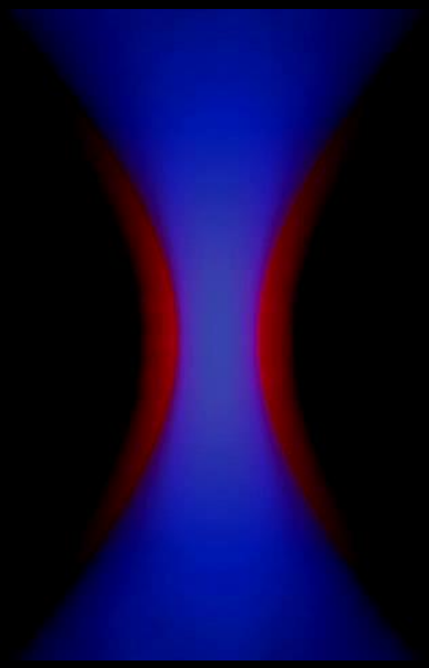
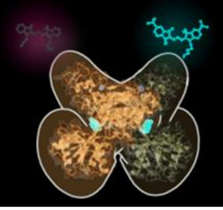
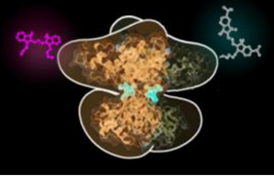


NUMBER AND BRIGHTNESS

Emmanuel MARGEAT
CNRS
Centre de Biochimie Structurale – Montpellier

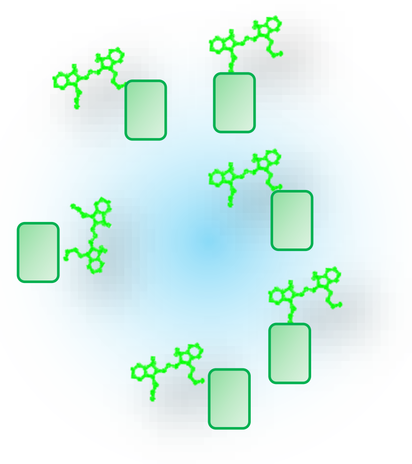


Measuring oligomerization

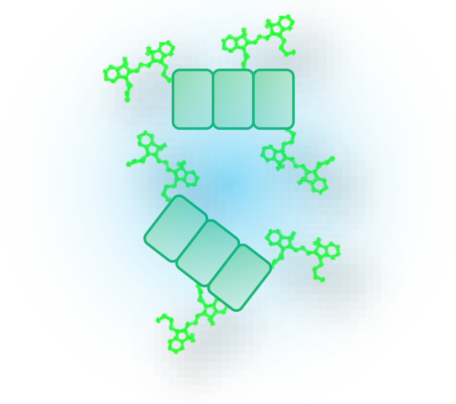


In an observation volume (« a pixel »), the fluorescence signal I is proportional to the number of fluorophores

$n = 6$
$\varepsilon = 1$



$\mathbf{I \sim 6}$



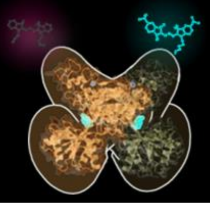
$n = 2$
$\varepsilon = 3$

We define n=number of diffusing particles in the observation volume, and ε=their molecular brightness

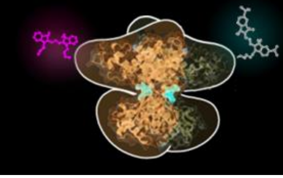
$$\mathrm{I} = \varepsilon . n$$

In the « small ensemble » regime, ε and n are extracted from the fluctuation of I

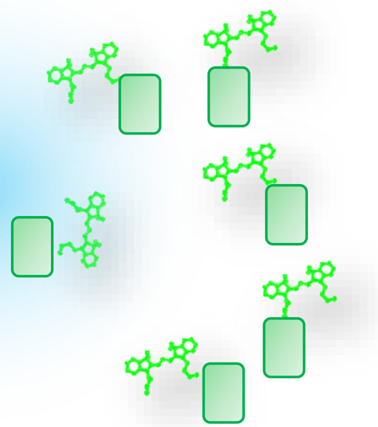
Digman, … Gratton, Biophysical J., 2008

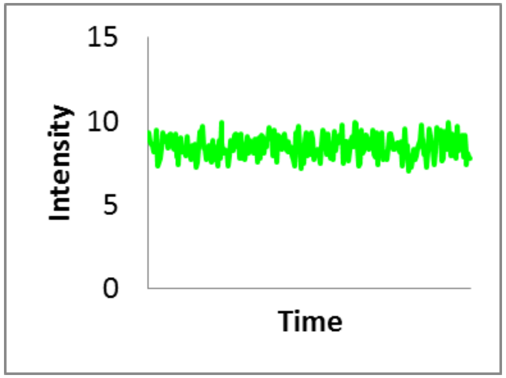


Measuring oligomerization

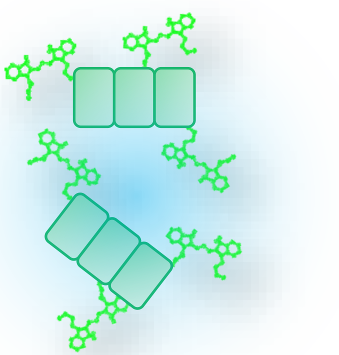


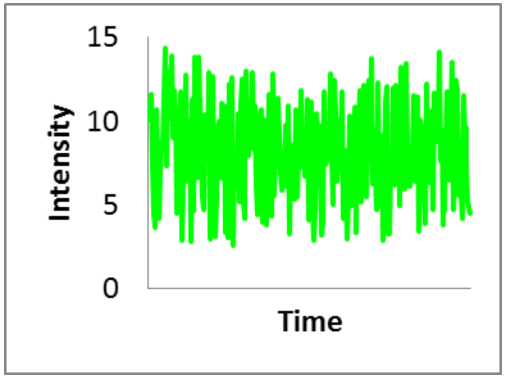
$n = 6$
$\varepsilon = 1$

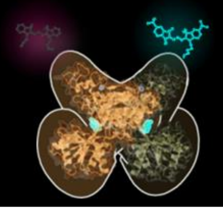




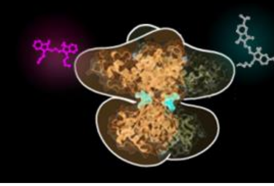
$n = 2$
$\varepsilon = 3$





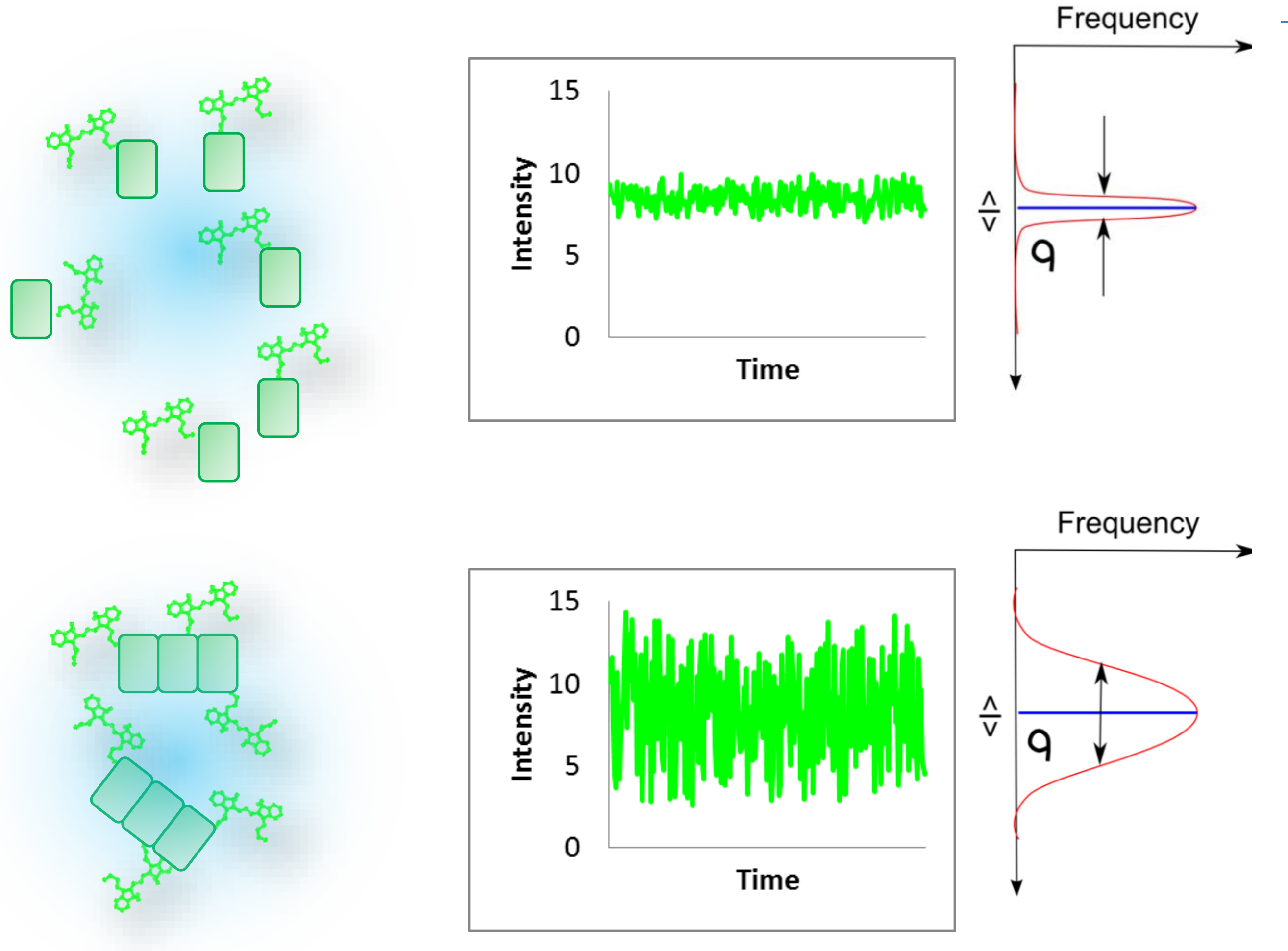


Measuring oligomerization



$n = 6$
$\varepsilon = 1$

$n = 2$
$\varepsilon = 3$



Same <I>

Different σ

$$\varepsilon = \frac{\sigma^2 - \langle I \rangle}{\langle I \rangle}$$

$$n = \frac{\langle I \rangle^2}{\sigma^2 - \langle I \rangle}$$

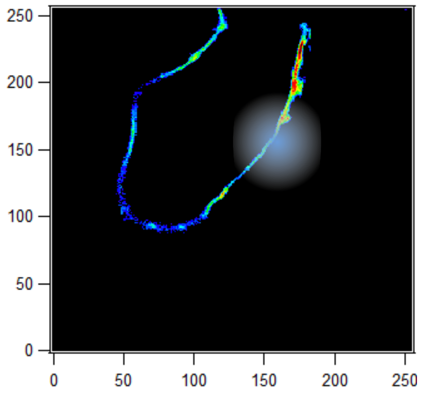
Change in the number of diffusing species (***n***) and their brightness (ε)

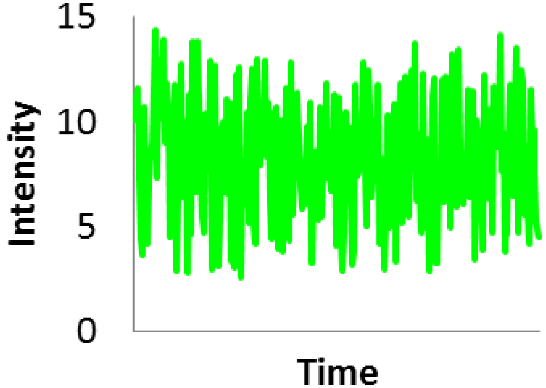
Digman, … Gratton, Biophysical J., 2008

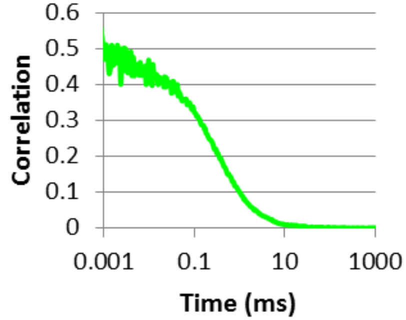
Data acquisition I : FCS

For one pixel

For one pixel



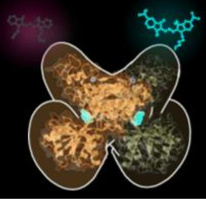




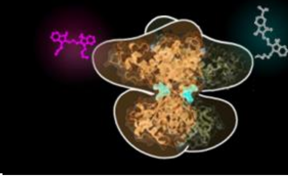
- Obtain simultaneously n, ε, t_d with great accuracy

- Time consuming (several seconds / pixel)
- Photobleaching is a big issue
- Difficult to obtain an image of n, ε, t_d (only a few points)

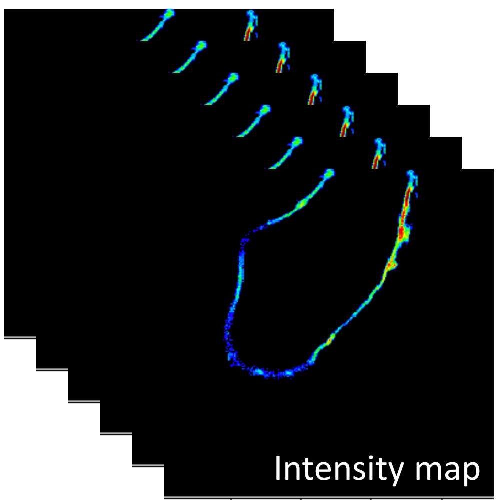
Qian et Elson, Biophysical J., 1990



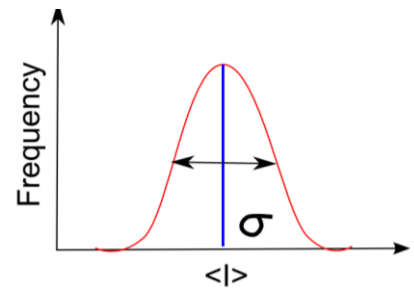
Data acquisition II : N&B



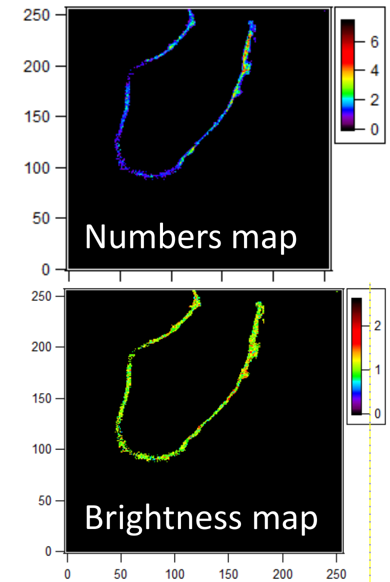
Stack of 100 images



For each pixel



Calculation of n & ε



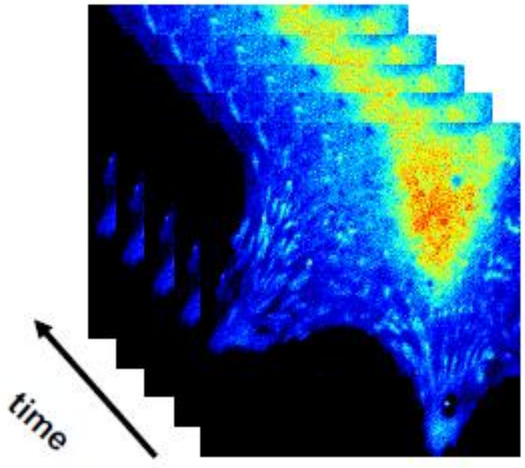
- Obtain an image of *n* & *ε*
- Photobleaching is reduced
- Implemented on commercial CLSM

- Timing information (t_D) is loss
- Necessary to have high speed scanning, photon counters preferred

Digman, … , Gratton, Biophys J, 2006

Principle of the N&B analysis

For a stack of k images, we define for each pixel !



$$F = \frac{\sum_K F(x,y)}{K}$$

$$\sigma_F^2 = \frac{\sum_K \left(F(x,y) - F\right)^2}{K}$$

Therefore we calculate: $$\frac{\sigma_F^2}{\langle F \rangle^2} = \frac{1}{\langle N \rangle} \Rightarrow \boxed{\langle N \rangle = \frac{\langle F \rangle^2}{\sigma_F^2}}$$

$$\langle F \rangle = B \langle N \rangle \Rightarrow \boxed{B = \frac{\sigma_F^2}{\langle F \rangle}}$$

Qian et Elson, Biophysical J., 1990
Digman, … , Gratton, Biophys J, 2006

Principe of the N&B analysis

Contributions to the variance

Fluctuations of the number of particles $\sigma_n^2 = \varepsilon^2 n$

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

n = true number
ε = true brightness

Total variance of the signal $\boxed{\sigma^2 = \sigma_n^2 + \sigma_d^2}$

$$B = \frac{\sigma_F^2}{\langle F \rangle} = \frac{\varepsilon^2 n + \varepsilon n}{\varepsilon n} = 1 + \varepsilon$$

$$\langle N \rangle = \frac{\langle F \rangle^2}{\sigma_F^2} = \frac{\varepsilon^2 n^2}{\varepsilon^2 n + \varepsilon n} = \frac{\varepsilon n}{\varepsilon + 1}$$

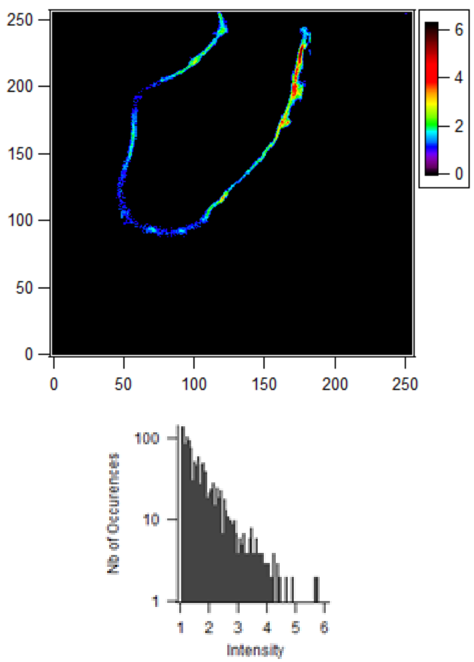
$$\varepsilon = \frac{\sigma_F^2 - \langle F \rangle}{\langle F \rangle}$$

$$n = \frac{\langle F \rangle^2}{\sigma_F^2 - \langle F \rangle}$$

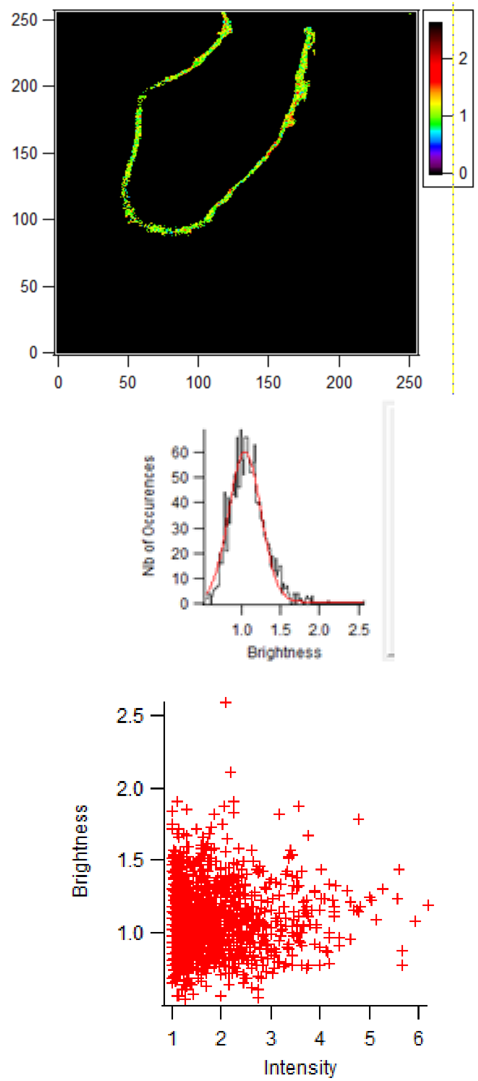
If the molecules are immobile, only the detector noise contributes to the variance (ie $\sigma^2 = \varepsilon n$). Thus, n cannot be calculated, and B=1

N&B data representation

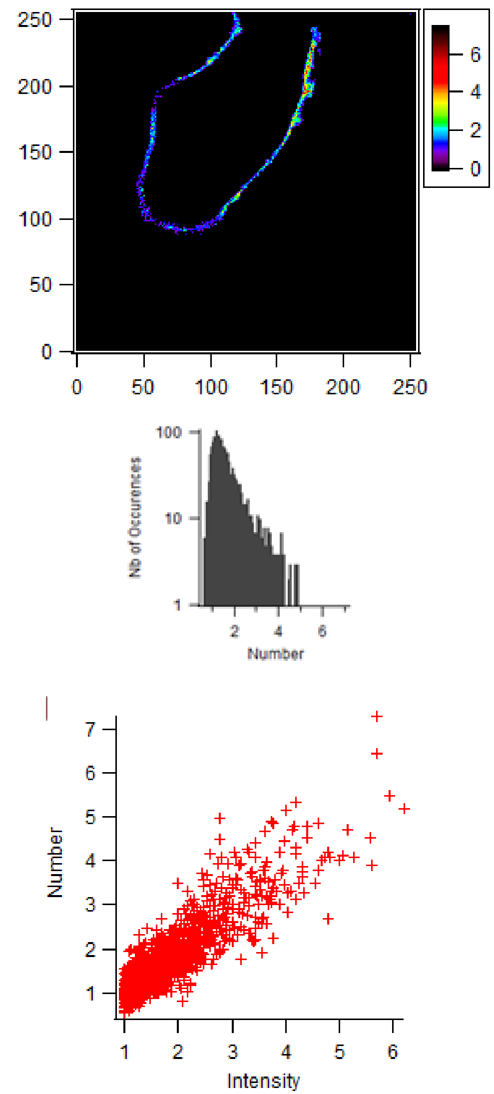
Intensity map



Brightness map



Number map



N&B : Experimental considerations

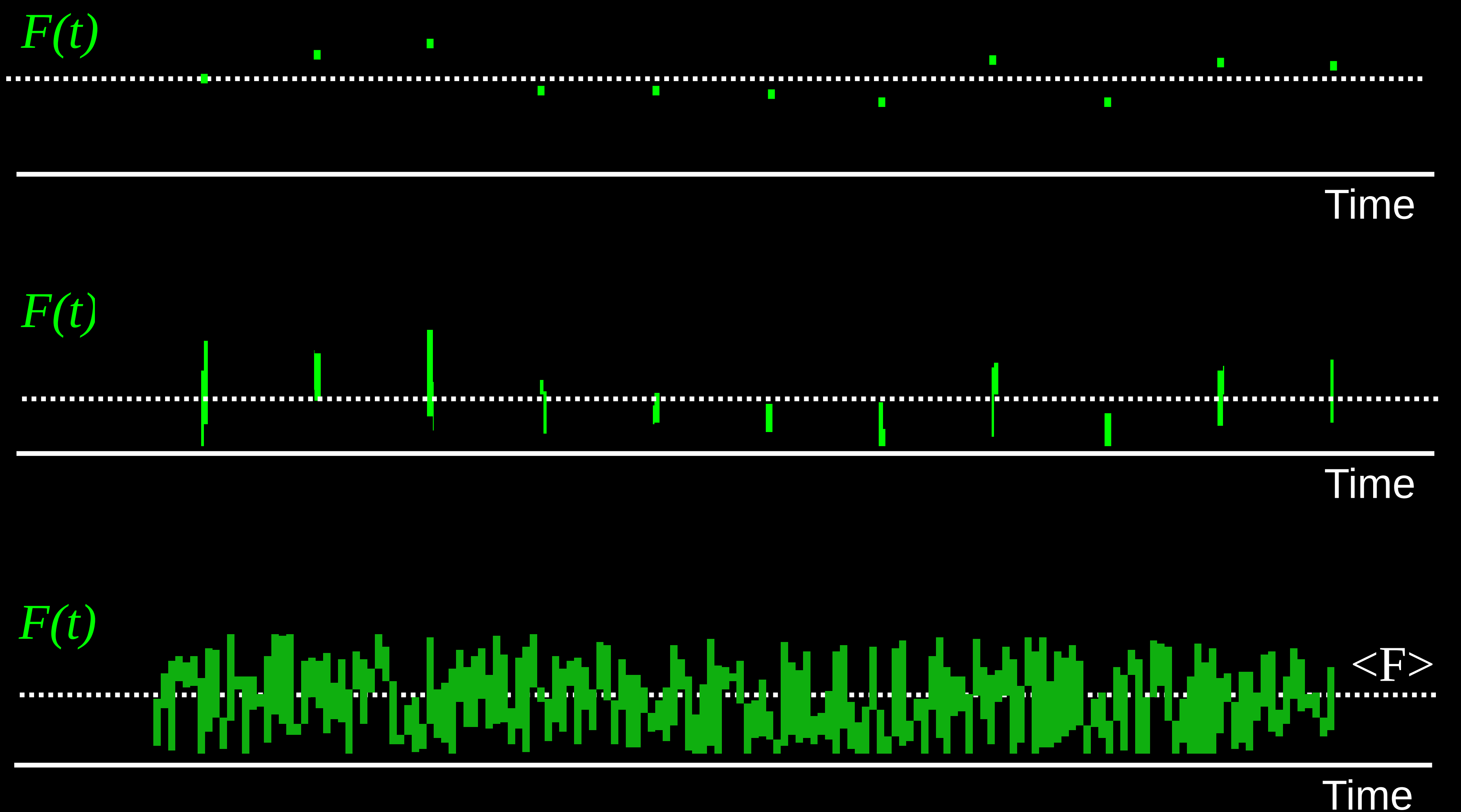
Imaging system

→ In principle, any microscope with laser scanning can be used
- Sensitivity
- Photon counting detectors
- Fast scanning

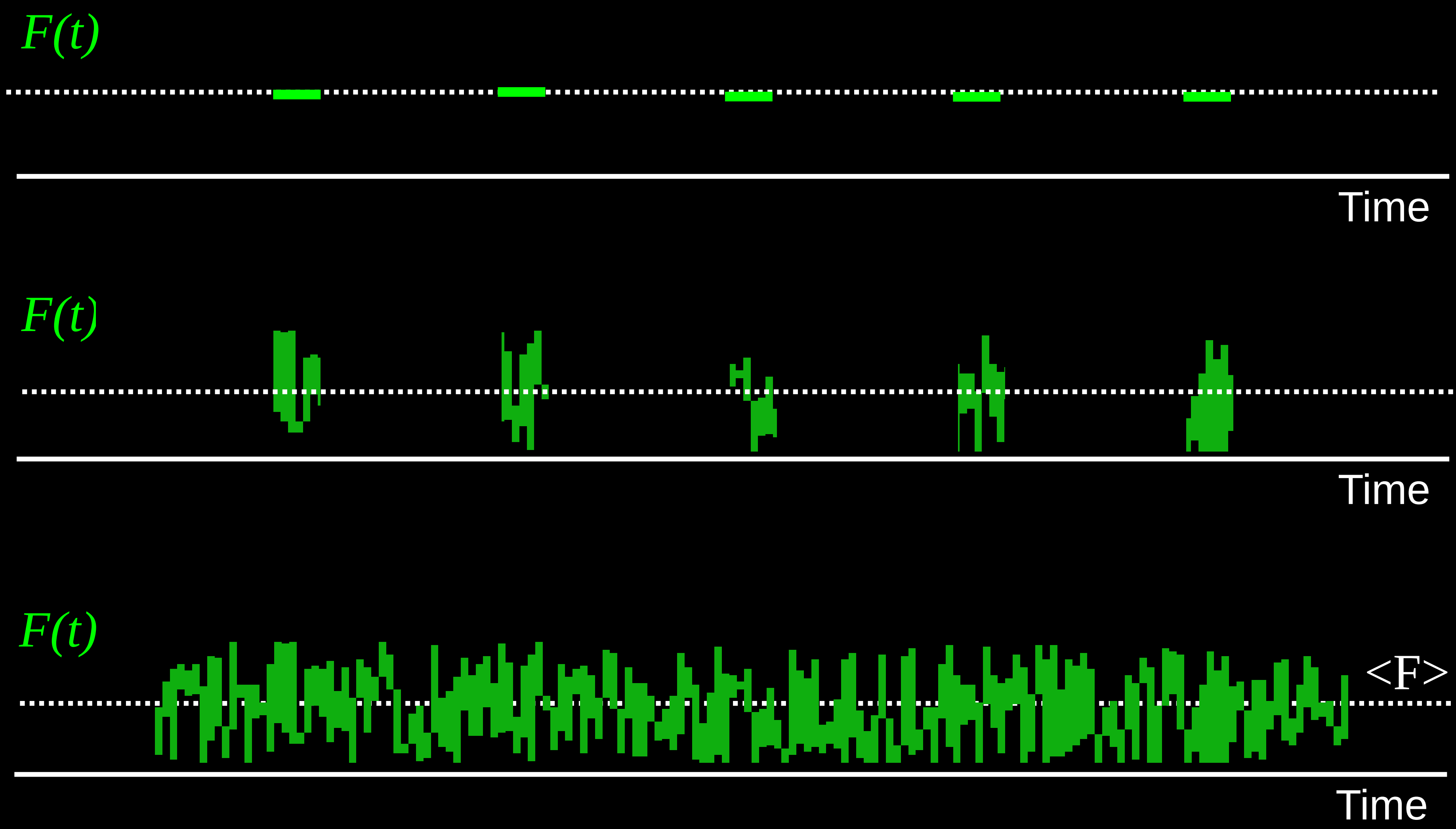
In the lab we use a semi-commercial system

- Femtosecond IR laser for 2-photon excitation
  - Low background
  - No out-of focus photobleaching
  - Small observation volume
  - Multicolor excitation (cross-correlation)
- Inverted microscope, high NA objective
- Detector ISS ALBA : scanning mirrors et 2 channel detection

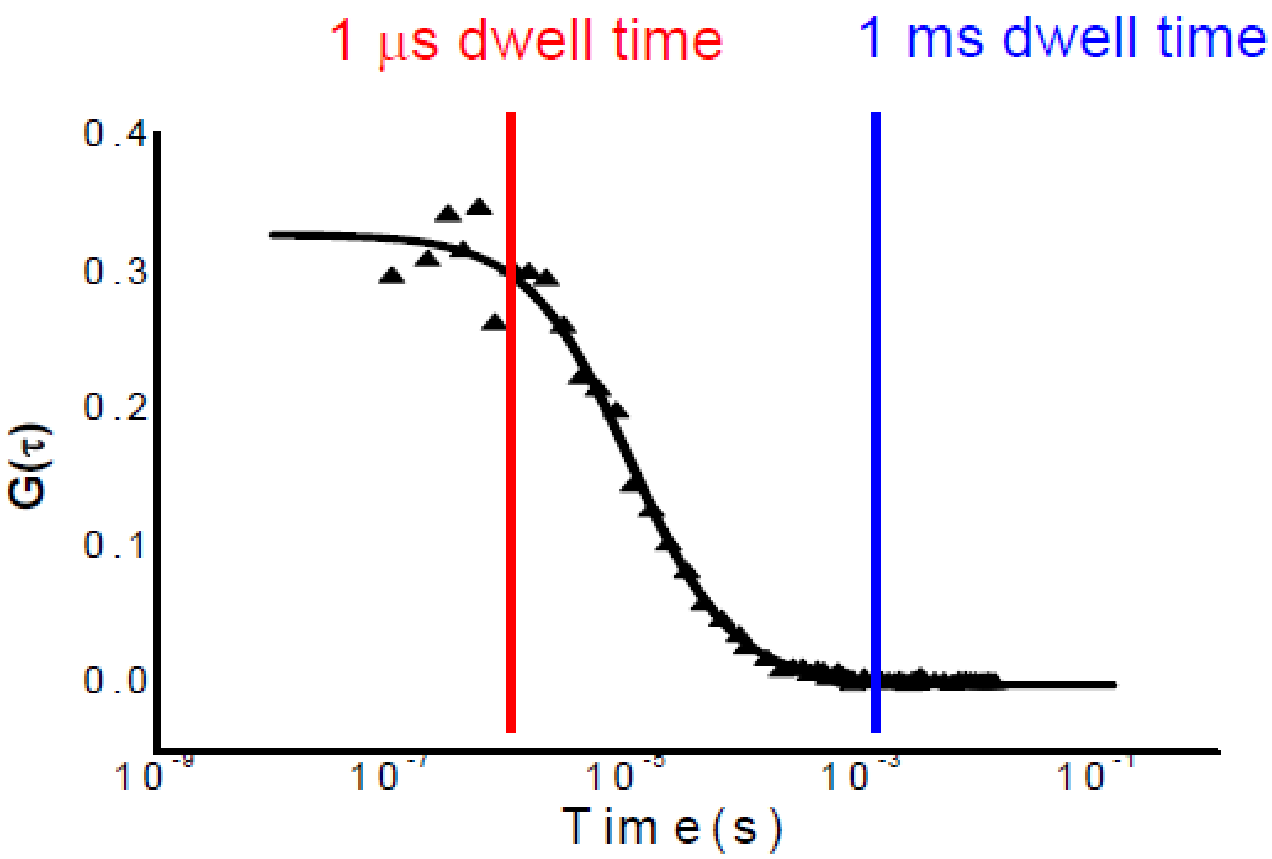
Fast Scanning



Slow scanning



Increasing the dwell time decreases the amplitude of the fluctuation.



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.

N&B : en résumé

- N&B allows the quantification of the number of molecules, and their brightness, pixel by pixel

  → An « image » of N and B is obtained

- Aquisition can be done with a conventional LSCLM

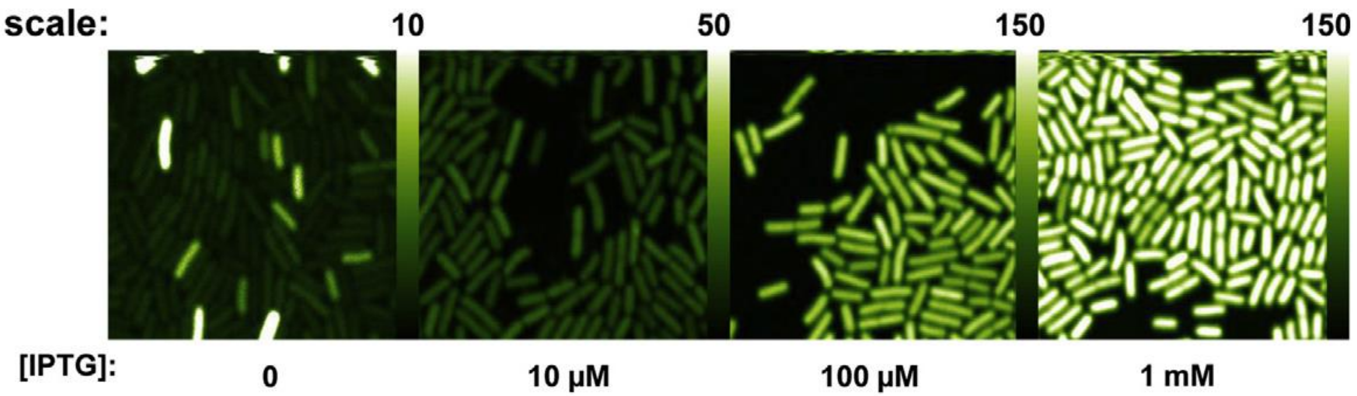
- An immobile fraction can be detected (B=1)

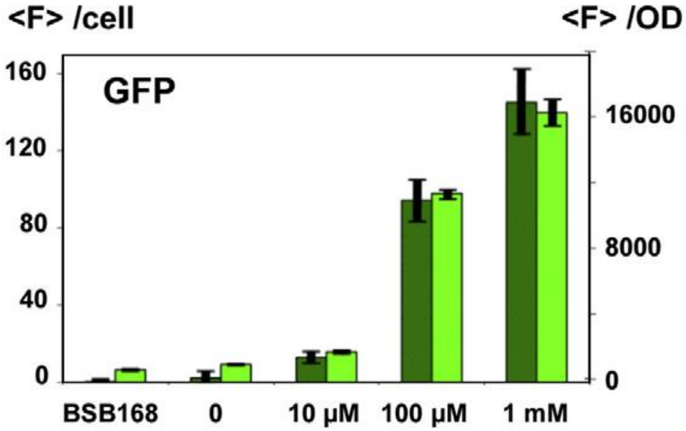
- Sample photobleaching can be reduced as compared to FCS, but temporal information is lost.

- Fluctuations in N are needed !!!
  - Low concentration (< μM)
  - Low background noise
  - Low photobleaching

Quantification of noise in gene expression in bacteria

Expression of Gfpmut2 under control of an inducible promoter in *B. Subtillis*



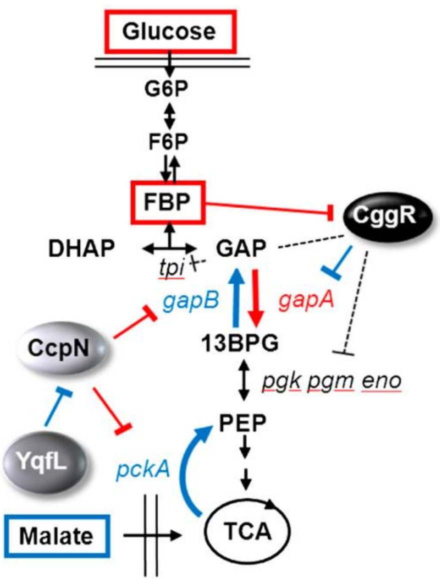


Correlation between the expression determined by 2P-microscopy and ensemble fluorescence

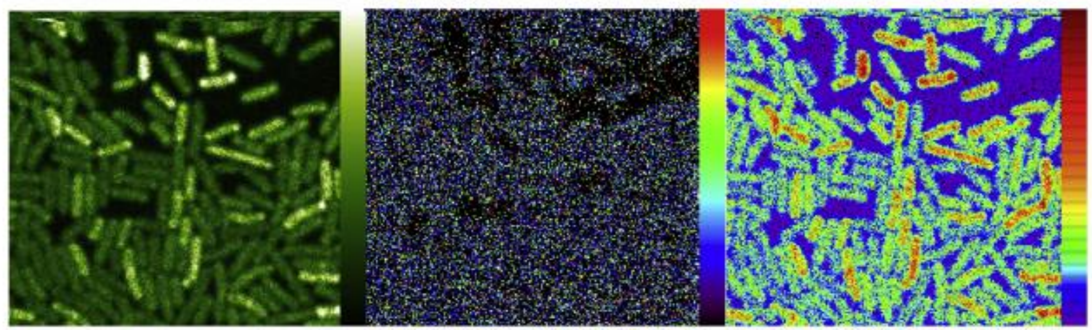
Ferguson et al., Anal. Biochem. 2011

Quantification of noise in gene expression in bacteria

Expression of Gfpmut3 under control of *gapB* promoter in *B. Subtillis*

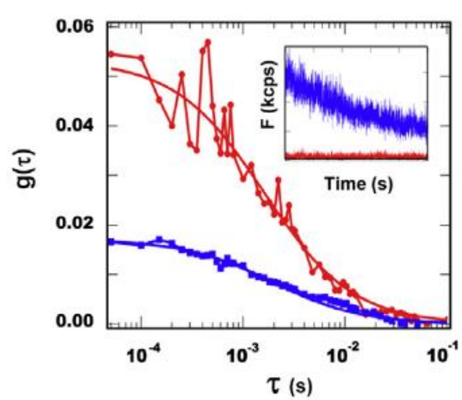


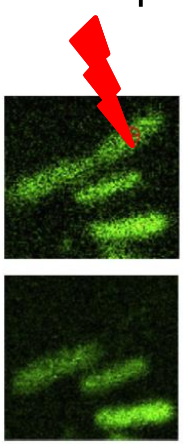
Glucose



Variability in gene expression in a population

Glucose Malate





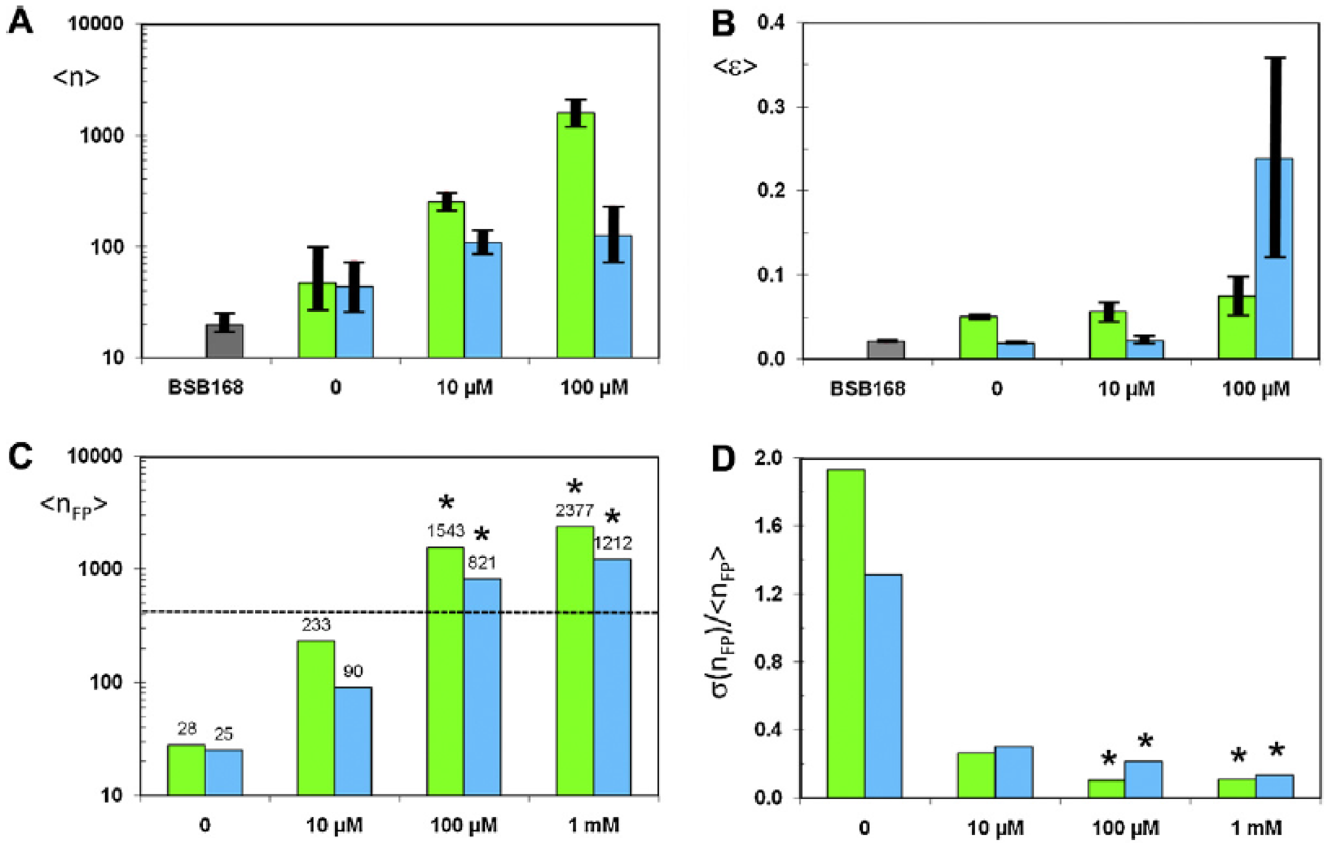
Before FCS

After FCS

Ferguson et al., Anal. Biochem. 2011

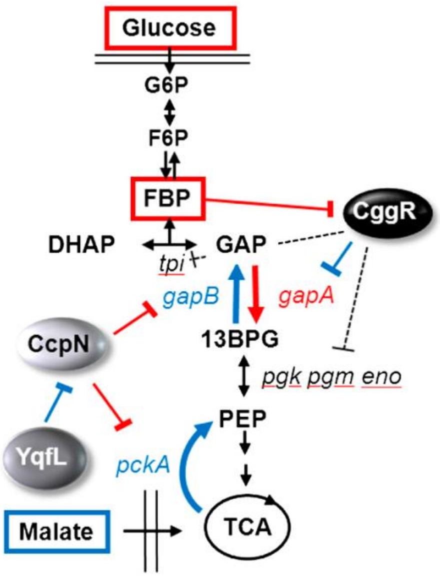
Quantification of noise in gene expression in bacteria

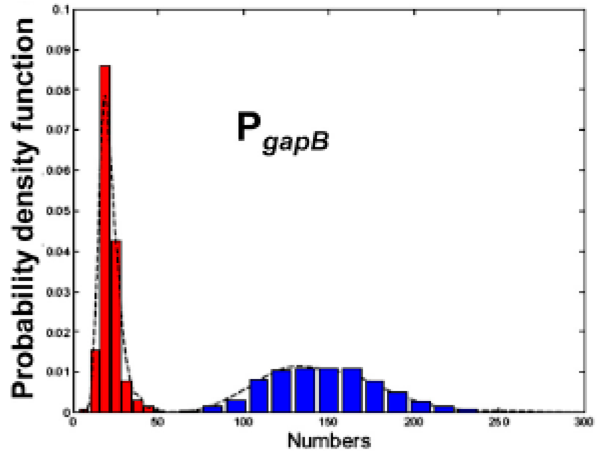
Expression of **Gfpmut3** and **CFP** under control of *gapB* promoter in *B. Subtillis*

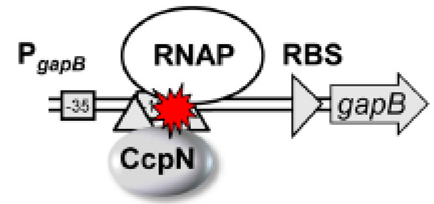


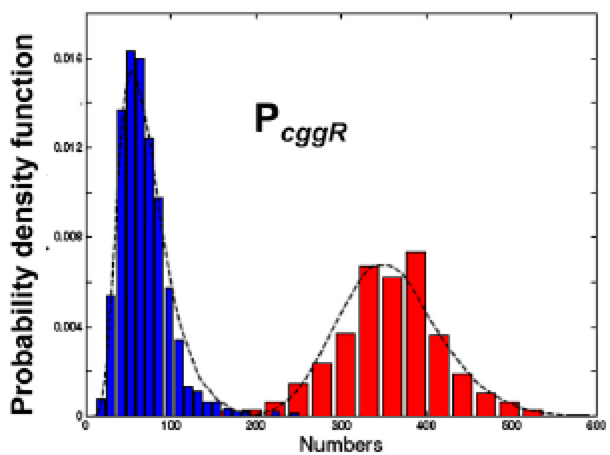
Ferguson et al., Anal. Biochem. 2011

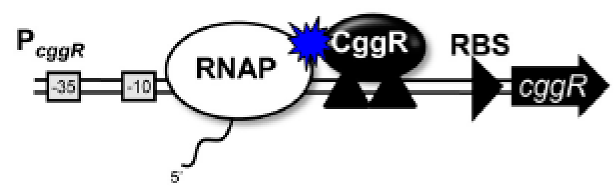
Quantification of noise in gene expression in bacteria





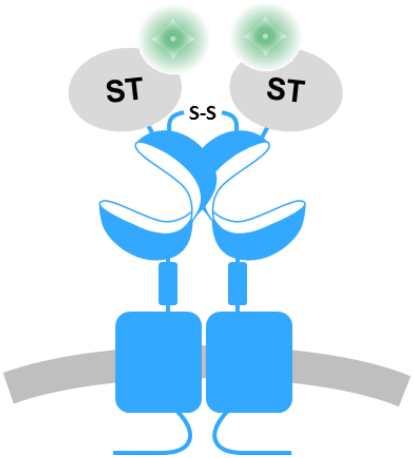


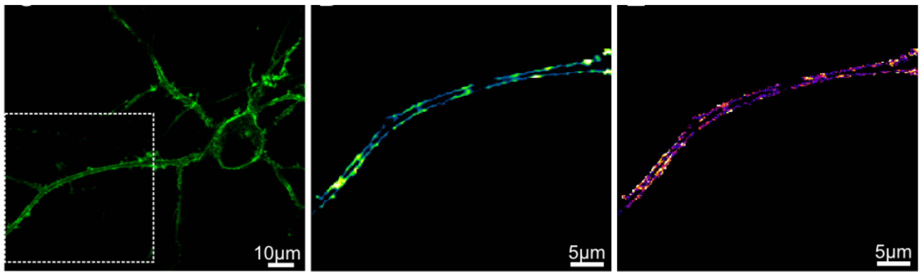




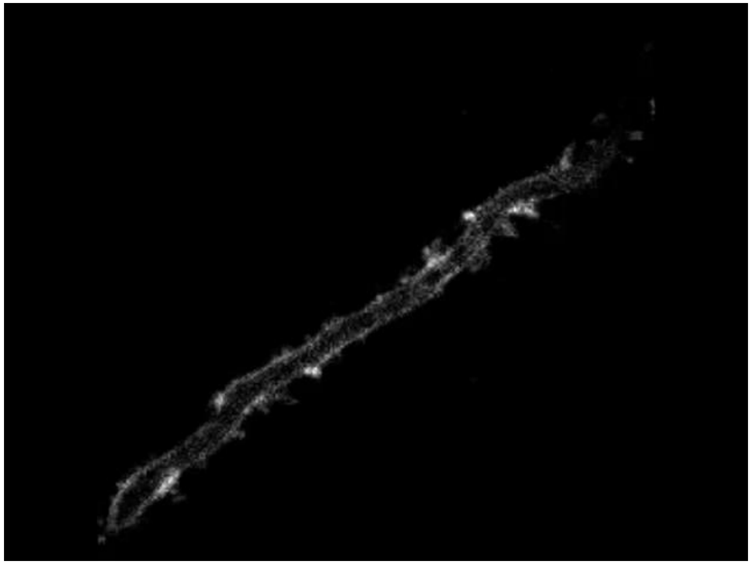
Ferguson et al., PNAS 2012

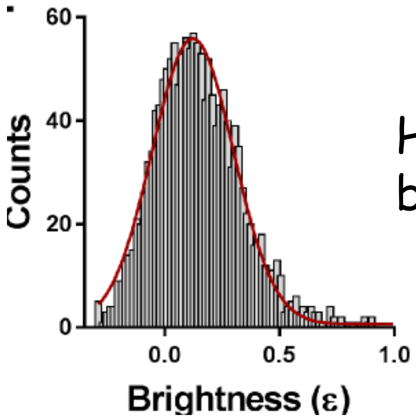
mGluR and GABA$_B$ oligomerization





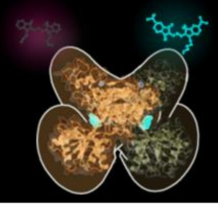
Neuron ROI Bmap



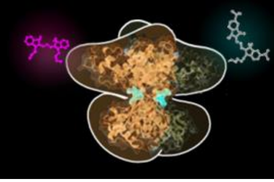


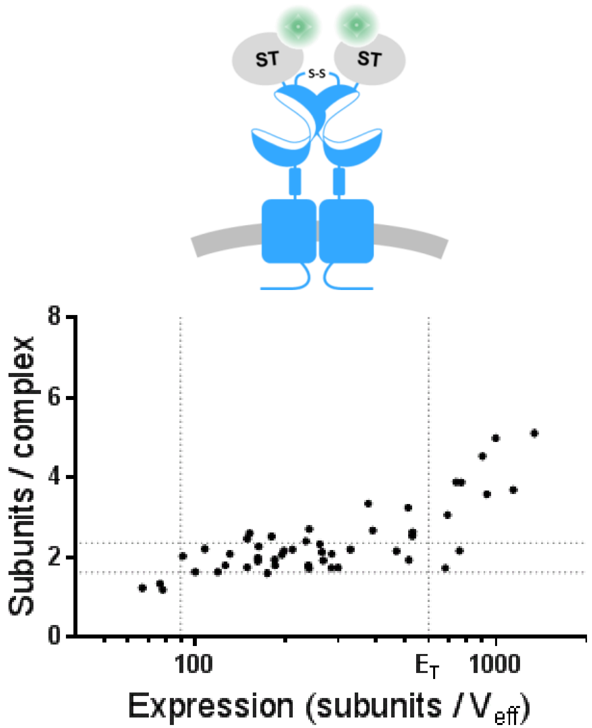
Histogram of brightness values

→ Compare with the value of a single Alexa488

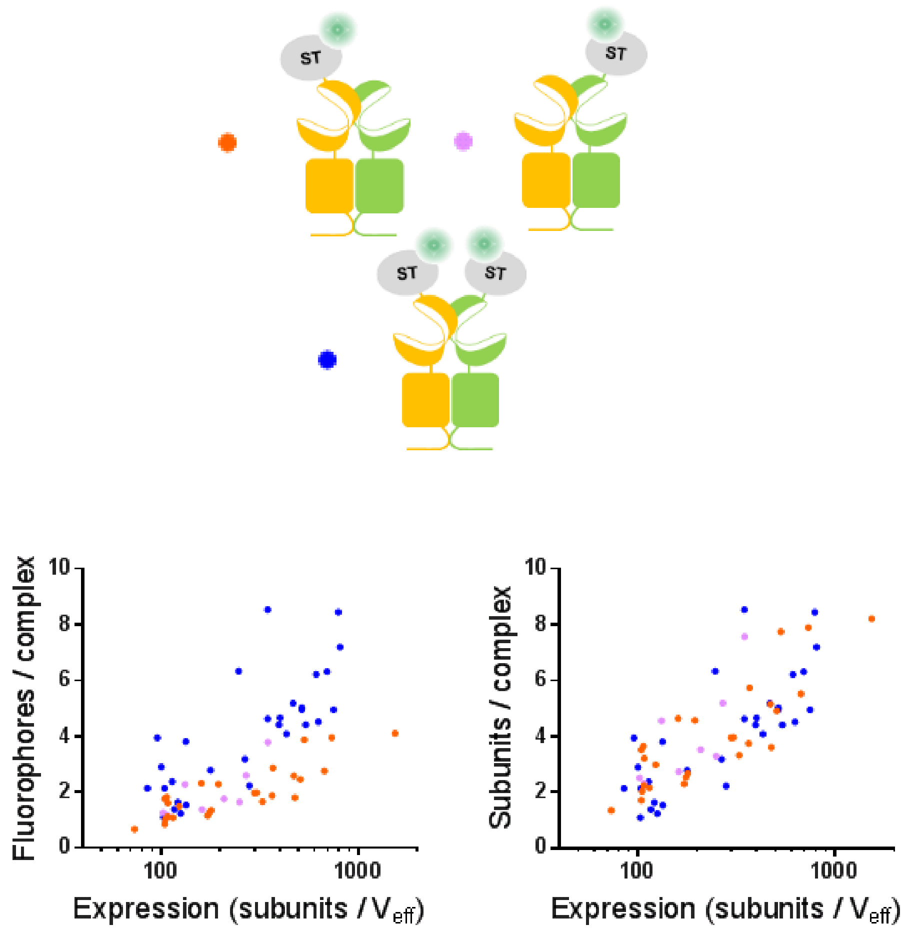


mGluR and GABA$_B$ oligomerization



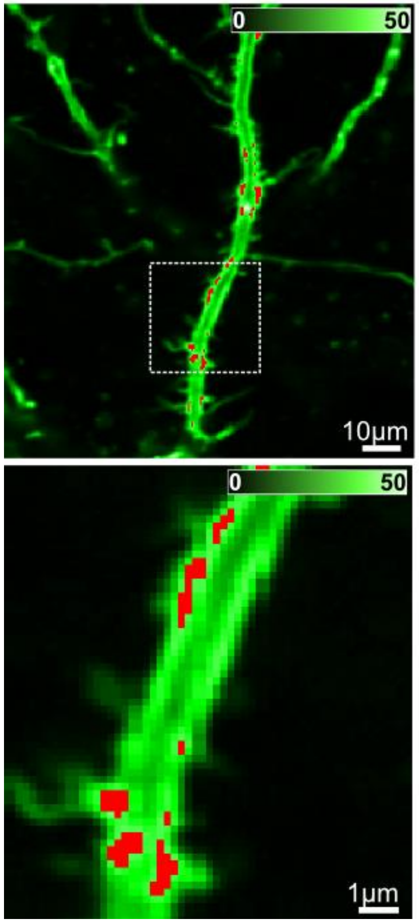


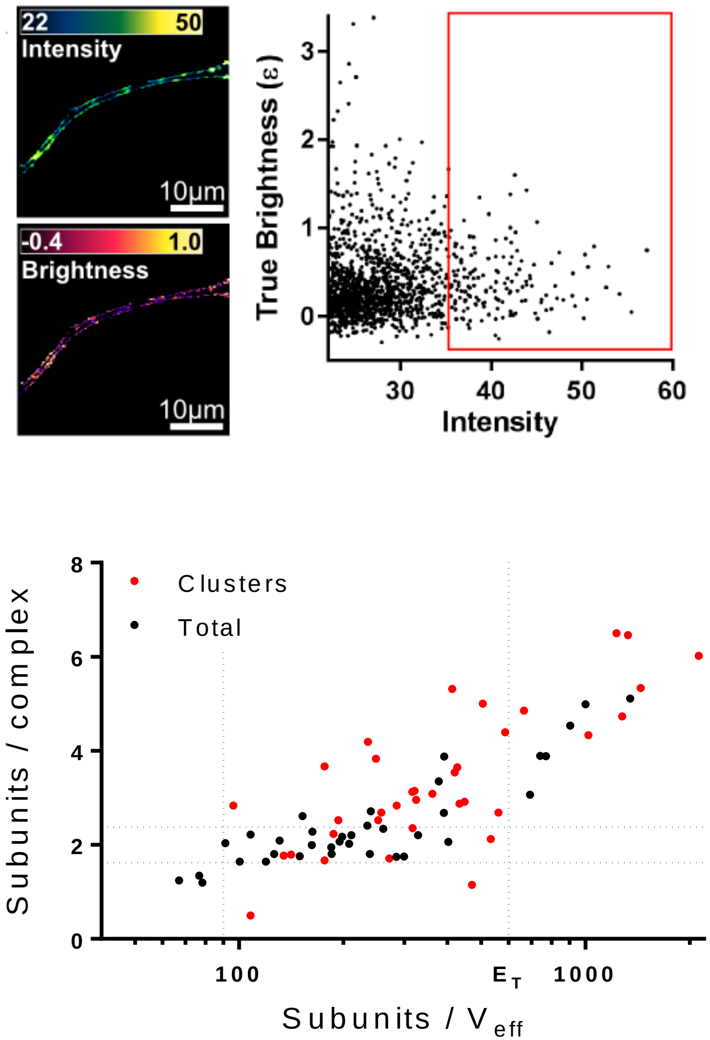
→ mGlu2 appears mainly as a dimer



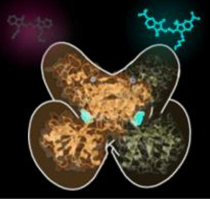
→ GABA$_B$ forms higher order oligomers

Spatial distribution

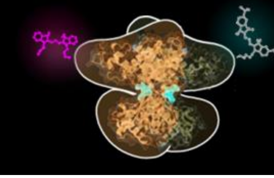


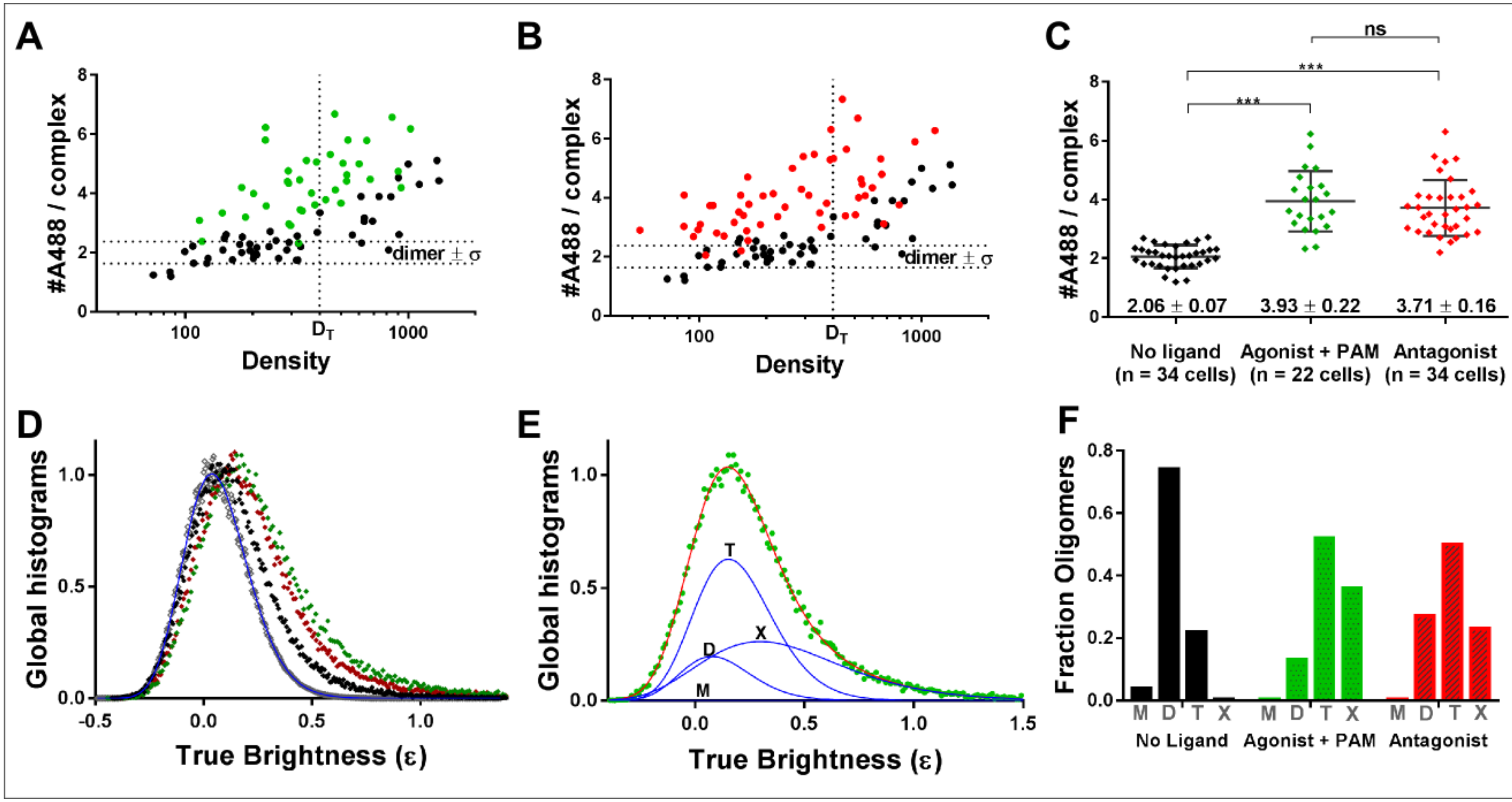


→ regions of higher intensity represent higher expression, not clusterisation



Ligand effects on mgluR oligomerization





→ mGlu2 makes dimers of dimers in the presence of agonists and antagonists