Jan 13, 2025

4. Data Generation - Systems with a Point Detector

In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.j8nlk8176l5r/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.j8nlk8176l5r/v1

External link: https://quarep.org/

Protocol Citation: Andre Zeug, Valeria Berno, Luis-Francisco Acevedo-Hueso, Gert-Jan Bakker, Mariana T Carvalho, Yann Cesbron, Orestis Faklaris, Ayse Aslihan Koksoy, Gabriel G Martins, David McFadden, Glyn Nelson, Roland Nitschke, Britta Schroth-Diez, Sathya Srinivasan, Kees van der Oord 2025. 4. Data Generation - Systems with a Point Detector. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.j8nlk8176l5r/v1**

Protocol status: Working

Created: September 08, 2024

Last Modified: January 13, 2025

Protocol Integer ID: 107125

Keywords: detection system, acquisition, in-homogeneous, characterization, point detector, PMT, APD, measurement, noise, HyD

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 inhomogeneous illuminated images with a scanning light microscope system equipped with point 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.

Setup 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 the 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**.

Adjust the detector settings

3 Select the appropriate bit depth

Note

For **Aim 1**, according to the experiment, for **Aim 2 and 3**, use the highest bit depth.

4 Set the light path (mirrors, opto-mechanics)

Note

For **Aim 1:** use settings according to your experiment

For **Aim 2:** use filter settings optimized for the test sample used. For the yellow fluorescent marker test sample, use FITC settings (e.g., 488 nm excitation, 500-550 nm emission range).

For **Aim 3:** with higher gain settings: use sub-optimal filters to avoid over exposure.

5 Adjust the scanner settings

Note

For **Aim 1:** use scanner settings according to your experiment

For **Aim 2 and 3:** use 512 x 512 pixels, set line speed/frequency around 2 µs pixel dwell time and set the zoom factor to 1.

6 Set the detector gain

Note

For **Aim 1**: use gain similar to your experiment settings.

For **Aim 2**: use the same fixed setting each time the calibration is performed, according to the optimal working range of your point detector.

For **Aim 3**: use a wide range of gain settings. Exception: one fixed gain setting should be used for point detectors in single photon counting mode.

Live mode adjustments

7 Focus your test sample

Note

Bring the test sample in focus and adjust its position such that the boundary between the dark and bright sample area is parallel to the fast axis of the scanning system (typically horizontal). The bright part of the sample should fill approximately one-third of the field of view.

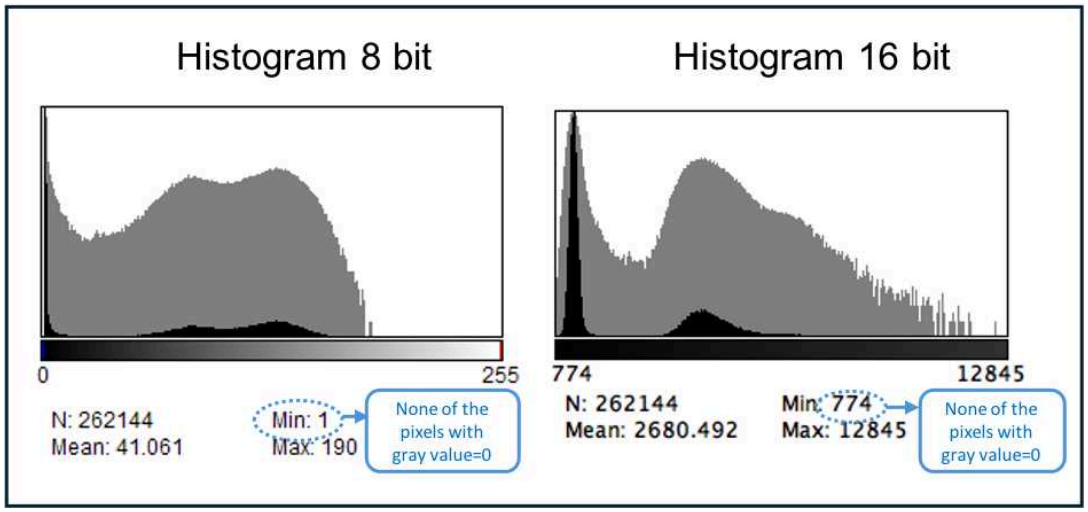
Image of the test sample, with approximately 1/3 of the image filled with bright pixels.

8 Set the detector offset

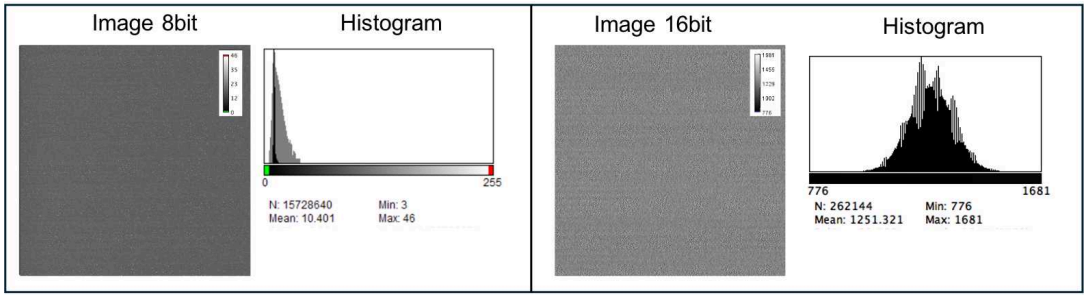
Note

Make sure that all pixels in the image have a value above zero. This should be valid for each gain setting, for the bright as well as for the dark area in the image, for the bright as well as the dark image series.

If the detector offset value is too low, there will be areas in the image where no signal or information is recorded. Exception: single photon counting detectors (HyD, APD, ...). For these detectors this step can be omitted because no offset value can be adjusted.



Bright images: pixel intensity histograms of images generated with acceptable detector offset (black: linear scaling; grey: logarithmic scaling). All pixels in the image have a value above 0.

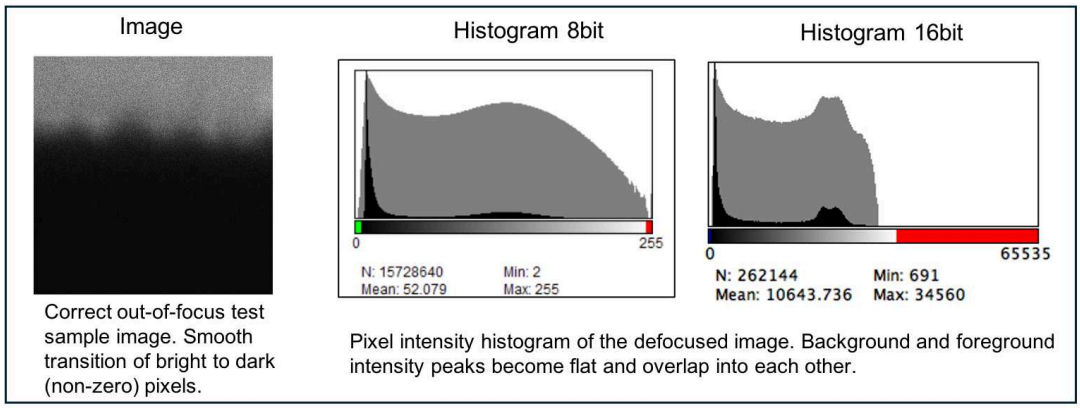


Dark images (left, 8-bit and right, 16-bit) and their histograms with linear (black) and logarithmic (grey) scaling. The lookup tables of the images and the x-axis of the 16-bit histogram are scaled to show background noise of the detection system in detail.

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

Note

Defocus by moving the objective focal plane away from the sample until the pixel intensity histogram becomes flat while the darkest pixels remain close to the dark background level.



Defocused test sample image (left) and representative 8-bit (middle) and 16-bit (right) pixel intensity histograms (black: linear scaling; grey: logarithmic scaling). In comparison to histograms of in focus images, the distributions are more flattened out.

Note

For **Aim 1**: For high (3-10x) zoom factors, defocus by about 50 um and use pinhole settings of about 1 AU to maintain sufficiently dark pixels in the darkest image area.

Note

For **Aims 2 and 3**: for zoom factor 1 in the scanner settings, a more open pinhole (5 Airy Units or more) can be selected in combination with more defocusing, to obtain a smoother transition from the bright upper area to the dark lower area.

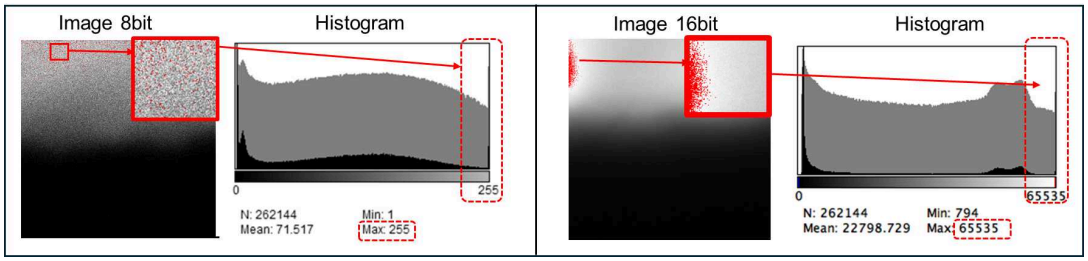
10 Choose between saturation-level calibration and arbitrary-level calibration. Adjust the excitation intensity accordingly.

Note

To change pixel intensities for a used gain setting, it is generally easier for systems with point detectors to change excitation intensity than adjusting other acquisition settings (such as scan speed, pixel dwell time, or other parameters).

Note

Saturation-level calibration: Increase excitation intensity until a small fraction (around 1%) of the pixels becomes saturated. If data is created in this manner, the analysis software is able to determine the dynamic range of the detection system.



8-bit (left) and 16-bit (right) saturated images and their pixel intensity histograms with linear (black) and logarithmic (grey) scaling. Inset: saturated pixels (red).

Note

Alternative: Arbitrary-level calibration
In case the total number of detected photons in the image needs to be determined, a so-called 'Arbitrary-level calibration' should be performed. Excitation intensity is adjusted such that all pixel intensities are well below the saturation limit. The total number of detected photons can be useful for comparison of detectors in the same microscope. In this case, there must be NO saturated pixels in the image.

Data Acquisition

11 Acquire a series of bright images and a series of dark images.

Note

The recommended number of images taken depends on the number of pixels being acquired. We suggest a series of at least 30 images for the initial measurement.

Some detection systems show noticeably different characteristics during the first frame (so-called "run-in" time) caused by for instance changes in offset or sensor temperature. The "run-in" time corresponds to the time until the intensity between pixels or frames stays constant, apart from noise. Take this into account when choosing the number of frames being recorded.

Note

Feedback from the analysis software (see section Analysis) can be used to improve data acquisition. For instance, the pixel intensity histogram of the dark images should contain bins with zero values on the left side of the peak in the histogram (see below).

Note

Make sure that all acquisition parameters (metadata) are recorded with each image series. 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). This will in most cases ensure storage of all the required metadata.

11.1 **Bright image series:** turn the excitation light **ON** and capture at least **30 images** using the same settings selection as determined in the previous steps.

Note

To minimize the contribution of electronic and laser fluctuations during recording of the images, acquire in time series mode with minimum time interval.

11.2 **Dark image series:** turn the excitation light **OFF** and capture at least **30 images** using the same settings as for the bright image series.

Note

To ensure that there is no any ambient light contributing to the signal, like for instance light coming from the system's monitor, either the full detection light path needs to be blocked or covered, or the entire microscopy room must be darkened.

12 For **Aim3:** Repeat go to step #3 to go to step #11 for all relevant detector, gain and scanner setting combinations.

Analysis

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

Acknowledgements

Funding acknowledgement.pdf 50KB CRediT_author_contributions_4_Dat…