TELEDYNE PHOTOMETRICS

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# PRIME BSI™

## Customer Applications Handbook

TELEDYNE PHOTOMETRICS Everywhereyoulook™
Part of the Teledyne Imaging Group

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## Prime BSI Technical Notes



## Camera Test Protocol



## Supported Software

# High Resolution BSI Scientific CMOS


Prime BSI delivers the perfect balance between high resolution imaging and sensitivity with an optimized pixel design and near perfect 95% Quantum Efficiency to maximize signal detection.

A 4 Megapixel camera with 6.5µm pixels, it capture higly detailed images with great quality while acquiring data at high frame rates. This ensures that all data is collected and no event goes undetected.

Prime BSI delivers a 100% pixel fill factor and does not rely on micro-lensing technology to increase detection, resulting in a 30% increase in sensitivity over previous sCMOS cameras.

This perfect balance in performance makes the Prime BSI the most versatile imaging camera for live-cell imaging with:

- Highest Sensitivity
- High Resolution
- Large Field of View
- High Frame Rates
- Large Dynamic Range

**Primary applications:**

**TIRF Microscopy**

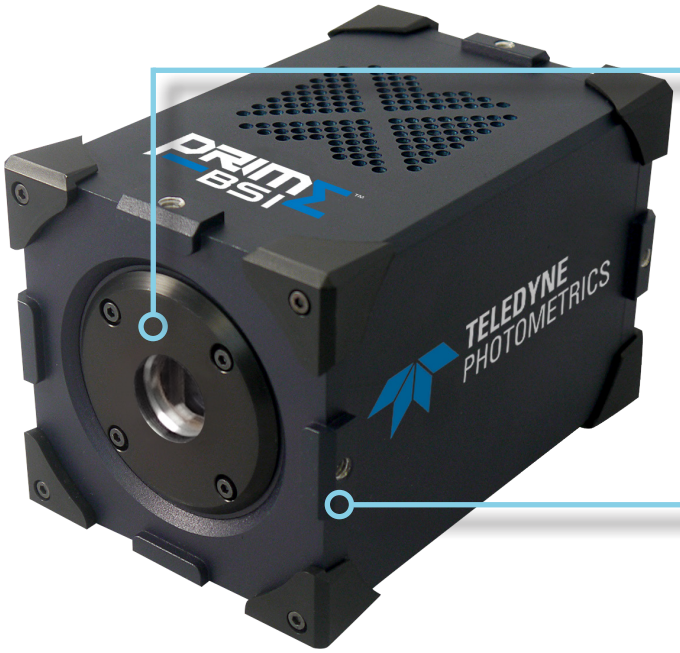
**Ratiometric Imaging**

**Cell Motility**

**Light Sheet Microscopy**

- **95% Quantum Efficiency**
- **6.5µm x 6.5µm Pixel Area**
- **1.0e⁻ Read Noise with Correlated Multi Sampling (CMS)**
- **43.5 fps @ 16-bit/ 12-bit (CMS)**
- **63fps @ 11-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 |
| Optimized 6.5µm Pixel SIze | Maximize light collection while maintaining proper spatial sampling at 60X |
| Extremely Low Read Noise | Maximize your ability to defect 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 |
| Prime Enhance™ | 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 25,000:1 Dynamic Range (91 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 |
| Programmable Scan Mode | Easily synchronize and control acquisitions with the rolling shutter readout |




## 4.2 Megapixel BSI CMOS Sensor

Backside Illuminated Sensor
1.0e- Read Noise (Median)
>95% peak QE
45,000e- full well
6.5 x 6.5µm pixels
18.8mm diagonal

## Easily Mounted and Secured

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

## Convenient Interfaces

16-bit / 12-bit Data
- 43.5 fps

11-bit Data
- 63 fps


## Multiple Cooling Options

Forced Air Cooling
- -20°C Cooling
- Selectable Fan Speed

Liquid Cooling
- -30°C Cooling
- Leak-proof, quick-disconnect ports

## Advanced Triggering Capabilities

Effective Global Shutter
Up to four selectable expose-out lines

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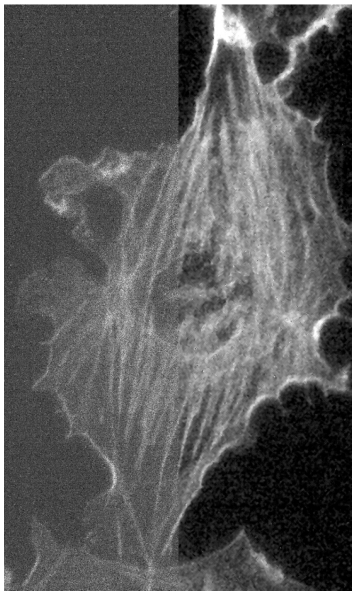
# Real-Time Application Optimization

## PrimeEnhance™



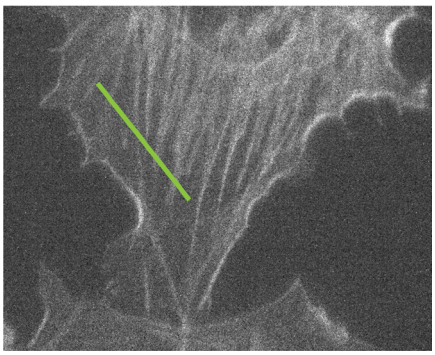
With the near-perfect sensitivity of Backside Illuminated Scientific CMOS sensors, the latest generation of scientific cameras have enabled imaging using only a few photons per pixel. Unfortunately, these minute signals are dominated by the natural Poisson variation in light levels preventing useful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

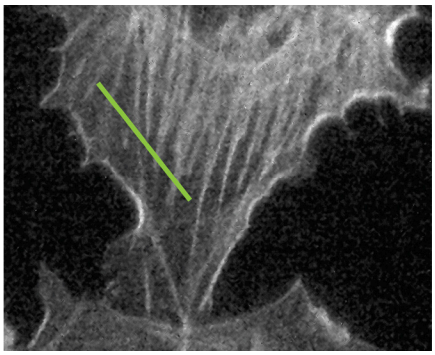
PrimeEnhance uses a quantitative SNR enhancement algorithm used in Life Science imaging to reduce the impact of photon shot-noise present in acquired images, leading to an increase in Signal to Noise Ratio (SNR) by 3x to 5x with equivalent exposure times.
With PrimeEnhance, the exposure times can be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

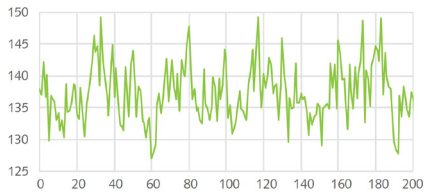
Invented at INRIA and further optimized for fluorescence microscopy at the Institut Curie, the denoising algorithm used in PrimeEnhance uses a patch based evaluation of image data and knowledge of the each individual camera's performance parameters to reduce the effects of photon shot-noise. The patches 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.

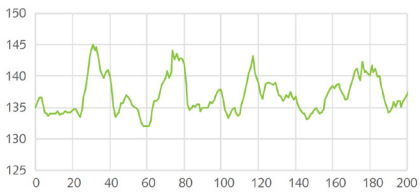
- Increase SNR 3x to 5x at low light levels by reducing photon shot-noise
- Preserve signal intensities ensuring quantitative measurements
- Extend cell lifetimes with reduced phototoxicity and photobleaching
- Extremely useful for low light imaging applications dominated by noise

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.







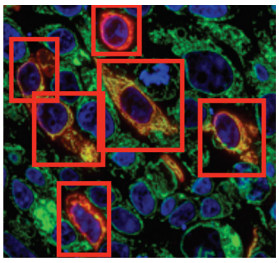



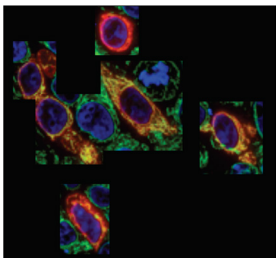
# Real-Time Application Optimization

## 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

| Specifications | | Camera Performance |
|---|---|---|
| Sensor | | Gpixel GSENSE2020BSI Scientific CMOS Sensor |
| Active Array Size | | 2048 x 2048 (4.2 Megapixel) |
| Pixel Area | | 6.5µm x 6.5µm (42.25µm²) |
| Sensor Area | | 13.3mm x13.3mm<br>18.8mm diagonal |
| Peak QE% | | >95% |
| Read Noise: | Correlated Multi-Sampling (CMS) | 1.0e⁻ (Median)<br>1.1e⁻ (RMS) |
| | Combined/High Gain | 1.6e⁻ (Median)<br>1.8e⁻ (RMS) |
| Full-Well Capacity | | 45,000e⁻ (Combined Gain)<br>10,000e⁻ (High Gain)<br>1,000e⁻ (CMS) |
| Dynamic Range | | 25,000:1 (Combined Gain) |
| Bit Depth | | 16-bit (Combined Gain)<br>12-bit (CMS)<br>11-bit (High Gain) |
| Readout Mode | | Rolling Shutter<br>Effective Global Shutter<br>Programmable Scan Mode (PCI-E only) |
| Binning | | 2x2 (on FPGA) |
| Linearity | | >99.5% |

| Cooling Performance | Sensor Temperature | Dark Current |
|---|---|---|
| Air Cooled | -20°C @ 30°C Ambient | 0.5e⁻/pixel/second |
| Liquid Cooled | -30°C @ 30°C Ambient | 0.12e⁻/pixel/second |

| Specification | Camera Interface |
|---|---|
| Digital Interface | PCIe, USB 3.0 |
| Lens Interface | C-Mount |
| Mounting Points | 2x 1/4"-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 | Any Row: Expose signal is high while any rows acquiring data<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<br>First Row: Expose signal is high while first row is acquiring data.<br>Line Output: Expose signal provides rising edge for each row advanced by the rolling shutter readout |
| Output Trigger Signals | Expose Out (up to four signals), Read Out, Trigger Ready |

| Programmable Scan Mode | Function |
|---|---|
| Scan Modes | Auto: Normal camera operation<br>Line Delay: Control rolling shutter propagation rate by adding delays to the line time<br>Scan Width: Control number of rows between reset and readout signal in the rolling shutter |
| Scan Direction | Down: Rolling shutter readout begins at the top of the sensor<br>Up: Rolling shutter readout begins at the bottom of the sensor<br>Down/Up Alternate: Rolling shutter readout alternates direction after starting at the top of the sensor |

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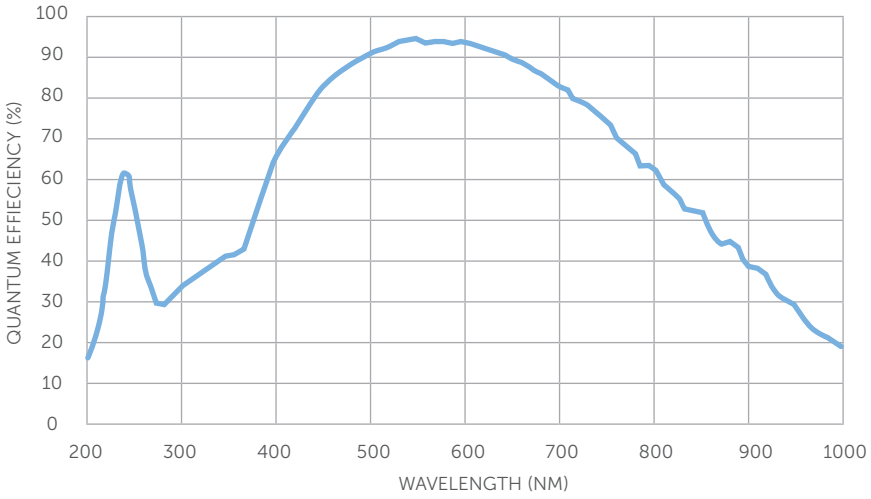



## Accessories (Included)

USB 3.0 Cable

Trigger Cable

Power Supply

Quickstart Guide

## Frame Rate

| | PCI-Express | | USB 3.0 | |
|---|---|---|---|---|
| Array Size | 16-bit / 12-bit | 11-bit | 16-bit / 12-bit | 11-bit |
| 2048 x 2048 | 43 | 63 | 43 | 58 |
| 2048 x 1024 | 87 | 125 | 87 | 125 |
| 2048 x 512 | 173 | 249 | 173 | 249 |
| 2048 x 256 | 345 | 493 | 345 | 493 |
| 2048 x 128 | 681 | 964 | 681 | 964 |

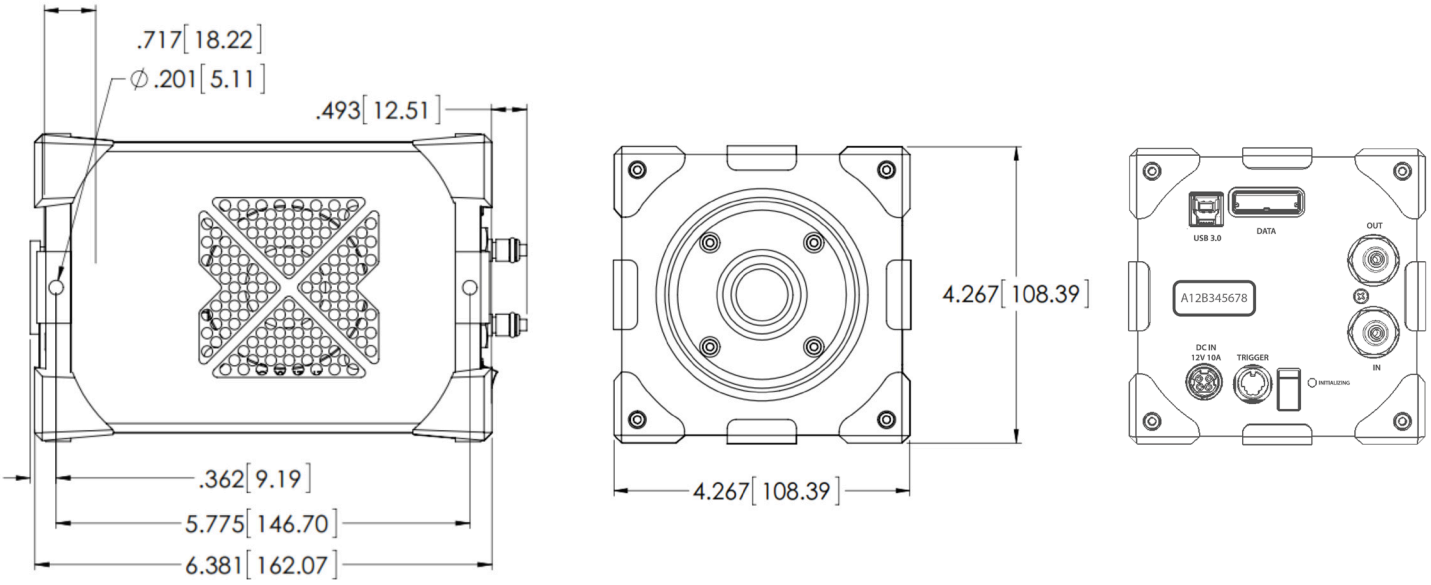
## Accessories (Additional)

PCIe Card/Cable

Liquid Circular

Liquid Cooling Tubes

Distance from C-mount to sensor



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# Mouse Brain Imaging and Electrophysiology

**Prof. David Attwell**

University College London

## BACKGROUND

The Attwell lab is interested in understanding the interactions that occur between neurons, glial cells and the vasculature of the brain through the use of electrophysiology and imaging techniques.

For years it was believed that brain blood flow, which provides the energy used for neural computation, was controlled solely by constriction and dilation of arterioles in the brain. However, the Attwell lab have since shown that contractile cells called pericytes, located at 30-micron intervals along brain capillaries, also play a major role (Hall *et al.*, 2014, Nature 508, 55; Mishra *et al.* 2016, Nature Neuroscience 19, 1619).

Capillaries are extremely small (around 4-5 microns in diameter) and therefore red and white blood cells need to change shape in order to pass through them. It is these changes in shape that the Attwell group are interested in imaging.

> " The camera has allowed us to acquire images that would otherwise be impossible to obtain, providing a basis for future analysis of how the properties of capillaries, and of red and white blood cells, determine brain blood flow. "

## CHALLENGE

The problem with imaging such changes of red and white blood cell shape is that the cells themselves move very quickly (~1mm/sec). They are also only 5 microns across, so they pass any given point in only a few milliseconds. They also change shape very quickly. Consequently, to capture images of the changes of shape, a rapid and high sensitivity imaging camera is needed.

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## SOLUTION

The Attwell lab required a high sensitivity and rapid acquisition camera, and thus are now using the Teledyne Photometrics Prime BSI with their imaging system.

Prof. Attwell told us that, "Based on prior interactions, we selected Teledyne Photometrics as a reliable source of information on this and ended up purchasing a Prime BSI for our imaging demands."

Prof Attwell went on to say that, "The Prime BSI is ideal for our purposes. By aligning a capillary with the camera image x-axis and choosing a smaller field of view in the y-axis, we can acquire images at ~1kHz, allowing us to image changes of the shape of the cells as they pass any given point in the capillary network. The camera has allowed us to acquire images that would otherwise be impossible to obtain, providing a basis for future analysis of how the properties of capillaries, and of red and white blood cells, determine brain blood flow".

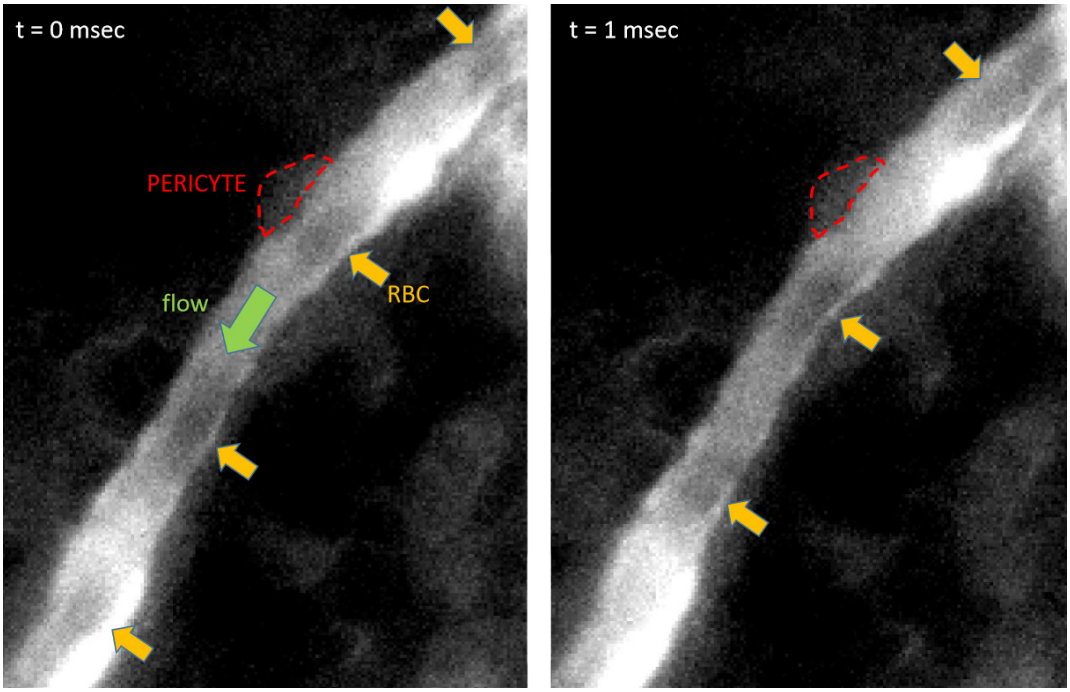


**Figure 1** Pictures taken 1 ms apart with the Prime BSI, showing the passage along a capillary (in an anaesthetised mouse brain) of red blood cells (RBCs; hazy black objects in the middle of the capillary, with orange arrows pointing at them - the bottom RBC moves out of the field of view during the time between the pictures). The Attwell group are interested in how the RBCs interact with, and have their movement affected by, the processes of contractile cells called pericytes which sit on the capillary wall (a pericyte is outlined in red).

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# Live Cell Imaging

**Jessica Kehrer, Dipl. Ing. & Prof. Friedrich Frischknecht**

Frischknecht lab, Centre for Infectious Diseases, Parasitology

Heidelberg University Medical School

## BACKGROUND

The Frischknecht lab aims to understand transmission of the malaria parasite between host and mosquito. The lab focuses on the two motile stages of the life cycle - the ookinete and the sporozoite using the rodent model parasite Plasmodium berghei. The movement of this parasite is particularly interesting because the cells are able to move at very high speed without changing their shape.

For their standard experiments, they are using reverse genetics to either remove proteins or to label them with fluorescent markers followed by characterization of the resulting parasite line with state of the art microscopy techniques.

> " Weak fluorescent proteins, which had been impossible to observe with our previous camera, can now be readily visualized. "

## CHALLENGE

Motility can be studied using sporozoites freshly isolated from the salivary gland of infected mosquitoes. Wild-type sporozoites generally show a circular movement which proceeds at a speed of 1.5-2 µm/s for up to one hour.

The current imaging solution in the Frischknecht lab is a front illuminated CCD sensor with a very limited field of view (FOV) and low sensitivity. Sensitivity limitations result in long exposure times which in turn make the analysis of the obtained data – involving tracking of individuals and studying their motion – difficult. Typically, exposure times of 300-400 ms or more for the individual channels is common. Therefore, it is currently impossible to image the localization of weak fluorescent proteins.

The relatively low FOV also results in a lower sample throughput, reducing the quality of the statistical information - especially for mutants with low infection rates.

(continued...)

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**CHALLENGE**

If sensitivity could be increased, the temporal resolution could also be improved. This would be crucial for improving tracking of the individual parasites, producing higher quality data that better represent physiological behaviour.

**SOLUTION**

The Prime BSI back-illuminated sCMOS allows the Frischknecht lab to image up to 20x faster with the same signal to noise ratio. With the increased sensitivity, lower exposure times allow for an increase in speed which produces a more accurate image of multiple channels, enhancing the ability to track individual Plasmodium parasites more reliably. This also means that their movement can be studied more accurately. Jessica told us, "Weak fluorescent proteins, which had been impossible to observe with our previous camera, can now be readily visualized."

In addition to the increase in speed, the Prime BSI has a much larger field of view resulting in the detection of nearly 3x more cells. This substantiates the produced data, solidifies statistics and makes the quality of scientific output much higher. In addition, this reduces the time needed to perform experiments, which reduces time at the microscope.

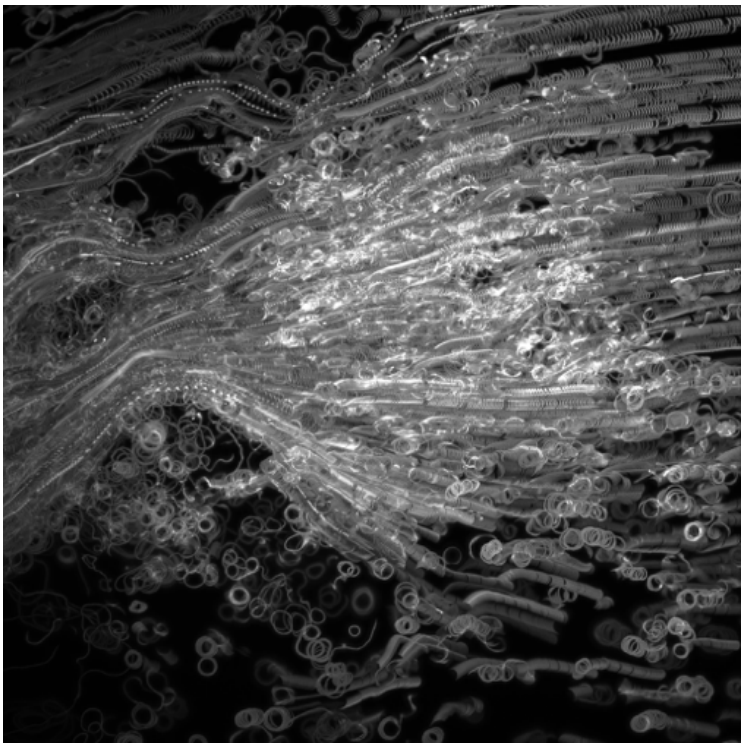

**Figure 1.** Maximum intensity projection of Plasmodia released from salivary glands. Cells were labelled with GFP. Imaging frequency was 250 fps.

Further details on: www.sporozoite.org

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# High Resolution Imaging of Bacterial Cell Division & Cytoskeleton Dynamics

**Dr Seamus Holden**

Centre for Bacterial Biology. Institute for Cell and Molecular Biosciences, Newcastle University.

## BACKGROUND

Dr. Holden's research lies between biophysics and microbiology, using super-resolution microscopy to study basic principles of bacterial spatial organization. In particular, the Holden Lab focuses on how the Gram-positive model bacterium *Bacillus subtilis* divides, and how the bacterial cytoskeleton guides the construction of a mid-cell cross-wall or septum. The Lab is currently working to understand these biological processes using novel methods based on microfabrication, microfluidics and single molecule and super-resolution microscopy.

> " The thing that really impressed me is how uniform the sensor is – far fewer hot pixels, noisy pixels and stripes than the last generation of sCMOS cameras. I hardly see a use for EMCCDs anymore. "

## CHALLENGE

Bacterial cell division takes place below the diffraction limit of microscopy, which is why the Holden Lab uses super-resolution and single molecule imaging to visualize aspects of the bacterial division process, such as the treadmilling dynamics of the bacterial cytoskeleton protein FtsZ during cell division.

The Lab's work depends on imaging either the dynamics of cytoskeletal protein filaments using only a few fluorescent protein labels on each filament, or individual cell wall synthesis enzymes in live bacteria samples at low illumination power. This combination of low fluorophore density and low illumination intensity means that they require a scientific camera to be sensitive and have a low noise profile.

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**SOLUTION**

The Holden Lab is now using the Teledyne Photometrics Prime BSI back-illuminated sCMOS to investigate how *B. subtilis* divide.

To make this decision, Dr Holden compared the Prime BSI to an EMCCD camera and was impressed with the equivalent level of sensitivity. He told us, "SNR levels are pretty indistinguishable for a tough sample of mNeonGreen-FtsZ in live cells at low illumination power."

Dr Holden went on to say that, compared to an EMCCD, "[the Prime BSI], is faster and offers a larger field of view with 2048x2048 pixels. The thing that really impressed me is how uniform the sensor is – far fewer hot pixels, noisy pixels and stripes than the last generation of sCMOS cameras. I hardly see a use for EMCCDs anymore."

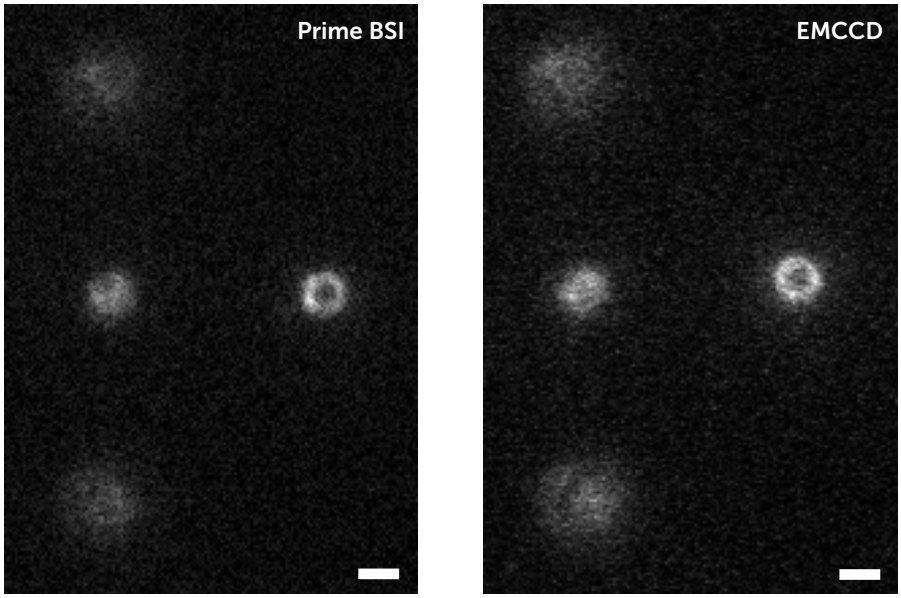


**Figure 1** Comparison of live cell imaging of FtsZ ring organization during bacterial cell division using a Prime BSI and an EMCCD camera. Images of FtsZ-mNeonGreen fluorescence in live *B. subtilis*, immobilized vertically in nanofabricated chambers, excited at 488 nm by HILO illumination, were acquired sequentially with a 100x objective using the same effective pixel size on each camera. Each image minimum value cropped to camera baseline of 100. Scale bar, 1 µm.

Additional Information:
https://threadreaderapp.com/thread/966001234573479937.html?refreshed=yes

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# Super-Resolution Standing Wave Microscopy

**Professor Gail McConnell**

Department of Physics, University of Strathclyde, Glasgow, UK

## BACKGROUND

The McConnell group are focused on the development of new optical microscopy methods for biomedical research and imaging.

Previously, the McConnell group were using mostly fixed cell imaging in their biological investigations, but they are now focusing on the development of techniques that will improve their live cell imaging capabilities. The group is currently working on the development of a new type of super-resolution microscopy based on the concept of standing wave microscopy.

Standing wave microscopy employs a wide field configuration that is combined with axial structured illumination to improve the resolution beyond the diffraction limit. The excitation light is composed of two beams which interfere giving rise to a standing wave, creating a sinusoidal excitation field in the axial plane. Only fluorescence molecules that coincide with the maxima of the standing wave will give rise to fluorescence.

> "The Prime BSI, with a 6.5 µm pixel, offers the most flexibility for use with the range of objectives we are using as well as improving the resolution."

## CHALLENGE

The McConnell group acquired a new Nikon Eclipse Ti2 widefield microscope as part of their tools and resources development project and so they started looking for a camera that would allow them to get the most out of their new system.

Professor McConnell told us, "We began to explore new options for suitable cameras to maximize the data quality from the new Ti2 instrument. We knew we wanted to use brightfield, fluorescence and DIC imaging. The microscope would also be used for live cell imaging using various magnification objectives, so we also required flexibility from the camera to be compatible with a range of objectives from 10X to 60X and compatibility with the new NIS-Elements software".

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**SOLUTION**

The McConnell group is now using the Teledyne Photometrics Prime BSI on the Nikon Eclipse Ti2 widefield system. Professor McConnell shared "Following the demo of the Prime BSI, we were very happy with the camera, and have been using it every day since."

Professor McConnell told us, "The Prime BSI, with a 6.5 µm pixel, offers the most flexibility for use with the range of objectives we are using as well as improving the resolution".

Professor McConnell went on to say, "The customer service from Teledyne Photometrics was excellent. All our queries were responded to quickly and Teledyne Photometrics were very available and always happy help and to offer training. I was very happy to buy from them."

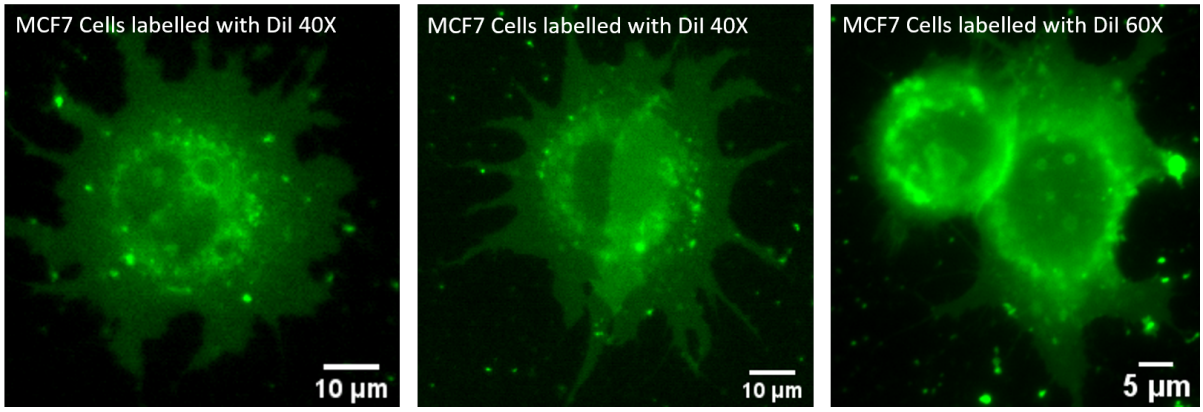


**Figure 1.** MCF-7 breast cancer cell line stained with either a 10.7µM concentration of 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (Invitrogen) [10µL of DiI to 1mL of Hepes Buffered Saline (Gibco)] or a 5.35µM concentration [5µL DiI to 1ml HBS]. Imaged with a 100ms exposure, excited with 525 nm CoolLED light source. Imaged using a 40X (air) or 60X (oil) objective on a Nikon Eclipse Ti2 with Teledyne Photometrics Prime BSI camera.
Cells plated in Ibidi dishes by Gill Robb, imaged by Gill Robb and Shannan Foylan of the McConnell group.

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# Single-Molecule Fluorescence Imaging

**Alexander R. Carr, Postdoctoral Researcher**
**Steven F. Lee, Principal Investigator**

TheLeeLab, Department of Chemistry, University of Cambridge

## BACKGROUND

TheLeeLab, at the University of Cambridge, focuses its research on developing biophysical tools to answer fundamental biological questions, primarily using single-molecule fluorescence imaging techniques. Recently the group has established single-molecule spectroscopic imaging, facilitating local hydrophobicity mapping (1), as well as implemented cutting-edge point-spread function engineering for large-volume single-particle tracking in live T cells (2).

> " The Prime BSI Back-illuminated sCMOS gives EMCCD-level sensitivity at high frame rates and with a very large field of view, speeding up our single molecule tracking acquisition. "

## CHALLENGE

As with all single-molecule techniques, sensitivity is vital as the photon budget of individual fluorophores can be limited. For single-particle tracking applications, detecting more photons allows for longer trajectories to be recorded and more robust statistical analysis to be conducted.

Additionally, the motion of a fluorophore must be adequately sampled in order to accurately record its behaviour. For fast-moving targets, such as many cytoplasmic and nucleic proteins, short exposure times are required and thus the emission is spread over a greater number of frames.

In order to achieve live single-particle tracking of fast-moving targets, both high sensitivity and fast acquisition rates are vital. Although EMCCDs have previously been used to achieve both of these factors, high speed came at the cost of a much reduced field of view, which made data collection inefficient.

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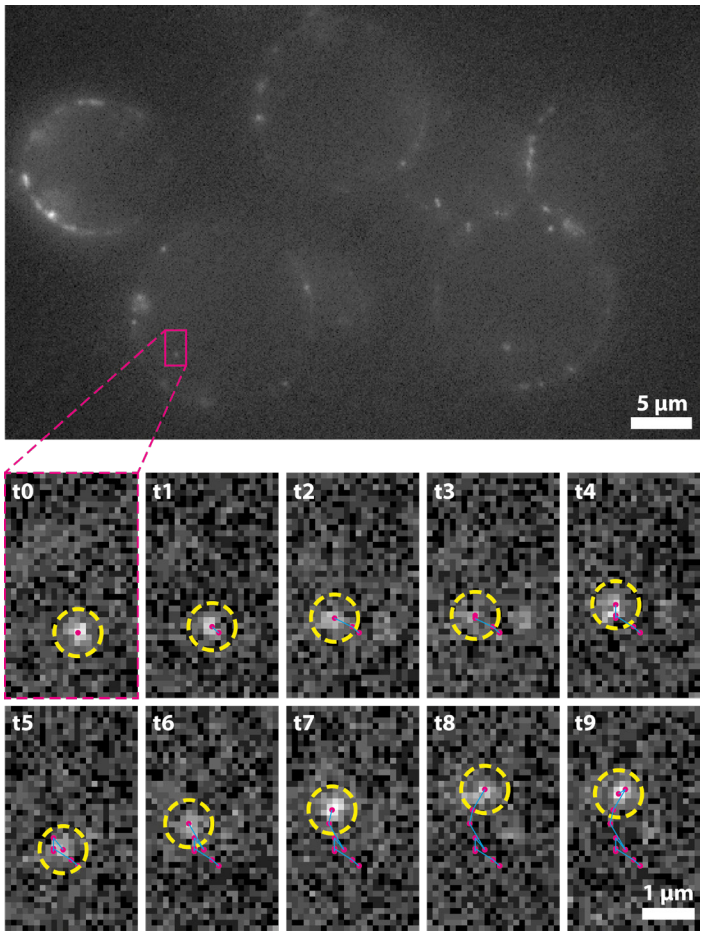
**SOLUTION**

The high quantum efficiency and low noise of the Prime BSI sCMOS combines EMCCD-level sensitivity with a fast acquisition rate and much larger field of view. The Prime BSI speeds up data collection as multiple cells can be imaged within the same field of view at >100 fps with high contrast.




Fast single-particle tracking in multiple live T cells. *Top* Six Jurkat T cells were imaged in a single field-of-view at 100Hz. The cytoplasmic protein Zap70 was fluorescently labeled with Tetramethylrhodamine (TMR) using the HaloTag enzyme. *Bottom* Sequential images of a single Zap70 molecule diffusing within the cytoplasm of a Jurkat T cell (highlighted region from *top*).

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# Imaging Mitotic Dynamics

**Dr Gary Gorbsky, W.H. and Betty Phelps Chair in Developmental Biology**
Program Chair, Cell Cycle & Cancer Biology Research Program
Oklahoma Medical Research Foundation

## BACKGROUND

Research in the Gorbsky lab focuses on the basic mechanisms of how chromosomes assemble and move during cell division in normal cells and in cancer cells, the process termed mitosis. A major emphasis is the mitotic spindle checkpoint pathway that makes sure that each copy of the 23 pairs of chromosomes is distributed equally to each of the daughter cells.

This checkpoint system detects chromosomes that have failed to align at the metaphase plate and delays chromatid separation at anaphase until alignment is complete. Failure of the checkpoint pathway may underlie chromosome abnormalities which are hallmarks of cancer and defects in embryo development.

> "The Prime BSI coupled with our light sheet microscope enables us to capture images every few seconds and still have cells successfully completing mitosis with no ill effects. With our previous instrumentation we were at best able to capture images at minute intervals."

## CHALLENGE

The use of fluorescent proteins in live cell microscopy has revolutionized the study of mitosis. However, there are many pitfalls that stem from the tendency of the fluorescent proteins to be photobleached and from cellular damage due to phototoxicity. Mitosis is particularly sensitive to phototoxicity and substantial efforts are taken by the lab to minimize the amount of light reaching the sample.

At the same time, the lab is interested in mapping events at the highest temporal and spatial resolution. Measuring fluorescent protein location during mitosis is challenging, as structures assemble and disassemble quickly. High illumination and emission intensities perturb the mitotic process which requires them to use short exposure times to limit cell damage.

Low excitation light and short integration times result in low photon numbers arriving at the camera, so high camera detection efficiency and low camera noise is key to generating good data.

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**SOLUTION**

The Photometrics Prime BSI, with its small pixels and high quantum efficiency, allows the Gorbsky lab to sample at high spatial resolution with high sensitivity. This allows them to image events in mitosis with finer time resolution or over longer duration which gives them better sampling with less total light input and lower overall photobleaching and photodamage. This is critical with their new light sheet imaging system, where they might acquire through focus series at frequent intervals.

For example, where their previous high resolution microscope and camera could at most image cells at one to five minute intervals for the entire process of mitosis, the Prime BSI coupled to their light sheet microscope enables them to capture images every few seconds, with cells successfully completing mitosis with no ill effects.

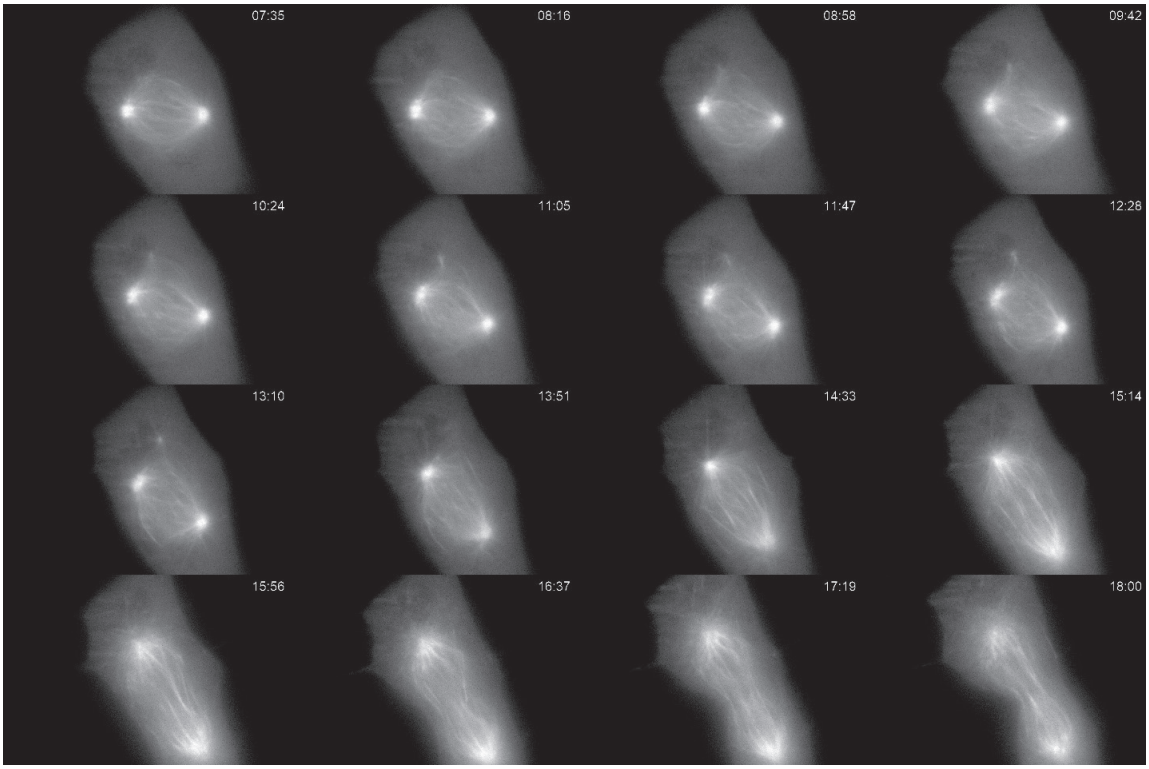


**Figure 1:** Cultured Xenopus cell expressing GFP-tubulin undergoing mitosis.
Imaged every ten seconds for over 18 minutes using light sheet illumination and a high resolution oil immersion objective. Selected frames are shown depicting major changes in the mitotic spindle as the cell progressed from prometaphase through metaphase, and anaphase. The high sensitivity of the Prime BSI allowed for greater total number of samples at lower illumination without noticeable photobleaching or photodamage allowing us to track changes in the mitotic spindle microtubules.

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# Live Vesicle Trafficking

**Dr George Sirinakis, Senior Research Associate**

St Johnston Lab, part of the Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, UK

## BACKGROUND

The St Johnston Lab is primarily interested in how cells become asymmetric so that they can perform distinct functions on opposite sides of the cell. This is known as cell polarity.

In particular, the lab are interested in epithelial cells, which form the sheets that line our organs. Epithelial cells must have an apical side which contacts the lumen, for example the inside of your small intestine, lateral membranes where cell-cell contacts are made, and a basal surface that maintains contact with the extracellular matrix.

Loss of polarity, especially of epithelial cells, is a hallmark of cancer. In this project the lab aims to image the movement of vesicles with cargo proteins destined for these different membranes.

> "The high sensitivity and low read noise of the Prime BSI is transformative in these experiments as it enables us to use low levels of excitation power and significantly reduce photo-damage to the tissue."

## CHALLENGE

The St Johnston Lab are aiming to image fast moving, small vesicles that contain a limited number of cargo proteins which results in a very low fluorescence signal. What is more, the lab are imaging in tissue, which causes further reductions in brightness due to aberrations. Finally, the tissue needs to be kept alive for imaging so the laser power must be kept to a minimum to reduce any phototoxic effects.

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## SOLUTION

The Prime BSI back-illuminated sCMOS camera was a great solution for the St. Johnston Lab, combining high, 95% quantum efficiency and low read noise with the fast speed expected of an sCMOS device.

Dr Sirinakis shared with us, "The high quantum efficiency and dynamic range of the Prime BSI enables us to visualize even the dimmest vesicles that contain limited numbers of cargo molecules and track them with high speeds in live tissue."

Dr Sirinakis went on to say, "The high sensitivity and low read noise of the Prime BSI is transformative in these experiments as it enables us to use low levels of excitation power and significantly reduce photo-damage to the tissue. With the Prime BSI we are now able to ask more meaningful questions about vesicle trafficking and better understand cell polarity."

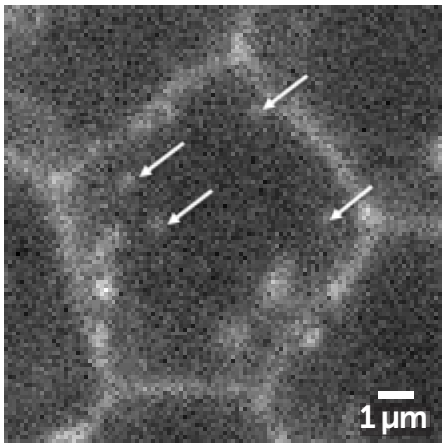


**Figure 1** Drosophila epithelial cells. The arrows indicate vesicles containing a cargo protein, cell adhesion molecule Fasciclin III (FAS3) tagged with Halo and labeled with SiR dye.

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# Single Molecule Imaging

**Redmar Vlieg, PhD Student**

John van Noort Group, Leiden Institute of Physics, Leiden University, The Netherlands.

## BACKGROUND

Redmar Vliegs research, within the group of John van Noort, primarily involves the use of two-photon microscopy to investigate biological processes in zebrafish embryos and *in vitro* measurements on gold nanorods (GNRs).

Due to their localized surface plasmon resonance (LSPR), GNRs have unique optical properties which allow them to be used as single-molecule sensors or very bright luminescent markers. The resonance frequency is dependent on the refractive index in the near-field of the rod, hence perturbations by small molecules can be detected by measuring this frequency shift. By exciting the GNRs via the non-linear excitation mechanism of two-photon microscopy, sensitivity can be increased to detect even smaller molecules.

Besides detection of single molecules, GNRs are used as luminescent markers. The LSPR mediates a significant increase in absorption of the excitation light as it couples with the incoming EM waves, making them as bright as quantum dots. Moreover, GNRs have the added benefit that they do not bleach and blink, and their surface can be easily functionalized for biological applications. Hence, the group investigates the merits of using GNRs as two-photon contrast agents for *in vivo* measurements.

> " The low readout noise, high quantum efficiency and large sensor size makes the Prime BSI all we need from a camera. "

## CHALLENGE

One issue that the group face when using GNRs is that when the temperature of the rods is increased the ends start to diffuse, even at relatively low temperatures. Although gold melts around 3000°C, the tips start diffusing at much lower temperatures. As gold nanorods start to lose their shape they begin to lose their aspect ratio, which causes the LSPR to shift and renders them useless for imaging.

(continued ...)

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## CHALLENGE

The group initially were using an EMCCD camera with a 60x TIRF oil lens for their *in vitro* studies. However, oil objectives have a very short working distance which is not appropriate for *in vivo* imaging of thick samples such as Zebrafish embryos. For this reason, they have since changed the setup to have a single 25x objective to permit a longer working distance and larger field of view, which required a more suitable camera to maximize speed and resolution.

The group therefore want to image with the lowest excitation light possible to prevent destruction of their GNR markers, as well as imaging the *in vivo* samples with high speed to permit single molecule tracking. For this reason, the group was interested in back-illuminated sCMOS cameras as they have high QE combined with large sensors and fast readout speeds.

## SOLUTION

The van Noort Group is now using the Teledyne Photometrics Prime BSI back-illuminated sCMOS with 25x magnification on their multifocal multiphoton custom microscope, using LabVIEW to control all components of the system. Redmar Vlieg told us that "The LabVIEW drivers provided with the Prime BSI allowed us to integrate the camera exactly the way we want in our in-house build microscope."

Redmar Vlieg went on to say, "We decided on the Prime BSI as the smaller pixel size, compared to other back-illuminated cameras on the market, made it best suitable for high resolution tracking. Moreover, our EMCCD camera, the QuantEM:512SC, was also from Photometrics, which always performed very adequately".

Having a high QE permits the reduction of exposure time which is ideal for his applications, permitting imaging of the gold nanorods without modifying their structure. Redmar Vlieg also stated that the camera offers great benefits such as no excess noise and no gain decay.

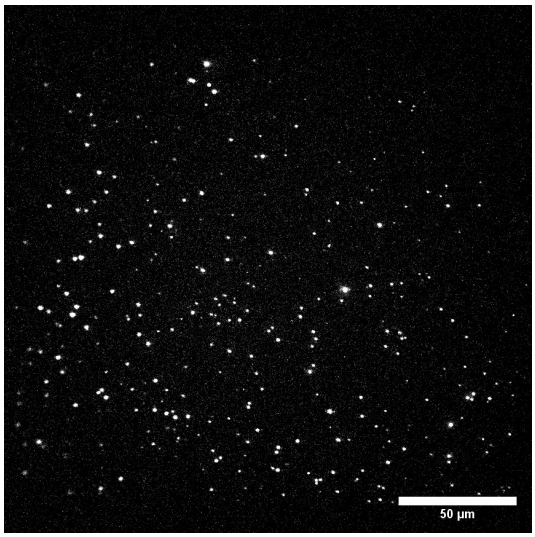


**Figure 1** Multifocal two-photon image of gold nanorods randomly dispersed on a glass coverslip. Differences in signal intensity are explained by either exciting the rods at their LPSR, or rod aggregates.

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# Live-Particle Tracking

**Dr. Adam Wexler, Post-Doctoral Researcher**

Arie Zwijnenburg Laboratory for Advanced Microscopy and Optical Metrology,

Wetsus - European Centre of Excellence for Sustainable Water Technology, Leeuwarden, Netherlands.

## BACKGROUND

Dr. Wexler's research interest is to bring the power of photonics to the field of sustainable water technology. Currently he, along with collaborators at the University of Twente, are focused on preventing outbreaks of waterborne viruses by understanding and improving water filtration technologies.

The virus particles investigated are small, roughly 20-30 nm in size, and can sometimes pass through water filtration systems and remain infectious. There is currently no practical way to investigate the effectiveness of these filtration devices in real time.

Dr. Wexler is working towards building a microscope that permits tracking of these particles in real time to detect viable viruses in the water and assess the efficiency of these filtration processes. The ultimate aim is to couple the real-time imaging to automated imaging algorithms that can detect problems in the water and alert water production managers before an outbreak begins.

> " The increase in QE was huge, and really helped with our application. "

## CHALLENGE

The primary challenge is the low fluorophore labelling densities, Dr. Wexler explains that they try not to modify the viruses too much so there are very few fluorophores (<40) per virus.

Dr. Wexler and his colleagues realized that when there are 40 fluorophores on a virus capsid they exhibit stable fluorescence but when the viruses are fragmented they blink. This allows differentiation between whole and broken virus particles. The combination of low fluorophore density, rapid blinking, and flow means that low noise, camera speed, and sensitivity are important.

Dr. Wexler looked at several sCMOS cameras and told us that one of the biggest factors for them was the quantum efficiency (QE). He sometimes uses far-red dyes so a high QE at these wavelengths is of additional importance.

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**SOLUTION**

Dr. Wexler is now using the Teledyne Photometrics Prime BSI back-illuminated sCMOS camera on his widefield microscope to image these virus particles in real-time under flow at 40x magnification. He shared, "The increase in QE was huge, and really helped with our application."

Dr. Wexler also mentioned that the larger field of view was a welcome improvement because they are tracking and counting particles, so the increased throughput really improves the quality of statistics from the population.

Dr. Wexler concluded, "So far the camera performs well and has a lot of potential for our future work." They also hope that the high speed will be useful for quantification measurements using FRET to distinguish between whole and fragmented viral particles.

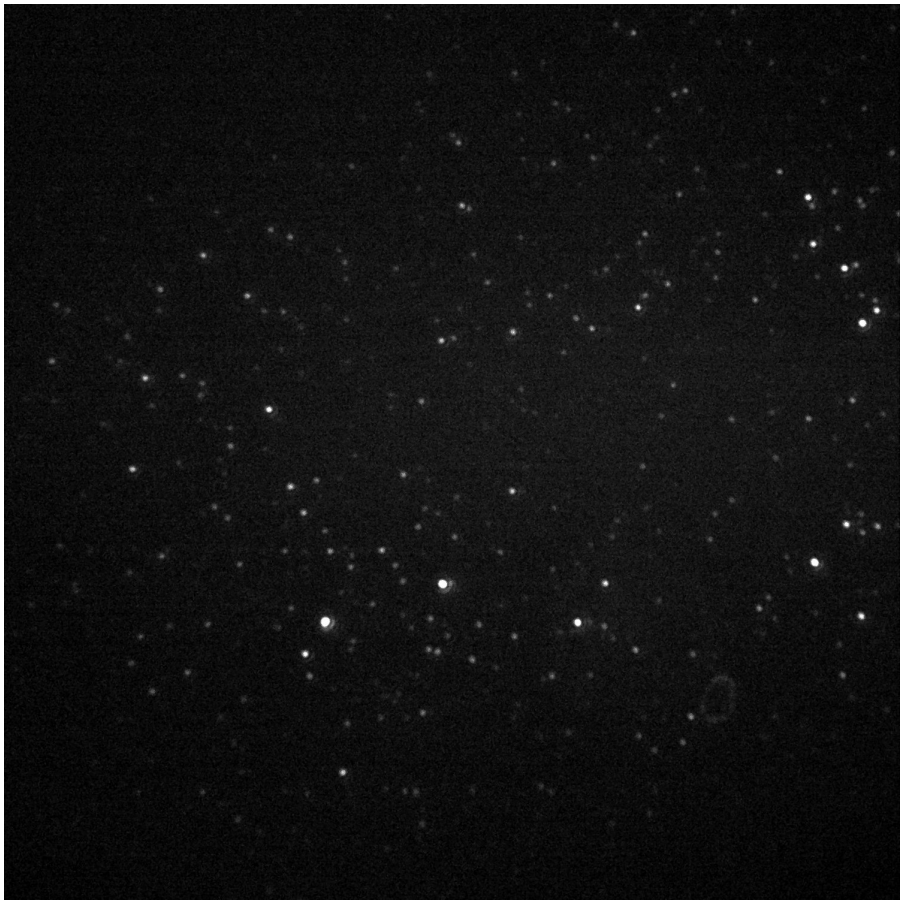

**Figure 1** Fluorescent image of CCMV (cowpea chlorotic mottle virus) particles. The brightest and largest objects are aggregates of many viruses while the dimmer points are single virus particles while the dimmest are fragments of capsids.

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# Single Molecule TIRF Imaging

**Dr. Biswajit Pradhan, Post-Doctoral Researcher**

Cees Dekker Group, Department of Bionanoscience, Kavli Institute of Nanoscience at Delft University of Technology, The Netherlands.

## BACKGROUND

Dr. Pradhan's research is concerned with understanding how DNA supercoils with a family of proteins called structural maintenance of chromosome (SMC) proteins.

When cells are not dividing, the chromosomes are not properly organized. However, during cell division DNA becomes organized. Just prior to cell division, following DNA replication, chromosomes condense and become tightly wound via supercoiling. Chromosomes then pair up along the center of the cell and are pulled to either side of the cell by fibers attached to the centromeres.

SMC proteins play a role in some of these events and Dr Pradhan is trying to study the interactions that occur between DNA and proteins at the single molecule level, to determine how the DNA becomes supercoiled by proteins during this process. Dr Pradhan is building a custom single molecule TIRF system to study these interactions.

> "The Prime BSI offers several advantages such as speed, field of view and price when compared to the EMCCD. The service at Teledyne Photometrics is also very good, I was always updated, and my questions were answered quickly, Teledyne Photometrics were really willing to help out."

## CHALLENGE

Dr Pradhan told us, "Our biggest challenge is the limited number of photons that a single molecule emits. Our molecules emit approximately $10^4$-$10^5$ photons before they bleach so the molecules can only be observed for a limited amount of time. If we want to use lower exposure times, the excitation light intensity needs to be increased which means the molecules bleach even more quickly."

Dr Pradhan was previously using an EMCCD camera with 120-150x magnification. As a result of this high magnification and relatively small field of view of the EMCCD camera, he was not able to image a very large sample area. He has since built a new system with 60x magnification to increase the sample area. However, the larger pixel size of the EMCCD does not allow for Nyquist sampling at 60x.

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**SOLUTION**

Dr. Pradhan is now using the Teledyne Photometrics Prime BSI back-illuminated sCMOS with his home-built single molecule system.

Dr Pradhan shared, "The Prime BSI offers several advantages such as speed, field of view and price when compared to the EMCCD." Dr Pradhan continued, "The service at Teledyne Photometrics is also very good, I was always updated, and my questions were answered quickly, Teledyne Photometrics were really willing to help out."

Dr Pradhan went on to say, "The Prime BSI has the advantage of a small pixel size, so I don't have to expand my beam. This makes the optical system much easier to work with. Without zooming I can get to the maximum resolution that I want. The high speed of the camera also really helps during my acquisitions."

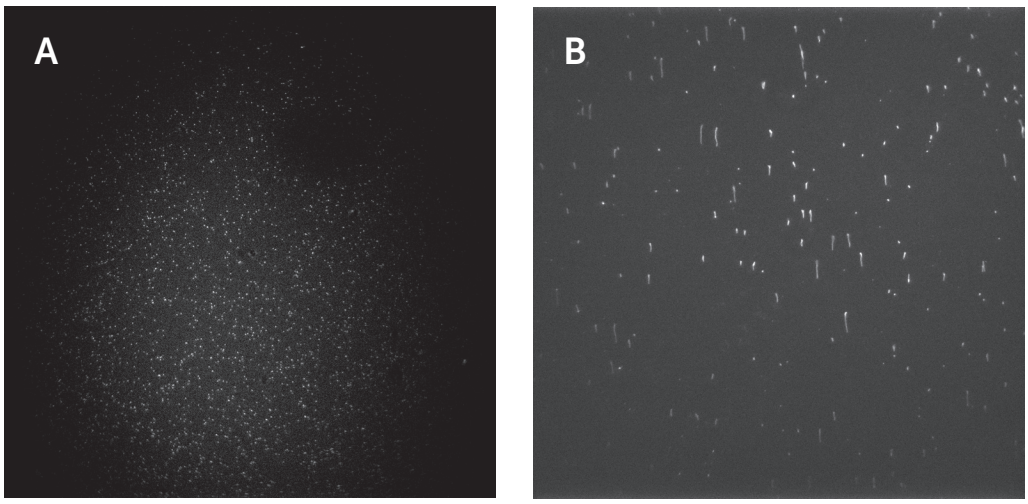


**Figure 1** Images of A) Single Molecules of ATOO647N and B) DNA Chains taken with the custom single molecule TIRF system using the Teledyne Photometrics Prime BSI.

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# Calcium Imaging

**Prof. Michael J.M. Fischer, PhD**

Institute for Molecular Physiology, Medical University

## BACKGROUND

Dr. Fischer´s research addresses nociception, the nervous system's response to harmful or potentially harmful stimuli, with focus on the peripheral nervous system. A particular interest is the role of transient receptor potential (TRP) channels, which play an important role in sensing pain.

Typically, imaging experiments in the Fischer lab involve the use of fluorescent indicators in cultured primary neurons or cell lines. Sensors for calcium ions ($Ca^{2+}$) and voltage are used as activity readouts for spontaneous and induced signal transduction – with sensors being either ratiometric or non-ratiometric.

> "The direct control of other devices by TTL output allowed reliable high-speed measurements triggered by the camera without a separate real-time controller. The combination of high sensitivity and the ability for fast measurements making use of this sensitivity became very useful."

## CHALLENGE

A typical experiment performed by the group uses a chemical cue to generate rolling $Ca^{2+}$ waves along a cellular extension which spread across the cell. Depending on the traveling speed, this requires a very high sampling rate. High-speed image acquisition, as well as structural-functional correlation, present important imaging challenges faced by the lab.

For conventional imaging techniques, observation of these $Ca^{2+}$ waves requires a compromise between the acquisition speed necessary to obtain any meaningful kinetics and the low magnification needed to achieve substantial output. This is especially important when obtaining precise $Ca^{2+}$-concentrations using ratiometric sensors such as Fura-2, which require switching between two excitation wavelengths.

In the best-case scenario, the $Ca^{2+}$ signals will need to be imaged at close to 1000 frames per second (fps) and triggering through a software solution can be too slow to achieve this.

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## SOLUTION

The Prime BSI enables the Fischer lab to image the progression of $Ca^{2+}$ waves across neurites with unprecedented speed, accuracy, and precision.

Even ratiometric Fura-2 imaging can be achieved with an effective speed of up to 460 fps (>920fps, alternating 340/380 nm excitation) using the SMART (Sequenced Multiple Acquisition in Real Time) streaming feature implemented on Photometrics Prime cameras.

SMART streaming allows the camera to directly hardware-trigger up to 4 individual excitation channels with individually adjustable exposure times and intensities.

When imaging at these speeds, every single photon counts. The Prime BSI, with 95% quantum efficiency and very low read noise, produces results with an excellent signal-to-noise ratio and a very high dynamic range, which makes the camera the perfect match for the Fischer lab´s application.

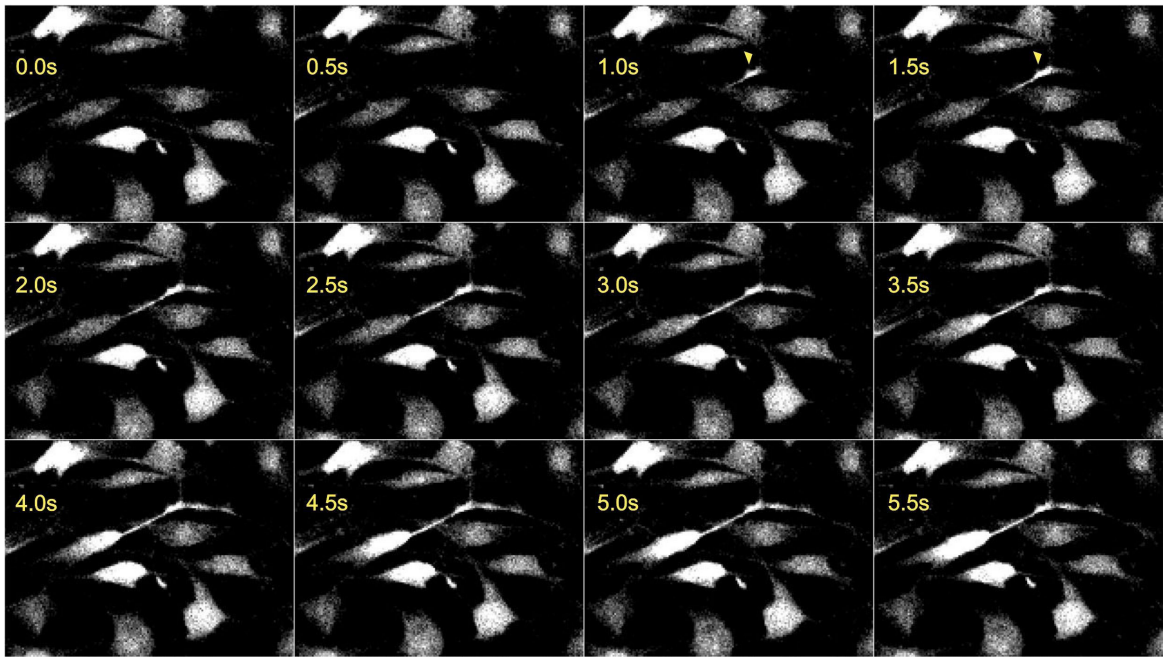


**Figure 1.** Montage of a sequence of grey scale images depicting a slow traveling calcium wave resolved at high temporal and spatial resolution with the Photometrics Prime BSI. Upon application of a receptor agonist, calcium influx started in a cellular extension (yellow arrowheads), followed by a spreading wave that reaches all cytosolic areas.

Cells were stained with Fluo-8 and excited at 455 nm using an Omicron LED HUB. The receptor distribution is compared to the start of the initiation of the calcium wave by subsequent confocal imaging of immunocytochemistry.

For more information visit www.meduniwien.ac.at

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# TIRF Microscopy

**Facility Technician: Marko Kamp, PhD Student: Christian Niederauer, Group Leader: Dr. Kristina Ganzinger**

Physics of Cellular Interactions Group at AMOLF

## BACKGROUND

The group of Dr. Ganzinger is interested in the basic physical principles of immunological signaling. The group uses synthetic biology to reconstitute signaling pathways, and single molecule imaging to enable them to understand how receptors and ligands work together to transmit signals through the cell.
In particular, they are interested in how the length of time the ligand is associated with the receptor can provide a means for a receptor to differentiate between multiple ligands. By labeling the receptor subunits their individual diffusion can be visualized prior to ligand binding events. Once the ligand is added, colocalization of the subunits can be observed and receptor interactions inferred, as well as the kinetics of different ligand binding.

> “It used to be that people would go for EMCCDs for their high quantum efficiency. However, back-illuminated sCMOS cameras now offer the same quantum efficiency but with the larger field of view which, for us, was essential for our multicolor acquisitions.”

## CHALLENGE

The group uses site-specific organic fluorophores to label the different subunits of the receptor of interest to ensure they have a large enough photon budget to track the molecules with enough signal. As they are monitoring multiple receptors and ligands, the experiments involve three color imaging, potentially including the far-red, and thus a camera with a high quantum efficiency over a broad spectral range is essential for their research.

The subunits can also diffuse quickly, meaning a camera with a high frame rate is needed to permit accurate tracking. Likewise, in order to track the molecules simultaneously, the sensor must be split into three. As such, a camera with a large field of view is also essential.

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**SOLUTION**

The group is now using the Teledyne Photometrics Prime BSI on their custom home built TIRF system to simultaneously image in up to three colors at high frame rates.

Christian told us, "We picked the Teledyne Photometrics Prime BSI camera partly due to the large field of view, which is essential as we split the sensor to permit imaging with two/three colors simultaneously, but also for the broad quantum efficiency suitable for imaging at multiple wavelengths. We also needed a camera that could offer high frame rates as the receptor subunits diffuse quite quickly." He went on to say that, "The Prime BSI also offers the ideal pixel size to match Nyquist with our 60X objective offering us the best resolution possible".

Christian concluded, "It used to be that people would go for EMCCDs for their high quantum efficiency. However, back-illuminated sCMOS cameras now offer the same quantum efficiency but with the larger field of view which, for us, was essential for our multicolor acquisitions."

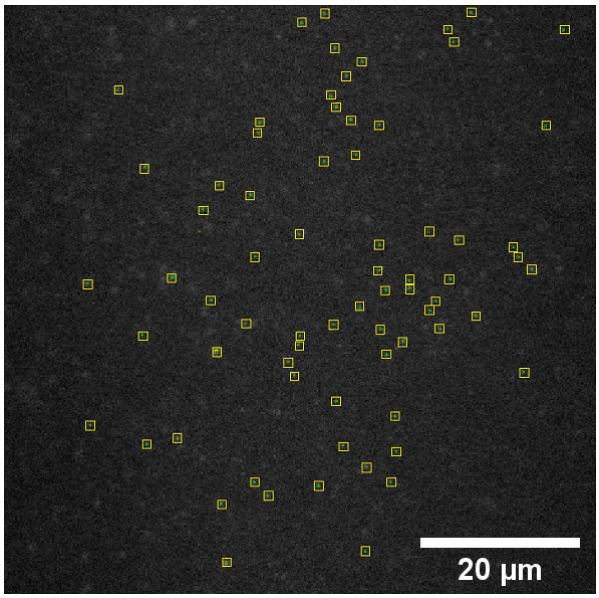



**Figure 1** Snippet of a movie taken with one of the receptor-proteins on a membrane. The single molecules are localized and fit with a Gaussian to find their position with sub diffraction-limited precision."

# 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 is able to 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. An easily repeatable experiment is also outlined to evaluate camera sensitivity.

## Quantum Efficiency

Quantum efficiency can be pretty well 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.

### *Photon shot noise*

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.

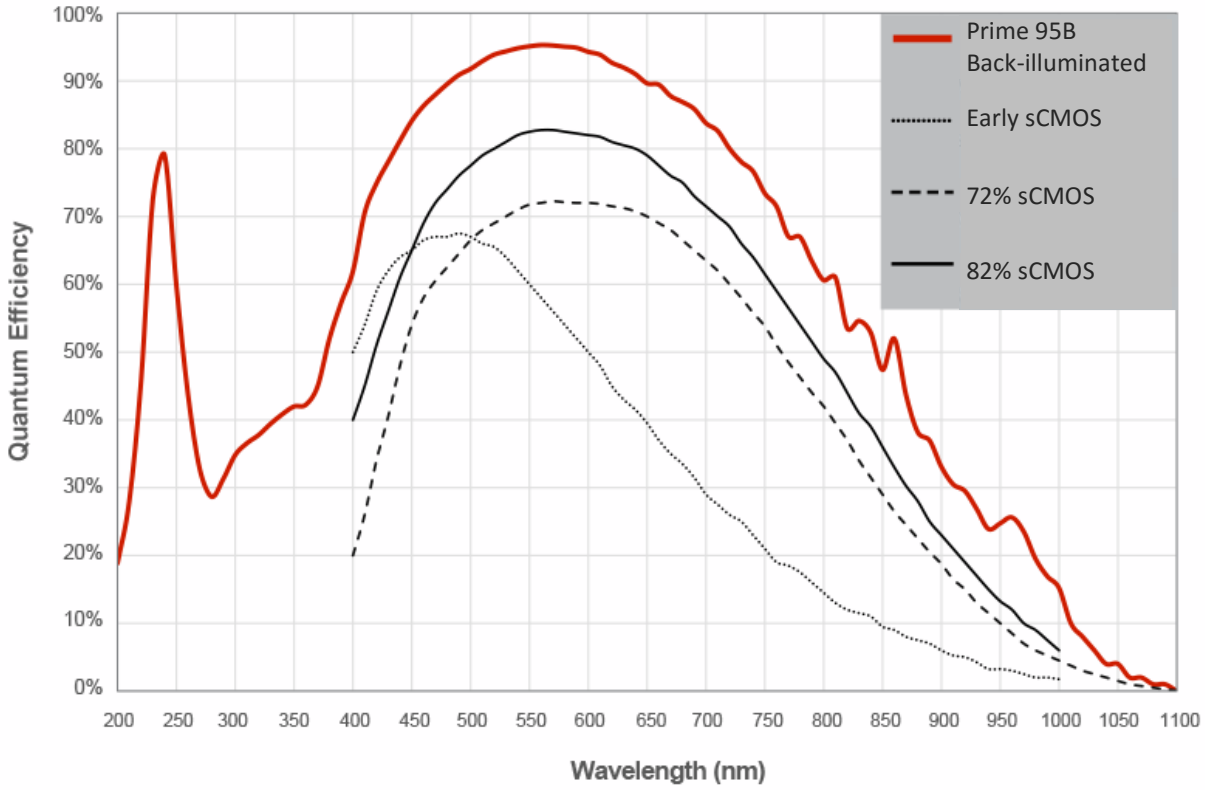
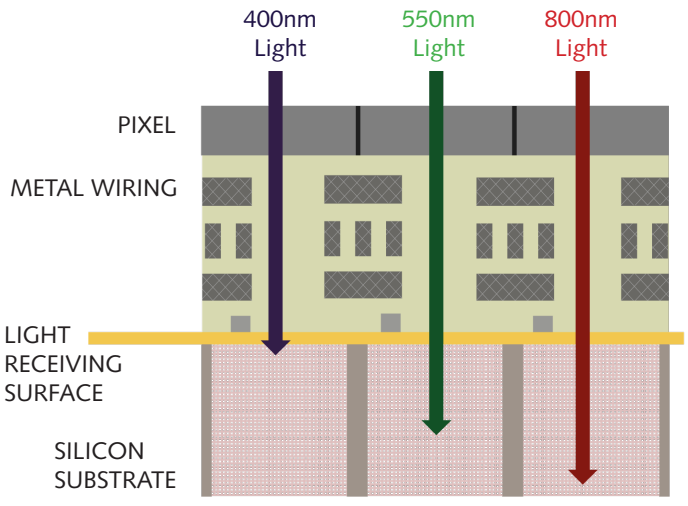



**Figure 1: Comparison of quantum efficiency curves of some typical CMOS cameras**
Adapted from Princeton Instruments, Kuro sCMOS.

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).




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.

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

*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

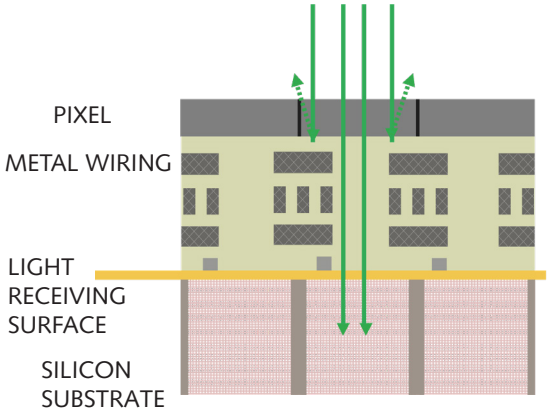



**Figure 3:** Photons unable to pass the metal tracks, wiring and transistors present in front of the CMOS sensor.

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.

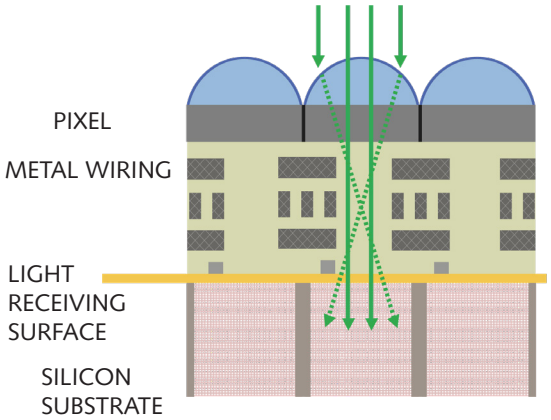



**Figure 4**: The use of microlenses to increase quantum efficiency of CMOS sensors.

## 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 To allow the photons to penetrate deep enough into the silicon substrate to be converted into

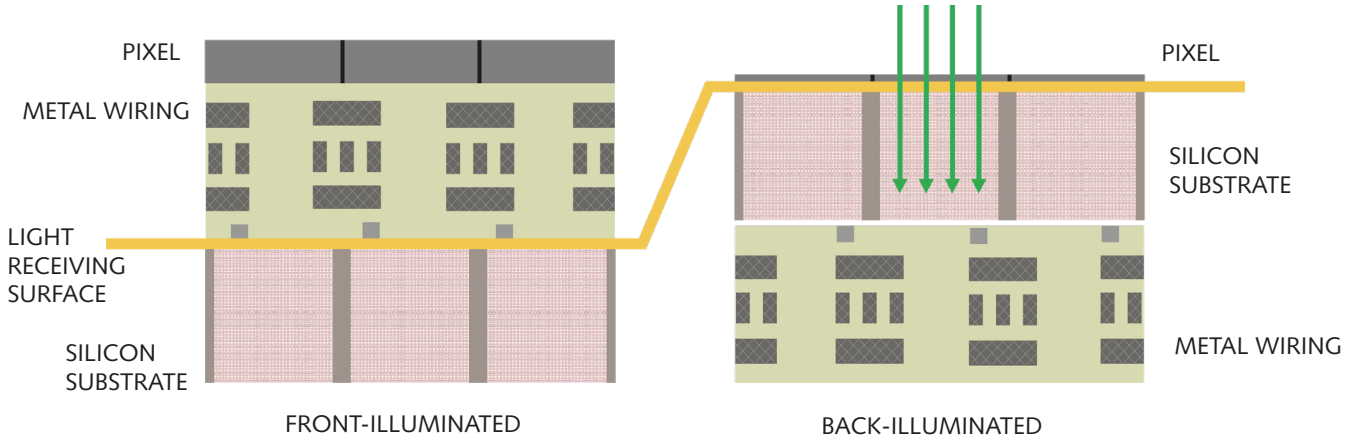



**Figure 5:** Comparison of front and back illuminated sensors.

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.

## Summary

Camera sensitivity is determined by quantum efficiency, pixel size and noise characteristics. Increasing pixel size comes with the disadvantage of reducing resolution so increasing quantum efficiency is a more attractive method of increasing camera sensitivity. Recently, back-illuminated CMOS sensors have been developed which allow quantum efficiency to reach up to 95%.

# 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 for. 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:

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

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.

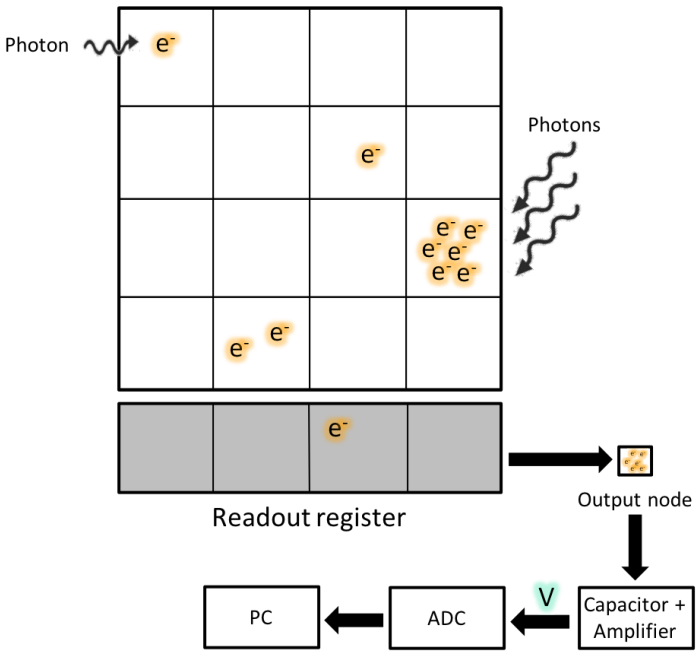



**Figure 1: CCD architecture.**
The number of electrons created is directly proportional to the number of photons hitting the pixel. After exposure, electrons are moved down, row by row, until they reach the readout register. The readout register shuttles the row of electrons one at a time into the output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

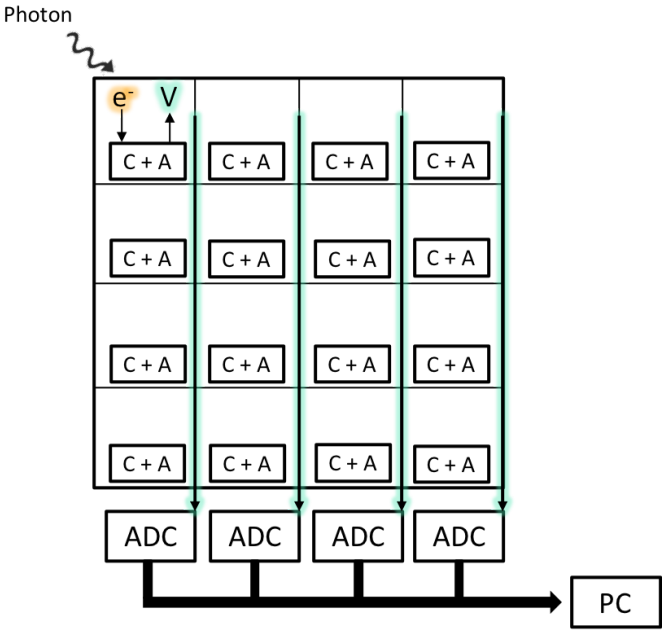



**Figure 2:** Scientific CMOS architecture. Photons hit the pixels and create electrons, individual capacitors and amplifiers are on every pixel. The generated voltages are sent down the whole column to the analogue to digital convertor (ADC) and the digital signal is read by a computer. This makes CMOS 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 3 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.

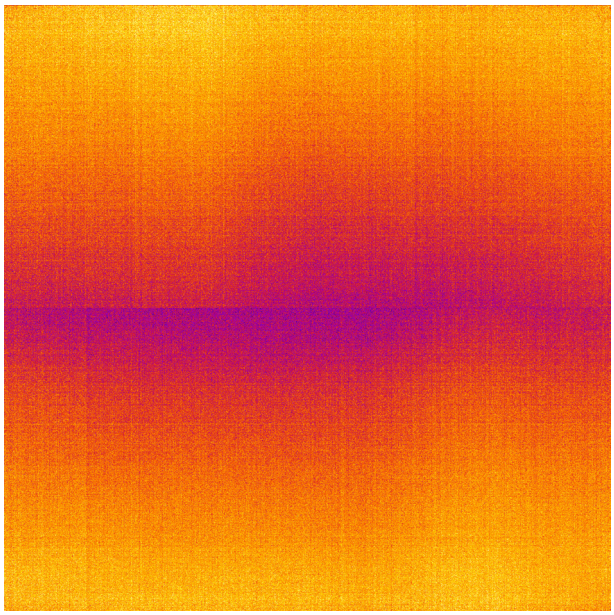


**Figure 3:** Fixed pattern noise. Fixed pattern noise on a typical scientific CMOS camera with a split sensor. Variation across the background is around 6 $e^-$.

### *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 Teledyne Photometrics scientific CMOS cameras (Figure 4B) prevents this problem from occurring and provides a better environment for quantitative imaging.

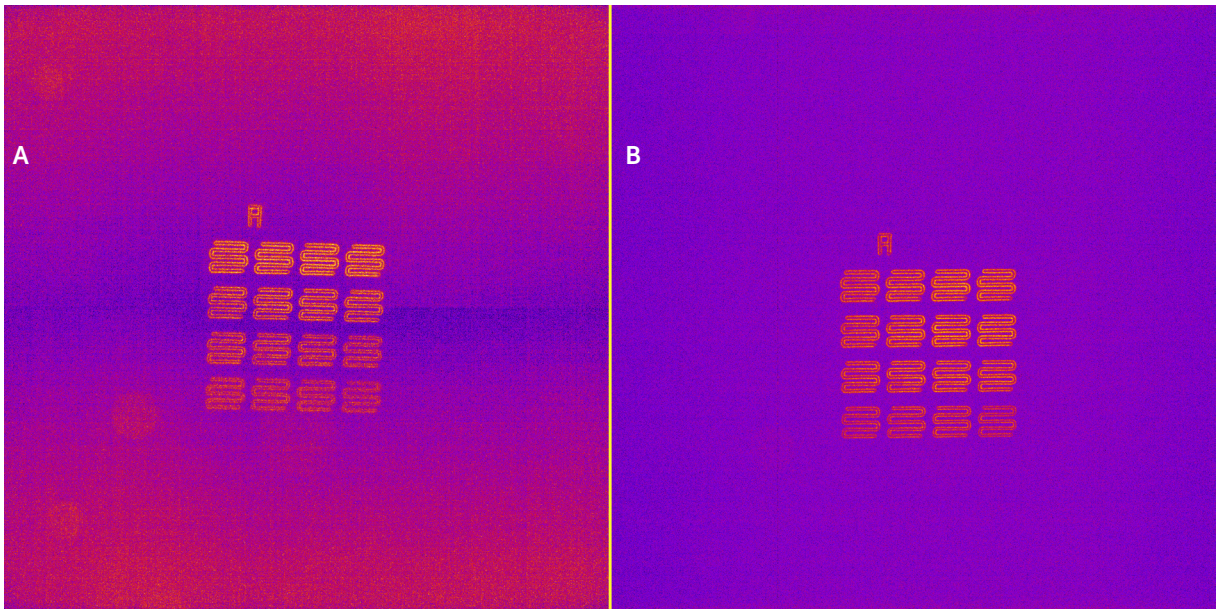



**Figure 4:** Roll off comparison
A) Current, standard, split sensor scientific CMOS camera and B) the GPixel GSENSE2020BSI sensor used in the Teledyne Photometrics Prime BSI.

### *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 Teledyne 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.

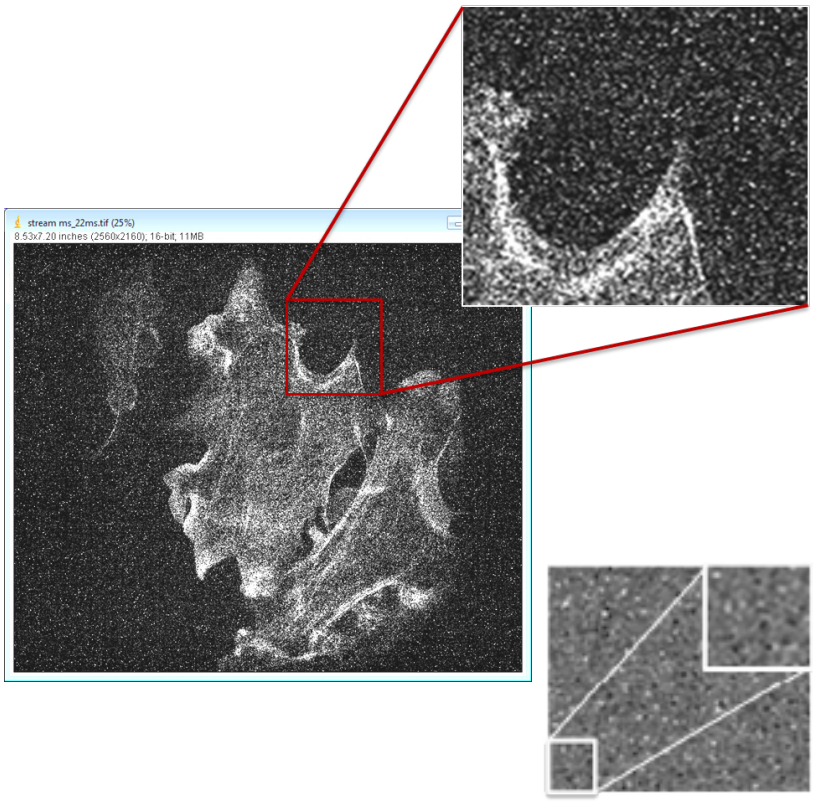



**Figure 5:** Random Telegraph Noise.
Example of random telegraph noise displaying the fluctuation between bright, average and dark pixel states.

## *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.

# 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:

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

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 Teledyne 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.

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.

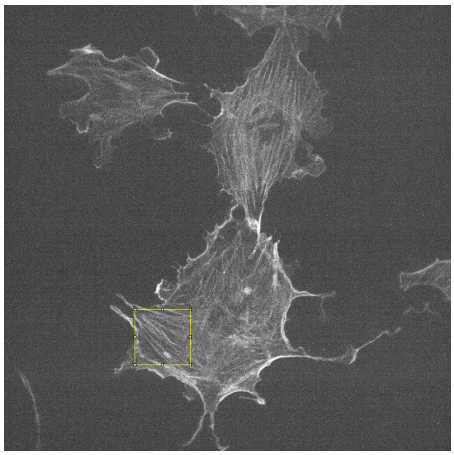


**Figure 1a**
Original Image

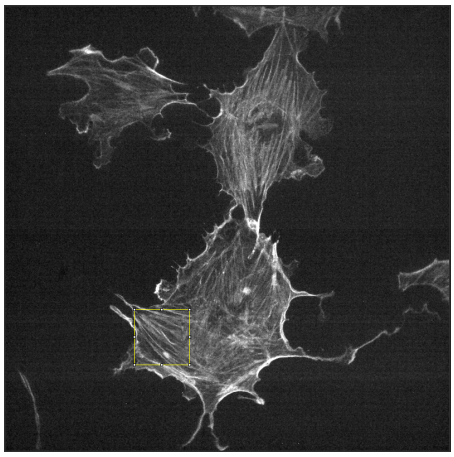


**Figure 1b**
PrimeEnhance

| | | 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**

## 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.

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.

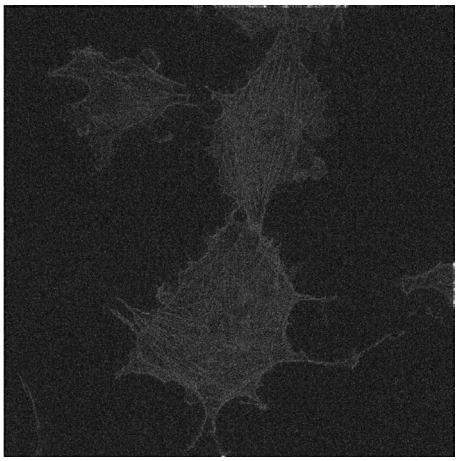


**Figure 1c**. Difference Image

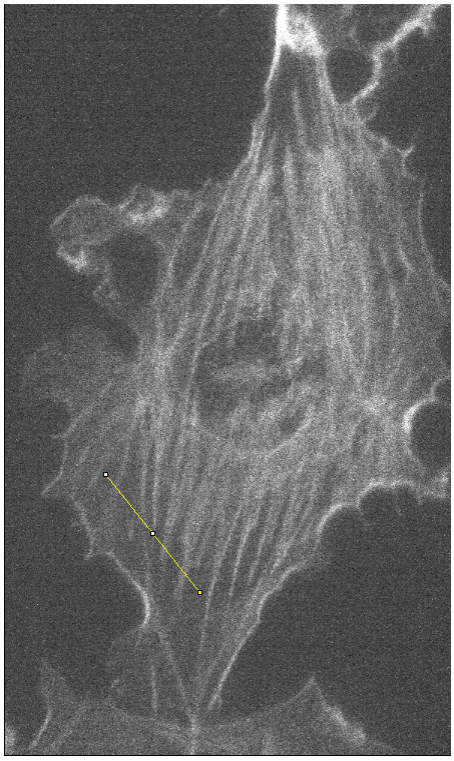


**Figure 2a.** Original Image

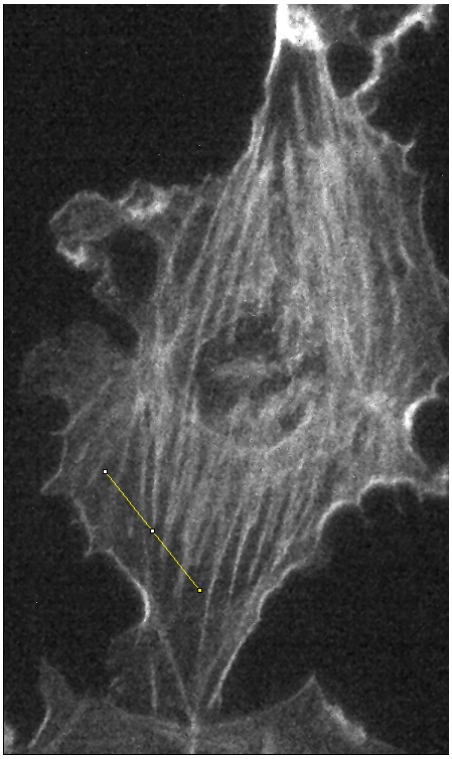


**Figure 2b.** PrimeEnhance

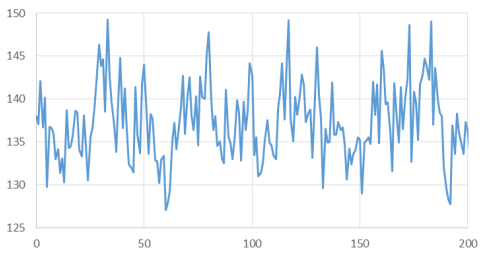



**Figure 2c.** Line Profile for Original Image

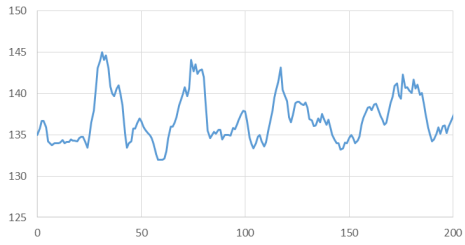



**Figure 2d.** Line Profile for PrimeEnhance

## 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.

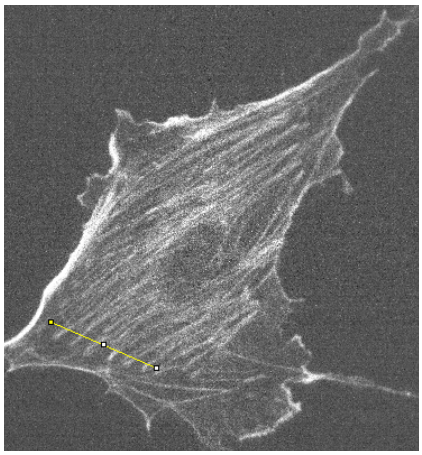


**Figure 3a.** Raw Image at 100ms exposure

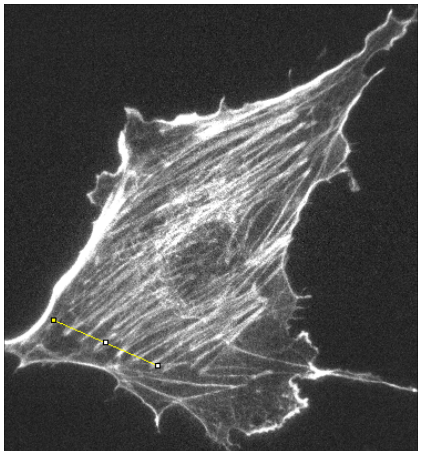


**Figure 3b.** PrimeEnhance at 100ms exposure

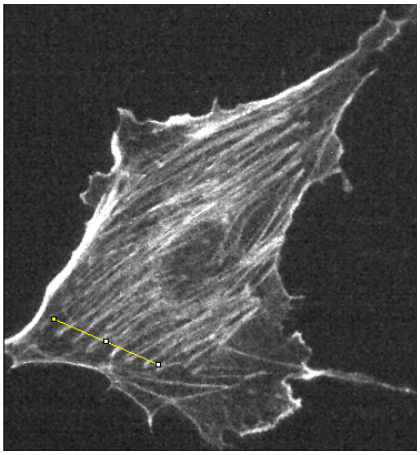


**Figure 3c.** Raw Image at 800ms exposure

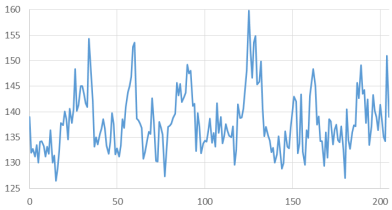



**Figure 3d.** Line Profile of Raw Image at 100ms

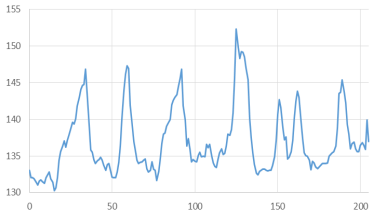



**Figure 3e.** Line Profile of PrimeEnhance Image at 100ms

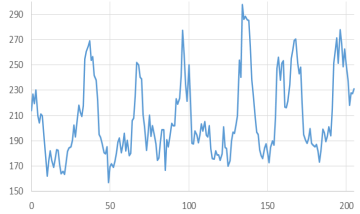



**Figure 3f.** Line Profile of Raw Image at 800ms

## 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.

Vision Systems Design 2016 Innovators Awards

# Lower Read Noise with Correlated Multi-Sampling

In the simplest of terms, pixel values in a CMOS image sensor represent photoelectrons collected in each pixel during an exposure. The collected electrons are first converted to a voltage inside the pixel which is subsequently measured using an Analog-to-Digital Converter (ADC).

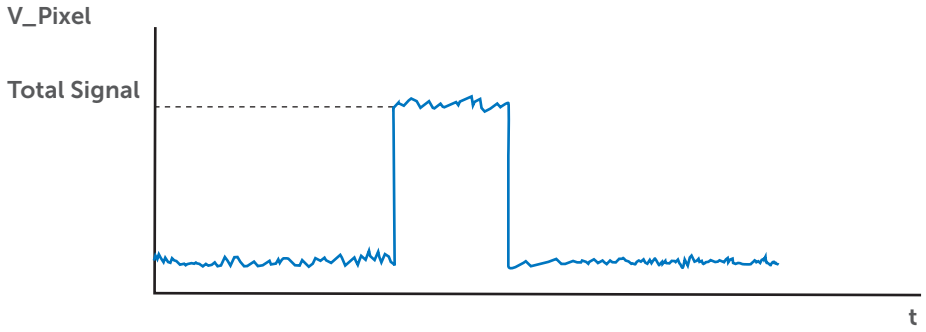



**Figure 1:** Peak of the voltage for the pixel indicates how much signal was measured. The pixel integrates the signal for the length of the exposure time, building up the voltage in the pixel

Unfortunately, the process of measuring the pixel contributes a small amount of additional noise known as read noise, impacting precision at low light levels.

Two of the main components of read noise are "reset noise" (also known as kTC noise) and "amplifier noise". Reset noise is a random offset created when a pixel is cleared of previous charge. Reset noise can largely be eliminated using Correlated Double Sampling (CDS). CDS measures both the offset and the signal level and subtracts the two in order to obtain an accurate measurement.

With reset noise largely eliminated by using CDS, amplifier noise becomes the dominant source of read noise. Amplifier noise has a high frequency white noise component and a low frequency "1/f" component. This low frequency component is particularly difficult to remove. One solution is to sample the offset and signal levels multiple times and average the results. This special implementation of CDS is called Correlated Multi-Sampling (CMS), and can be done with two or more samples.

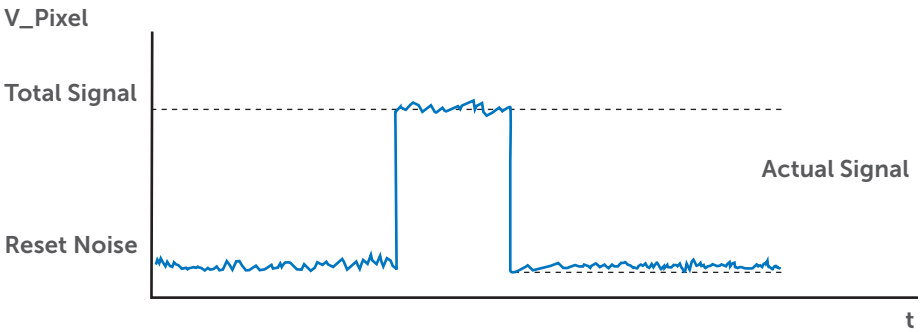



**Figure 2:** The actual measured signal is the voltage of the signal minus the reset noise.
Actual Signal = Total Signal – Reset Noise

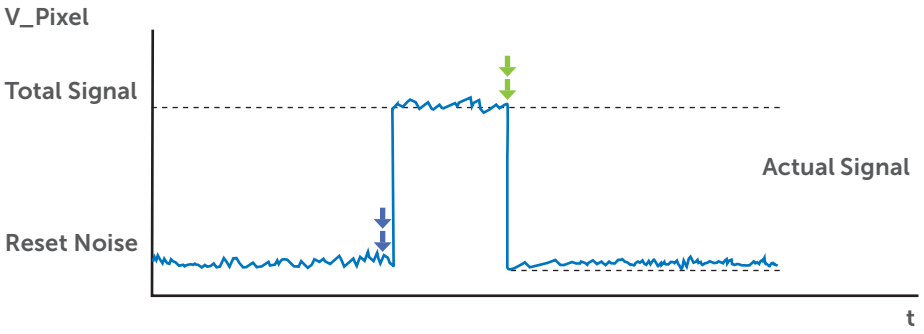



**Figure 3:** Sampling the reset and the integrated signal twice

Using the Signal to Noise equation to determine the impact on read noise, the calculation for a CDS based acquisition is as follows. Other noise sources such as shot noise are omitted as they do not contribute and are not affected by CDS.

$$SNR_{1x\ CDS} = \frac{Signal}{\sqrt{Noise^2}}$$

Using CMS and sampling the measured signal twice results in the signal to noise calculation becoming:

$$SNR_{2x\ CMS} = \frac{2 \times Signal}{\sqrt{2 \times Noise^2}}$$

$$SNR_{2x\ CMS} = \frac{1.414 \times Signal}{\sqrt{Noise^2}}$$

A two-times sampling of the signal provides a theoretical $\sqrt{2}$ improvement in the SNR of the measurement and, as the signal level itself is not changing, this corresponds primarily to the reduction of the amplifier noise component of the read noise. This makes it possible to achieve read noise levels close to 1 electron with CMS capable sCMOS sensors.

Typically, sampling the integrated signal level multiple times comes with a negative impact to frame rate, as each sampling introduces a delay to the readout process. This delay is equal to the time it takes to digitize a pixel. To minimize this delay, some CMOS sensors can use two separate signal chains to simultaneously perform 2X CMS.

Typically, these two signal chains are used for sampling with high sensitivity (high-gain) for low signal levels, and a low sensitivity (low-gain) for higher signal levels, to provide dynamic range. The high-gain and low-gain data is combined to provide an image with both high sensitivity and a large dynamic range.

In some sensors it is possible to reconfigure the low-gain amplifier as a second high-gain amplifier. Instead of using a high-gain and a low-gain to combine into an image, it is now two high-gain images being combined. This enables the multi-sampling of the signal level to reduce the read noise in the measurement without a trade-off in frame rate. The trade-off comes in the form of a lack of significant dynamic range as the full-well capacity in this implementation is limited.

# Programmable Scan Mode

## Introduction

Programmable Scan Mode provides increased control over the rolling shutter exposure and read-out functionality of CMOS sensors. The rolling shutter read-out behavior is a common implementation on CMOS sensors, and Programmable Scan Mode provides access to the sensor timing settings to allow optimization around imaging requirements.

Rolling shutter readout implementations digitize pixel intensities one row at a time. A "reset" signal starts at the first row of the sensor and resets or clears the row of pixels of any collected signal, effectively starting the exposure. This reset signal sweeps downwards towards the last row, propagating at a rate defined as line-time: the amount of time it takes to activate each row of pixels.

The reset signal is followed by a "readout" signal which causes the amount of light captured by each row of pixels to be digitized and read out of the sensor. The readout signal signifies the end of the exposure or light capture. The time between the reset and readout signals is defined as the exposure time, the amount of time each pixel is in the light collection phase.

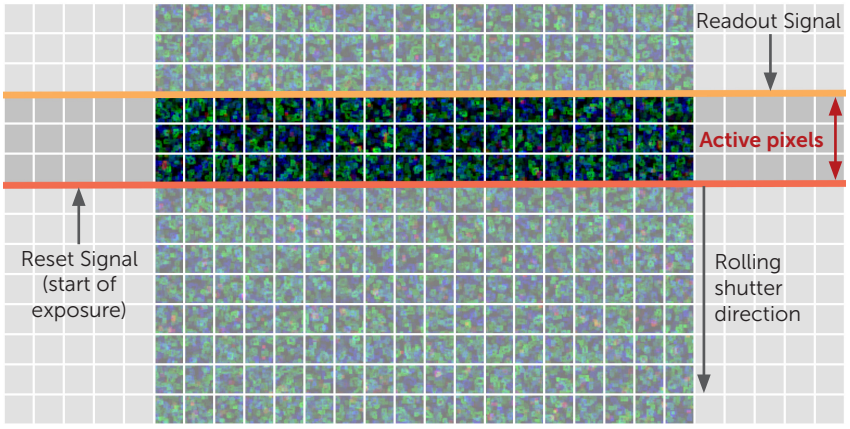



**Figure 1:** The highlighted region of the sensor between the reset signal and readout signal is the only area collecting light. As the rolling shutter proceeds down the sensor, the highlighted region sweeps from the top to the bottom. This can be synchronized to the sweep of an illumination laser for scanning light sheet microscopy.

The readout signal always follows the reset signal by a delay equal to the exposure time. This exposure time is always a multiple of the line-time of the sensor. If the exposure time is set to be equal to 10 line-times, the readout signal is behind the reset signal by 10 rows.

## Increasing the Line-Time

Modifying and increasing the line-time of a sensor gives greater flexibility to control the number of rows between the reset and readout signals and the exposure time, allowing for both an optimized experimental setup and adequate signal levels.

Controlling the line-time makes synchronization between the illumination and acquisition simpler as well, providing control over the rate at which the exposing rows propagate down the sensor.

Programmable Scan Mode does not change the line-time of the sensor but provides the ability to add delays to the reset and readout signals. The addition of these delays delivers the same effect as if the line-time of the sensor has been increased (Figure 2).

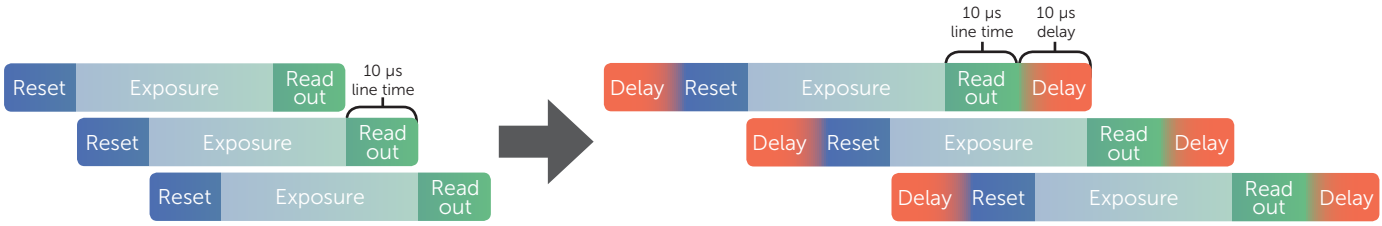



**Figure 2:** Delays added to the reset and readout signals allow the line-time of the sensor to be increased. In this case, the line time is doubled by adding an extra 10 µs delay to the readout signal.

## Modes

Programmable Scan Mode provides access to three modes:

### 1. Auto

When Programmable Scan Mode is set to Auto, the line-time is set to 1 line. This provides the highest framerates and minimal control over the ability to set the width between the reset and readout signals. There is no delay added after the line time. This is the default mode and represents normal sensor operation.

### 2. Line Delay

When Programmable Scan Mode is set to Line Delay, a delay can be added after the line-time, slowing the propagation of the reset and readout signals. This causes the effective line time of the sensor to be increased. This delay is added onto the default line time in increments equal to the line-time.

$$\text{Line Time}_{\text{Effective}} = \text{Line Time}_{\text{Sensor}} + (\text{Line Time}_{\text{Sensor}} \times \text{Line Delay Value})$$

A value of 1 adds a delay equal to 1 line-time. This results in an effective line-time equal to double the value of line time.

$$\text{Line Time}_{\text{Sensor}} = 10\mu s$$

$$\text{Line Delay Value} = 1$$

$$\text{Delay} = 10\mu s \times 1 = 10\mu s$$

$$\text{Line Time}_{\text{Effective}} = 10\mu s + 10\mu s = 20\mu s$$

A value of 10 adds a delay of 10 line-times which results in an effective line-time equal to 11 times longer than the default line-time.

$$\text{Line Time}_{\text{Sensor}} = 10\mu s$$

$$\text{Line Delay Value} = 10$$

$$\text{Delay} = 10\mu s \times 10 = 110\mu s$$

$$\text{Line Time}_{\text{Effective}} = 100\mu s + 10\mu s = 110\mu s$$

The frame rate when imaging in this mode is determined by the number of rows being imaged and the Effective Line Time.

$$\text{Readout Time}_{\text{Image}} = \text{Line Time}_{\text{Effective}} \times \text{N}_{\text{Rows}}$$

$$\text{Frame Rate} = \frac{1}{\text{Readout Time}_{\text{Image}}}$$

The minimum line delay value is 1. This is also the default value when Line Delay mode is selected.

When in Line Delay mode, Scan Width, the number of rows between the reset and readout signals will be calculated automatically.

$$\text{Scan Width} = \frac{\text{Exposure Time}}{\text{Line Time}_{\text{Effective}}}$$

A Scan Width parameter is available and reports the number of rows between the reset and readout signals.

### 3. Scan Width

When Programmable Scan Mode is set to Scan Width, the number of rows between the reset and readout signal can be set. It gives direct control to set the size of the imaging region.

Scan Width = Number of Rows between Reset and Readout

When the Scan Width is set, the effective line time required is automatically calculated.

$$\text{Line Time}_{\text{Effective}} = \frac{\text{Exposure Time}}{\text{Scan Width}}$$

A Scan Line Time parameter is available and reports the Effective Line Time in nanoseconds.

The three scan modes can be selected in the Device Property Browser dialog box of Micro-Manager as shown in Figure 3. The line time of Teledyne Photometrics cameras that can use Programmable Scan Mode can be found in Appendix I.

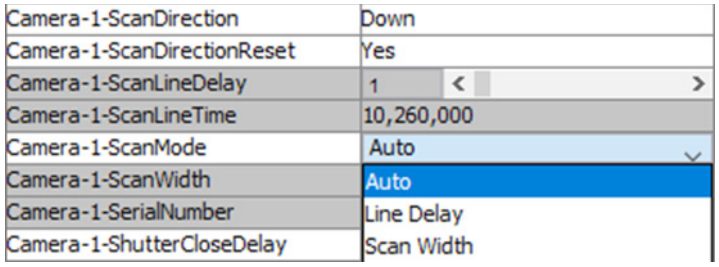


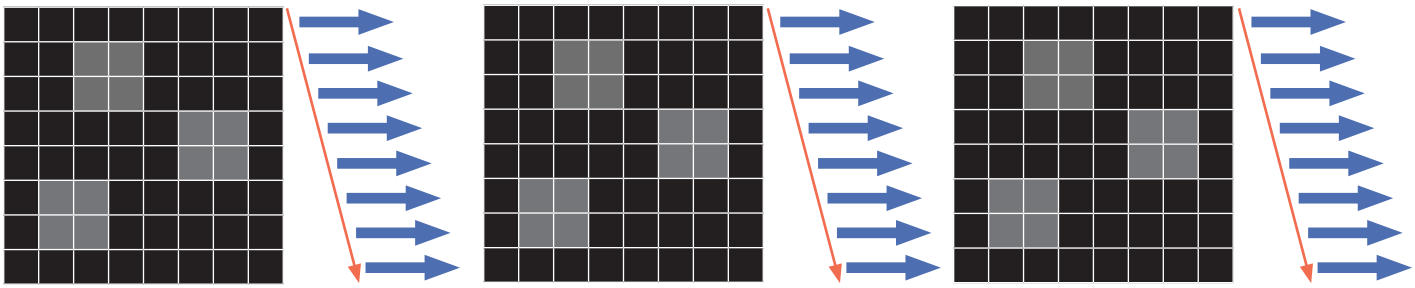

**Figure 3:** Scan Mode selection as displayed in Micro-Manager

## Scan Directions

To help with integrating sCMOS cameras into imaging systems, Programmable Scan Mode also provides control over the direction of readout for the rolling shutter. Programmable Scan Mode provides three options to select from.

### 1. Down

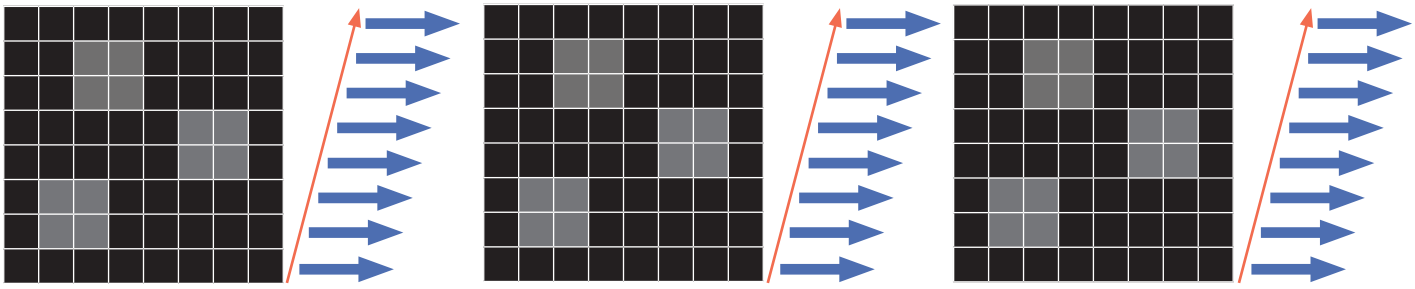
A scan direction of Down is the default readout direction for all sCMOS cameras. The rolling shutter starts at the topmost row of the sensor and propagates downwards towards the bottommost row.



Each subsequent frame acquisition restarts at the topmost row.

### 2. Up

A scan direction of Up inverts the direction of read out. The rolling shutter starts at the bottommost row and propagates upwards towards the topmost row.

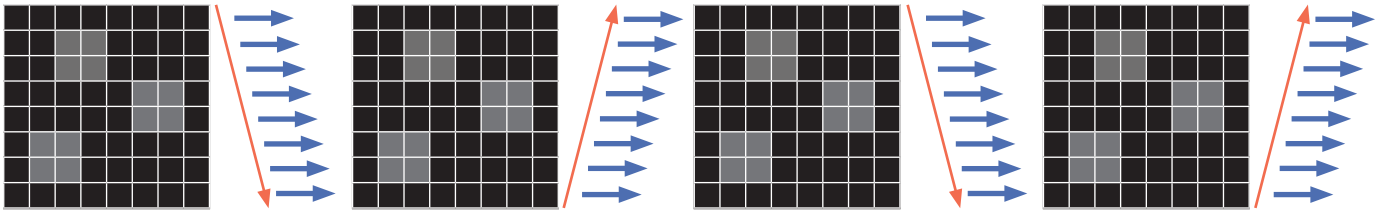


Each subsequent frame acquisition restarts at the bottommost row.

The image orientation when acquired in this mode will not be inverted and will be consistent with the Down scan direction.

### 3. Down-Up Alternate

A scan direction of Down-Up alternates the direction of acquisition. The rolling shutter starts at the topmost row and propagates downwards towards the bottommost row. For the next frame, the rolling shutter will begin at the bottommost row and propagate upwards towards the topmost row. The acquisition will continue to alternate the readout direction between frames.



The image orientation when acquired in this mode will have no inverted frames and will be consistent with the Down scan direction.
The three scan directions can be selected in the Device Property Browser dialog box of Micro-Manager as shown in Figure 4.

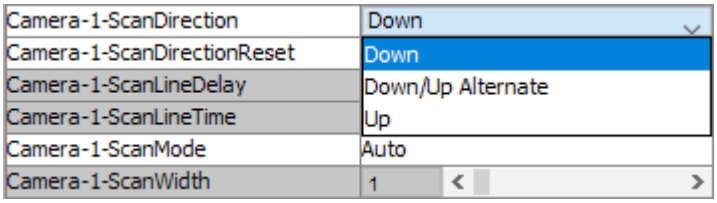



**Figure 4:** Scan Direction selection as displayed in Micro-Manager

A Scan Direction Reset parameter is also available (Figure 5). The default setting for this parameter is Yes. This ensures that the first frame of each new acquisition sequence will start at the topmost row and propagate downwards.
When the Scan Direction Reset parameter is set to False, the first frame for each new acquisition will start at the location of the last frame in the previous sequence. If the last frame ended at the bottommost row, the first frame of the subsequent acquisition will begin at the bottommost row and propagate upwards.

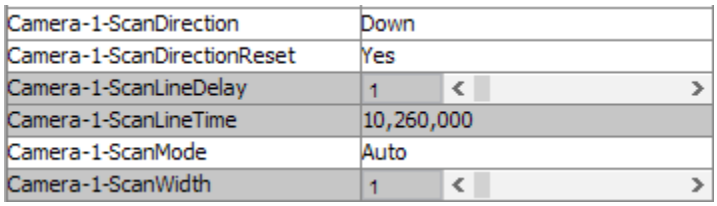
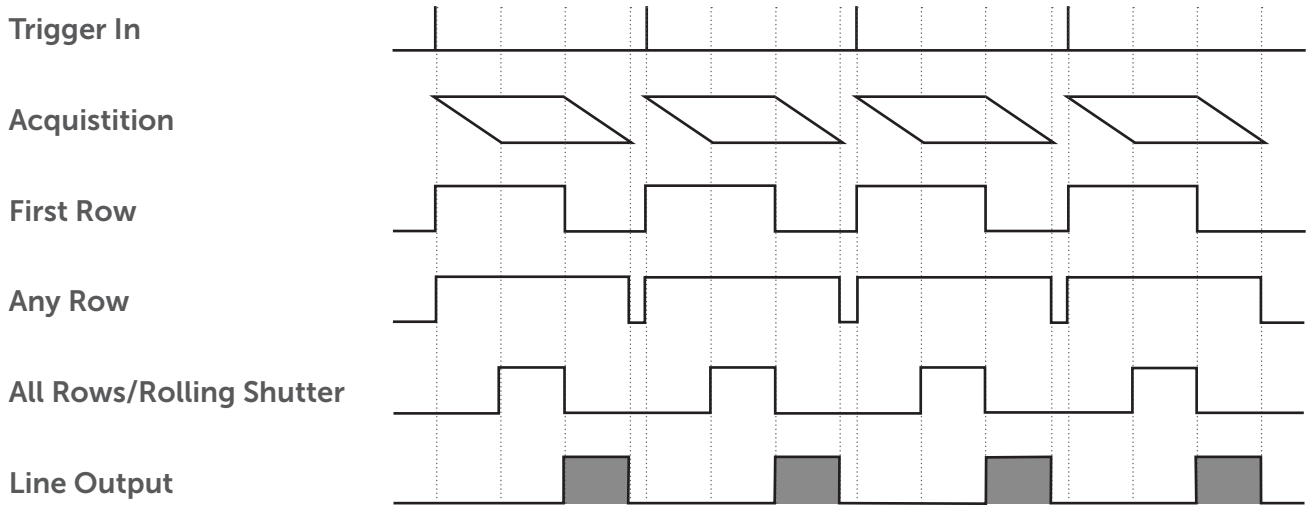



**Figure 5:** Scan Direction Reset selection as displayed in Micro-Manager

## Expose-Out Modes

A Line Output expose out mode has been added as an option. This expose out mode provides a rising edge for each row the reset signal propagates past. This provides a hardware signal that indicates the progress as the acquisition moves across the sensor.

The triggering and expose out mode behavior is shown in the diagram below:




## References

Optics Express, Vol. 20, Issue 19, pp. 21805 – 21814, 2012.

## Appendix I

| Camera | Interface | Mode | Line Time |
|---|---|---|---|
| **Iris 15** | PCI-Express | 16-bit HDR | 10.26 µsec |
| **Iris 9** | PCI-Express | 16-bit HDR | 10.26 µsec |
| **Prime BSI** | PCI-Express, USB 3.0 | 16-bit HDR, 12-bit CMS | 11.20 µsec |

# 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, Teledyne Photometrics has 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 (Figure 1) to get a peak representing the signal across the line in Grey Levels. Find the value at the top of the peak.

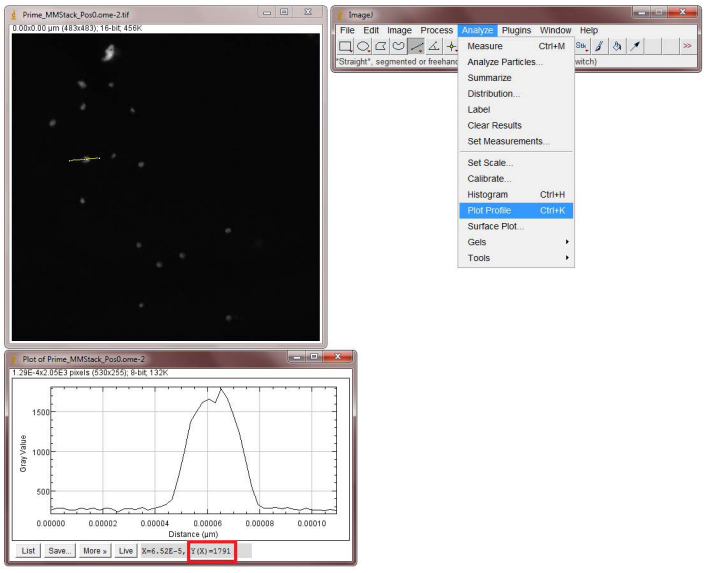



**Figure 1**

3. Subtract the camera bias from this Grey Level signal.
4. Multiply the result by the camera system gain.

The full equation is:

Signal in Electrons= (Signal in Grey Levels - Bias)*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 BSI™ which has a bias of ~100 and a gain of ~1.18. By inserting these values into the equation, we get the following result:

Signal in Electrons= (1791 - 100)*1.18

Signal = 1995 $e^-$

## 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 (Figure 2).

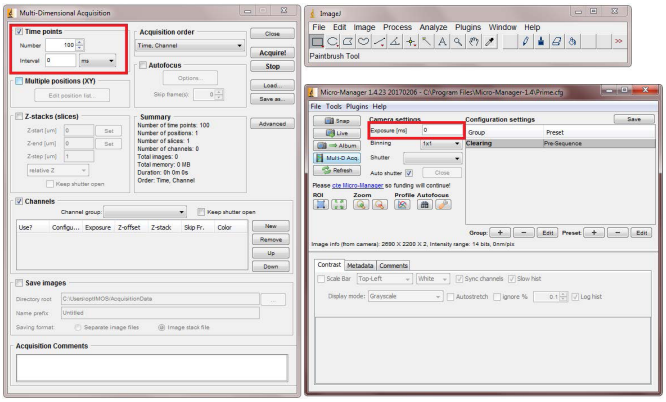


Figure 2

4. Calculate the mean of every frame by selecting Stacks from the Image menu and then clicking on Plot Z-axis profile (Figure 3). This should give you the mean values of every frame in the Results window.

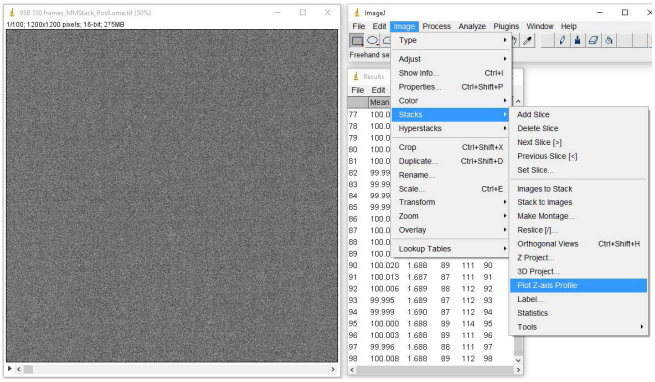



Figure 3

5. Calculate the mean of the 100 frame means by selecting Summarize in the Results menu (Figure 4).

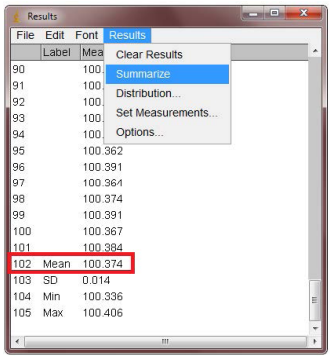



Figure 4

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.

## Calculating Camera Gain

# 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 5 ms exposure time.
3. In ImageJ, Measure the means of both images and average them. We'll call this $Mean_{Image1,\ Image2}$ (Figure 5).

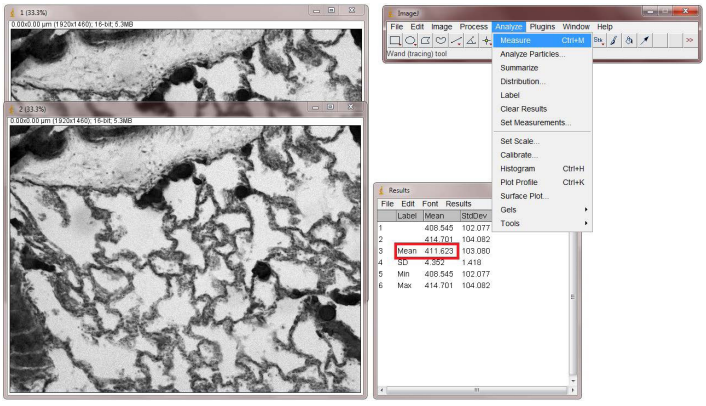



**Figure 5**

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 (Figure 6).

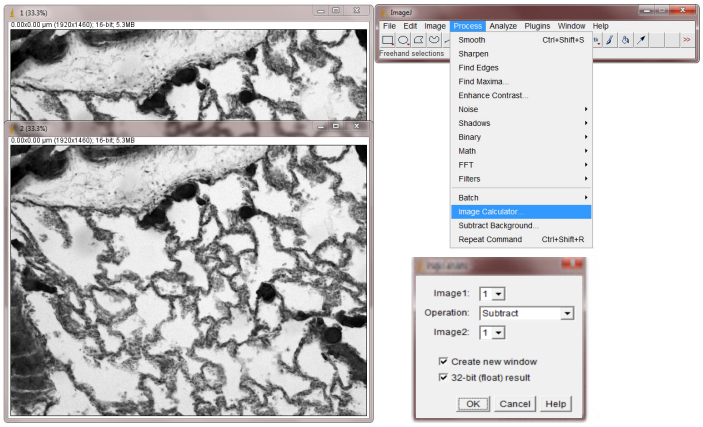



**Figure 6**

5. Measure the Standard Deviation of the diff image, we'll call this Standard deviation$_{\text{Diff image}}$ (Figure 7).

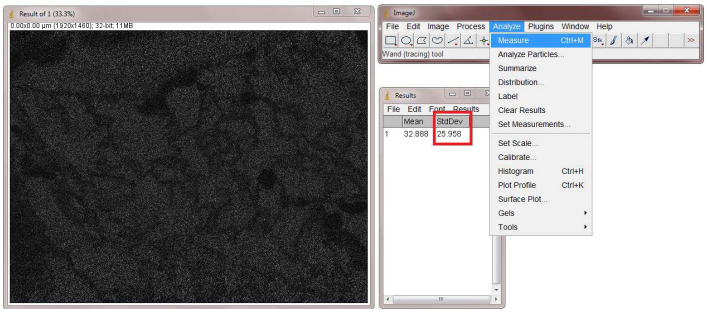



**Figure 7**

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 10 ms, 20 ms and 40 ms 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 Teledyne Photometrics on the website:
https://www.photometrics.com/learn/calculators

## 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:

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

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 Teledyne Photometrics on the website:
https://www.photometrics.com/learn/calculators

## 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 of an EMCCD can be calculated using the following equation:

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

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 Teledyne 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 (Figure 8).

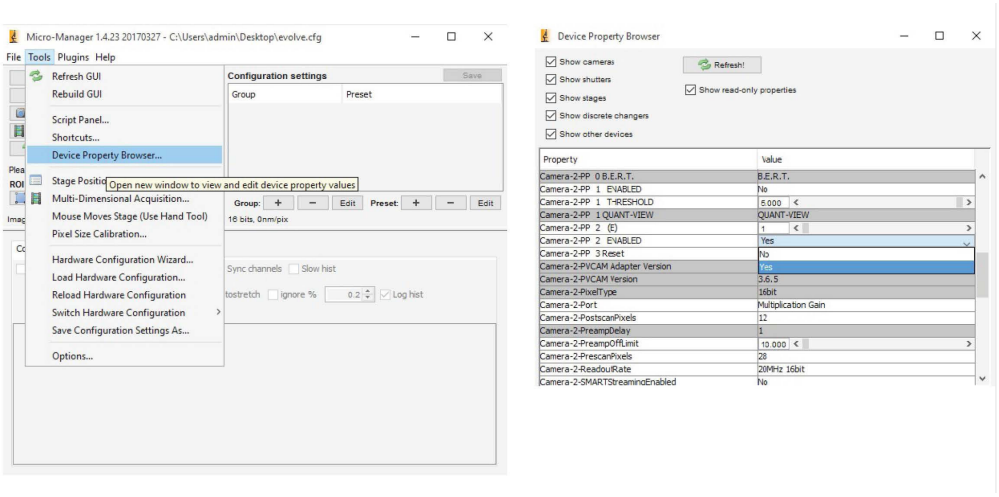



**Figure 8**

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 Teledyne Photometrics Cascade series, please see the following tech note:
https://www.photometrics.com/learn/calculators

# Part 2.
# Testing Camera Quality

## 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 Teledyne Photometrics is to produce a stable bias that doesn't deviate by more than one electron, which is shown here using the Prime BSI™ Scientific CMOS data (Figure 9).

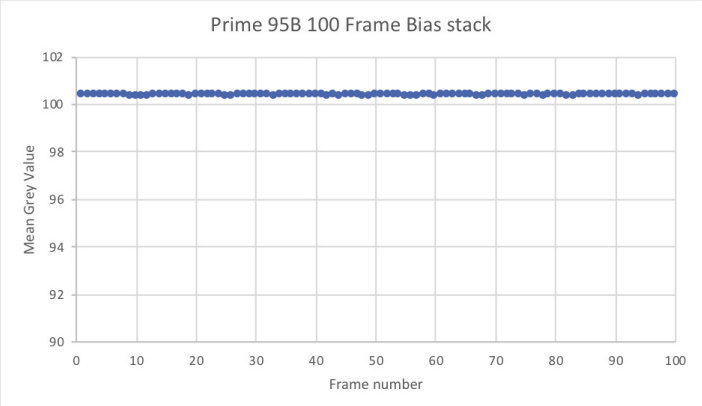



**Figure 9**

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 in figure 10 on the Prime BSI will give more accurate intensity data and produce higher quality images.

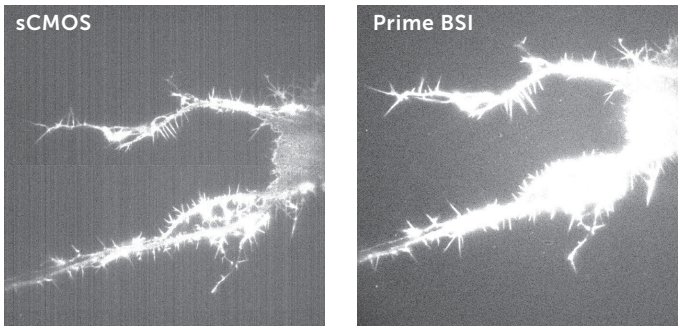



**Figure 10**

## 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

Teledyne Photometrics recommends that any deviation from the line be no more than 1%, as shown in figure 11 using the CoolSNAP™ DYNO data:

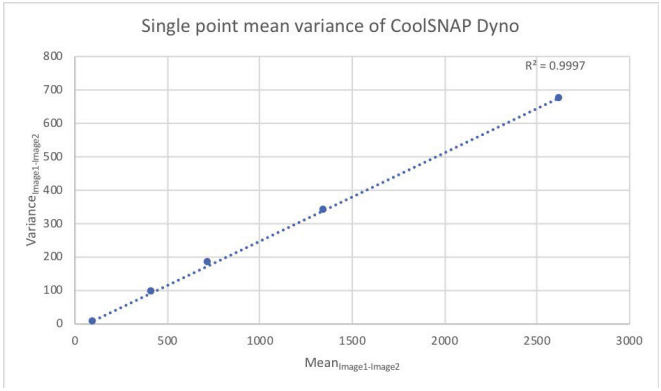



**Figure 11**

# 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:

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 (Figure 12).

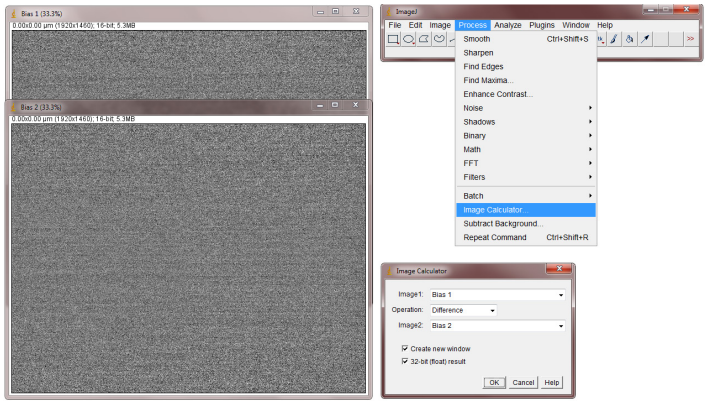



**Figure 12**

3. Measure the Standard Deviation of the diff image, we'll call this Standard deviation$_{\text{Diff image}}$ (Figure 13).

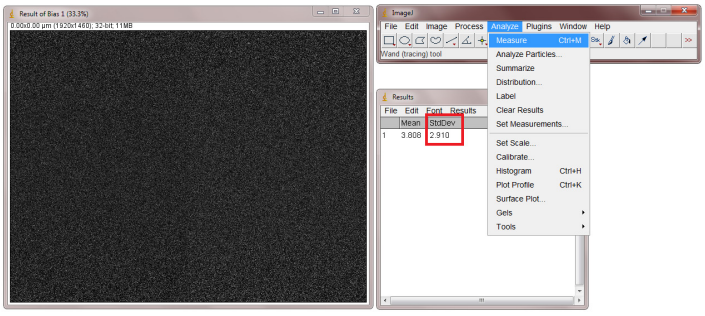



**Figure 13**

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 Teledyne Photometrics on the website:
https://www.photometrics.com/learn/calculators

## 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:

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

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 (Figure 14).

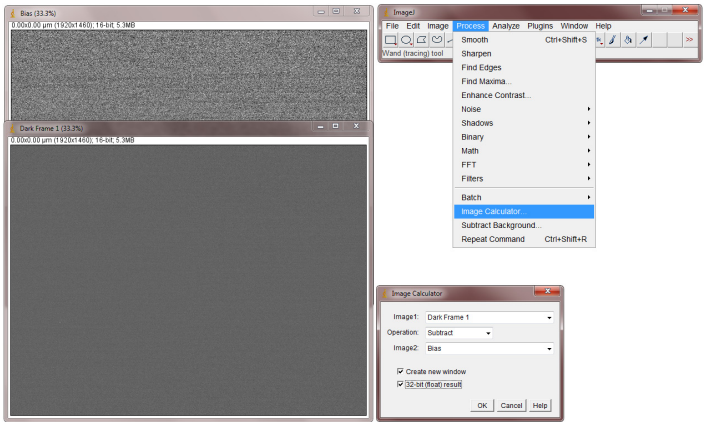



**Figure 14**

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 (Figure).

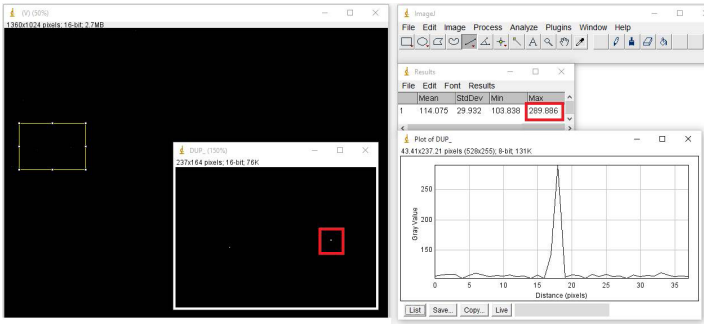



**Figure 15**

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 Teledyne 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 Teledyne 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 $SiO_2$ 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 Teledyne 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 Teledyne 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 (Figure 16).

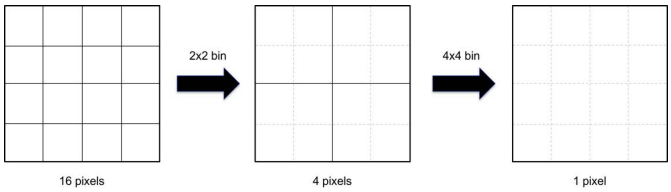



**Figure 16**

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 higher quality 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 collimated 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 $\mu m^2$ and an 11x11 µm pixel has an area of 121 $\mu m^2$ 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 Teledyne 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 | 509nm (GFP) | 4.8 µm | CoolSNAP DYNO (4.54 µm) |
| 60X | 1.4 | | 6.7 µm | Prime BSI (6.5 µm) |
| 100X | 1.4 | | 11.1 µm | Prime 95B (11 µm) |
| 150X | 1.4 | | 16.6 µm | Evolve 512 Delta 16 µm) |

**Table 1**

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 BSI even though the Prime BSI 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 BSI Software Support

## Supported Software

- ✓ Nikon NIS-Elements
- ✓ Zeiss Zen
- ✓ Olympus cellSens
- ✓ Molecular Devices MetaMorph
- ✓ Visitron VisiView
- ✓ Intelligent Imaging Innovations (3i) SlideBook 6
- ✓ Quorum Technologies Volocity
- ✓ National Instruments LabVIEW
- ✓ MathWorks MATLAB
- ✓ WaveMetrics Igor Pro 8
- ✓ Python 3.6
- ✓ Open CV
- ✓ Micro-Manager
- ✓ Teledyne Photometrics Ocular
- ✓ Camera driver SDK available for developers

## Supported Operating Systems

- ✓ Windows 7, 8, 10 64-bit
- ✓ Linux

## Notes

We recommend contacting your local third-party software office for minimum version numbers, support and relevant information.

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