0.1 Image analysis

Our analysis is based on the photon transfer curve method [1–3], with inhomogeneous illumination. This takes individual pixels and plots their variance against their mean intensity values, and is expected to follow a straight line given the Poissonian statistics of the photon shot noise. The intensity gradient in the bright exposure (figure **??**) provides the required range of pixel intensities without needing to change either the exposure time of the source brightness.

The photon transfer method would appear to assume a one-to-one relationship between charge carriers (photoelectrons) generated by the incident light and electrons detected by the read-out circuit. But this is not the case with an amplified imager such as an emCCD, as one photoelectron will generate a number of secondary electrons for the read-out. This turns out not to be a problem for interpreting the calibration results as long as the user is primarily interested in the signal-to-noise performance of the detector.[1] We will therefore use the term "effective photoelectrons" to distinguish the calibration term from physical photoelectrons.

There are a number of known pixel anomalies (hot pixels, defect pixels, noisy-pixels, saturations) which would bias the fit and which we therefore exclude from the analysis. To keep track of pixels which exhibit these types of anomalies, we create a 2D map corresponding to the sensor array size and successively add to it the location of the excluded pixels.

The first step is to mark as invalid pixels which measure at least one zero-valued or saturated value, as there is a high likelihood that the signal was clipped. If the images were not already in floating-point format, they are now converted to such.

[1] As the amplification process is stochastic in nature, it generates an additional fluctuation which we can no longer distinguish from photon shot noise. The effect of this is that we underestimate our gain (e^-/ADU), which makes us underestimate the number of generated photoelectrons. We cannot deduce the number of photoelectrons physically produced by the light from the image stack alone. But from the user's perspective, if the detector introduces noise which looks like shot noise, then it is as if the quantum efficiency of the camera had effectively dropped, even though no photoelectrons were physically lost. Note that this interpretation considers only the variance of the amplified signal distribution and does not accurately describe what happens with higher moments such as the skewness. As the photon flux of the calibration source is stable, it offers a convenient way of comparing this *effective* quantum efficiency with that of a unampflified detector. The user can now decide whether the trade-off in effective quantum efficiency is worth the benefit of an effective reduction in read-noise that is achieved by the amplification scheme.

Next, we look at the mean values of the individual exposures. While individual pixel samples will fluctuate from exposure to exposure, one would expect the mean signal of the ensemble of pixels from individual exposures to remain nearly constant, according to the law of large numbers, if the exposures were taken under identical conditions.

The mean signal of dark exposures is, however, often found to drift over time, which can be due to the temperature not yet having reached a steady state. Manufacturers have implemented offset-clamp technology (EMCCD) which attempts to mitigate this, but this can actually introduce an additional fluctuation on the pixel-level if these corrections are added as discrete digital values.

For evaluating the stack of background images, we therefore subtract the spatial mean value of each individual dark exposure frame from each pixel of this frame and add back the total mean over all dark exposures. By averaging all such offset-drift-corrected frames, we obtain an individual offset for each pixel and by calculating the variance over time, we obtain a pixel-wise variance estimation.

For the bright exposure stack, the dark offset is subtracted to obtain a stack of unbiased images. This can be done as a global value, on a pixel-by-pixel basis. CCDs perform well with a global value, but CMOS cameras should use the latter method. As a third option for CMOS sensors, one could perform column or half-frame column averages according to the CMOS detector readout scheme.

The relative brightness within the offset-subtracted exposure stack can also fluctuate. In particular, brightness fluctuations of the source can be detrimental to the gain estimation. This can be remedied, to an extent, by estimating the total amount of light within each exposure via the sum over all (offset-corrected) pixel measurements, dividing the data by the mean brightness of each frame, and then multiplying it by the overall mean brightness.

We now return to the pixel anomalies. We consider pixels to be hot or cold if their mean value in the dark exposure stack is more than four standard deviations removed from the mean value of the ensemble. These are added to the invalid map, and their count is reported in the summary.

A major limitation in CMOS imagers is a type of readout-noise known variously as random telegraph signal (RTS) noise, bi-stable noise, or popcorn noise [4,5]. It affects the noise level of pixels on an individual level. Whilst most pixels in a sensor array might exhibit a very low noise level, a small proportion of pixels will reside at the long tail of the distribution and exhibit an unusually high amount of noise. These noisy pixels tend to bias some of the low brightness bins, and if the illumination is weak then they can also dominate the high brightness bins. This is a purely stochastic effect.

This small proportion of pixels can have an outsized effect on the gain estimation, so we exclude them from the analysis (see figure **??**). We therefore set a threshold at the 98th percentile: The 2% of noisiest pixels in the background exposure stack are identified and added to the invalid pixel map. This cut-off is somewhat arbitrary and the effect becomes much weaker at higher illumination levels. It it is important to note that these noisy pixels may be detrimental for later applications such as single-molecule localization [6].

Calculating the mean projection of the unbiased image stack gives us an estimate for the light level for each pixel. While these values are adequate for CCD imagers, sCMOS cameras exhibit pixel-wise offset, gain and quantum efficiency [7], and this is detrimental to using the pixel mean as an estimate for the physical light level.

We therefore take advantage of the low sharpness of our calibration image: Blurring the image with a 7×7 Gaussian kernel leaves the local brightness levels mostly unchanged, but now each pixel includes the brightness of its close neighbours in its brightness estimate, thereby improving the statistics. In order for this to work, pixels that were previously mapped as invalid need to be substituted with reasonable value prior to the Gaussian convolution, and for this, we use the median value of the surrounding 7×7 kernel.

The variance projection of the unbiased stack provides a variance estimate for each pixel. No filtering is applied to the variance projection.

We now select each pixel which we did not mark as invalid and assign them to equally spaced bins based on their brightness estimate. For each bin, we then calculate the mean value of all pixels in the bin, as well as the mean variance of these pixels. These results are plotted as a photon transfer curve figure **??**.

Finally, we use the mean and variance data of the bins to perform an iteratively reweighted least squares linear fit. The weights correct for the brightness variance var_{poisson} predicted by the model given Poissonian statistics, but also account for the predicted *variance of the variances* within a bin: Each bin contains a limited number of variance estimates N. The predicted variance is therefore $var_{\mathrm{poisson}}/(N+1)$, which decreases with the number of estimates N within a bin.

The slope of the resulting fit corresponds to the camera gain. Note that the averaging within each bin enables us to neglect the uncertainty along the mean-value coordinate during the fitting procedure.

It should be noted that this method generalises over the ensemble of valid pixels and does yield gain information on the pixel level, as described by Huang et al [6].

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