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3. Data Generation - Systems with an Area Detector

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Mathias Hammer[1], Britta Schroth-Diez[2], Luis-Francisco Acevedo-Hueso[3], Gert-Jan Bakker[4], Laszlo Barna[5], Sebastian Beer[6], Valeria Berno[7], Yann Cesbron[8], Orestis Faklaris[9], Nathalie Gaudreault[10], Thomas Guilbert[11], Rainer Heintzmann[12], Gabriel G Martins[13], David McFadden[14], Glyn Nelson[15], Roland Nitschke[16], Kees van der Oord[17], Sathya Srinivasan[18], Andre Zeug[19], David Grunwald[20]

[1]UMASS Chan Medical School, RNA Therapeutics Institute, Worcester, MA, USA;
[2]Max Planck Insitute of Molecular Cell Biology and Genetics, Dresden, Germany;
[3]Neuro-AI Monitoring and Quantum Medical Diagnostics;
[4]Radboud University Medical Center, Medical BioSciences department, Nijmegen, the Netherlands;
[5]Institute of Experimental Medicine, Budapest; [6]Hamamatsu Photonics Deutschland GmbH;
[7]ALEMBIC Advanced Light and Electron Microscopy BioImaging Center, San Raffaele Scientific Institute;
[8]Institute of Science and Technology Austria;
[9]MRI, Biocampus, University of Montpellier, CNRS, INSERM, Montpellier, France;
[10]Allen Institute for Cell Science, Seattle, WA, USA;
[11]Institut Cochin, INSERM U1016, CNRS UMR 8104, Université Paris Cité, Paris, France;
[12]Leibniz Institute of Photonic Technology; [13]Gulbenkian Institute for Molecular Medicine;
[14]Friedrich Schiller University Jena; [15]Newcastle University, BioImaging Unit, UK;
[16]Life Imaging Center, University of Freiburg, Germany; [17]Nikon Europe BV; [18]ONPRC;
[19]Cellular Neurophysiology, Hannover Medical School, Hannover, Germany;
[20]UMass Chan Medical School, RNA Therapeutics Institute, Worcester, MA, USA

QUAREP-LiMi
Tech. support email: **info@quarep.org**

Britta Schroth-Diez
Max Planck Institute of Molecular Cell Biology and Genetics,...

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Disclaimer

This protocol collection was developed by members of **WG2 "Detection System Performance"** of the international consortium **QUAREP-LiMi.**

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This protocol collection has undergone the internal approval process of QUAREP-LiMi.

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Abstract

This protocol describes the measurement procedure to produce dark and inhomogeneously illuminated images with a light microscope system equipped with area detectors. The protocol can be followed according to the three different aims of microscope characterization described in the introduction of the protocol collection "Characterization of the Photon Conversion Factor, Noise, and Dynamic Range of Light Microscope Detection Systems". The protocol uses the microscope slide for inhomogeneous illumination described in protocol 2 and generates data which can be analyzed with protocol 5, to obtain the photon conversion factor, readnoise, and dynamic range of the detection system.

Guidelines

Please refer to protocol "**1. Introduction - Background and Aims**" of this collection for a more detailed description of a detection system, its parameters and various aims for performance monitoring.
This protocol is part of a collection of protocols developed by QUAREP-LiMi WG2 for characterising detection system performance.
We recommend to perform the described measurements one to two times a year, or if you notice unusual behaviour of your detector.

Materials

High dynamic range fluorescent test sample. E.g., marker pen slide as discribed in **protocol 2** of this collection.

Safety warnings

Ensure you follow general lab safety guidelines for radiation sources and chemicals as outlined within your organisation.

Laser safety and regulations

- Please refer to the documentation provided by the manufacturer for additional warnings and preventive, protective equipment (PPE) requirements (e.g. laser safety goggles). Always consult your local Laser Safety Officer or Radiation Safety Officer and refer to your laboratory safety documentation for more information.
- You can also consult your Laser Safety Standards ANSI Z136 in North America, SUVA 66049.D in Europe, and BS EN 60825-1 in the UK. Additionally, laser safety standards and regulations are covered by IEC norm 60825-1, and LED eye safety standards and regulations are covered by IEC norm 62471 in Europe.

Safety information

Hazardous, visible, or invisible radiation from lasers, lamps, and other light sources used for microscopy **can cause permanent damage to the retina, skin burns, and fire**. Always follow proper laser safety protocols for your equipment and situation.

Before start

This protocol can be used to determine experiment QC (Aim 1), instrument QC (Aim 2) or characterisation of the system (Aim 3). In each case, the notes describe the breakdown for each Aim. For further explanations of each aim, please refer to protocol "**1. Introduction - Background and Aims**" of this collection.
Make sure the microscope is turned on at least one hour prior to the measurements to allow the system to stabilize. All measurements in this protocol must be performed without any ambient light contributing to the signal.
For consistency, remember to always record your settings with your images. All settings must be kept unchanged to be able to reliably monitor system performance over time.

Set up the microscope

1 Select the appropriate objective.

Note

For **Aim 1 - experiment QC:** use the objective according to the settings of your experiment

For **Aim 2 - instrument QC and Aim 3 - system characterization**: use a 10x objective (or 20x if a 10x is not available); if measurements are done repeatedly, always use the same objective.

2 Install a high-dynamic range test sample on the stage.

Note

It is essential to have both, very bright as well as very dark, areas in the sample to ensure a high dynamic range signal distribution. A maximum contrast (signal-to-background) ratio of at least 100:1 is ideal.
Suitable test samples can be prepared following our **fluorescent test sample protocol**.

3 Focus and position your test sample.

Note

For the blurry edge test sample (prepared according to the protocol mentioned above), bring the test sample in focus and adjust its position such that the bright area fills about one-third of the field of view.

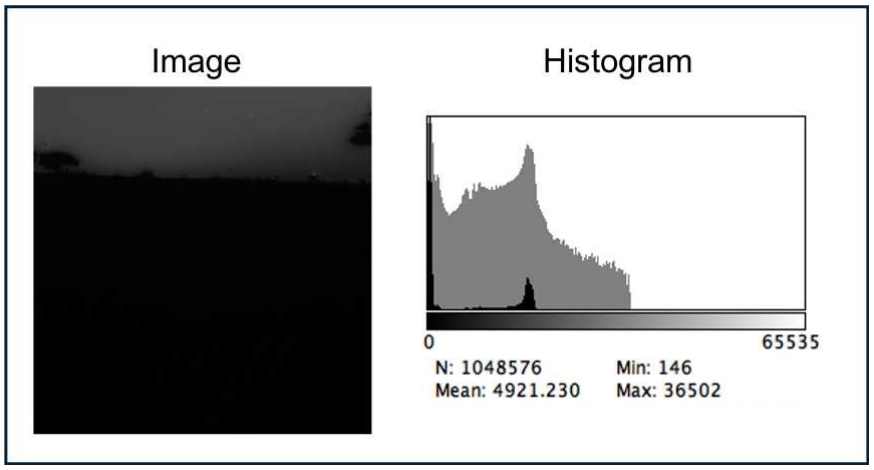


Image and histogram of the test sample (black: linear scaling; grey: logarithmic scaling).

4 Defocus the sample slightly to create a continuous intensity distribution.

Note

Defocussing by about one micrometer creates a blurry edge.

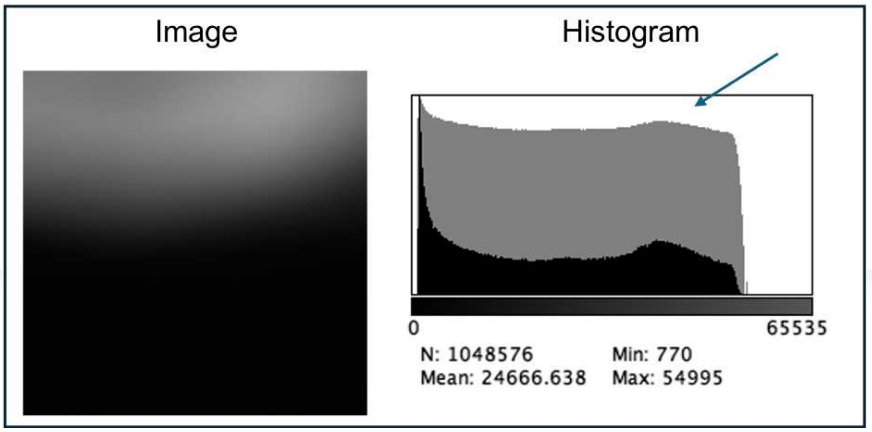


Image and histogram of the sample after defocussing. Histogram: linear (black) and logarithmic (grey) scaling. The intensity distribution in the histogram is flattened out in comparison to in focus images.

Adjust the detector settings

5 Adjust the acquisition parameters according to your requirements.

Note

Acquisition parameters include: detector settings such as exposure time, gain settings, temperature set point, read out rates, bit depth, binning, as well as other parameters, like the illumination power of the light source or the filters used in the optical path of the detection system.

Note

For **Aim 1**: Use your experiment settings.
For **Aim 2**: Use always the same analog gain setting, the same operational mode, and the same exposure times (between 50 and 100ms).
For **Aim 3**: We recommend to use all standard analog gain settings, standard operational modes, and an exposure time between 50 and 100ms.
For further considerations please see step 6 and respective sub steps.

Note

Check any other settings that may affect the image or image integrity. Pre-processing filters (such as pixel defect removal, camera autocontrast, or autodeblur) will affect the calibration results. We recommend turning these off for calibration and quality control to ensure repeatability and consistency, especially for Aims 2 and 3.

6 Choose between saturation-level calibration and arbitrary-level calibration. Adjust your acquisition settings accordingly.

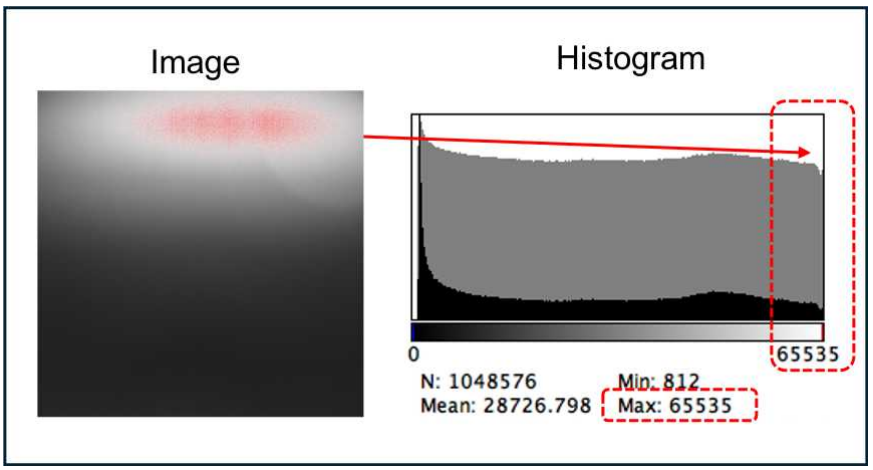
Note

1. **Saturation-level calibration:**
   The calibration is performed at intensities ranging from very low up to the saturation level, covering the entire dynamic range of the detector. A saturation-level calibration can be useful as a repeatable "one size fits all" approach. Saturation-level calibration will, in addition, yield information on the total dynamic range of the detector.
2. **Arbitrary-level calibration:**
   The calibration is performed at an arbitrary intensity level below the saturation level. Some experiments benefit from a calibration performed close to the intensities of the actual experiment since detectors often show significant performance differences depending on the charge load of its detection elements. Arbitrary-level calibration yields information about the total number of detected photons.

6.1 **For saturation-level calibration:** Adjust the parameters so that around 1% of the pixels are saturated.

Note

The precise number of saturated pixels is not important.
The percentage of saturated pixels can be determined after checking the results according to the analysis protocol.
Please be aware that oversaturation may harm the detector. This is especially true for EMCCDs.



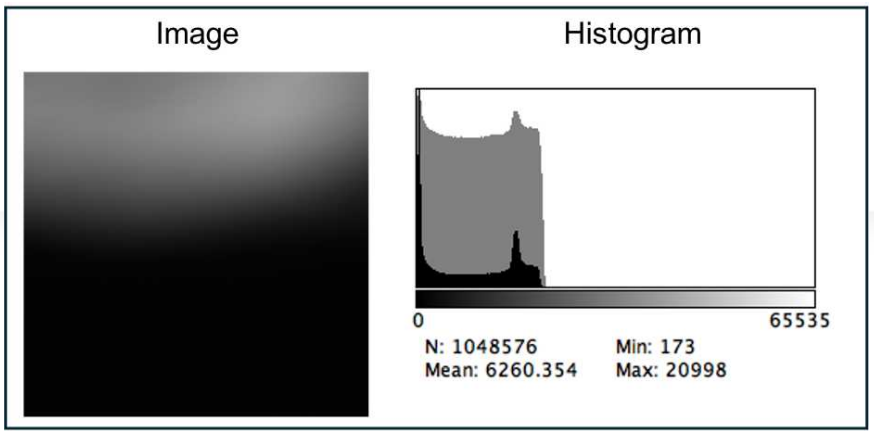
A saturated image and its histogram (black: linear intensity distribution; grey: logarithmic intensity distribution). Highlighted in red are the saturated pixels.

Note

For EM-CCDs grayscale values corresponding to saturation greatly depend on the camera settings. Detector saturation might occure before the A/D conversion. Saturation-level calibration is a good tool for determining this.

6.2 **For arbitrary-level calibration:** If you want to calibrate for an illumination range that is less than 50% of the detector saturation level, adjust the acquisition parameters so that the brightest pixels in the image are 20% higher than your target maximum intensity.

Note



This figure shows a non-saturated image and its histogram (black: linear intensity distribution; grey: logarithmic intensity distribution). There are no saturated pixels. The histogram spans across about a third of the 16bit range.

Data acquisition

7 Acquire a bright image series and a dark image series.

7.1 **Bright image series:** Take a series of at least **20 images** with the selected settings and with the respective illumination turned **ON**.

Note

Some detectors show noticeably different characteristics during a "run-in" period caused by for instance changes in offset or sensor temperature. The "run-in" time corresponds to the time until the intensity between images stays constant, apart from noise.

We suggest a series of at least 20 images for the initial measurement.
Adjust the number of images to the "run-in" time plus 20 images, remove the "run-in" images from the series before the analysis.

Note

Detectors can perform differently depending on the acquisition conditions (exposure time, temperature and illumination level).
If you wish to calibrate multiple conditions, you will need to acquire a new series for each set of conditions (**Aims 1 through 3**).

7.2 **Dark image series**: Take a series of at least **20 images** with the same respective settings, but with the illumination turned **OFF**.

Note

Dark images are necessary for determining a detector's offset value and dark noise.

Note

To ensure that there is no ambient light contributing to the signal, either the full detection light path needs to be blocked or covered, or the entire microscopy room must be darkened.

Note

If the detector settings are kept the same and only illumination or optical path settings are changed, the same 20 dark images can be used for analysis. No additional dark image series has to be acquired in this case.

8 Record all acquisition parameters (metadata) with each image series.

Note

Metadata are needed for further data handling and analysis and thus must be kept together with the image data. We recommend to store data in the microscope manufacturer's data format (where available). In most cases this will ensure storage of all the required metadata.

9 Repeat steps go to step #5 through go to step #8 for each additional set of acquisition parameters or illumination levels for which you want to perform a calibration.

Analysis

10 For respective analysis steps, please refer to the **Analysis protocol**.

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