The Number & Molecular Brightness (N&B) Method

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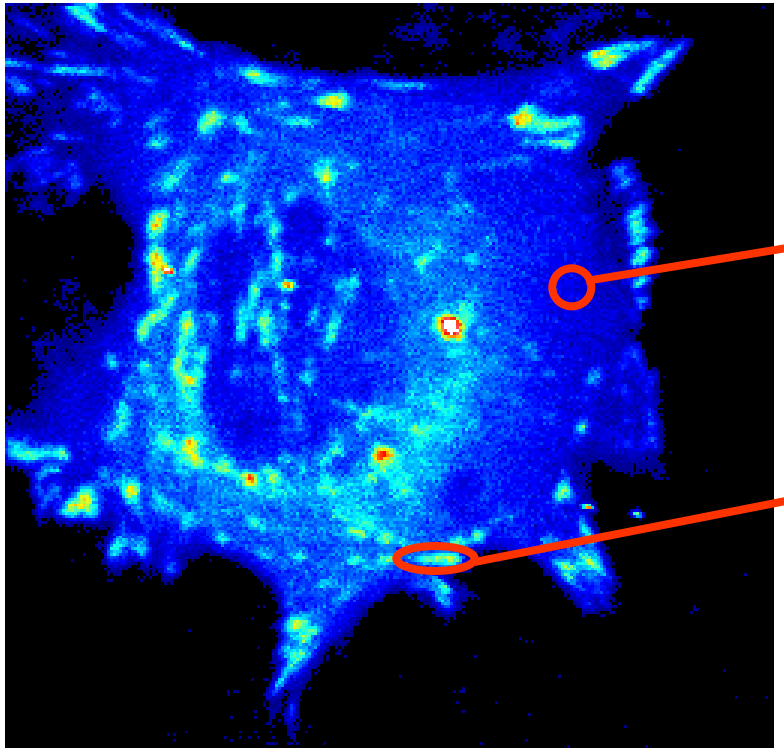
Existing Methods to determine protein concentration and aggregation of proteins in cells

1. Calibration of the free fluorophore based on intensity



Average intensity of MEF cells expressing Paxillin-EGFP

A **INTENSITY**

31,250counts/sec

B

93,750 counts/sec

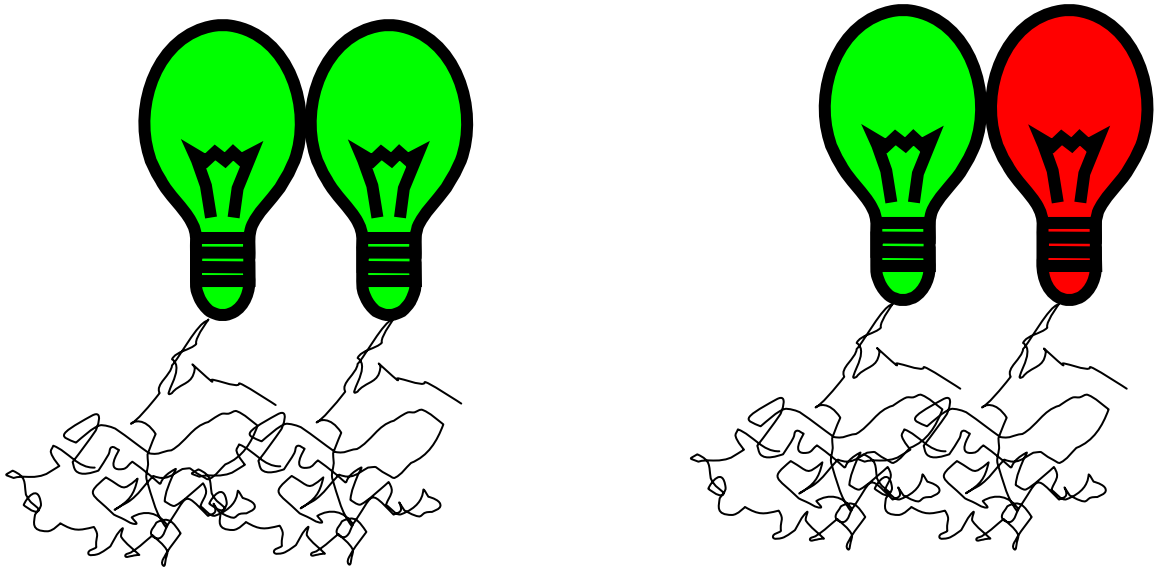
If "free" EGFP at 10nM gave 30,000 counts/sec then the conclusion would be that :

A =10nM

B = 30nM

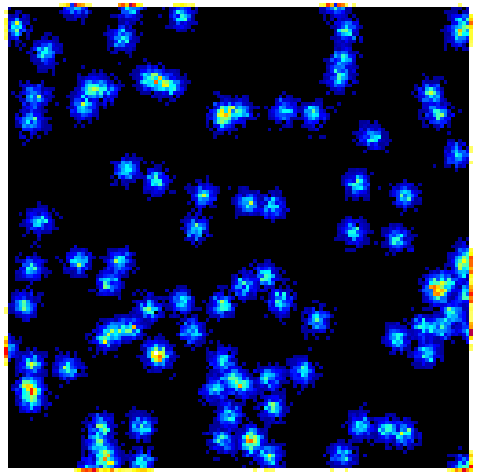
However, it doesn't give you the size distribution
Only concentration is given

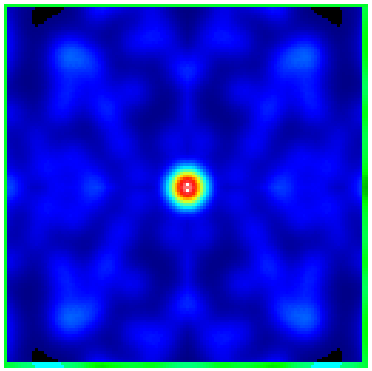
2. Förster resonance energy transfer (FRET)

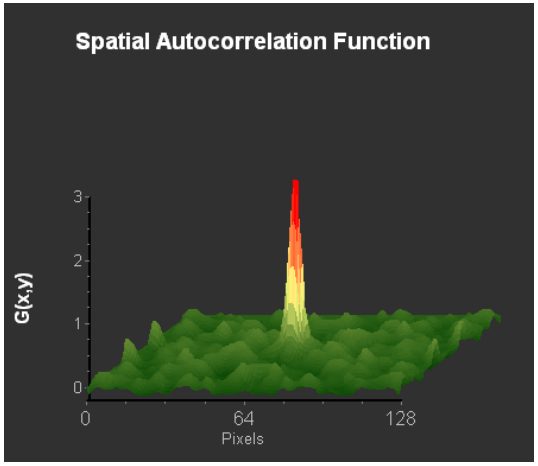


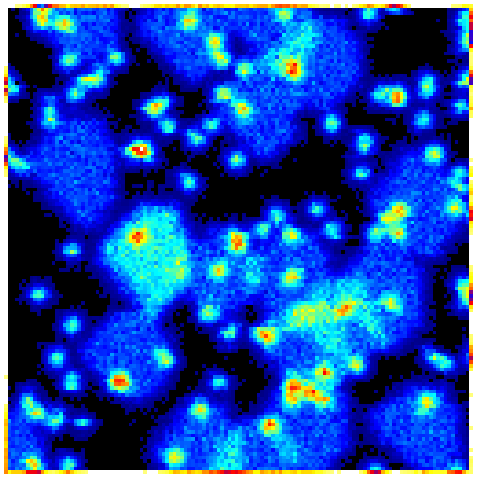
This method is very sensitive to detect the formation of pairs.

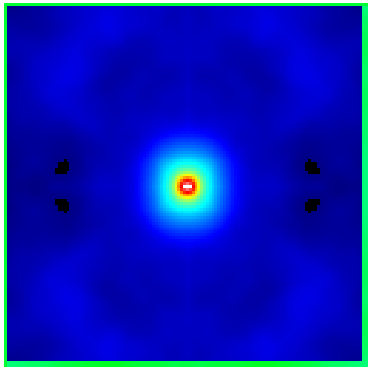
3. Image correlation Spectroscopy (ICS)

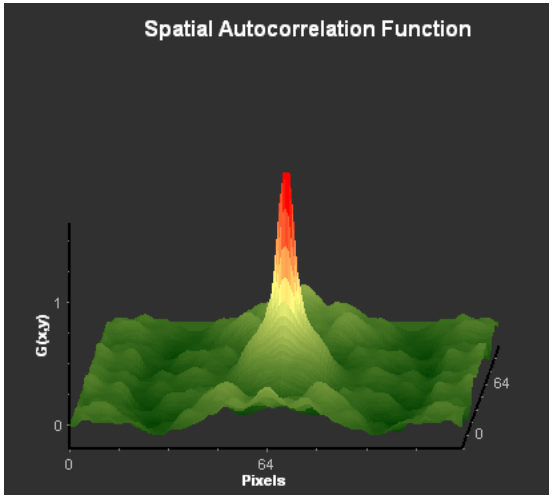












However, the events must be slow >1sec (no movement during one frame) and the aggregates must be large.

Petersen and Wiseman:Biophys J. 1999

The Number and Brightness (N&B) analysis

Purpose: Provide a pixel resolution map of molecular number and aggregation in cells

Method: First and second moment of the fluorescence intensity distribution at each pixel

Source: Raster scanned image obtained with laser scanning microscopes
TIRF with fast cameras
Spinning disk confocal microscope

Output: The N and B maps, B vs intensity 2D histogram

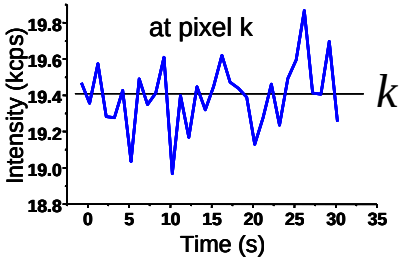
Tools: Cursor selection of pixel with similar brightness
Quantitative analysis of center and std dev of the *e* and *n* distribution
Tools for calibration of analog detectors

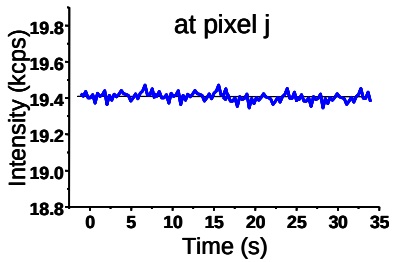
Tutorials: mathematical background, data import, analysis examples (our web site)

How to distinguish pixels with many dim molecules from pixels with few bright molecules?

Average (first moment)

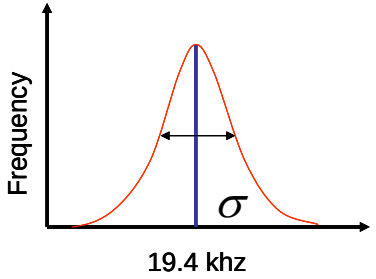
$$< k >= \frac{\sum_i k_i}{K}$$

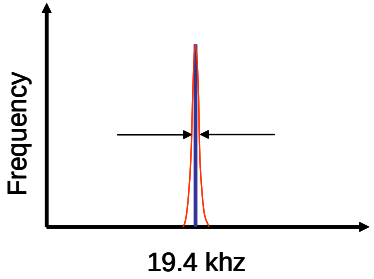


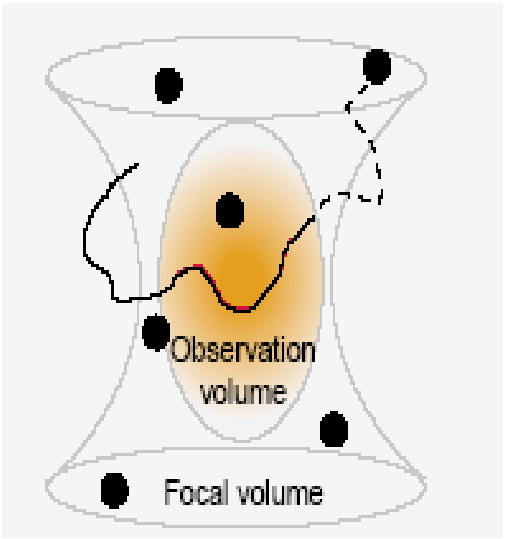


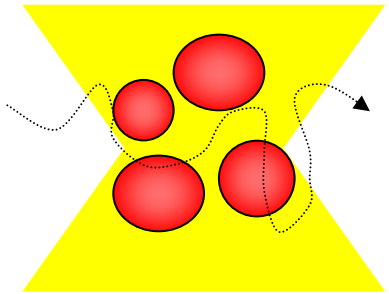
Variance (second moment)

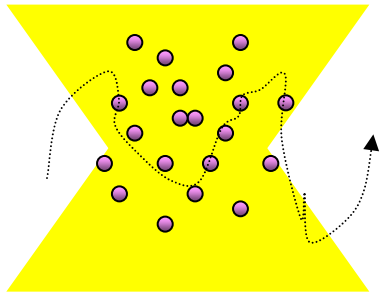
$$\sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$











$G(0) = \sigma^2/\langle k \rangle^2 = 1/N$

- **Given two series of equal average, the larger is the variance, the less molecules contribute to the average. The ratio of the square of the average intensity ($\langle k \rangle^2$) to the variance (σ^2) is proportional to the average number of particles <N>.**

* Originally developed by Qian and Elson (1990) for solution measurements.

Calculating protein aggregates from images

This analysis provides a map of <N> and brightness (B) for every pixel in the image. The units of brightness are related to the pixel dwell time and they are “counts/dwell time/molecule”.

$$< k >= \frac{\sum_i k_i}{K} \qquad \sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$

$$B = \frac{< k >}{< N >} = \frac{\sigma^2}{< k >}$$

$$< N >= \frac{< k >^2}{\sigma^2}$$

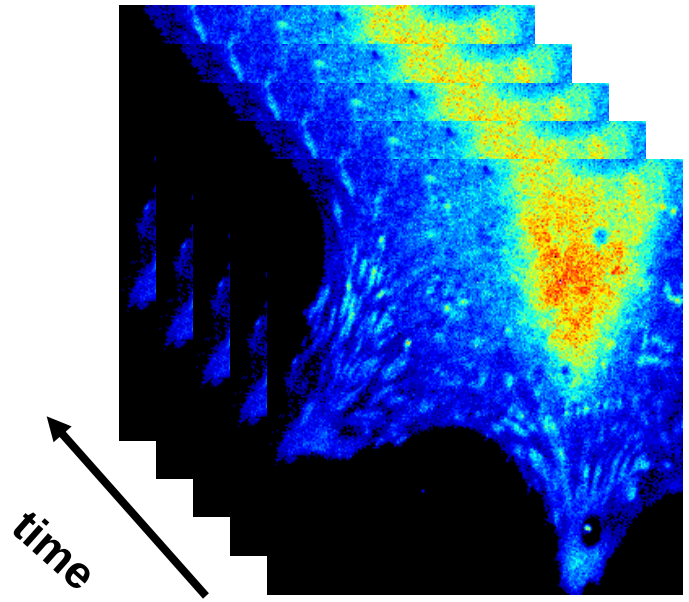
σ^2 = Variance

<k>= Average counts

N = Apparent number of molecules

B = Apparent molecular brightness

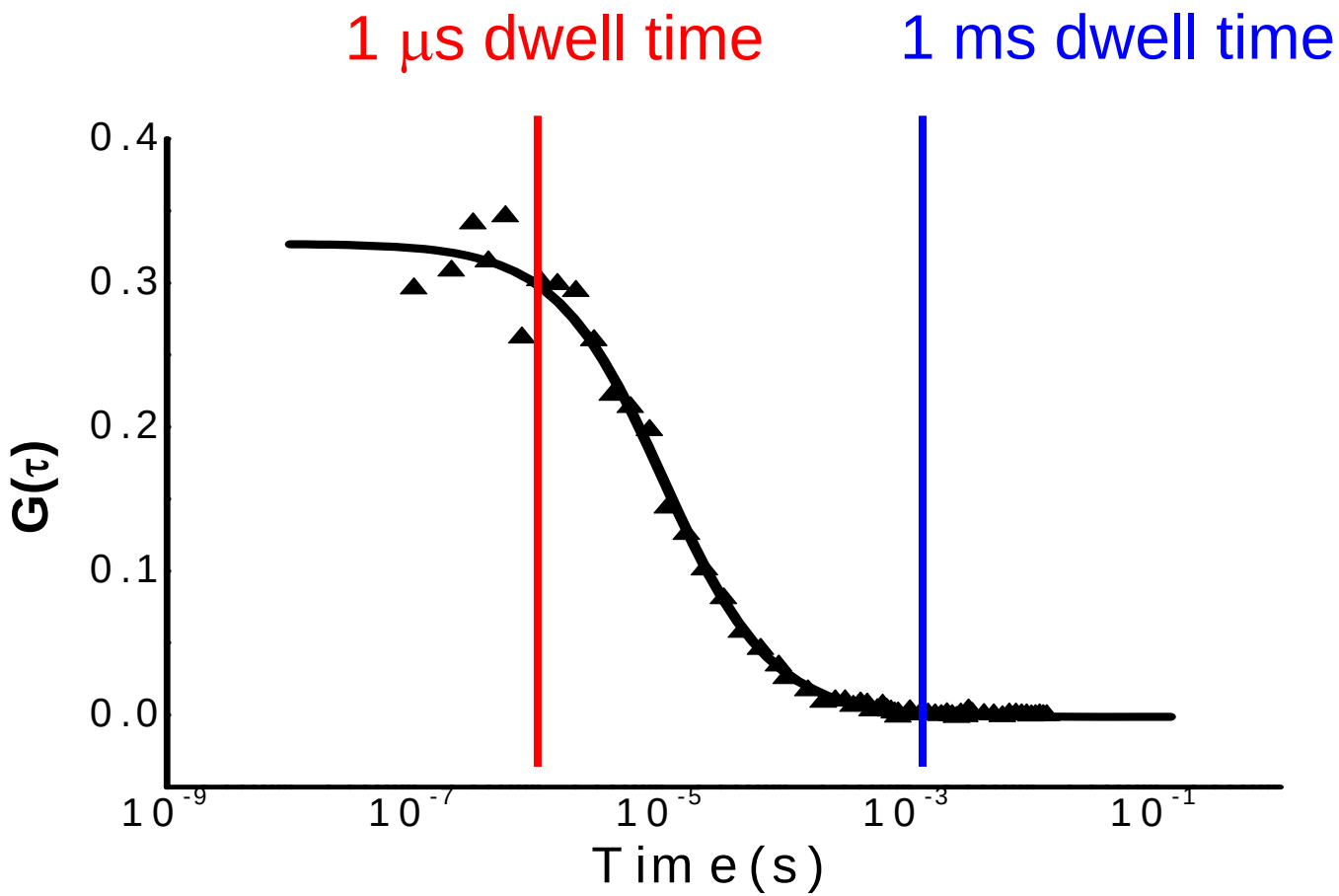
K = # of frames analyzed



Selecting the dwell time

To increase the apparent brightness we could increase the dwell time, since the brightness is measured in counts/dwell time/molecule.

Increasing the dwell time decreases the amplitude of the fluctuation.



What contributes to the variance?

Variance due to particle number fluctuations $\sigma_n^2 = \varepsilon^2 n$

Variance due to detector shot noise $\sigma_d^2 = \varepsilon n$

The measured variance contains two terms, the variance due to the particle number fluctuation and the variance due to the detector count statistics noise

$$\sigma^2 = \sigma_n^2 + \sigma_d^2$$

These two terms have different dependence on the molecular brightness:

$\sigma_n^2 = \varepsilon^2 n$ $\sigma_d^2 = \varepsilon n$ (for the photon counting detector)

Both depend on the intrinsic brightness and the number of molecules. We can invert the equations and obtain n and ε

n is the true number of molecules
ε is the true molecular brightness

How to Calculate n and ε

$$B = \frac{\sigma^2}{< k >} = \frac{\sigma_n^{\,2}}{< k >} + \frac{\sigma_d^{\,2}}{< k >} = \frac{\varepsilon^2 n}{\varepsilon n} + \frac{\sigma_d^{\,2}}{< k >} = \varepsilon + 1$$

This ratio identifies pixels of different brightness due to mobile particles.

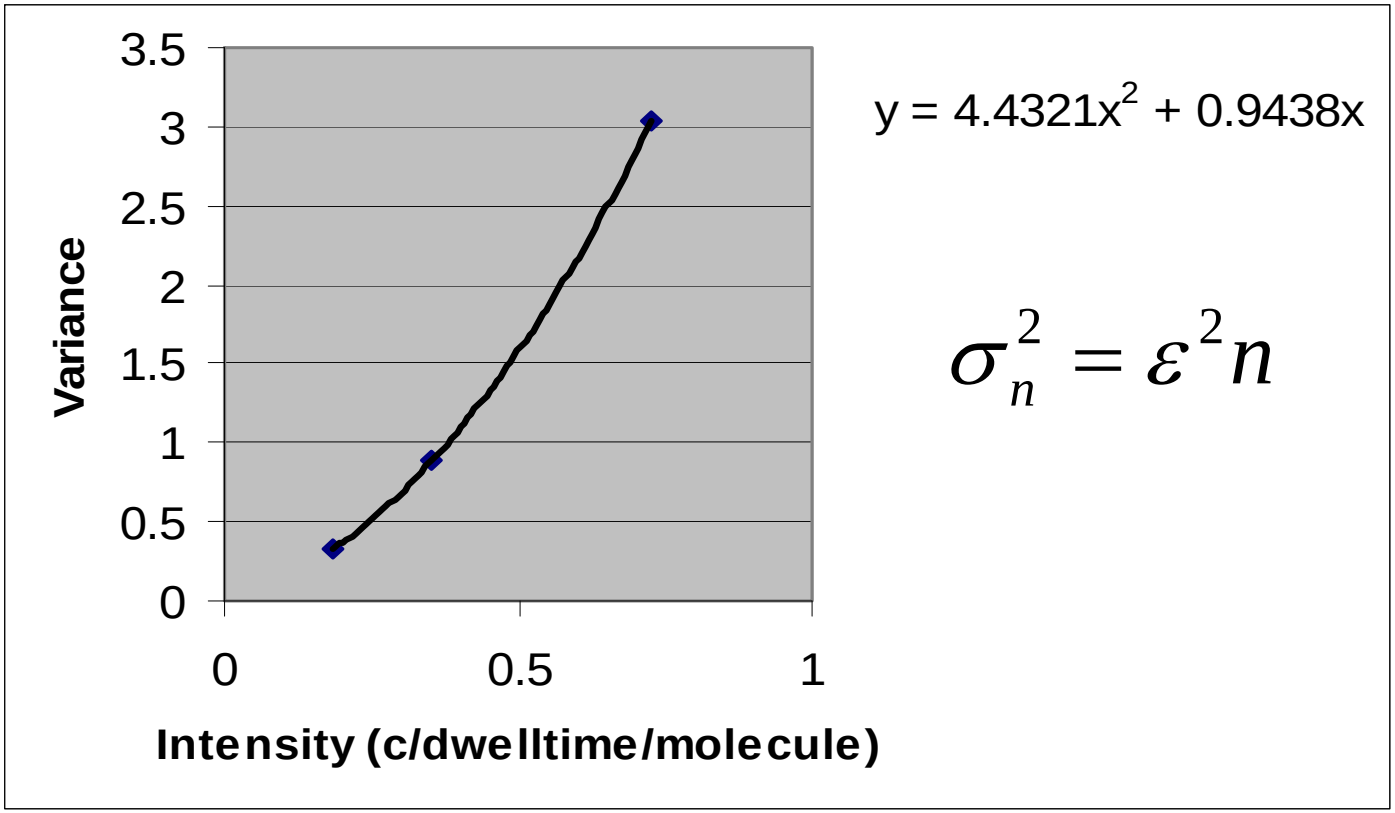
The “true” number of molecules n and the “true” molecular brightness for mobile particles can be obtained from

$$n = \frac{< k >^2}{\sigma^2 - < k >} \qquad \varepsilon = \frac{\sigma^2 - < k >}{< k >}$$

If there are regions of immobile particles, n cannot be calculated because for the immobile fraction the variance is = <k>. For this reason, several plots are offered to help the operator to identify regions of mobile and immobile particles. Particularly useful is the plot of N*vs*B.

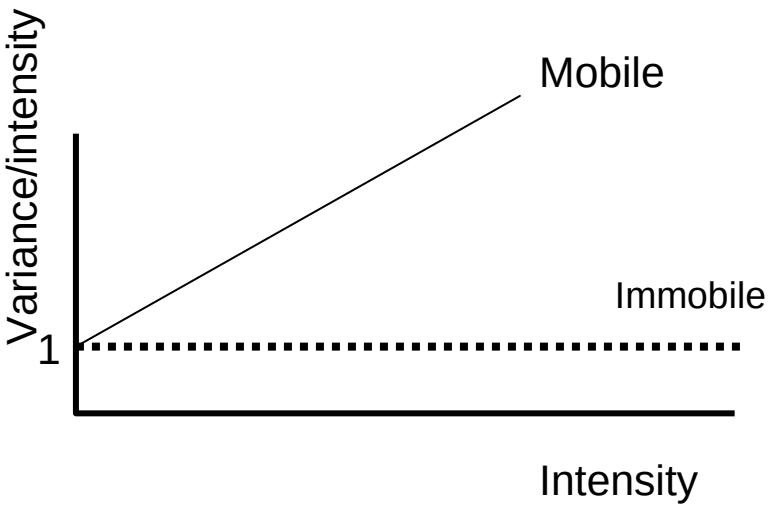
Quadratic dependence of the variance on particle brightness

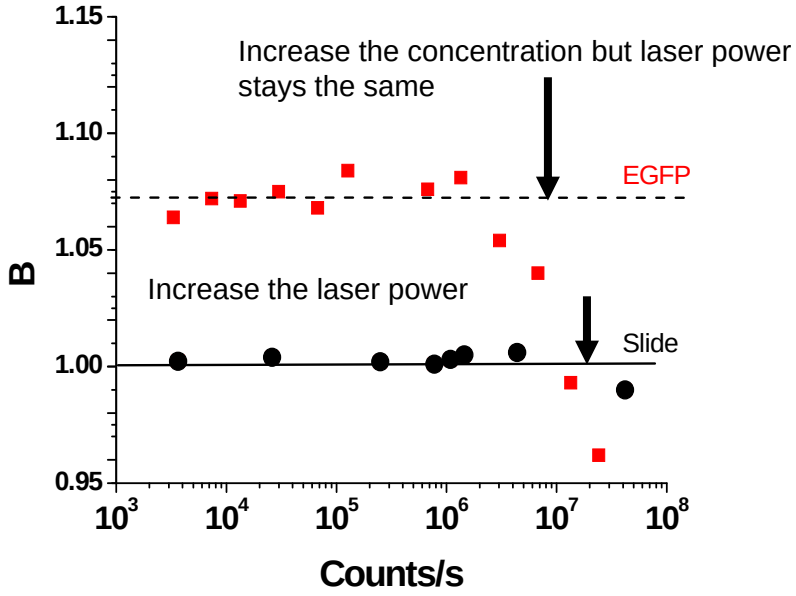
20nM EGFP in solution as a function of laser power



2-photon excitation using photon counting detectors

Identification of mobile and immobile molecules

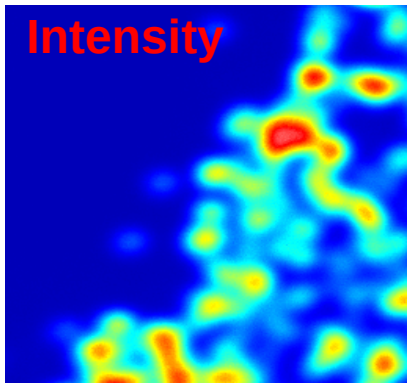


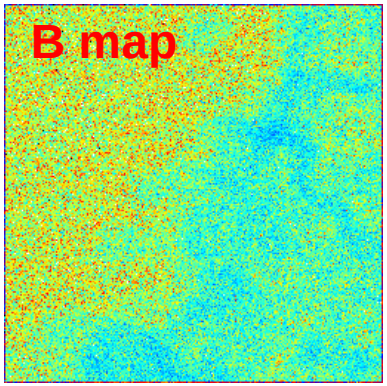


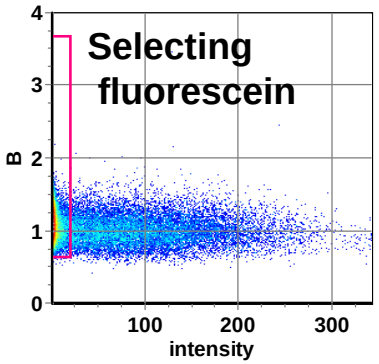
If we change the laser power, a plot of the ratio variance/intensity vs intensity can distinguish the mobile from immobile fraction. The two curves are for different pixel integration times.

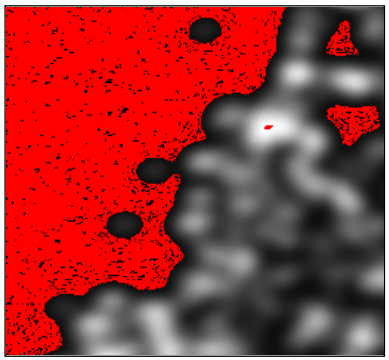
The effect of the immobile part: with photon counting detectors

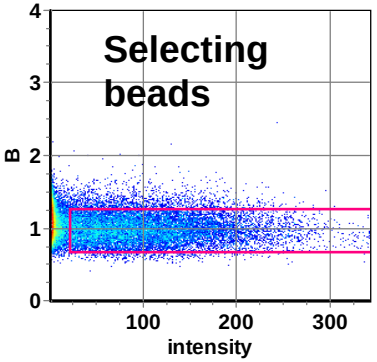
Fluorescent beads in a sea of 100nM Fluorescein.

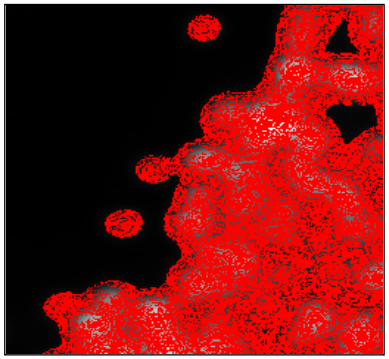


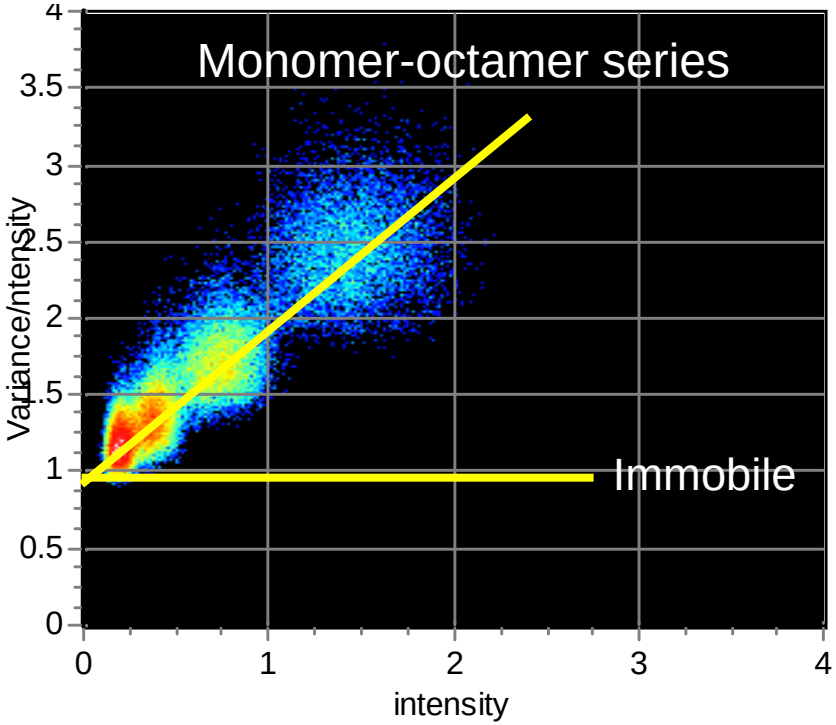






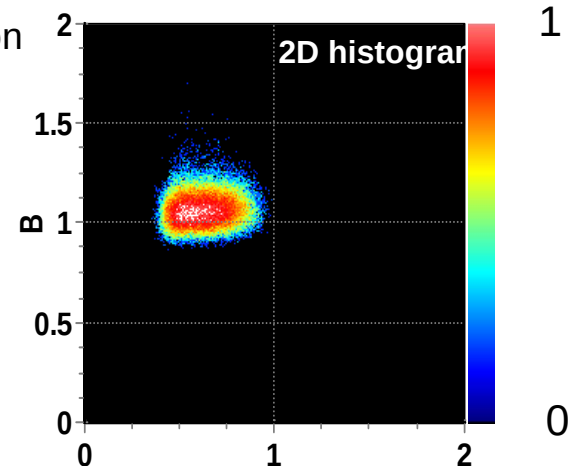


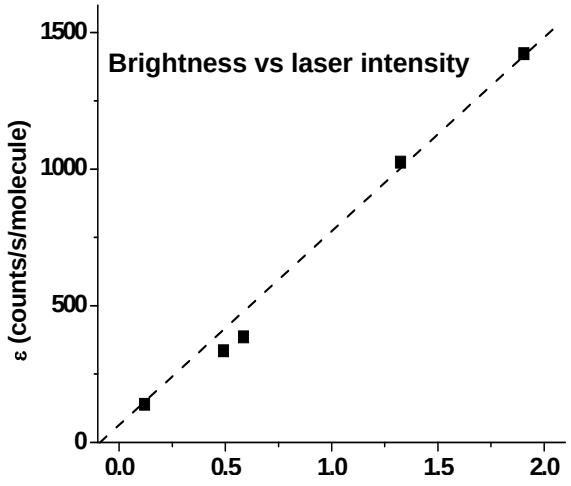


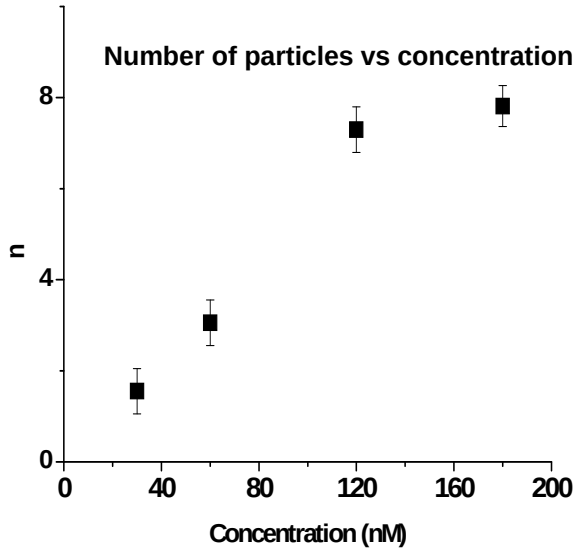


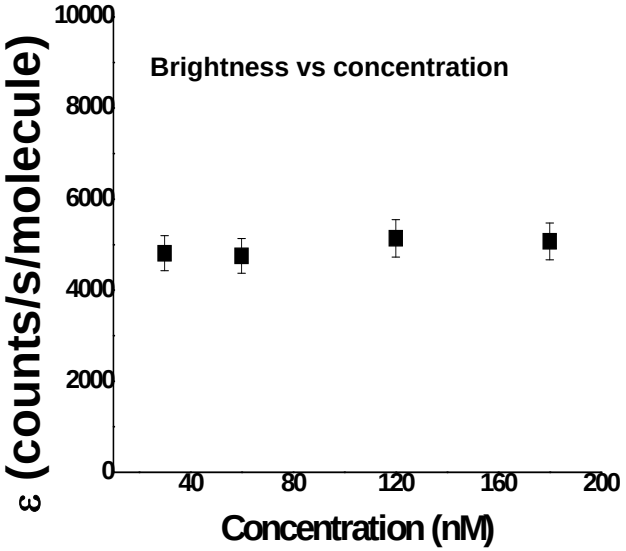
Brightness and number of molecules can be measured independently

EGFP in solution









What are the parameters for analog systems?

Detector Noise in Analog Systems

Additional considerations with analog detection systems:

- **digital levels are recorded (instead of photon counts)**
- **an offset is typically present**
- **additional detector variance at low currents**

d_{offset} = analog offset

$$< k >= \varepsilon n + d_{offset}$$

S = digital levels per photon

σ_0^2 = variance of analog detector

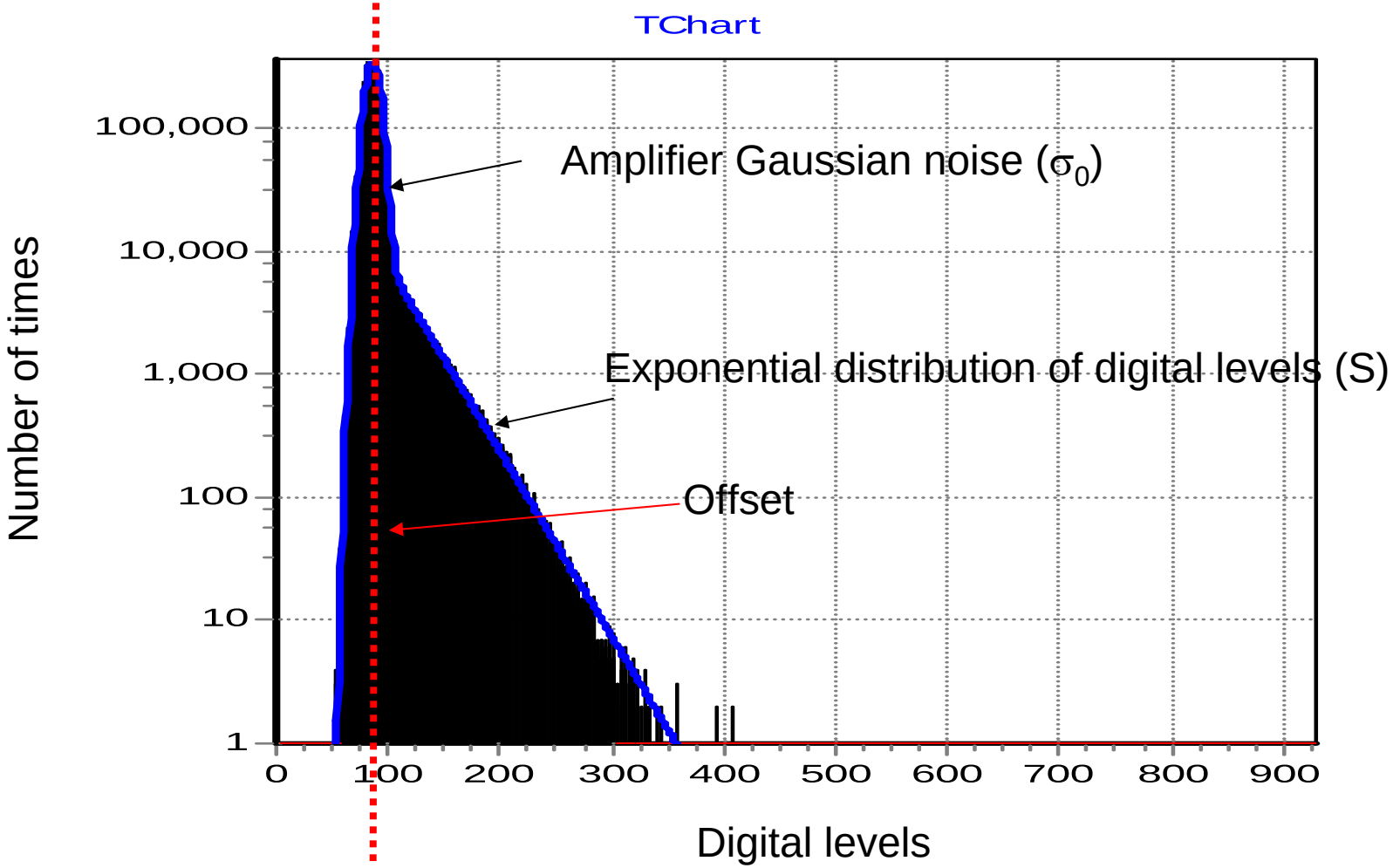
$$\sigma_d^2 = S\varepsilon n + \sigma_0^2$$

$$n = \frac{< N > B}{B - S}$$

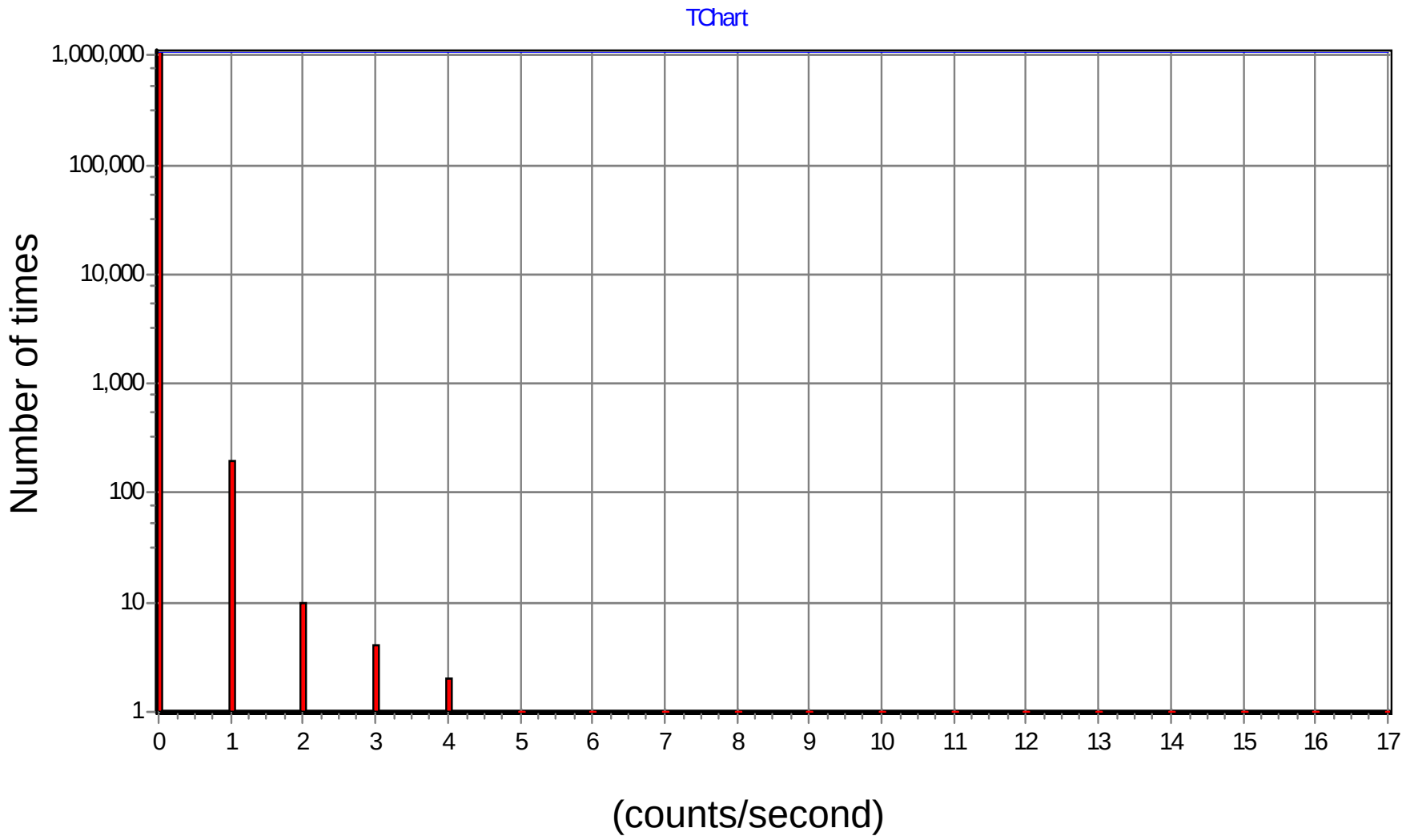
$$\varepsilon = B - S$$

If we fix the PMT settings (voltage and gain), then S and σ_0^2 should not change and need only be determined once.

Detector characterization
Analog detector response (dark current)

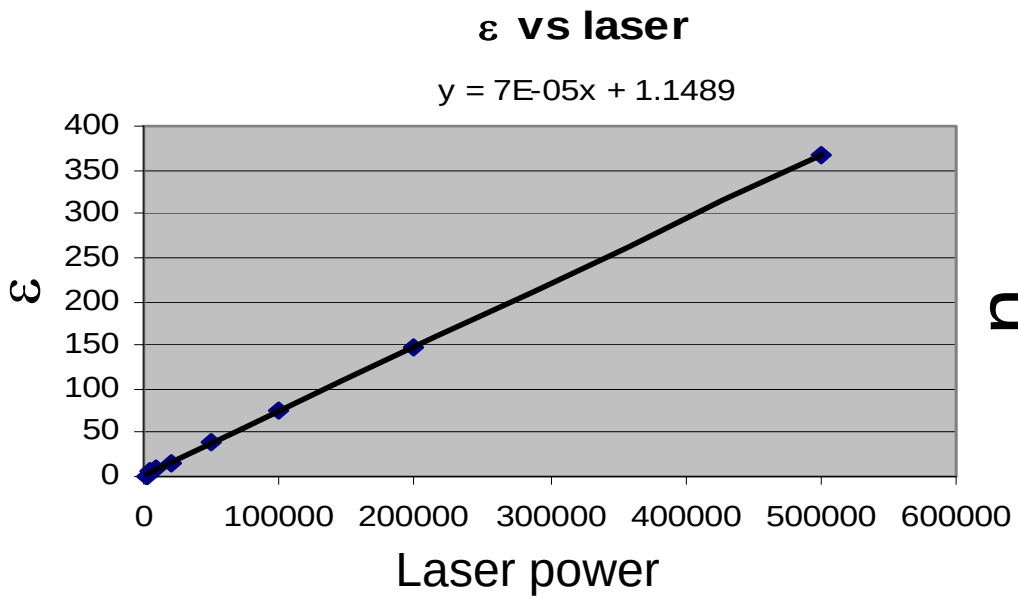


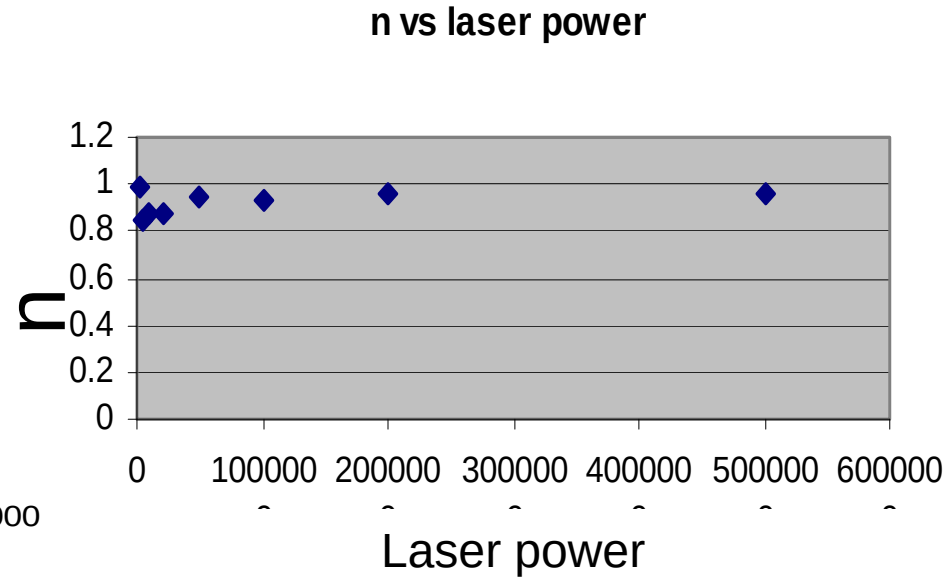
Detector characterization
Photon counting detector response (dark current)



Solution experiments: using analog detectors

Recovery of n and ε in the analog system for 20nM EGFP in solution



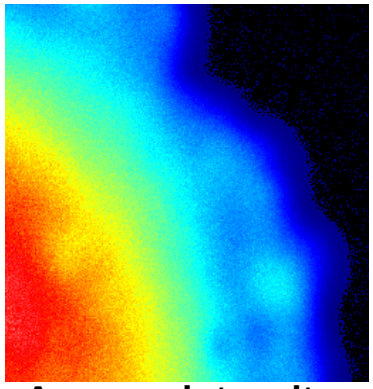


In the analog system, the recovery of relative values is good, for absolute values the calibration is more problematic. The best obtained so far is within a factor of 2

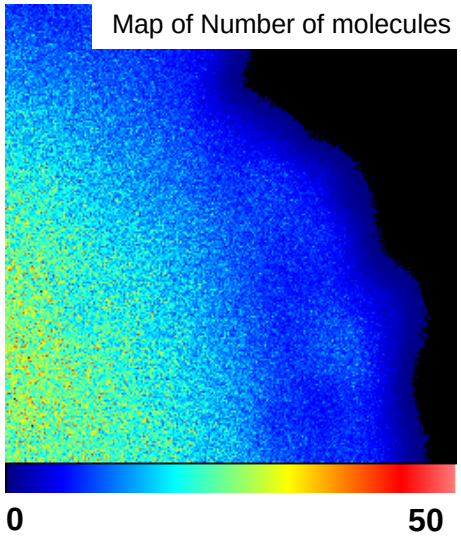
Courtesy of Valeria Vetri

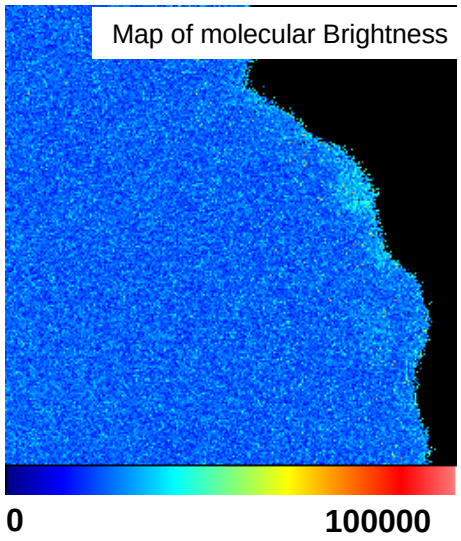
EGFP in CHO-k1 (1-Photon LSM)

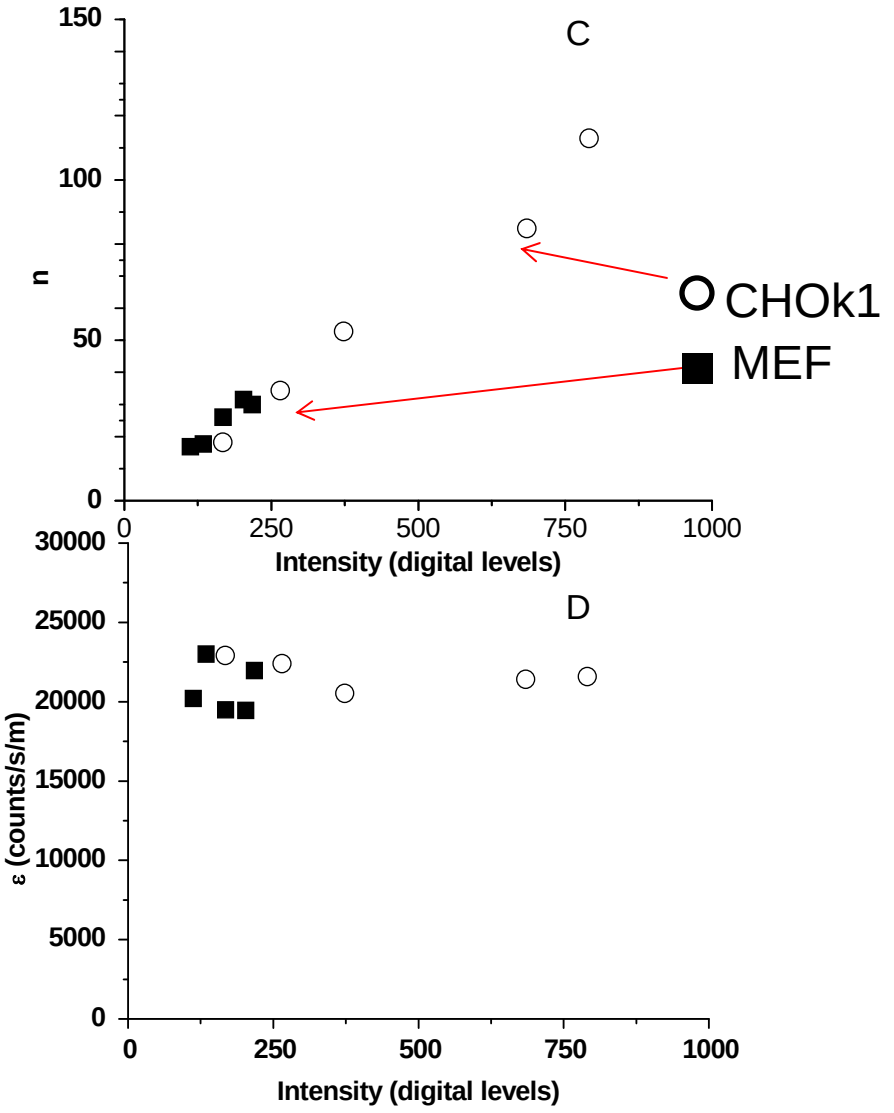
homogenous Brightness & heterogeneous Number of Molecules



Average intensity







Summary of N&B

- N&B distinguishes between number of molecules and molecular brightness in the same pixel
- The acquisition for the N&B can be done with a commercial Laser Scanning Microscope (LSM) and the same data used for RICS can be used to map N and B.
- The Immobile fraction can be separated since it has a Brightness value =1
- The N&B analysis of paxillin at adhesions shows large aggregates of protein during disassembly.

Additional Reading

1) Jay R Unruh and Enrico Gratton.Analysis of molecular concentration and brightness from fluorescence fluctuation data with an electron multiplied CCD camera.Biophys J. 2008; [epub ahead of print].

2) Michelle A Digman, Rooshin Dalal, Alan R Horwitz, and Enrico Gratton.Mapping the number of molecules and brightness in the laser scanning microscope.Biophys J. 2008; 94(6): 2320-2332.

3) Rooshin B Dalal, Michelle A Digman, Alan R Horwitz, Valeria Vetri, and Enrico Gratton. Determination of particle number and brightness using a laser scanning confocal microscope operating in the analog mode. Microsc Res Tech. 2008; 71(1): 69-81.

4) Yan Chen, Joachim D Müller, Qiaoqiao Ruan, and Enrico Gratton. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. Biophys J. 2002; 82(1): 133-44.

5) Alberto Garcia-Marcos, Susana A Sánchez, Pilar Parada, John S Eid, David M Jameson, Miguel Remacha, Enrico Gratton, and Juan P G Ballesta.Yeast ribosomal stalk heterogeneity in vivo shown by two-photon FCS and molecular brightness analysis. Biophys J. 2008; 94(7): 2884-2890.

6) Michelle A Digman, Paul W Wiseman, Colin K Choi, Alan R Horwitz, and Enrico Gratton. Mapping the stoichiometry of molecular complexes at adhesions in living cells.Proc Natl Acad Sci USA. 2008; [submitted].

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