These patches will be low-intensity compared to the brightest events and can be ignored. Should more than 512 events occur within a single frame, those events will be lost.

PrimeLocate does not attempt to localize events to precision better than a pixel, as this step is expected to be performed on the host. Leaving this step to the host allows choice of localization algorithm and configuration. The data transmitted to the host includes the patch data itself as well as the patch's (X,Y) location, so that the sparse image can be reconstructed at the host.

## PrimeLocate and µManager

µManager is an excellent open-source microscope control application that provides image acquisition and device control (www.openimaging.com). µManager supports PrimeLocate along with several new innovative features of the Prime family of cameras. Settings for PrimeLocate are found in the "Device Property Browser…", or as shown below, the "Configuration settings" dialog when using the Hardware Configuration provided with the Prime and Prime 95B cameras.
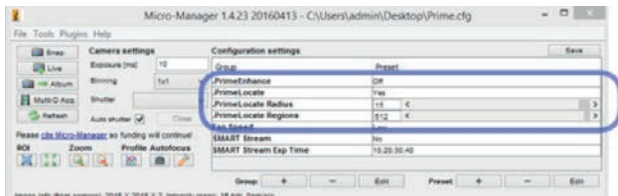


**Figure 4.** PrimeLocate settings in µManager

There are three highlighted controls:

| Name | Description | Selection |
| --- | --- | --- |
| PrimeLocate | Controls if PrimeLocate is enabled | Off/On |
| PrimeLocate Radius | Controls the size of the region transferred around each local maxima | 1 to 15 |
| PrimeLocate Regions | Controls the number of maxima transferred | 1 to 512 |

**Table 1.** Highlighted Controls

In the following example, a 512x512 portion of the sensor was used to acquire 1000 frames at 300 FPS. In each frame, 16 regions were selected for transfer using PrimeLocate with a size of 31x31 pixels (radius=15).
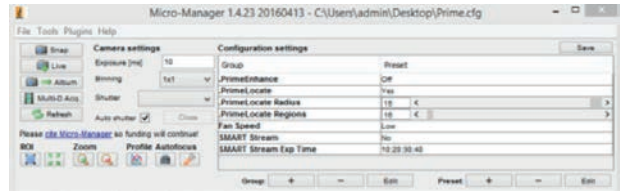


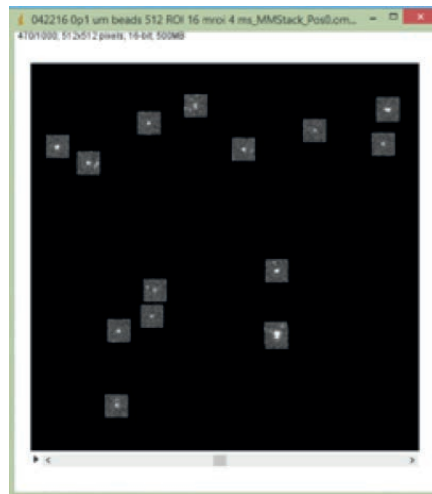**Figure 5a.** PrimeLocate settings in µManager



**Figure 5b.** Example of a 512x512 region, with PrimeLocate configured to find the 16 brightest regions, and return them as 31x31 pixels patches

## PrimeLocate and Data Reduction

Windows provides a convenient method for taking advantage of compression by saving images to a "compressed" folder on the hard disk. Windows will dynamically compress the image data as the images are written.

An uncompressed TIFF format image stack consisting of 1000 frames of 512x512, 16-bit image data without PrimeLocate enabled is 504MB uncompressed, and 346MB when saved to a compressed folder.

However, when using PrimeLocate configured for 16 regions of 31x31 pixels, the same file consumes only 54MB in the compressed folder as the blank-zero valued area will now efficiently compress using Windows compression algorithm.
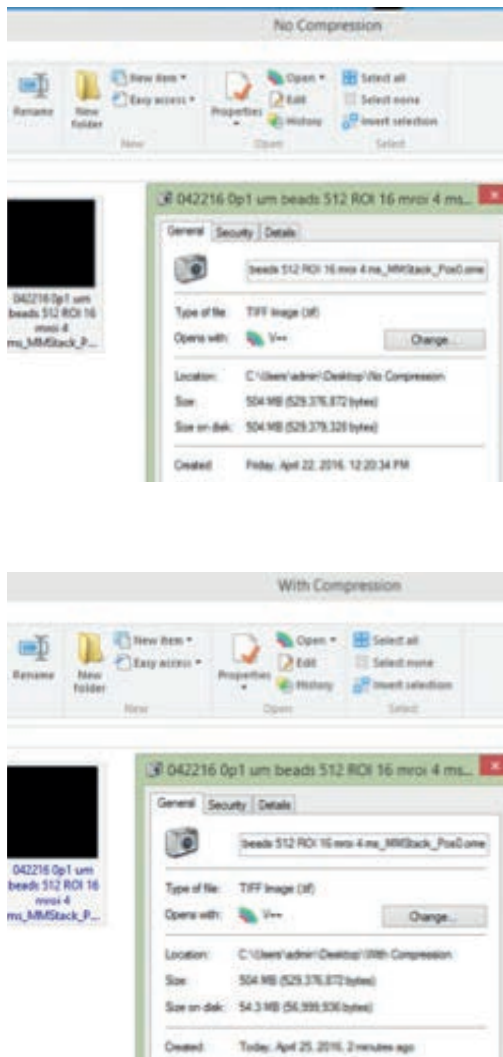
When using the maximum 512 regions, more data from the original image is transferred, and this same file is reduced to 257MB. These results are typical, the exact amount of compression depends on the image data and the number and size of stored regions.

## Conclusion

This brief introduction is designed to provide background on how PrimeLocate works. The next White Paper for PrimeLocate will focus on the results when using this feature for STORM imaging.

**Additional information is available on the Photometrics website:**

**www.photometrics.com**

**Figure 6.** PrimeLocate enables effective compression using Windows compressed folder mechanism, resulting in a 10x reduction in storage requirements in the 1000 frame example shown

# Live Particle Tracking

## Introduction

The Prime 95B™ Scientific CMOS camera is great for imaging situations requiring both extreme sensitivity as well as a high acquisition rate, making it ideal for techniques such as super-resolution localization microscopy and single-molecule tracking. The Prime camera platform incorporates a powerful FPGA and performs real-time image processing optimized for specific applications to provide more useful experimental data.

Live Particle Tracking is the latest addition to the real-time processing capabilities of the Prime 95B camera, and is designed to increase the efficacy of single molecule particle tracking experiments.

## How It Works

Live Particle Tracking, as its name suggests, is designed to identify individual single molecule particles and track them across the field of view by adapting a published algorithm tuned for two-dimensional tracking. The algorithm processes the images in the following order:

1. Image detection
2. Image restoration
3. Estimation and refinement of point locations
4. Non-particle discrimination
5. Trajectory linking
6. Image output with tracking meta-data

The image detection step requires the camera to determine only the dynamic portions of the image and disqualify anything static from detection. The data is then run through a restoration step which behaves as a bandpass filter, reducing both the high frequency and low-frequency noise, and allows the correction of any noise variation on a pixel-to-pixel basis as well as any background intensity modulations due to uneven illumination.

The points are then processed to determine the local-maxima within the radius of the evaluation kernel, and go through a refinement process to ensure a high efficiency in particle detection based on a threshold to
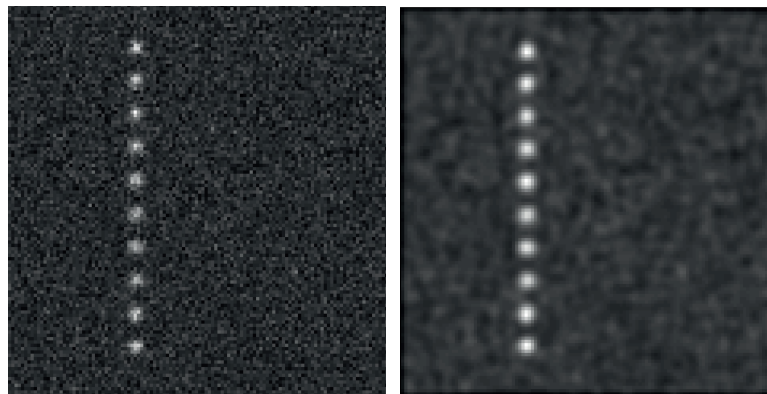


**Figure 1:** The input image of simulated single-particle data and the output of the image-restoration step to reduce image noise and pixel-to-pixel variation.

reduce the susceptibility to false positives. Any remaining artifacts are filtered out during the non-particle discrimination step, aimed at hot pixels and cosmic events.

Finally, the particles are tracked and linked through the acquired frame stack. The metadata included with all images is updated to include the particle data within each frame, providing particle IDs as well as the ability to display particle path traces as well as boxes to outline each detected particle.



**Figure 2:** The live particle tracking feature adds meta-data to each image providing the movement information of each particle

## Conclusion

The Live Particle Tracking feature of the Prime 95B Scientific CMOS camera provides the ability to evaluate the behaviour of single-molecules during the acquisition, ensuring that expected behaviour can be determined early in the experimental process. By reducing the number of experiments that do not meet the required or expected rates of movement by the particles, the efficacy of experiments can be increased significantly.

## References

*I.F. Sbalzarini, P. Koumoutsakos. 2005.* Feature point tracking and trajectory analysis for video imaging in cell biology
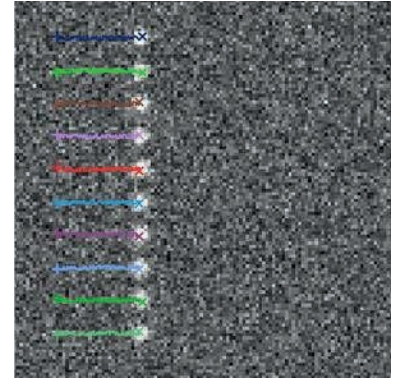
# Camera Test Protocol

## Introduction

The detector is one of the most important components of any microscope system. Accurate detector readings are vital for collecting reliable biological data to process for publication.

To ensure your camera is performing as well as it should be, Photometrics designed a range of tests that can be performed on any microscope. The results of these tests will give you quantifiable information about the state of your current camera as well as providing a method to compare cameras, which may be valuable if you're in the process of making a decision for a new purchase.

This document will first take you through how to convert measured signal into the actual number of detected electrons and then use these electron numbers to perform the tests. The tests in this document make use of ImageJ and Micro-Manager software as both are powerful and available free of charge.

## TABLE OF CONTENTS

# Part 1.
# Working With Photoelectrons

## Measuring Photoelectrons

### Background

A fluorescence signal is detected when photons incident on the detector are converted into electrons. It's this electron signal that's converted by the analog-to-digital converter (ADC) in the camera to the Grey Levels (ADUs) reported by the computer.

Although grey levels are proportional to signal intensity, not every camera converts electrons to the same number of grey levels which makes grey levels impractical for quantifying signal for publication.

Instead, signal should be quantified in photoelectrons as these are real world values for intensity measurement that allow for consistent signal representation across all cameras. This signal can then be compared against noise to assess the quality of images by signal to noise.

### Method

To convert signal in grey levels to signal in electrons:

1. Load an image into ImageJ, pick a fluorescent spot and draw a line across it
2. Select Plot Profile from the Analyse menu to get a peak representing the signal across the line in Grey Levels. Find the value at the top of the peak.
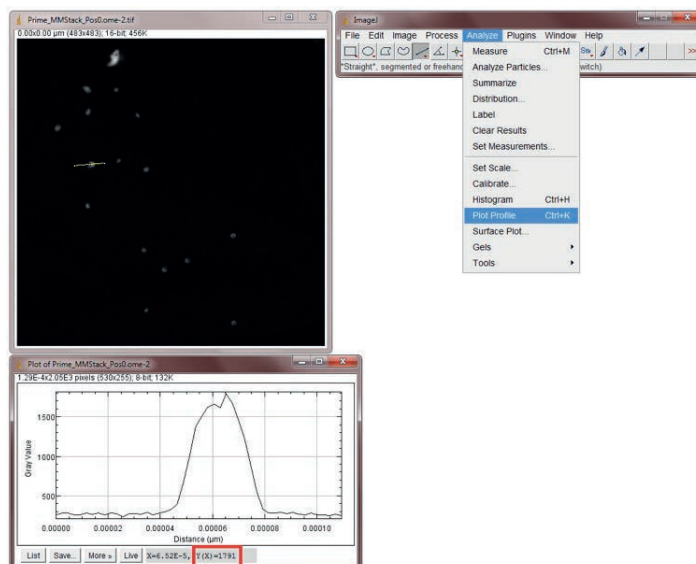
3. Subtract the camera bias from this Grey Level signal

4. Multiply the result by the camera system gain

The full equation is:

$$\text{Signal in Electrons} = (\text{Signal in Grey Levels} - \text{Bias}) * \text{Gain}$$

The camera bias and camera system gain can be found on the Certificate of Performance (COP) or other information provided with the camera or they can be calculated by tests explained below.

As an example, the data in the image above was taken with the Prime 95B™ which has a bias of ~100 and a gain of ~1.18. By inserting these values into the equation, we get the following result:

$$\text{Signal in Electrons} = (1791 - 100) * 1.18$$
$$\text{Signal} = 1995 \text{ e-}$$

## Measuring Camera Bias

### Background

When visualizing a fluorescence image, we would expect the intensity value of a pixel to correspond only to the intensity of fluorescence in the sample. However, every camera has a background offset that gives every pixel a non-zero value even in the absence of light. We call this the camera bias.

The bias value is necessary to counteract fluctuating read noise values which might otherwise go below zero. The value of the bias therefore should be above zero and equal across all pixels. The bias value doesn't contain any detected signal so it's important to subtract it from an image before attempting to calculate the signal in photoelectrons.

### Method

To calculate the camera bias:

1. Set your camera to a zero millisecond exposure time

2. Prevent any light entering the camera by closing the camera aperture or attaching a lens cap

3. Take 100 frames with these settings (see next page)
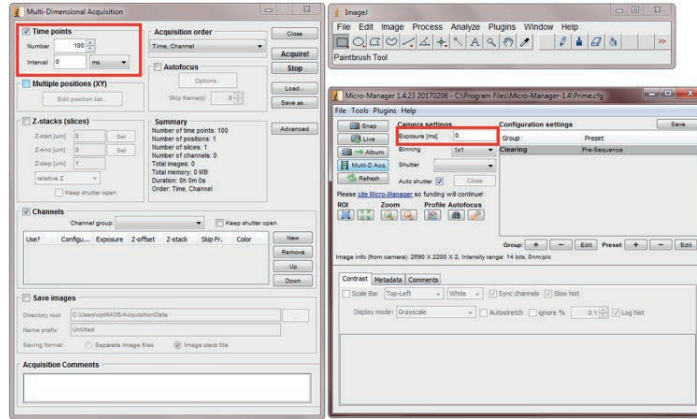
4. Calculate the mean of every frame by selecting Stacks from the Image menu and then clicking on Plot Z-axis profile. This should give you the mean values of every frame in the Results window
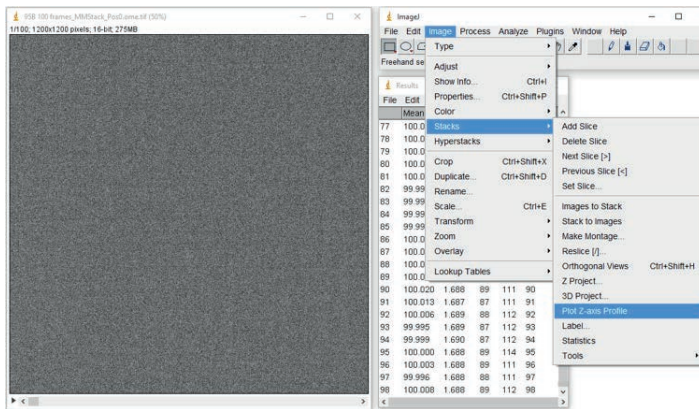


5. Calculate the mean of the 100 frame means by selecting Summarize in the Results menu
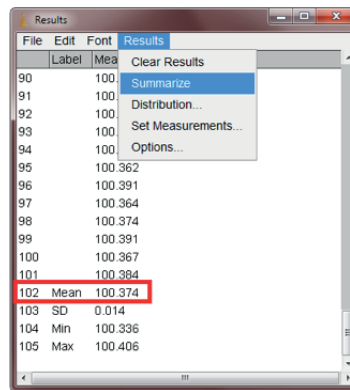


The bias is the mean of a single frame so by plotting the mean values of all 100 frames we calculate a more accurate bias.

## Calibrating Your Camera for Photoelectron Measurement

### Background

When the amount of light entering a camera is linearly increased, the response of the camera in grey levels should also linearly increase.

The gain represents the quantization process as light incident on the detector is processed and quantified. It varies from camera to camera depending on electronics and individual properties but it can be calculated experimentally. If a number of measurements are made and plotted against each other the slope of the line should represent the linearity of the gain.

### Method

Camera system gain is calculated by a single point mean variance test which calculates the linear relationship between the light entering the camera and the cameras response to it. To perform this test:

1. Take a 100-frame bias stack with your camera like in the previous section and calculate the mean bias

2. Take 2 frames of any image using the same light level with a 5ms exposure time

3. In ImageJ, Measure the means of both images and average them. We'll call this $\text{Mean}_{\text{Image1, Image2}}$
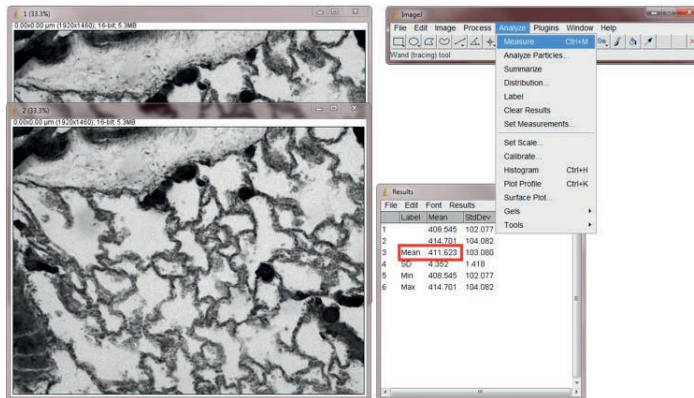


4. Calculate the difference between the two images by selecting Image Calculator from the Analyze menu. Select the two frames and Subtract, you will need to float the result. Press OK to generate the diff image.
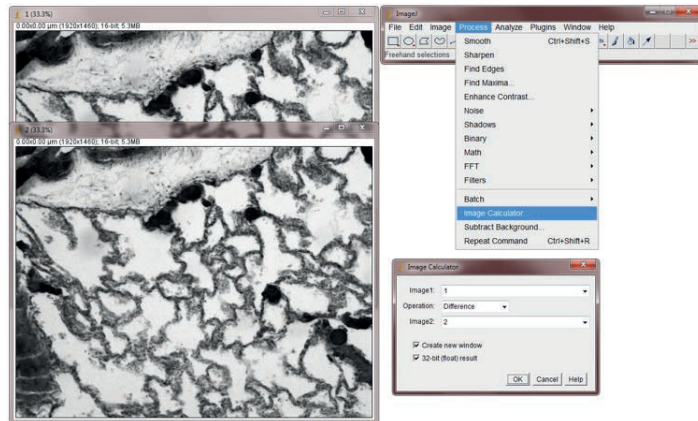
5. Measure the Standard Deviation of the diff image, we'll call this Standard deviation$_{\text{Diff image}}$.
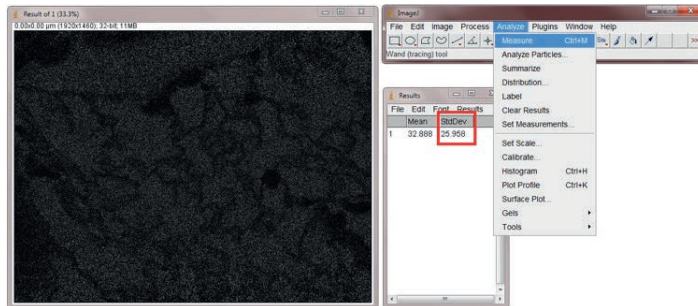


6. Calculate the variance of the two images with the following equation:

$$\text{Variance}_{\text{Image 1,Image 2}} \; = \; \frac{\text{Standard deviation}_{\text{Diff image}}{}^2}{2}$$

7. Calculate the gain from the variance using the following equation, remember to remove the previously calculated bias:

$$\text{Gain} = \frac{(\text{Mean}_{\text{Image 1,Image 2}}) - \text{bias}}{\text{Variance}_{\text{Image 1,Image 2}}}$$

Gain is represented as e–/grey level.

8. Repeat this process with 10ms, 20ms and 40ms exposure times to check that the gain is consistent across varying light levels.

9. You can also use the single-point mean variance (gain) calculator provided by Photometrics on the website: https://www.photometrics.com/resources/imaging-tools/mean-variance-calculator.php

## Calculating Signal to Noise Ratio (SNR)

### Background

The signal to noise ratio describes the relationship between measured signal and the uncertainty of that signal on a per-pixel basis. It is essentially the ratio of the measured signal to the overall measured noise on a pixel. Most microscopy applications look to maximise signal and minimize noise.

All cameras generate electron noise with the main sources being read noise, photon shot noise and dark current. These noise values are displayed on the camera data sheet and are always displayed in electrons. This means that the most accurate way to calculate the signal to noise ratio is by comparing signal in electrons to noise in electrons.

### Method

The signal to noise ratio can be calculated using the following equation:

$$SNR = \frac{S}{\sqrt{S + [Nd * t^2] + Nr^2}}$$

Where:
S = Signal in electrons.

The best way to calculate an electron signal for use in the equation is to use a line profile across an area of high fluorescence as described at the beginning of this document.

Nd = Dark current in electrons/pixel/second
Nr = Read noise in electrons
t = Exposure time in seconds

You can also use the signal to noise calculator provided by Photometrics on the website:
https://www.photometrics.com/resources/imaging-tools/signal-to-noise-calculator.php

## Calculating Signal to Noise Ratio (SNR) of an EMCCD Camera

### Background

EMCCD cameras are designed for very low light applications and function in the same way as a CCD but have additional electronics to multiply the captured electrons. This process occurs after the electron signal has been captured but before it's been read out.

The multiplication process means that the camera read noise is effectively reduced to less than 1 electron, allowing the detection of very low signal. However, this is not free in terms of signal to noise. The multiplication process is not a definitive event – there is a probability associated with gaining extra electrons and this uncertainty adds an extra noise source to the SNR calculation, Excess Noise Factor. Excess noise factor has a value of $\sqrt{2}$ and effectively cuts the sensors quantum efficiency in half. When calculating the SNR of an EMCCD camera, this must be added to the equation.

## Method

The signal to noise ratio can be calculated using the following equation:

$$\text{EMCCD SNR} = \frac{S}{\sqrt{[S * F^2] + [Nd * t^2 * F^2] + [\frac{Nr}{E}]^2}}$$

Where:
S = Signal in electrons
Nd = Dark current in electrons/pixel/second
Nr = Read noise in electrons
t = Exposure time in seconds
F = Excess noise factor
E = EM gain

To get accurate electron counts from EMCCD data we recommend you use the QuantView™ function of the Photometrics Evolve® Delta. QuantView converts Grey Level intensities into the number of electrons measured at the sensor so there are no calculations necessary to convert Grey Levels into electrons. To activate QuantView:

1. In Micro-Manager, open the Device Property Browser

2. Scroll down to QuantView and change it from off to on
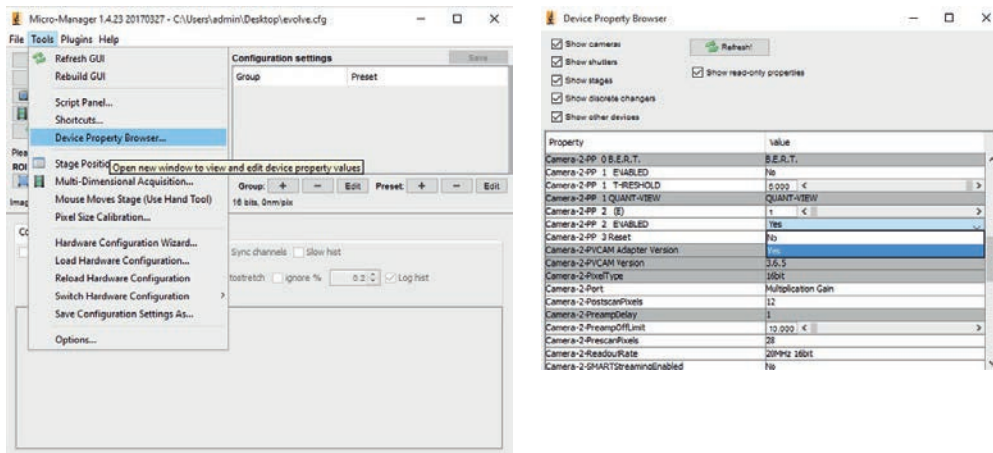


Alternatively, locate the gain value of the camera on the Certificate of Performance (CoP) or other information provided with the camera and perform the calculation given at the beginning of this document to convert Grey Levels to electrons.

To convert Grey Levels to electrons on non-linear gain EMCCDs such as the Photometrics Cascade series, please see the following tech note:
https://www.photometrics.com/resources/learningzone/calculating-electron-multiplication-gain.php

<div align="right">

# Part 2.
# Testing Camera Quality

</div>

## Evaluating Bias Quality

### Background

There are two important things to look for in a bias, the stability and the fixed pattern noise.

The stability is simply a factor of how much the bias deviates from its set value over time. A bias that fluctuates by a large amount will not give reliable intensity values.

Fixed pattern noise is typically visible in the background with longer exposure times and it occurs when particular pixels give brighter intensities above the background noise. Because it's always the same pixels, it results in a noticeable pattern seen in the background. This can affect the accurate reporting of pixel intensities but also the aesthetic quality of the image for publication.

### Method

To evaluate the bias stability:

1. Plot the mean values of all 100 bias frames taken in the previous section
2. Fit a straight line and observe the linearity

Our goal at Photometrics is to product a stable bias that doesn't deviate by more than one electron, which is shown here uing the Prime 95B™ Scientific CMOS data:
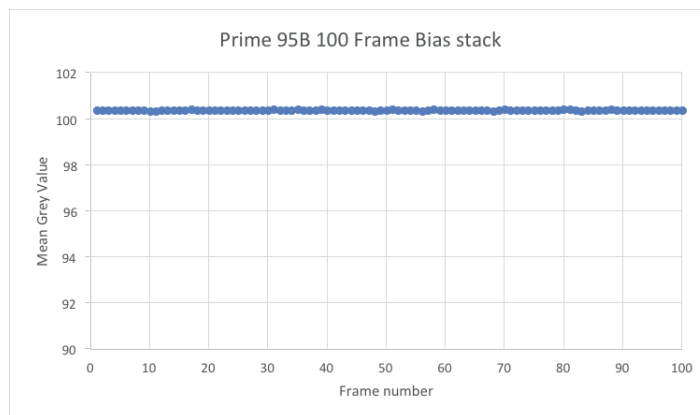
To evaluate fixed pattern noise:

1. Mount a bright sample on the microscope and illuminate it with a high light level

2. Set the exposure time to 100 ms

3. Snap an image

4. Repeat this experiment with longer exposure times if necessary

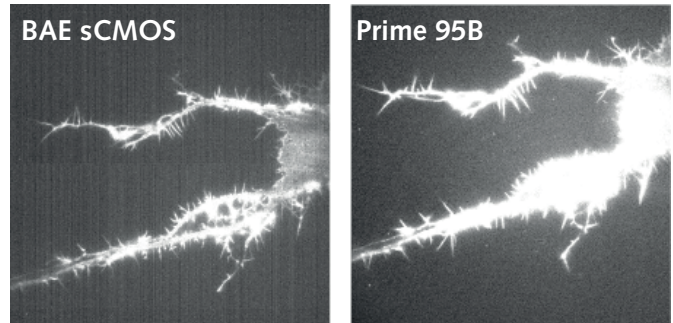A 'clean' bias such as that demonstrated at right on the Prime 95B will give more accurate intensity data and produce higher quality images.



## Evaluating Gain Quality

### Background

Gain linearity is very important as the gain directly influences how the electron signal is converted into the digital signal read by the computer. Any deviation from a straight line represents inaccurate digitization.

### Method

To evaluate the gain linearity:

1. Plot the Mean$_{Image1 – Image2}$ against the Variance$_{Image1 – Image2}$ data collected in the 'Calibrating your camera for photoelectron measurement' section

2. Fit a straight line and observe the linearity

Photometrics recommends that any deviation from the line be no more than 1%, as shown using the CoolSNAP™ DYNO data:
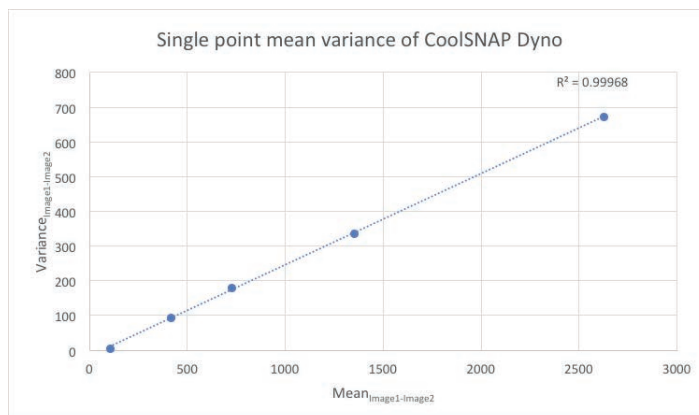
# Evaluating EM Gain Quality

## Background

All EMCCD cameras suffer from EM gain fall-off over time. This means that the EM gain multiplication of any EMCCD camera will be reduced with usage. Most modern EMCCD cameras have ways to recalibrate the EM gain multiplication so there will not be any noticeable change but eventually there will come a point when no more can be done.

This becomes a problem when, for example, 300x EM gain was used to overcome read noise but due to EM gain fall-off the camera can no longer reach this gain level. At this point the camera has lost it's EM gain functionality and the only option is to buy a new camera.

## Method

To test the EM gain multiplication of your camera:

1. Take a 100-frame bias stack with your EMCCD camera and calculate the mean bias
2. Take a long exposure (~1s) image of a dim sample *without* EM gain
3. Without changing anything about the sample, take a short exposure (~10ms) *with* EM gain

Note - It's necessary to lower the exposure time for point 3. to avoid saturating the pixels when using EM gain. We'll correct for time in point 4.

4. Subtract the bias value from both images and divide both by their respective exposure time in milliseconds to equalize them
5. The factor difference in signal per time unit should be the EM gain multiplication factor

If you're worried about EM gain fall-off, you can reduce its impact by following these guidelines:

1. Only use the EM gain necessary to overcome read noise. An EM gain of 4 or 5 times the root-mean-square (rms) read noise should be enough. It should almost never be necessary to go above an EM gain of 300 to achieve this.
2. If EM gain isn't necessary for your work, don't use it. Most EMCCD cameras have non-EM ports to read out the signal without using the EM register
3. Avoid over-saturating the EMCCD detector

# Calculating Read Noise

## Background

Read noise is present in all cameras and will negatively contribute to the signal to noise ratio. It's caused by the conversation of electrons into the digital value necessary for interpreting the image on a computer. This process is inherently noisy but can be mitigated by the quality of the camera electronics. A good quality camera will add considerably less noise.

Read noise will be stated on the camera data sheet, certificate of performance or other information provided with the camera. It can also be calculated as explained below.

## Method

Read noise can be calculated with the following method:

1. Take two bias images with your camera

2. In ImageJ, calculate the difference between the two images by selecting Image Calculator from the Analyze menu. Select the two frames and Difference, you will need to float the result. Press OK to generate the diff image.
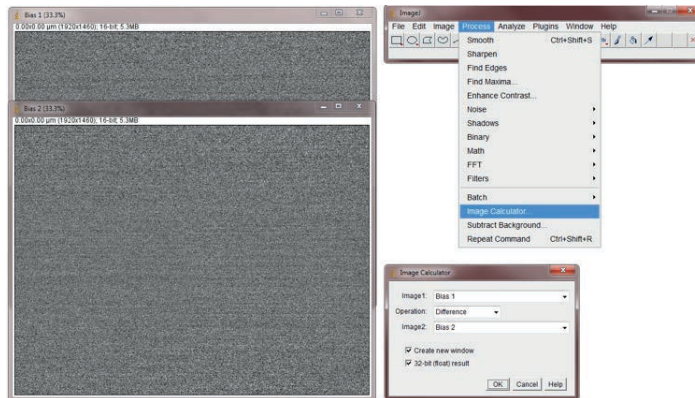


3. Measure the Standard Deviation of the diff image, we'll call this Standard deviation$_{Diff\ image}$

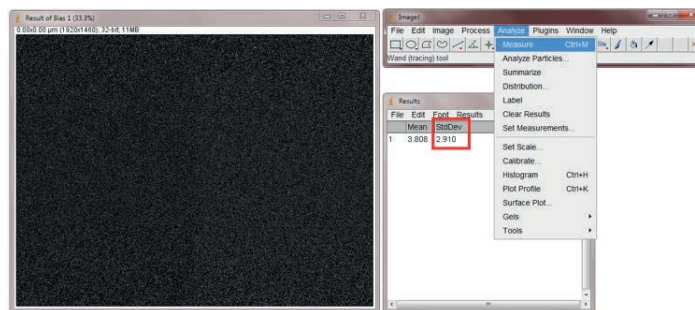4. Use the following equation to calculate system read noise, you'll need the previously calculated gain value or you can use the gain value given in the information provided with the camera:

$$\text{Read Noise} = \frac{\text{Standard deviation}_{\text{Diff image}} * \text{Gain}}{\sqrt{2}}$$

You can also use the read noise calculator provided by Photometric on the website: [https://www.photometrics.com/resources/imaging-tools/read-noise-calculator.php](https://www.photometrics.com/resources/imaging-tools/read-noise-calculator.php)

# Calculating Dark Current

## Background

Dark current is caused by thermally generated electrons which build up on the pixels even when not exposed to light. Given long enough, dark current will accumulate until every pixel is filled. Typically, pixels will be cleared before an acquisition but dark current will still build up until the pixels are cleared again. To solve this issue, dark current is drastically reduced by cooling the camera. You can calculate how quickly dark current builds up on your camera with the method below.

## Method

To calculate how much dark current is accumulating over differing exposure times, you need to create a dark frame. A dark frame is a frame taken in the dark or with the shutter closed. By creating multiple dark frames with varying exposure times or acquisition times, you can allow more or less dark current to build up.
To do this:

1. Prevent any light entering the camera and take images at exposure times or acquisition times you're interested in. For example, you may use a 10ms exposure time but intend to image for 30 seconds continuously. In this case, you should prepare a 30 second dark frame.

2. Take two dark frames per time condition

3. In ImageJ, calculate the difference between the two dark frames by selecting Image Calculator from the Analyze menu. Select the two frames and Difference, you will need to float the result. Press OK to generate the diff image.

4. Measure the Standard Deviation of the diff image, we'll call this Standard deviation$_{\text{Diff image}}$

5. Use the following equation to calculate system read noise and dark current:

$$\text{Read Noise} + \text{Dark current} = \frac{\text{Standard deviation}_{\text{Diff image}} * \text{Gain}}{\sqrt{2}}$$

Note - the equation remains the same as in the previous section but because we've allowed the camera to expose for a certain amount of time, dark current has now built up on top of the read noise.

6. Subtract the number of electrons contributed by read noise - calculated in the previous section - to be left with the noise contributed by dark current

7. Compare the calculated dark current value to the acquisition time to determine how much dark current built up per unit time

8. This experiment can be repeated at differing exposure times and temperatures to determine the effect of cooling on dark current build-up

## Counting Hot Pixels

### Background

Hot pixels are pixels that look brighter than they should. They are caused by electrical charge leaking into the sensor wells which increases the voltage at the well. They are an aspect of dark current so the charge builds up over time but they are unable to be separated from other forms of dark current.

### Method

To identify hot pixels:

1. Take a bias frame with your camera

2. Prevent any light entering the camera and take a 10-frame stack with a long (~5 sec) exposure

3. In ImageJ, subtract the bias frame from one of the long exposure frames by selecting Image Calculator from the Analyze menu. Select the two frames and Subtract, you will need to float the result. Press OK to generate the image.
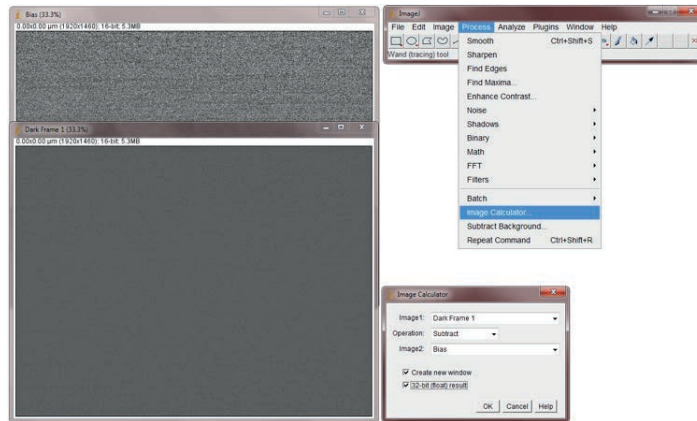
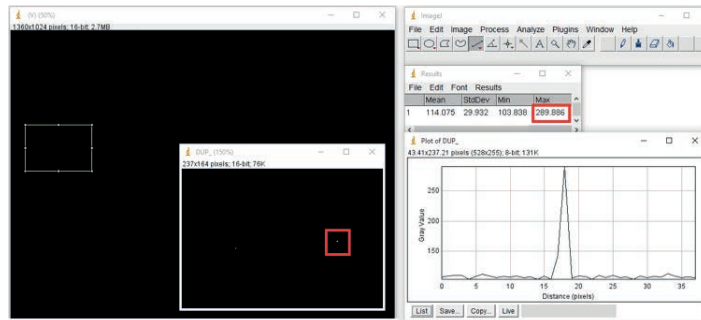4. Hot pixels should immediately be visible as bright white spots on the dark background. Draw line profiles over individual hot pixels to measure the intensity



5. Compare hot pixels between all 10 long exposure frames

The advantage of hot pixels is that they always stay in the same place so once they are identified these pixels can be ignored for data processing.

Like normal dark current, camera cooling drastically reduces hot pixel counts. If you are still having issues with hot pixels you may be able to adjust the fan speed of the camera to provide more cooling or even switch to a liquid cooled system.

# Part 3.
# Other Factors to Consider

## Saturation and Blooming

### Saturation

Saturation and blooming occur in all cameras and can affect both their quantitative and qualitative imaging characteristics.

Saturation occurs when pixel wells become filled with electrons. However, as the pixel well approaches saturation there is less probability of capturing an electron within the well. This means that as the well approaches saturation the normally linear relationship between light intensity and signal degrades into a curve. This affects our ability to accurately quantify signal near saturation.

To control for saturation, we call the full well capacity before it starts to curve off the *linear full well* capacity. A high-quality camera will be designed so that the linear full well capacity fills the full 12-, 14- or 16-bit dynamic range so no signal is lost. At Photometrics, we always restrict the full well capacity to the linear full well so you'll never experience saturation effects.

### Blooming

An additional saturation problem is that when the pixel reaches saturation, the extra charge can spread to neighbouring pixels. This spread is known as blooming and causes the neighbouring pixels to report false signal values.

To control for blooming Photometrics cameras feature the anti-blooming technology, *clocked anti-blooming*. In this technique, during an exposure two of the three clock-voltage phases used to transfer electrons between neighbouring pixels are alternately switched. This means that when a pixel approaches saturation, excess electrons are forced into the barrier between the Si and SiO2 layers where they recombine with holes. As the phases are switched, excess electrons in pixels approaching saturation are lost, while the electrons in non-saturated pixels are preserved. As long as the switching period is fast enough to keep up with overflowing signal, electrons will not spread into neighbouring pixels. This technique is very effective for low-light applications.

## Speed

### Types of Speed

Biological processes occur over a wide range of time scales, from dynamic intracellular signalling processes to the growth of large organisms. To determine whether the speed of your camera can meet the needs of your research, you need to know which aspects of the camera govern its speed. These aspects can be broken down to readout speed, readout rate, readout time and how much of the sensor is used for imaging.

Readout speed tells you how fast the camera is able to capture an image in frames per second (fps). For a camera with a readout speed of 100 fps for example, you know that a single frame can be acquired in 10 ms. All latest model Photometrics cameras are able to show hardware generated timestamps that give much more reliable readout speed information than the timestamps generated by imaging software. This can be shown in PVCAMTest provided with the Photometrics drivers or turned on in Micro-Manager by enabling metadata. The .tiff header will then show the hardware generated timestamps.

Readout rate tells you how fast the camera can process the image from the pixels. This is particularly important for CCD and EMCCD cameras which have slow readout rates because they convert electrons into a voltage slowly, one at a time, through the same amplifier.

CMOS cameras have amplifiers on every pixel and so are able to convert electrons into a voltage on the pixel itself. This means that all pixels convert electrons to voltage at the same time. This is how CMOS devices are able to achieve far higher speeds than CCD and EMCCD devices, they have far higher readout rates.

Readout rate is typically given in MHz and by calculating 1/readout rate you can find out how much time the camera needs to read a pixel.

Readout time is only relevant for sCMOS devices and tells you the readout rate of the entire pixel array. This can be calculated as 1/readout speed, so if the readout speed of the camera is 100 fps, the readout time is 10 ms.

### Binning and Regions of Interest (ROI)

When speed is more important than resolution pixels can be binned or a region of interest (ROI) can be set to capture only a subset of the entire sensor area.

Binning involves grouping the pixels on a sensor to provide a larger imaging area. A 2x2 bin will group pixels into 2x2 squares to produce larger pixels made up of 4 pixels. Likewise, a 4x4 bin will group pixels into 4x4 squares to produce larger pixels made up of 16 pixels, and so on.
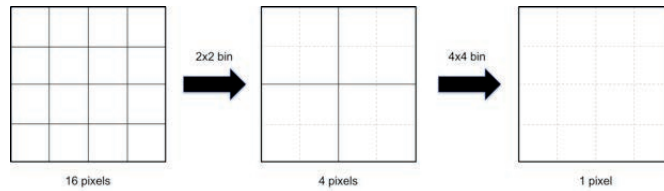
On a CCD or EMCCD, binning increases sensitivity by providing a larger area to collect incident photons as well as increasing readout speed by reducing the number of overall pixels that need to be sent through the amplifier.

Binning on an sCMOS also increases sensitivity but cannot increase readout speed because electrons are still converted to voltage on the pixel. Binning is therefore only useful to increase sensitivity and reduce file size.

Both devices can benefit from setting an ROI as this limits the number of pixels that need to be read out. The less pixels to read out, the faster the camera can read the entire array.

## Camera Sensitivity

### Quantum Efficiency

Sensitivity is a function of both quantum efficiency and pixel size.

Quantum efficiency (QE) tells you what percentage of photons incident on the sensor will be converted to electrons. For example, if 100 photons hit a 95% QE sensor, 95 photons will be converted into electrons.

72% QE sCMOS was made 82% quantum efficient with the addition of microlenses. By positioning microlenses over the pixels, light from wider angles was able to be directed into the active silicon. However, it's important to make a photoelectron detection comparison with both types of sCMOS as most light used in biological applications is columnated which gives limited light collection advantage to the microlenses.

### Pixel Size

Pixel size on the other hand tells you how large an area the pixel has for collecting photons. For example, a 6.5x6.5 µm pixel has an area of 42.25 µm2 and an 11x11 µm pixel has an area of 121 µm2 which makes the 11x11 µm pixel ~2.86x larger than the 6.5x6.5 µm pixel. So, if the 11x11 µm pixel collects 100 photons, the 6.5x6.5 µm pixel only collects ~35 photons.

This means that, as far as sensitivity is concerned, a high QE and a large pixel are preferred. However, larger pixels can be disadvantageous for resolution.

## Pixel Size and Resolution

The optical resolution of a camera is a function of the number of pixels and their size relative to the image projected onto the pixel array by the microscope lens system.

A smaller pixel produces a higher resolution image but reduces the area available for photon collection so a delicate balance has to be found between resolution and sensitivity. A camera for high light imaging, such as CCD cameras for brightfield microscopy, can afford to have pixel sizes as small as 4.5x4.5 µm because light is plentiful. But for extreme low light applications requiring an EMCCD or scientific CMOS camera, pixel sizes can be as large as 16x16 µm.

However, a 16x16 µm pixel has significant resolution issues because it can't achieve Nyquist sampling without the use of additional optics to further magnify the pixel.

In light microscopy, the Abbe limit of optical resolution using a 550 nm light source and a 1.4 NA objective is 0.20 µm. This means that 0.20 µm is the smallest object we can resolve, anything smaller is physically impossible due to the diffraction limit of light. Therefore, to resolve two physically distinct fluorophores, the effective pixel size needs to be half of this value, so 0.10 µm. Achieving this value is known as Nyquist sampling.

Using a 100x objective lens, a pixel size of 16x16 µm couldn't achieve Nyquist sampling as the effective pixel size would by 0.16 µm. The only way to reach 0.10 µm resolution would be to use 150x magnification by introducing additional optics into the system.

This makes it very important to choose the camera to match your resolution and sensitivity requirements. The table below outlines which Photometrics cameras achieve Nyquist under which magnification:

| Magnification | NA of objective | Wavelength of light | Required Pixel Size for Nyquist | Ideal camera (pixel size) |
|---|---|---|---|---|
| 40X | 1.3 | | 4.8 µm | CoolSNAP DYNO (4.54 µm) |
| 60X | 1.4 | | 6.7 µm | Prime™ (6.5 µm) |
| 100X | 1.4 | 509nm (GFP) | 11.1 µm | Prime 95B (11 µm) |
| 150X | 1.4 | | 16.6 µm | Evolve 512 Delta 16 µm) |

Note – It's often the case that sensitivity is more important than resolution. In this case, choosing the Prime 95B for use with a 60x objective is far superior to choosing the Prime even though the Prime matches Nyquist. This is where the researcher will need to balance the demands of their application with the best available camera. Additional optics can always be used to reduce the effective pixel size without changing the objective.

# Prime 95B Software Support

## Supported Software

- ✓ **Nikon NIS-Elements**

- ✓ **Zeiss Zen**

- ✓ **Olympus cellSens**

- ✓ **Leica LASX**

- ✓ **Molecular Devices MetaMorph**

- ✓ **Visitron VisiView**

- ✓ **Intelligent Imaging Innovations (3i) SlideBook 6**

- ✓ **National Instruments LabView**

- ✓ **MathWorks MatLab**

- ✓ **WaveMetrics Igor Pro**

- ✓ **Python**

- ✓ **Micro-Manager**

- ✓ **Photometrics Ocular**

- ✓ **Camera driver SDK available for developers**

## Supported Operating Systems

- ✓ **Windows 7, 8, 10 64-bit**

- ✓ **Linux**

## Notes

Photometrics recommends contacting your local third-party software office for minimum version numbers, support and relevant information