Brightness

N&B

Number

Overview

① Introduction, basic concept and math
② A step-by-step demonstration
③ Errors, corrections and pitfalls
④ Some examples
⑤ Conclusion, outlook, what’s next.

Molecular Interactions

Methods to look at molecular interactions:

- Pull-down assays, immunoprecipitation, etc… very indirect
- NMR, XRD, EM: not in a living cell
- Co-localization: not enough spatial resolution
- FCS, PCH: limited to single spot or small area measurement
- ICS: works on very slow (immobile) objects
- FRET: limited applicability, systems & experiment

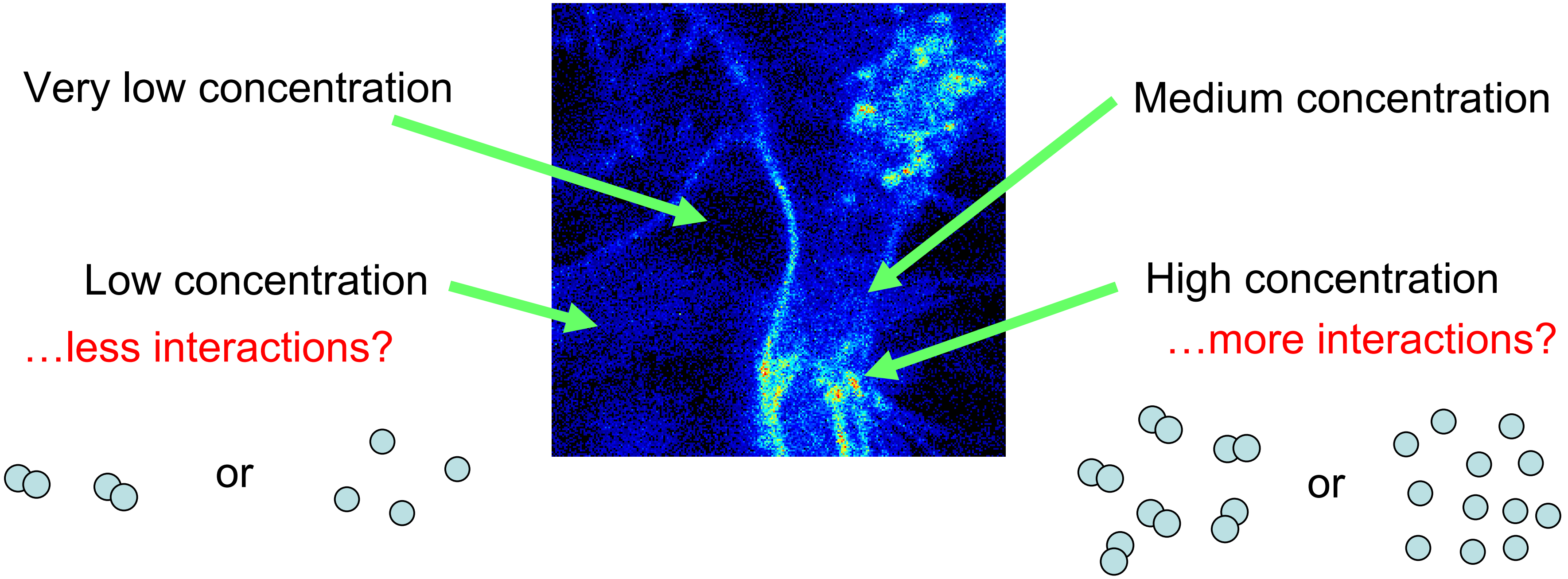
Is there a way to observe the interactions on images?

N&B

①

The problem in imaging

Less fluorescence only means fewer fluorophores – nothing is said about interactions.
More fluorescence only means more fluorophores – nothing is said about interactions.



Very low concentration

Medium concentration

Low concentration

…less interactions?

High concentration

…more interactions?

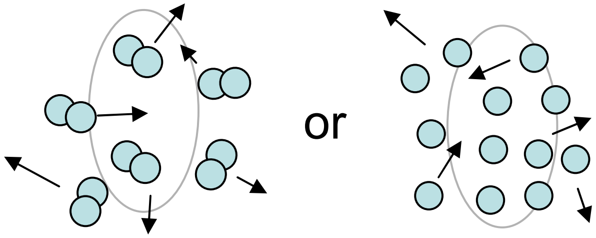
or

or

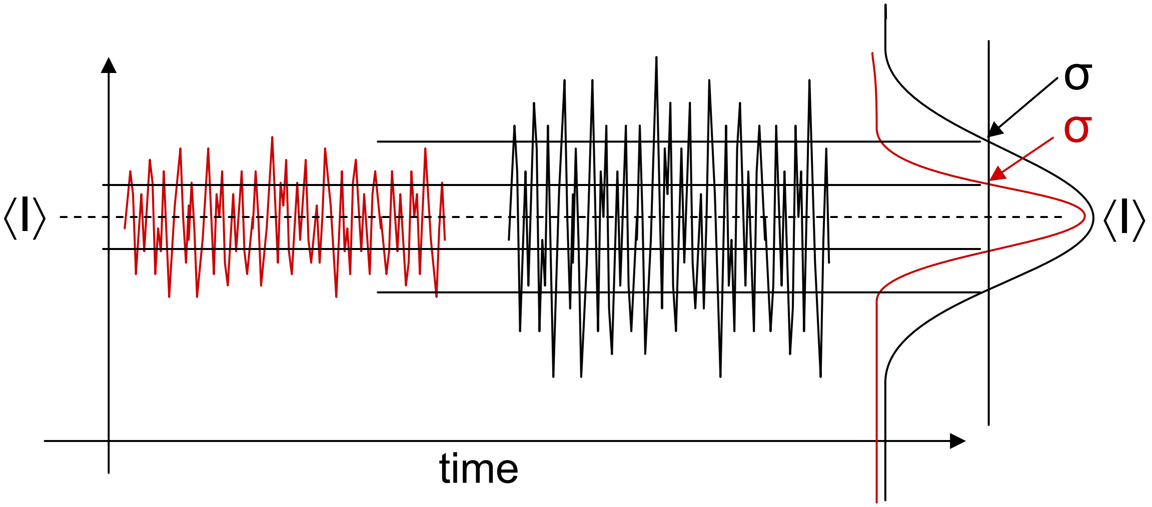
* The “proportionality” is weakened by taking into account, for example, that different moieties may lead to different quantum yields, spectral shifts, etc.

①

The problem in imaging



If we recall some of the PCH theory we know that we have a chance to sort those out, if we can catch the objects’ fluctuations in and out of a known volume:

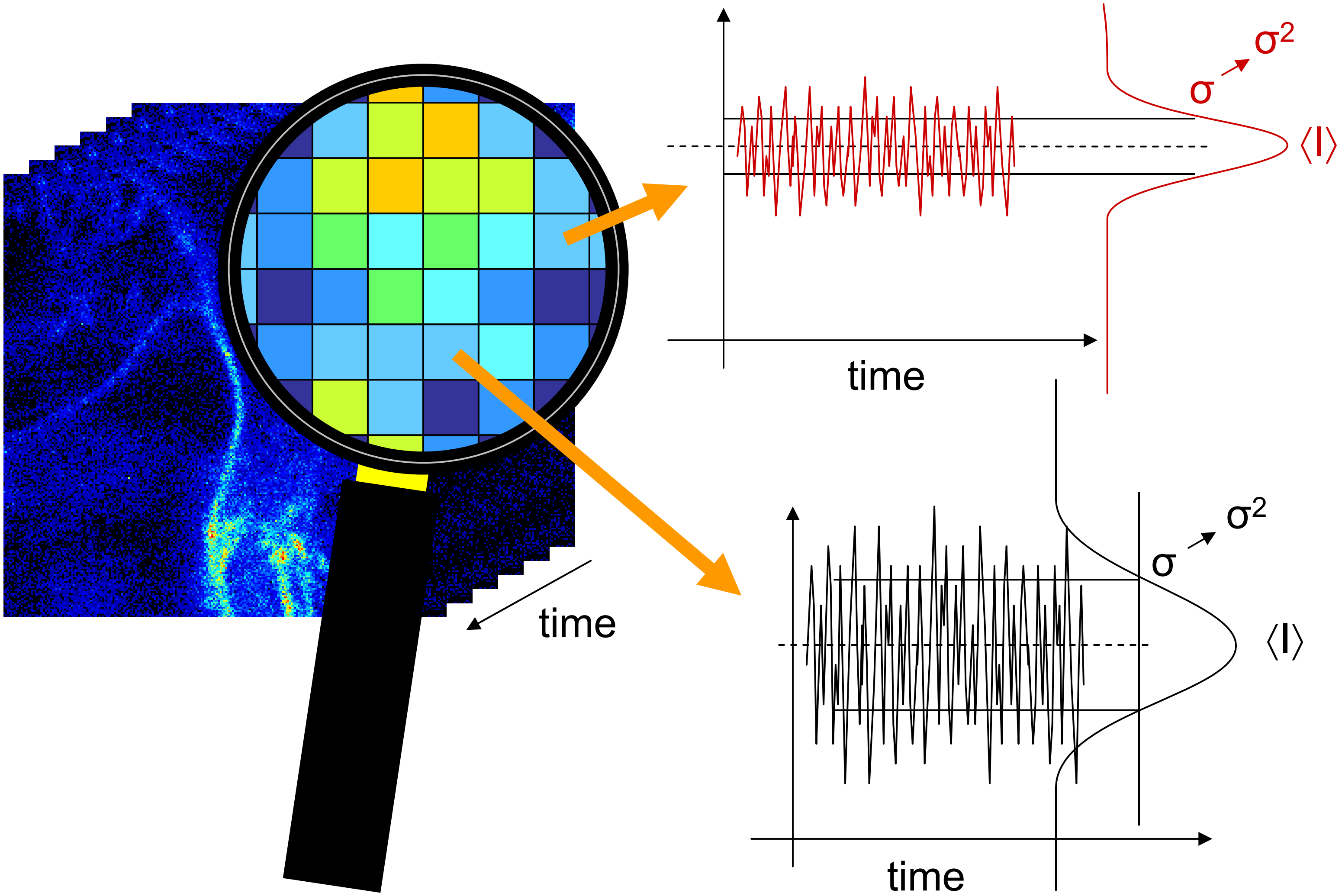


$G(0) \sim 1/N = \sigma^2/\langle I \rangle^2$

Qian and Elson, PNAS, **87** (14), 5479 (1990)

①

The problem in imaging



①

The basic maths

Schoolbook definitions:

$\langle I \rangle = n^{-1} (I_1+I_2+I_3+\dots I_n)$; mean in n frames

$\sigma^2 = \langle I^2 \rangle - \langle I \rangle^2$; variance

Qian & Elson contribution:

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

$N = \langle I \rangle^2/ \sigma^2$

Gratton (& common sense) definition:

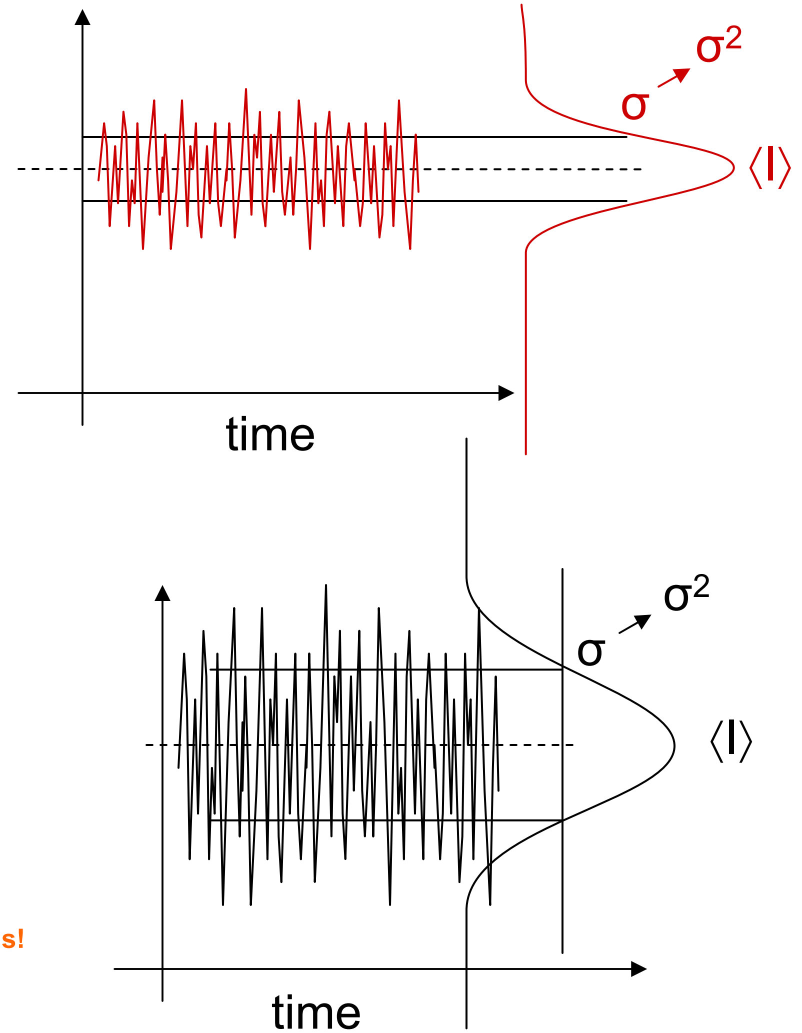
$B = \langle I \rangle / N = \sigma^2/\langle I \rangle$

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

detector $\sigma_d^2 = \langle I \rangle$ $\sigma_o^2 = \varepsilon\langle I \rangle$ object

reasonable assumptions!

$B = \langle I \rangle / N = \sigma^2/\langle I \rangle = \varepsilon + 1$



①

The basic maths

all things with no variance

$B = \varepsilon + 1$

ε is in units of:

$\langle I \rangle / N$ counts per observation time per object

or: counts per molecule and dwell time

Note that ε is also a function of instrument parameters, like:
γ-factor, quantum yield

→ Callibrate your equipment
→ To keep things simple, use the same settings between samples.

dwell time = t_d

If I want ε in “per second”:

$\varepsilon_{td} / t_d = \varepsilon_{sec}$

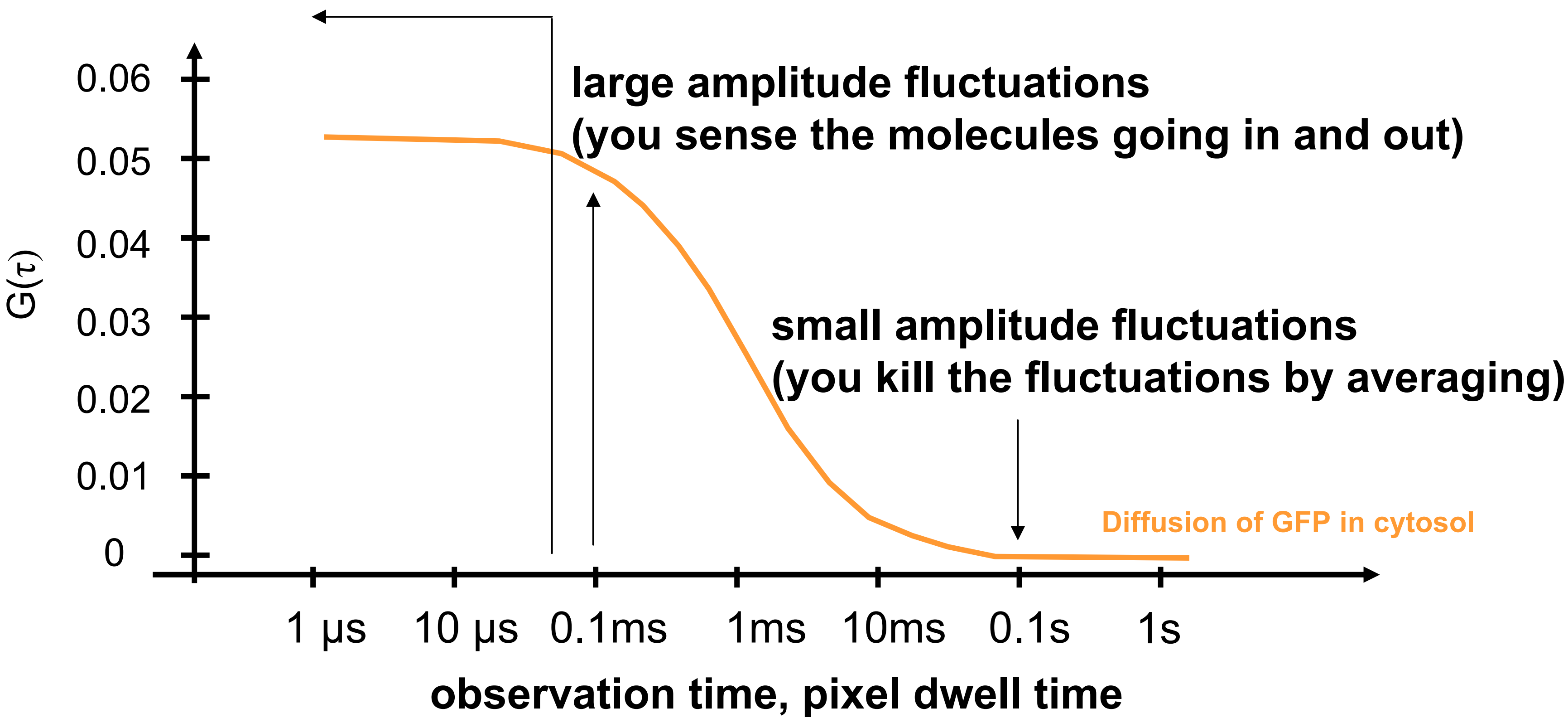
Examples using the top-most brightness of dyes, before they saturate:
Alexa $1 \cdot 10^6$. At 10% collection efficiency to get B = 2.0 → t_d has to be 10 μs.
eGFP $5 \cdot 10^4$. At 10% collection efficiency to get B = 2.0 → t_d has to be 200 μs.

Coming up: Dwell-time considearations!

①

The basic maths

Dwell-time considerations – You still have to pick up the fluctuation!



OK-range (in cells) is dwell time < 50 µs

large amplitude fluctuations
(you sense the molecules going in and out)

small amplitude fluctuations
(you kill the fluctuations by averaging)

①

The basic maths

What kind of B-values do we expect?

Assuming a good (10%) efficiency of the set-up…

Settings	B-Values		Remarks
	eGFP	Alexa	
max. power (before saturation) max. dwell time (50 µs) 5.5 min	1.25	6.0	high photobleaching and phototoxicity issues
mid power (half saturation) mid dwell time (32 µs) 3.5 min	1.08	2.6	Compromise setting
low power (tenth of saturation) short dwell time (4 µs) 26.2 sec	1.002	1.04	B may be too low

↑

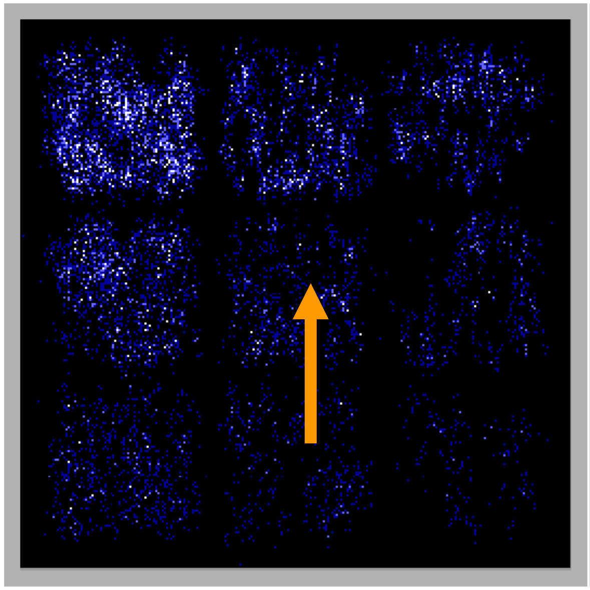
Total time for 100 scanned images of 256x256…

Every photon counts!!!

②

Step-by-step example

movie (400 frames)

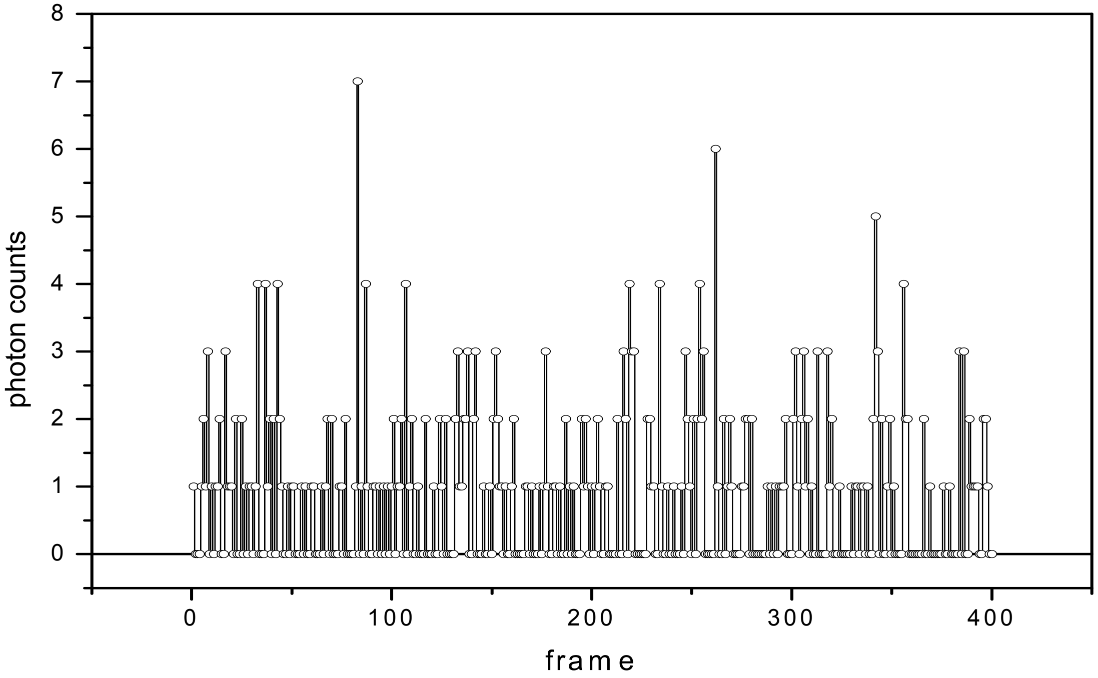


$\langle I \rangle = 0.8125$

$\sigma = 0.9624$

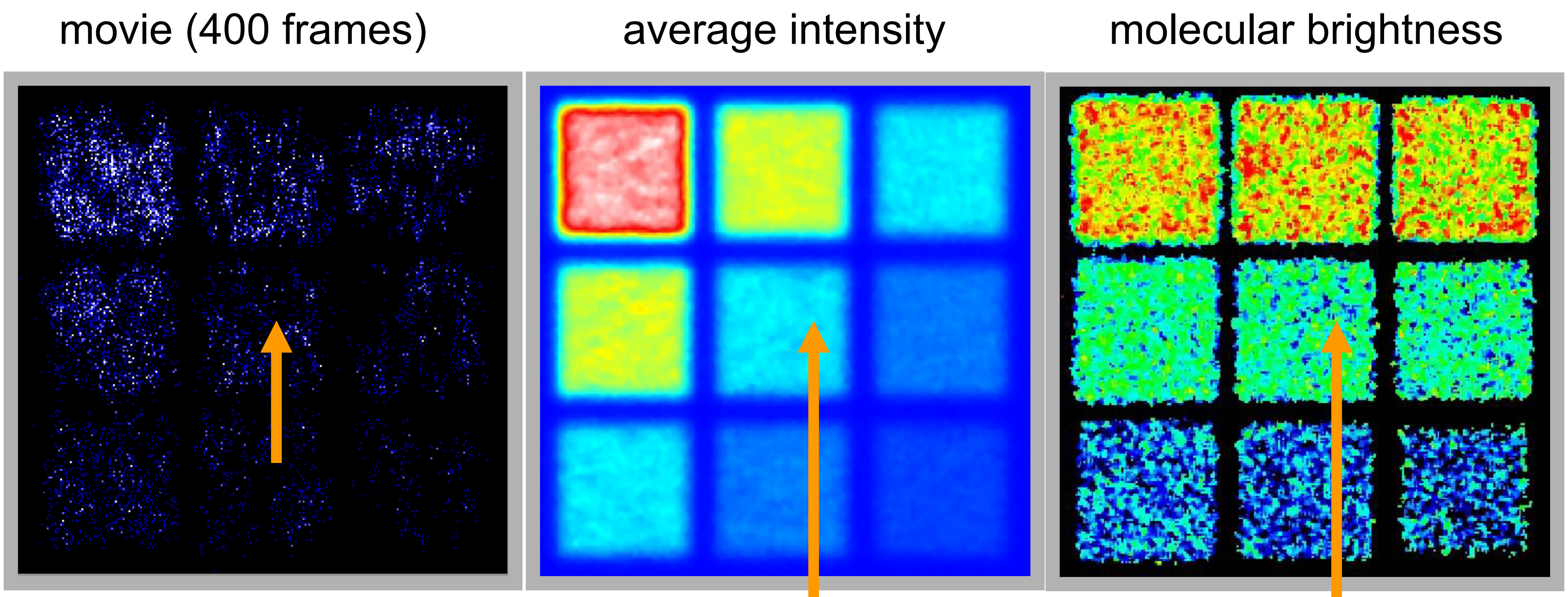
$\sigma^2 = 0.9262$

$\sigma^2/\langle I \rangle = 1.1399$



②

Step-by-step example



movie (400 frames)

average intensity

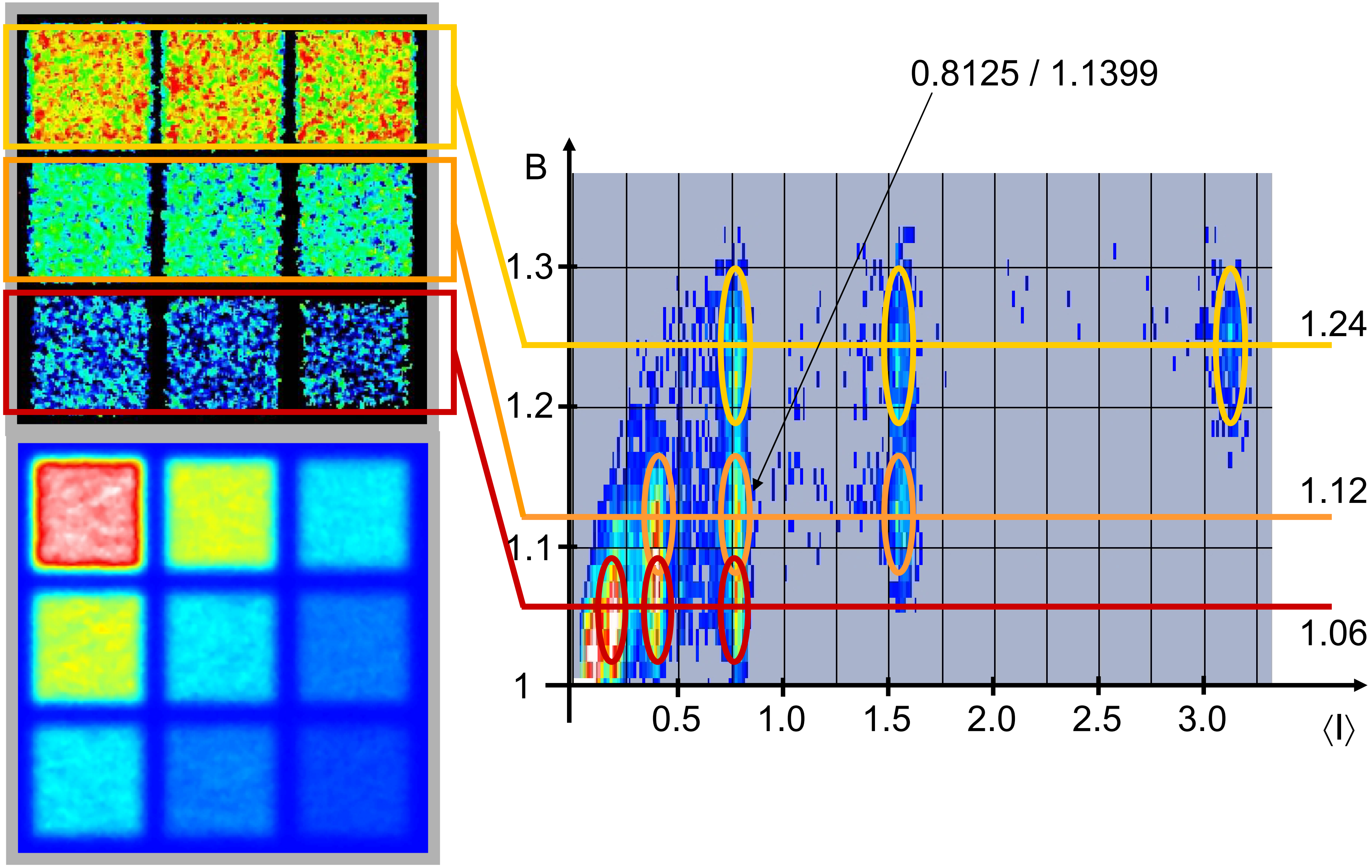
molecular brightness

$\langle I \rangle = 0.8125$

$\sigma^2/\langle I \rangle = 1.1399$

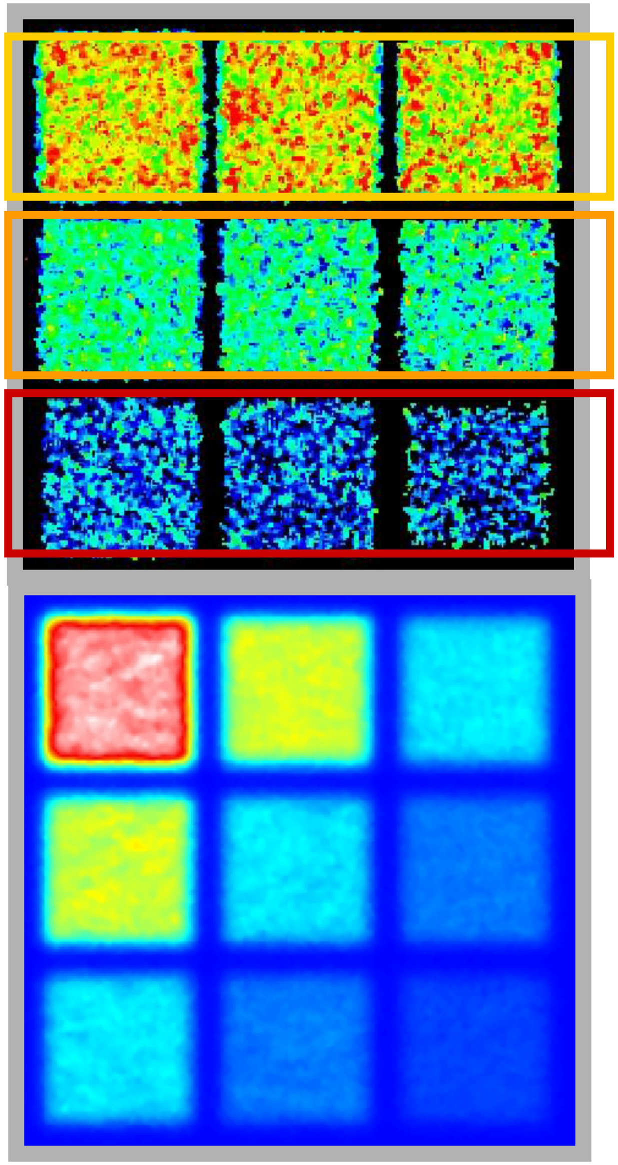
②

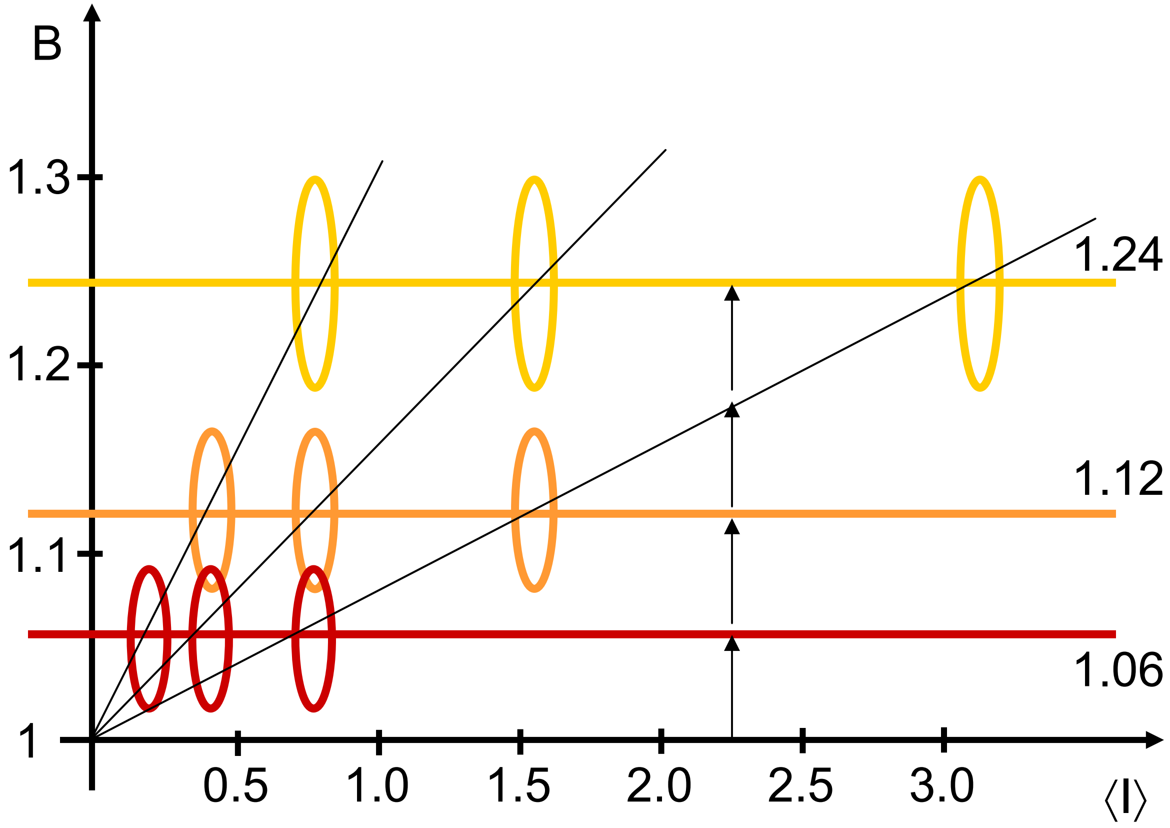
N&B graphic representation



②

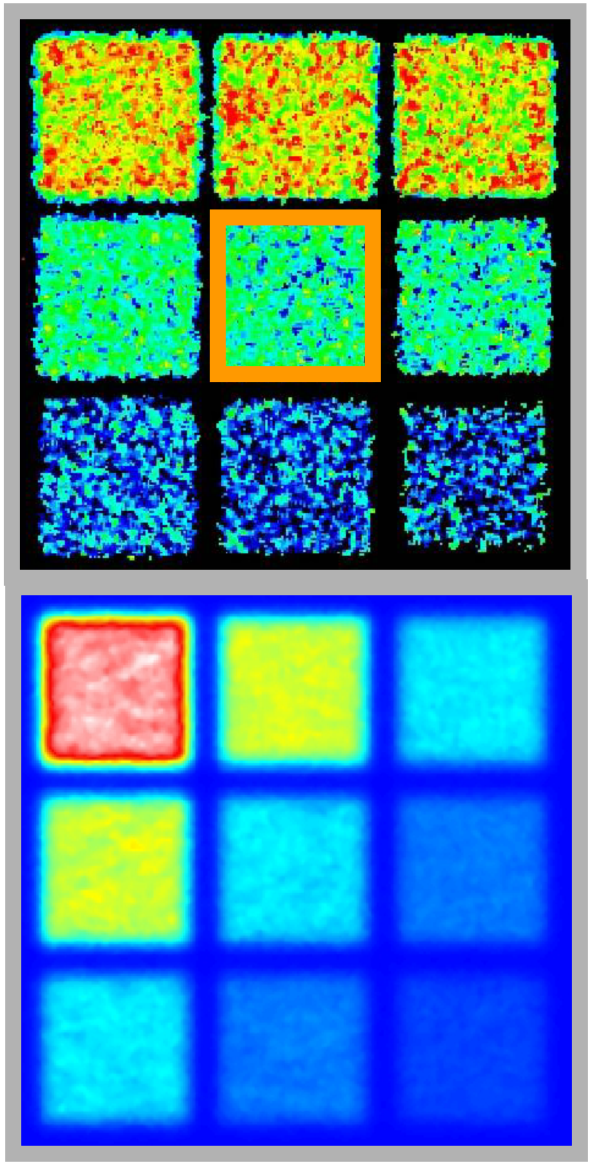
N&B graphic representation

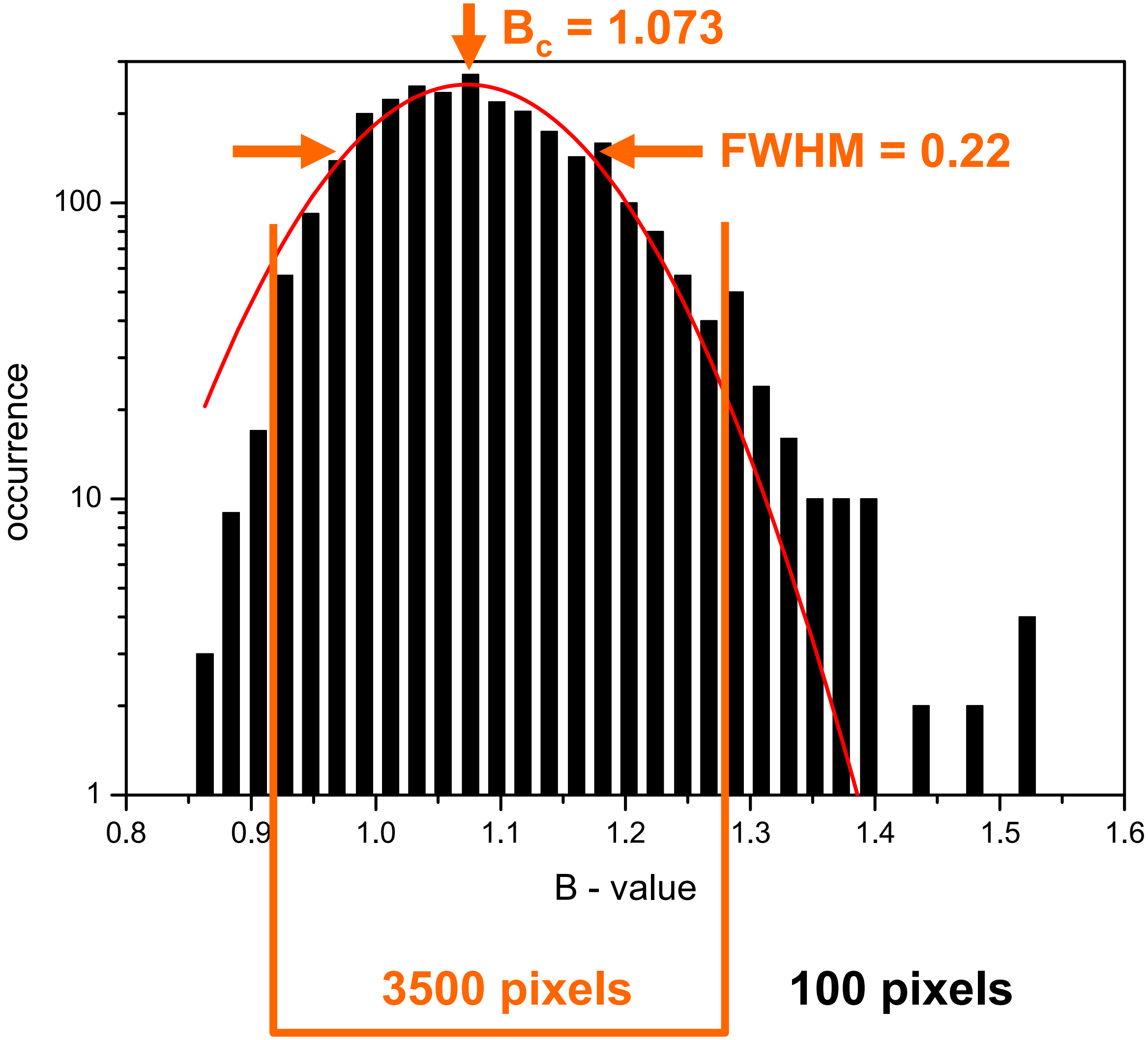




③

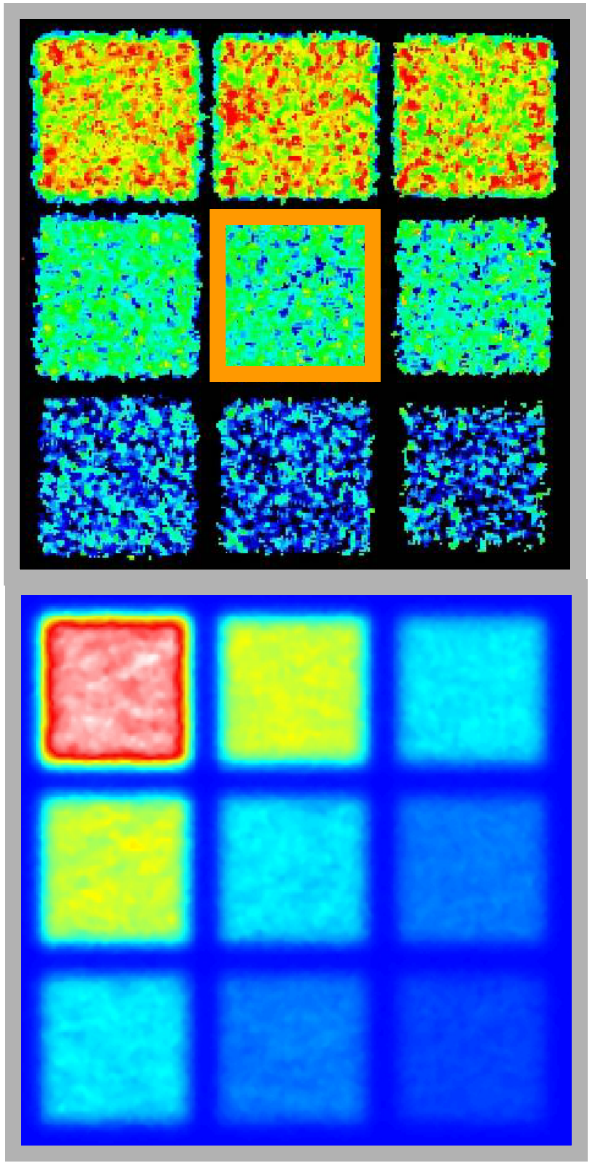
Error discussion: B-Value distrubution

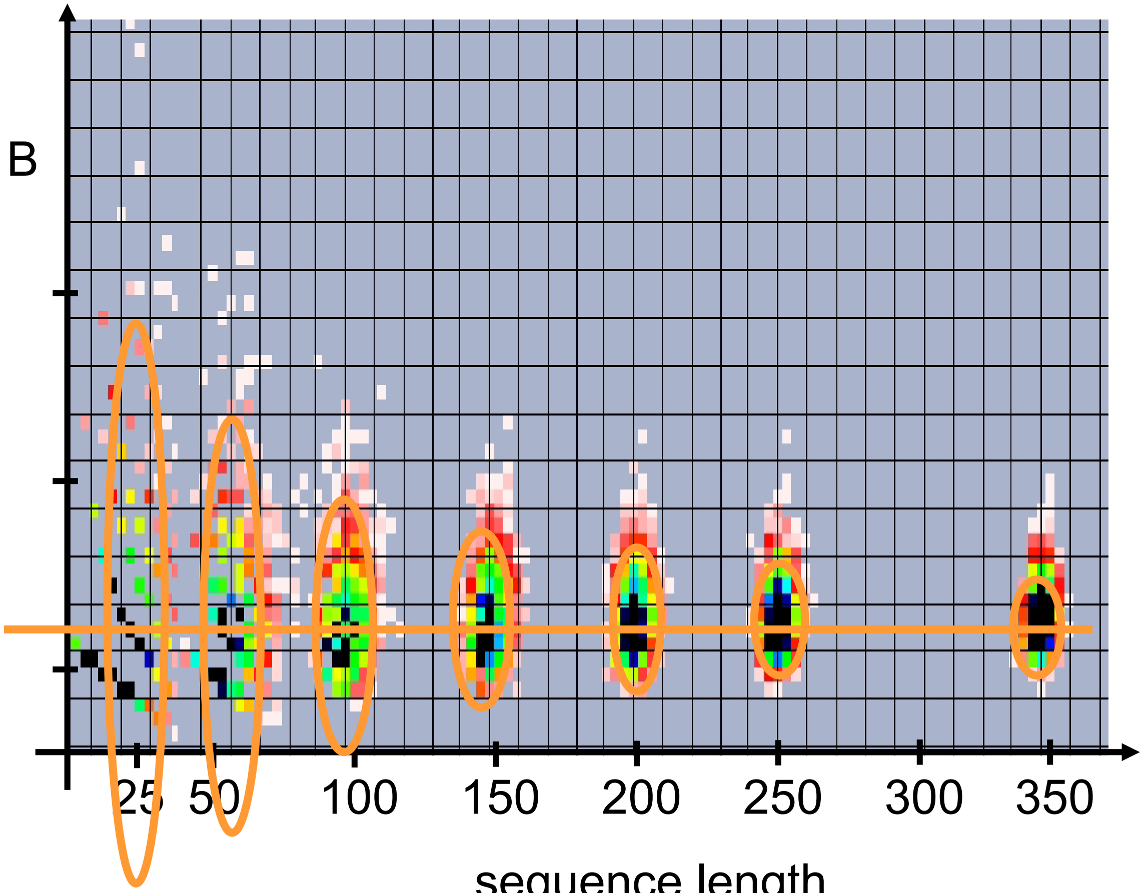




③

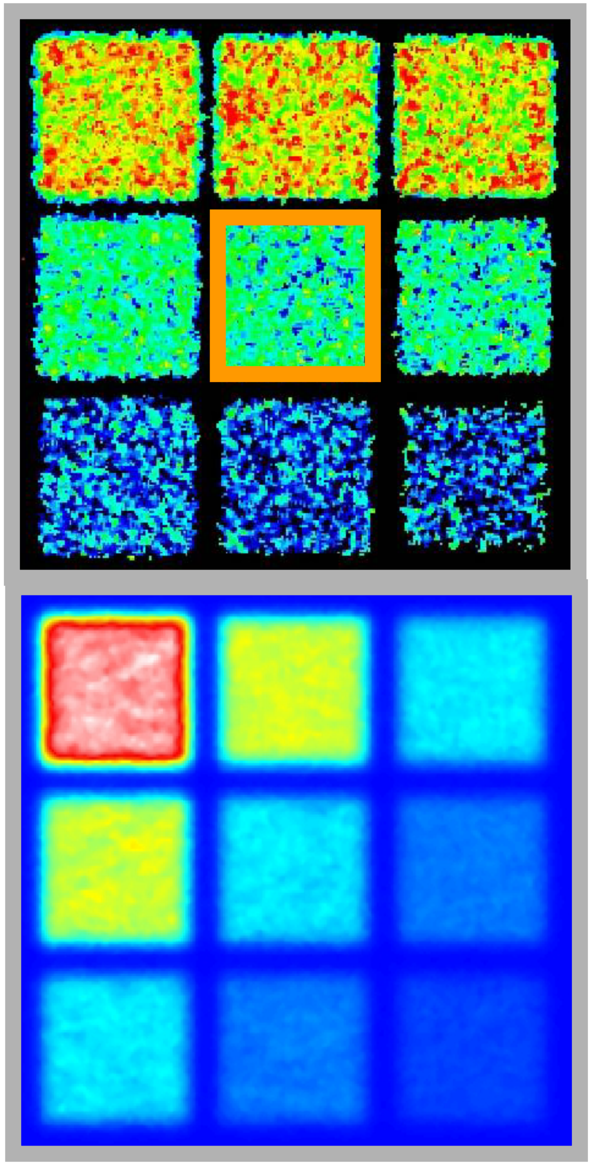
Error discussion: Sequence Length

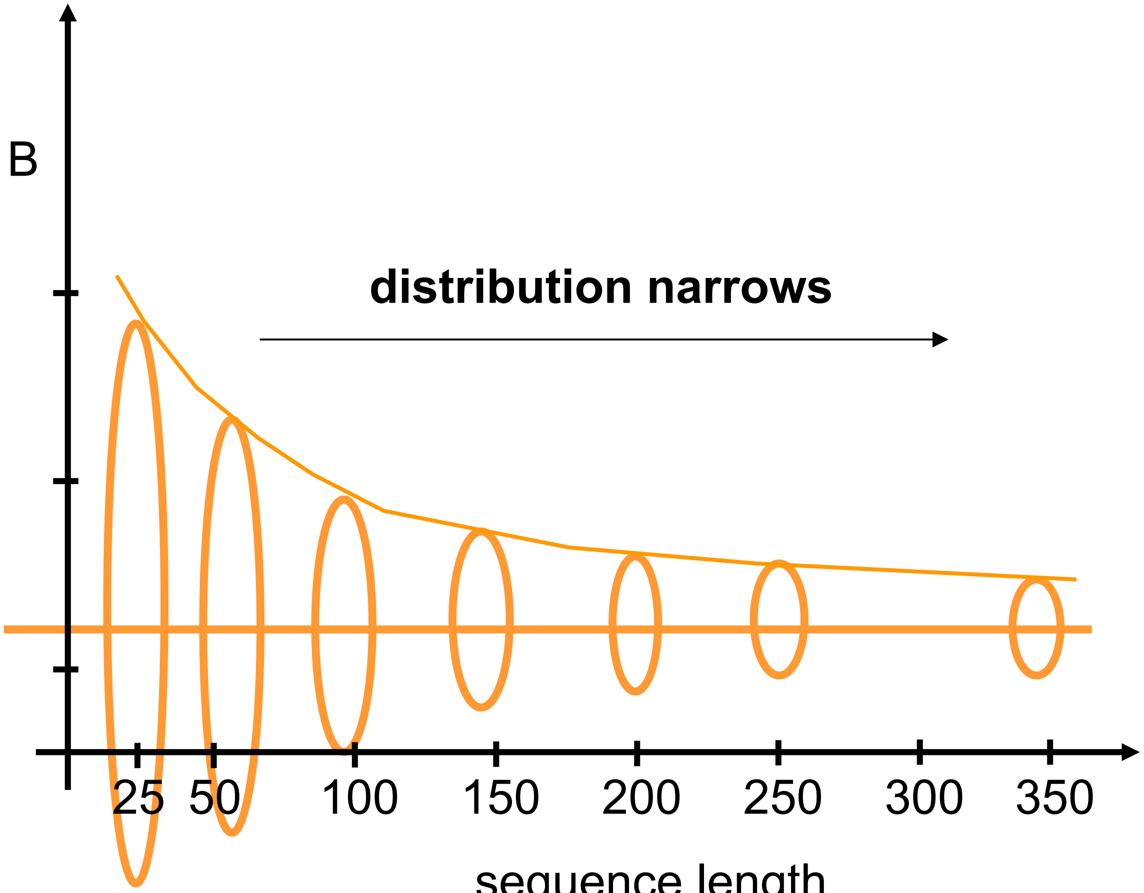




③

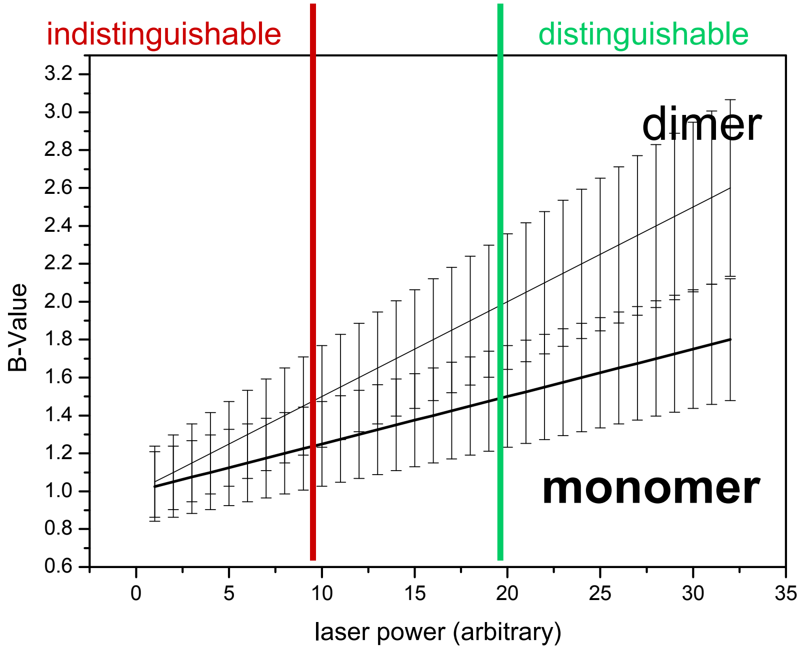
Error discussion: Sequence Length

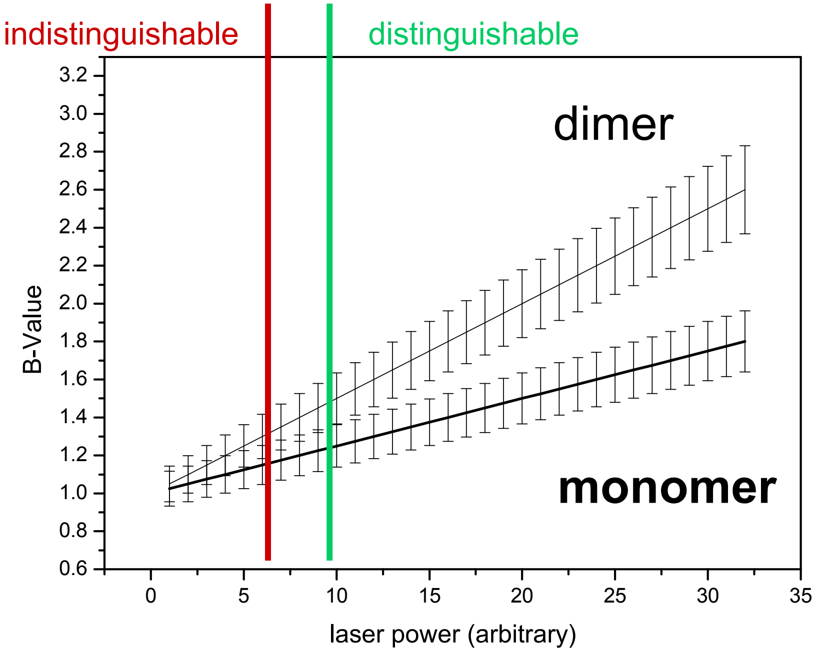




③

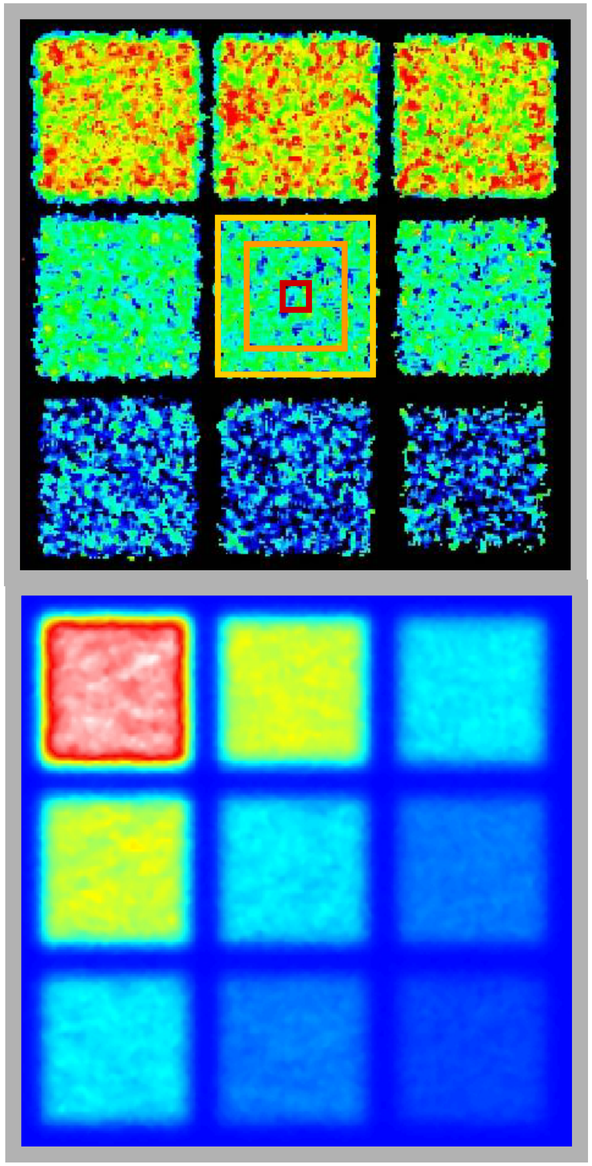
Error discussion: Distinguishability

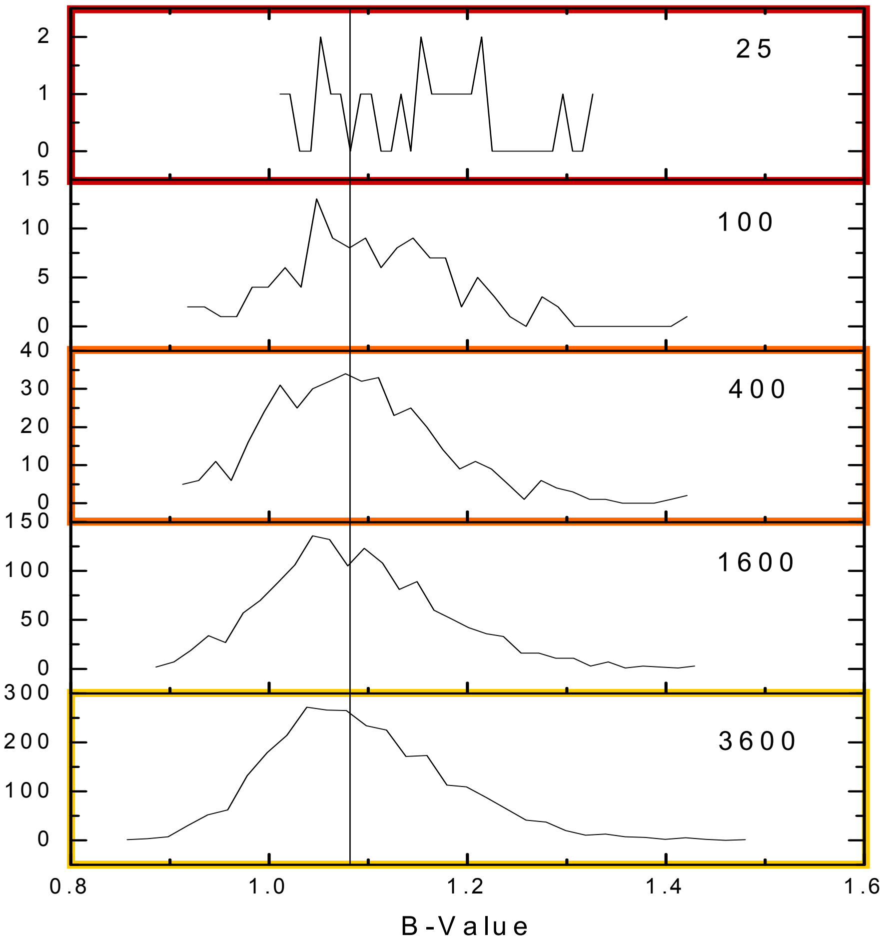

B-Value and FWHM for 100 frames


B-Value and FWHM for 200 frames

③

Error discussion: Population Size

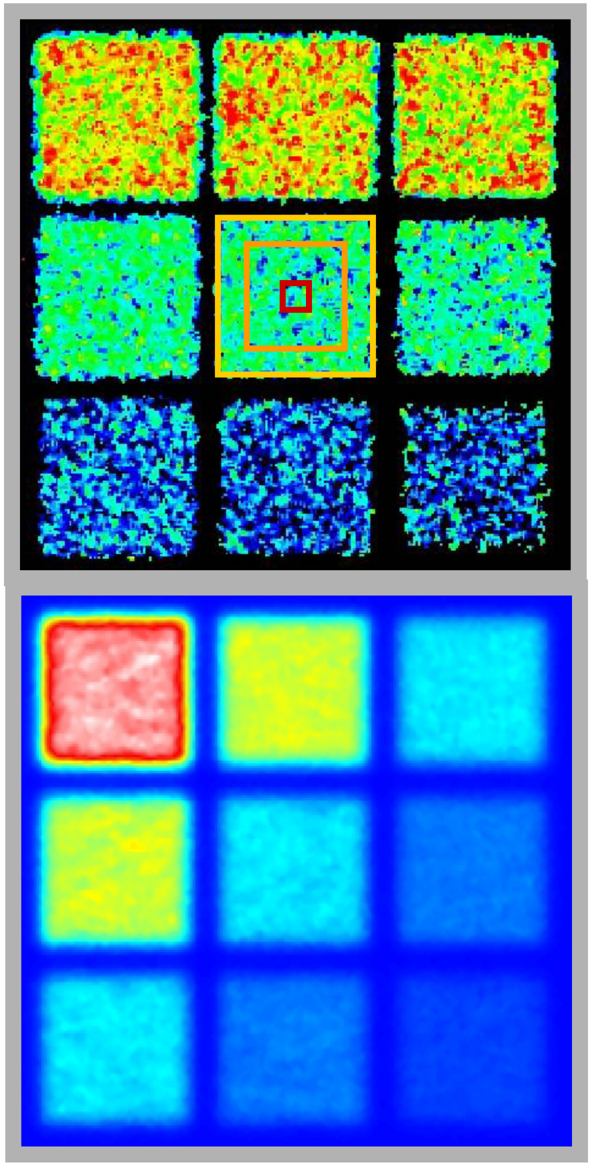


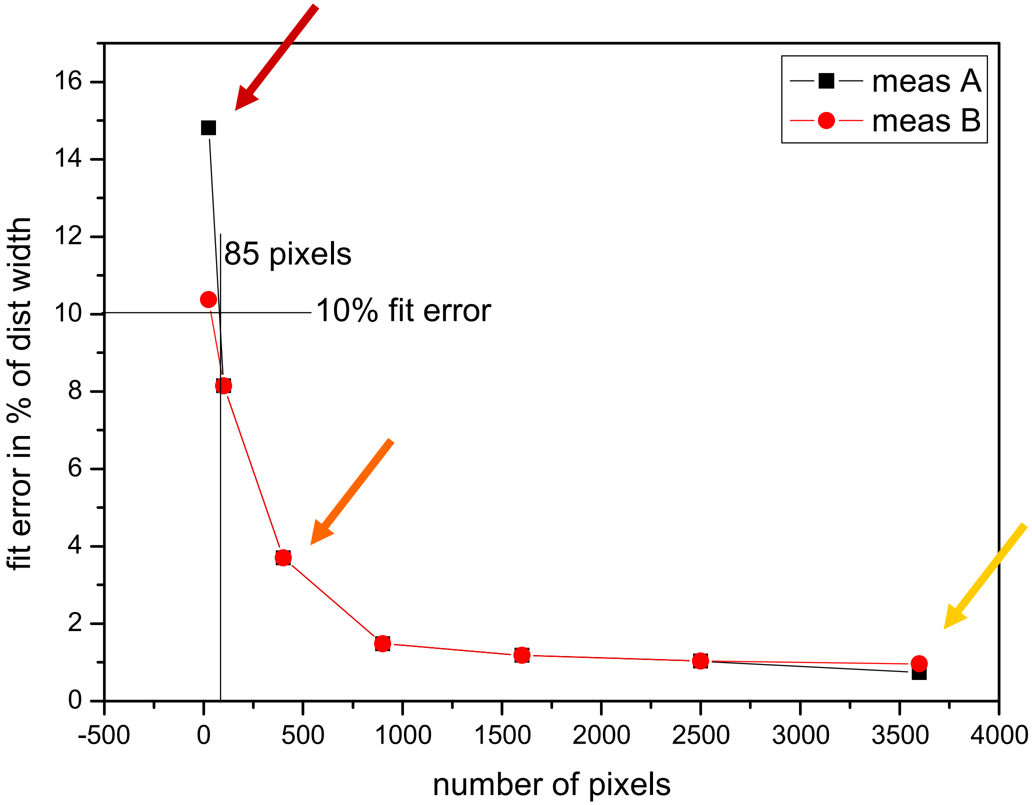


Distribution does not narrow! It gets less noisy!

③

Error discussion: Population Size

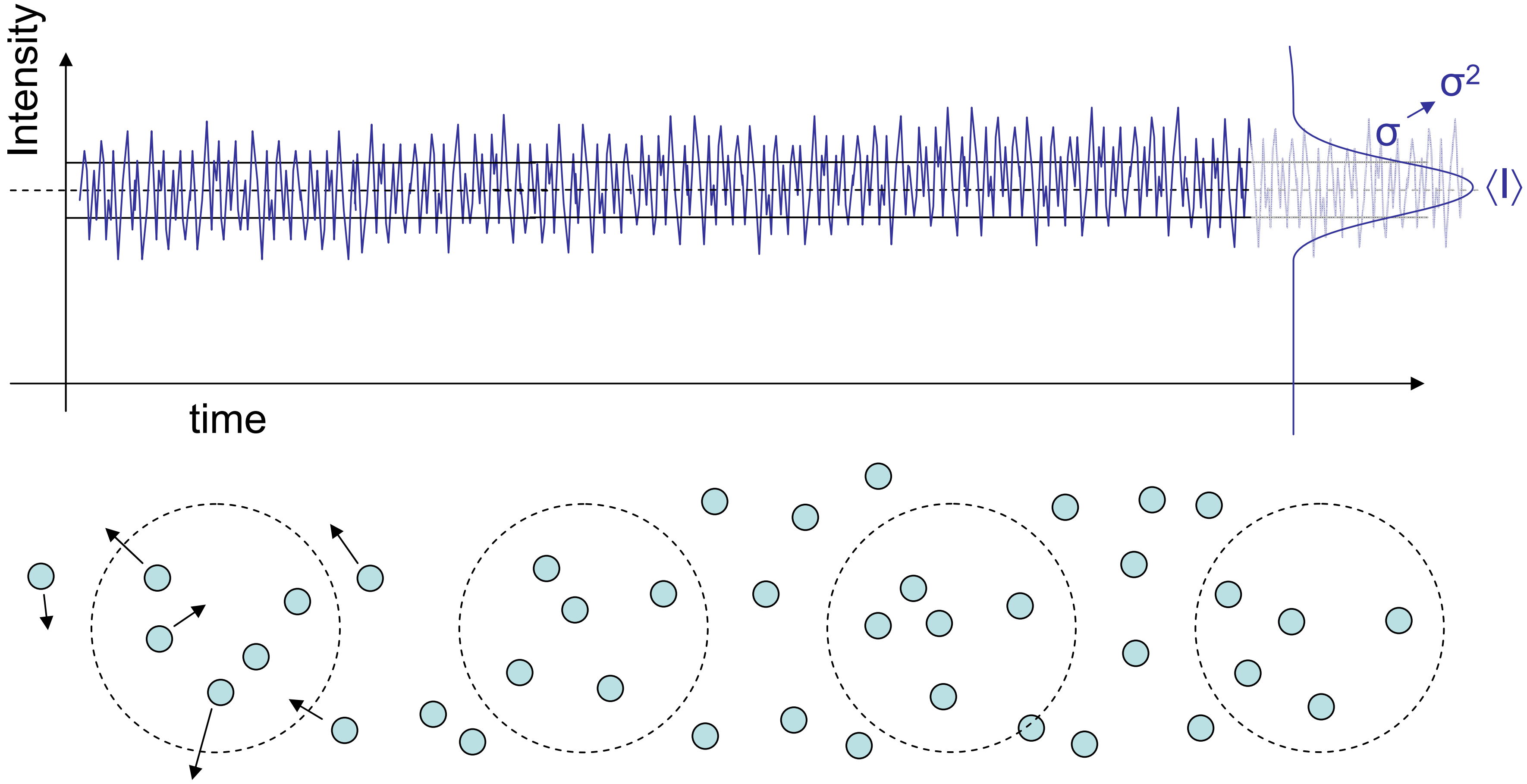




Distribution does not narrow! It gets less noisy!

③

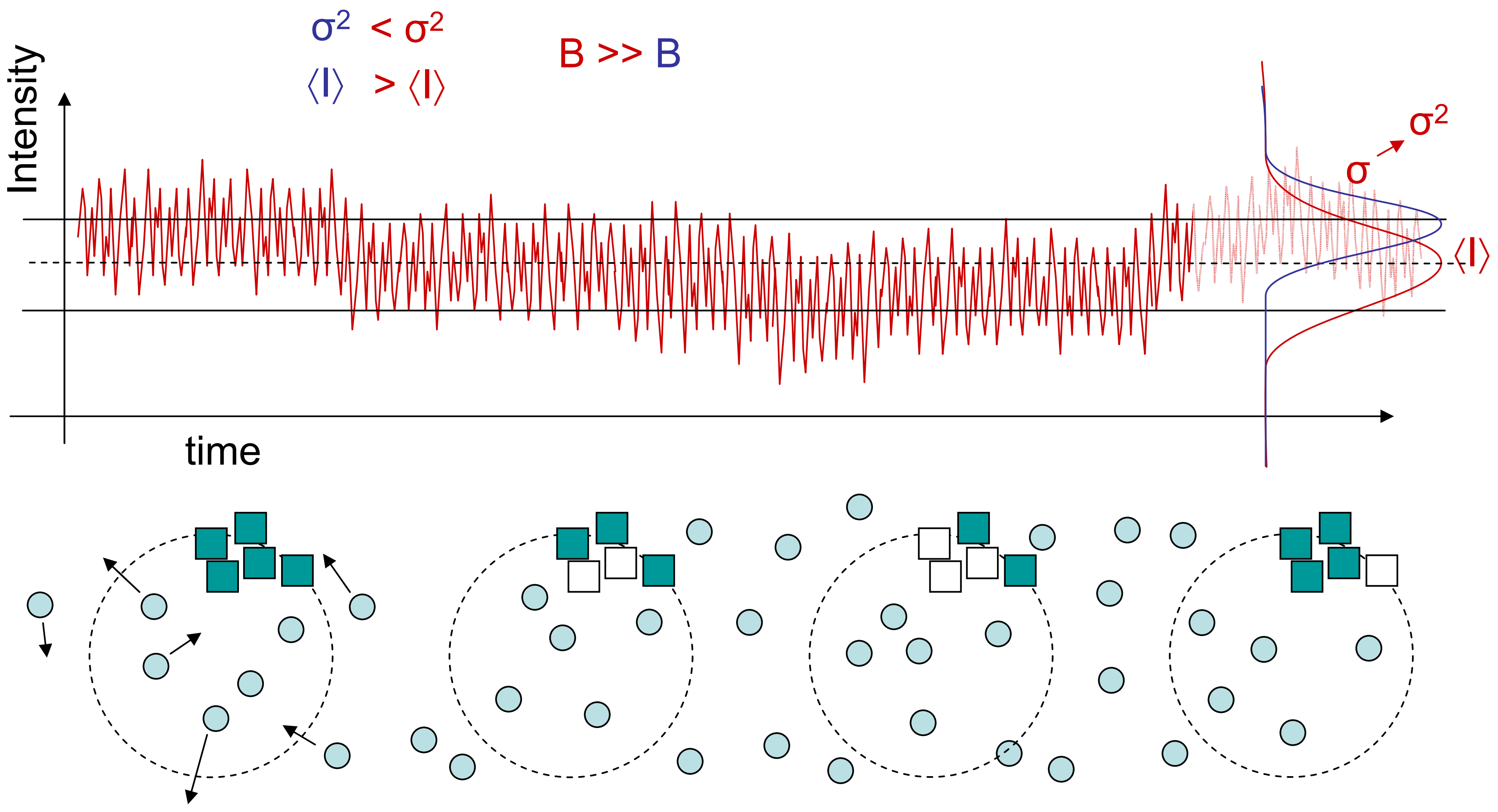
Pitfall 1: Slow dynamics



without slow dynamics

③

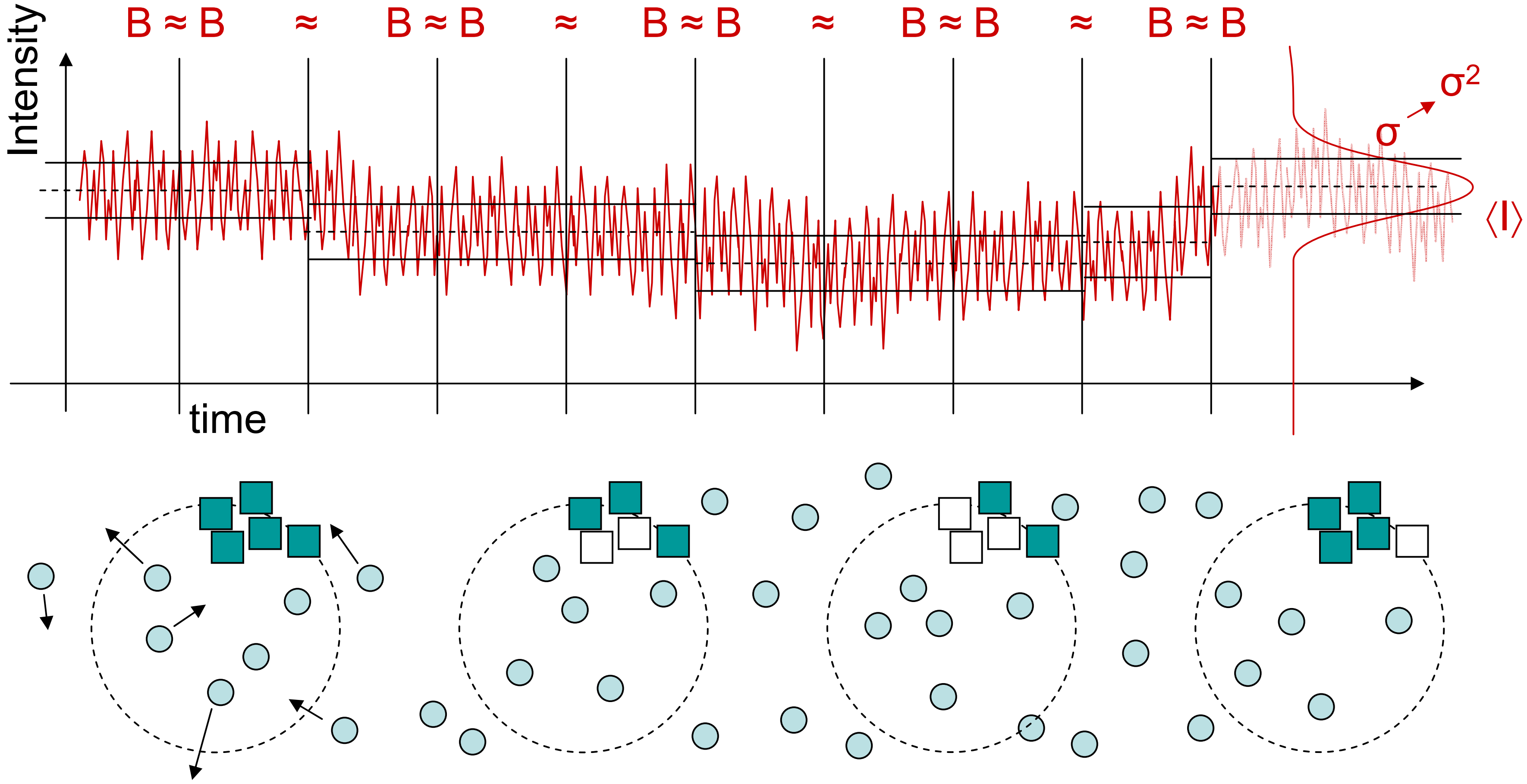
Pitfall 1: Slow dynamics



with slow dynamics – B is overestimated

③

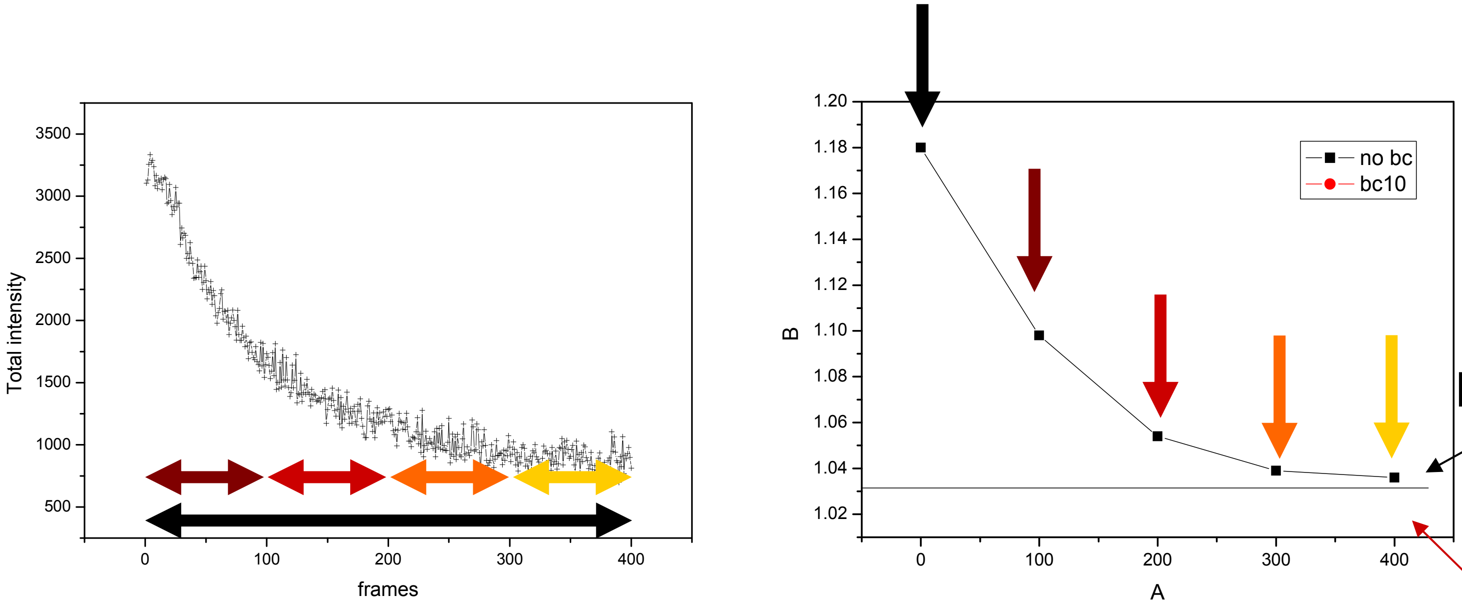
Pitfall 1: Slow dynamics



Effect of slow dynamic is minimized on segment-by-segment analysis

③

Pitfall 1: Slow dynamics

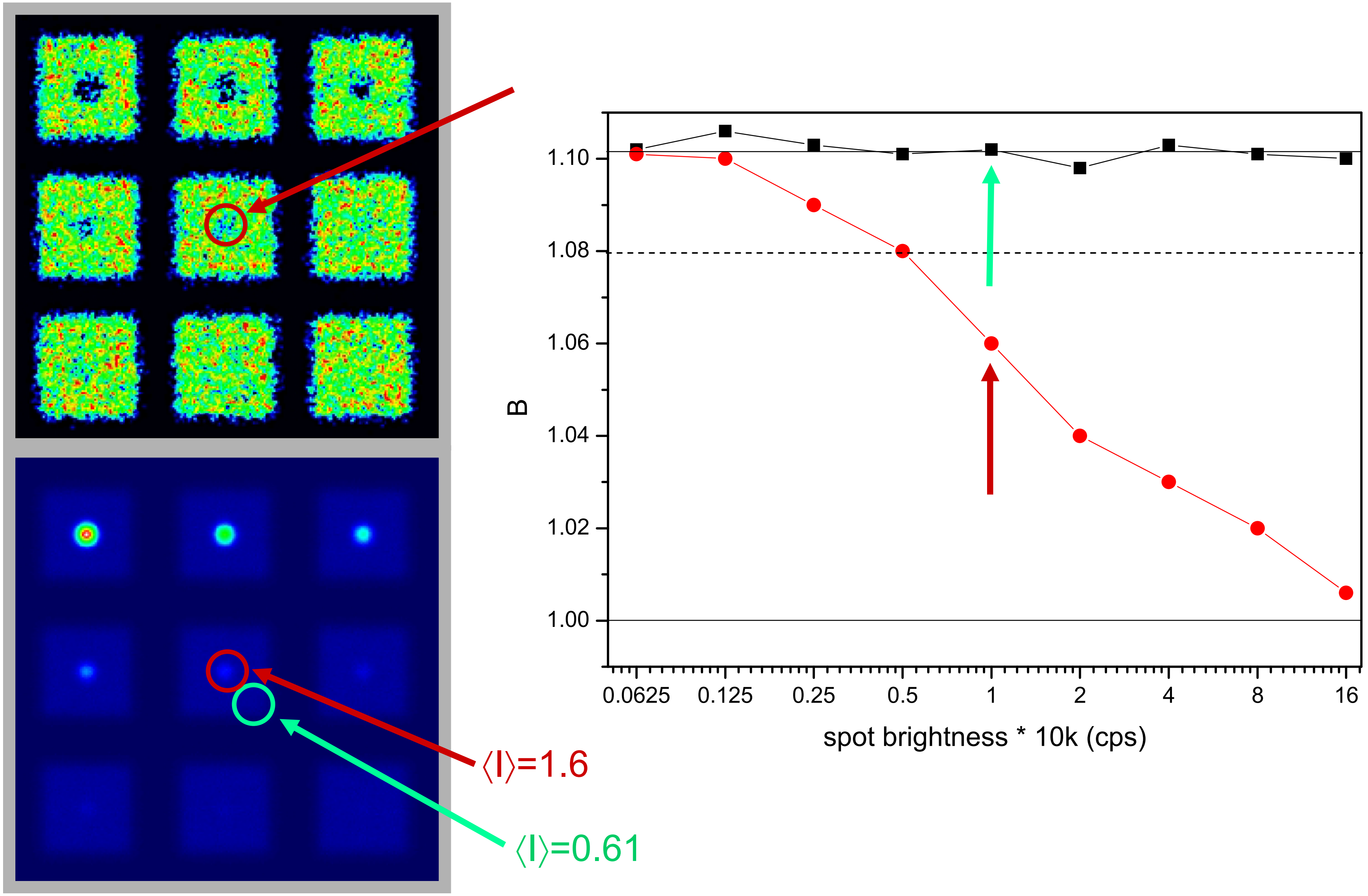


N&B as-is

N&B segment-by-segment

③

Pitfall 2: Mixed Populations



④

Examples

First Examples: Mobile versus Immobile / Aggragates / TIRF

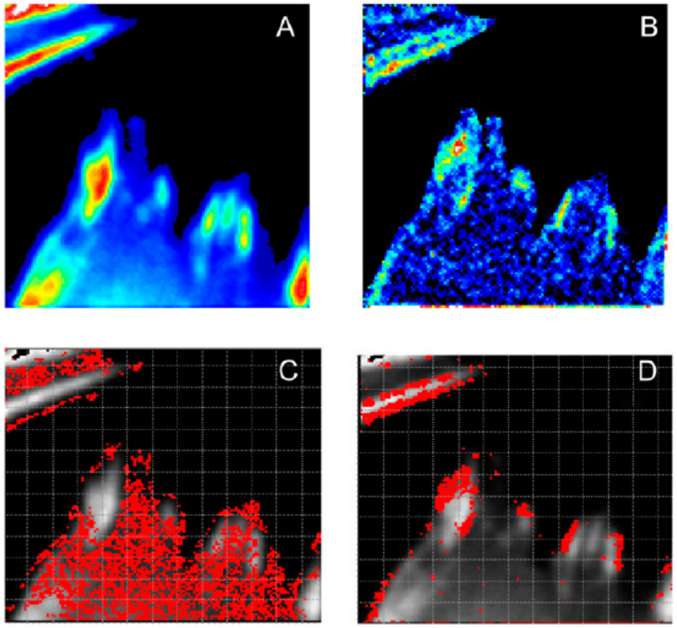


FIGURE 10 Paxillin-EGFP in CHO-K1 cells. (*A*) Average intensity. Paxillin accumulates at focal adhesions. (*B*) The *B* image shows that the larger values of *B* are seen at the borders of some adhesions. (*C*) Using the two-dimensional histogram of *B* versus intensity, all points with brightness of 1150 counts/s/molecules (corresponding to EGFP monomers were selected. These points accumulate in the cytosol. In panel *D*, all pixels with brightness of 11,500 counts/s/molecule were selected. These pixels accumulate at the border of the adhesions. Four-hundred frames were collected for this measurement. Motion artifacts were corrected using the high-pass filter procedure. The image size is 31 μm × 31 μm.

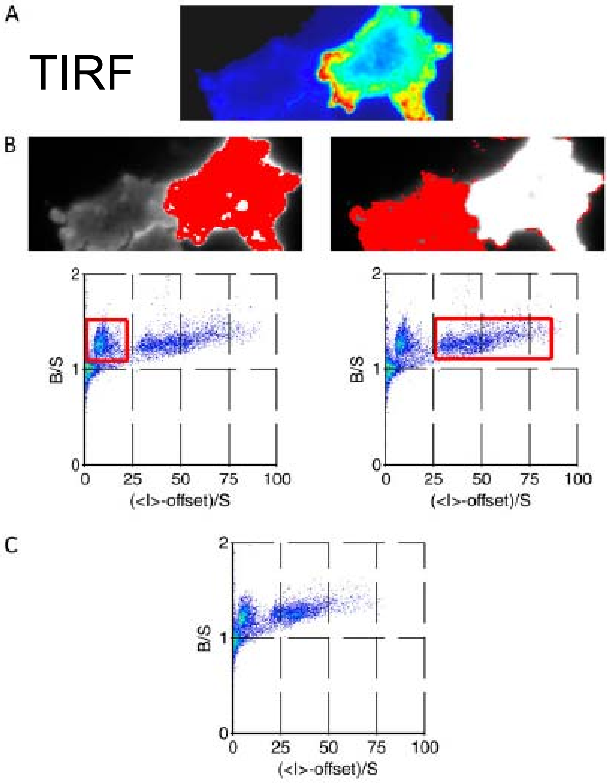


FIGURE 12 (*A* and *B*) Images of CHO K1 cells transfected with GAP-EGFP. (*B*) Red regions in *A* correspond to selected pixels from the B/S versus $(\langle I \rangle - offset)/S$ histograms. (*C*) B/S versus $(\langle I \rangle - offset)/S$ histograms after 50 s of illumination.

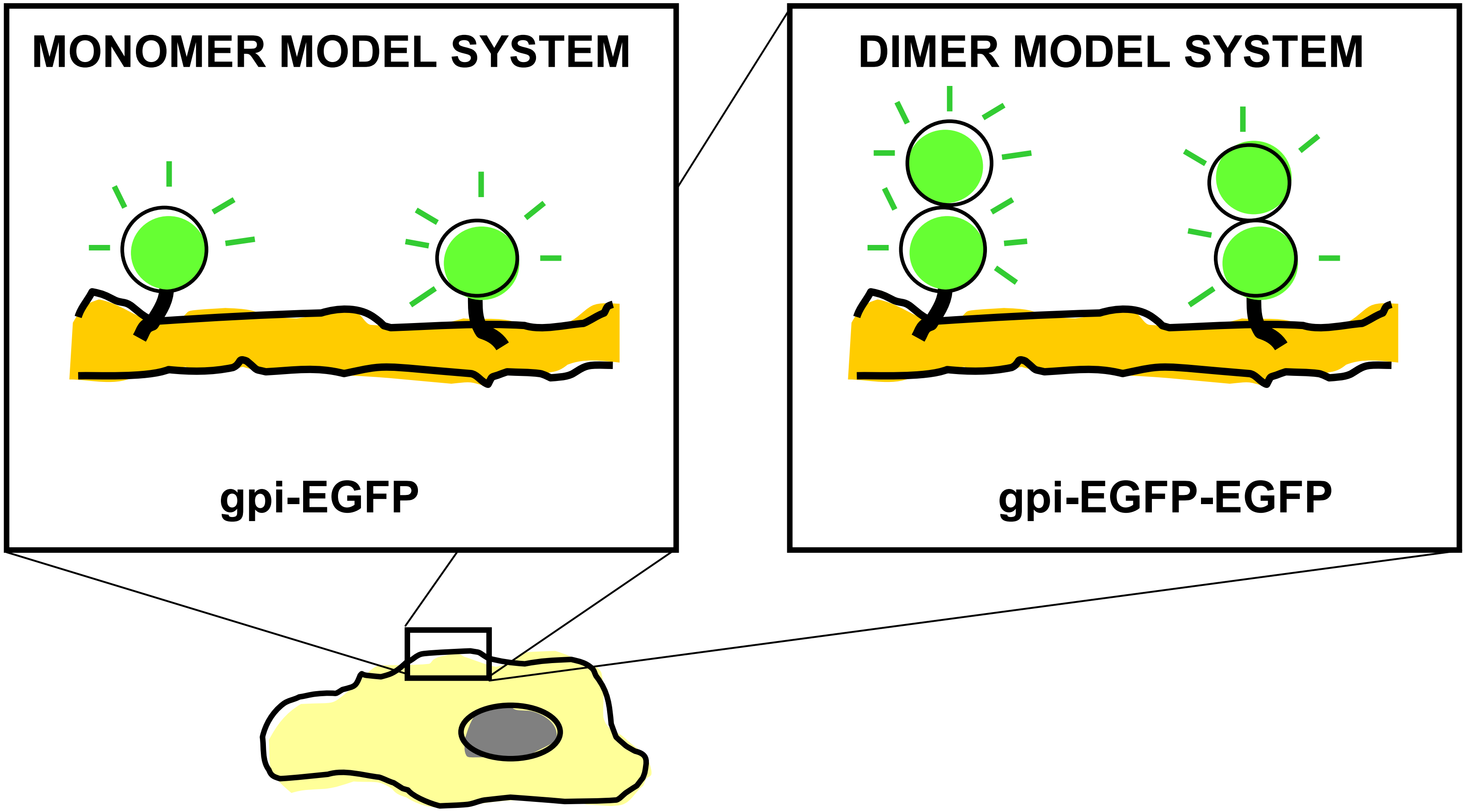
Digman et al. *Biophys J* **2008** *94*(*6*) 2320.

Unruh et al. *Biophys J* **2008** *95*(*11*) 5385.

④

Examples

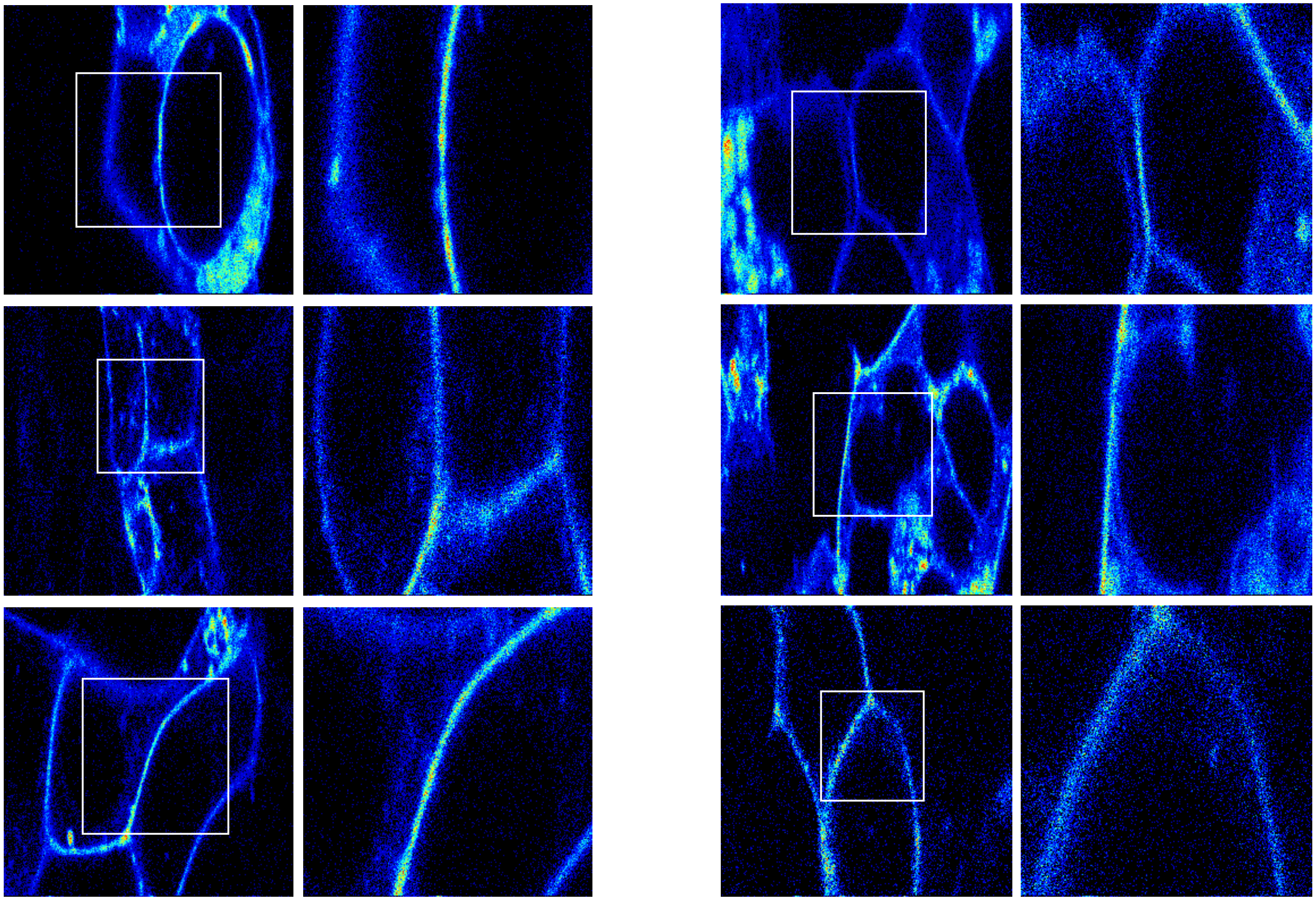
Advanced Example: Can we distinguish between monomers and dimers?



④

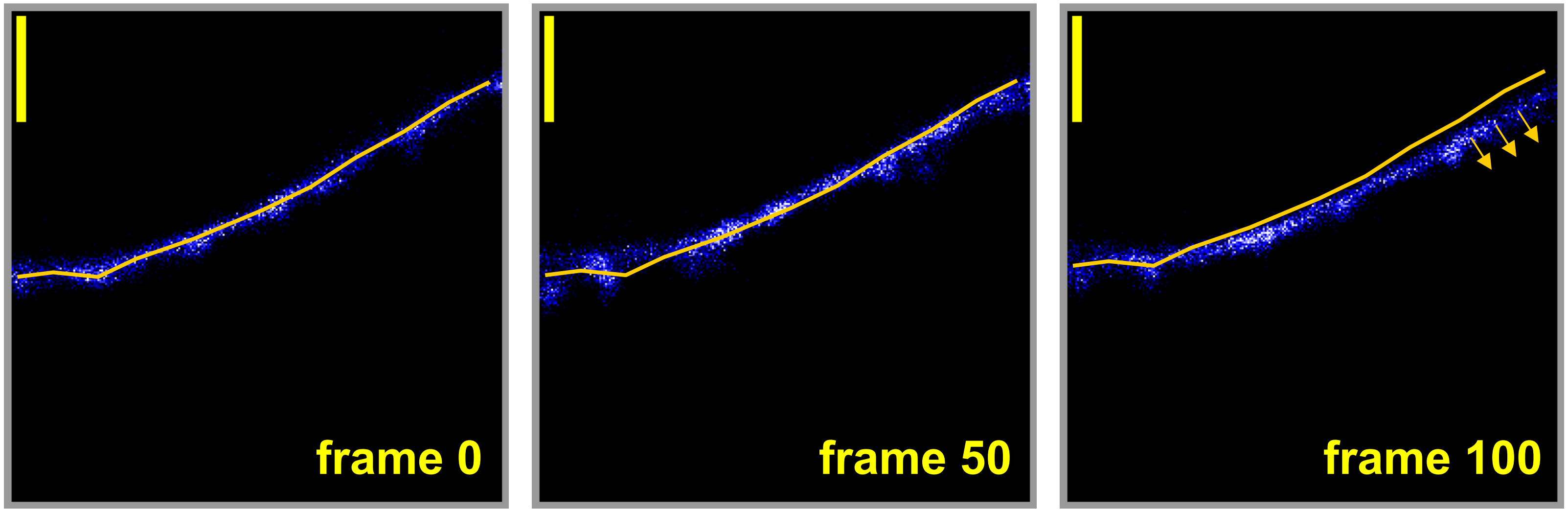
Examples

Can we distinguish between monomers and dimers?



④

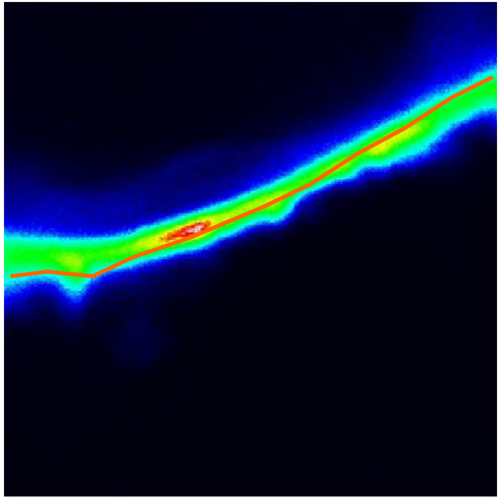
Examples

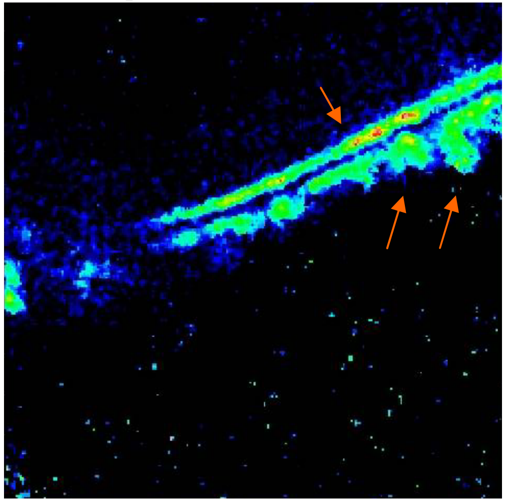


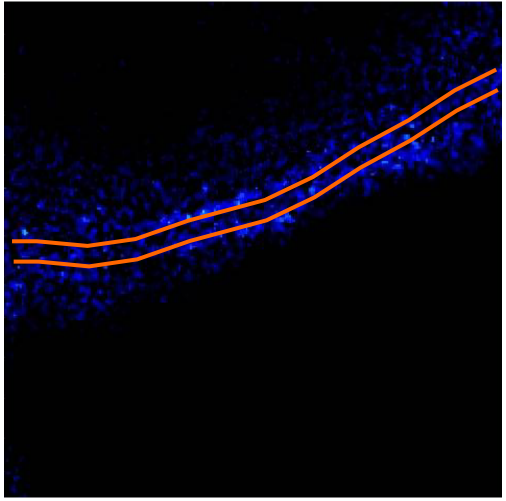
Time sequence (200 frames total):

a) Membrane is highlighted (fluorophore goes where it should)
b) Membrane has undulations and other visible features
c) Membrane moves slowly in the course of the sequence

Examples


average intensity


B-Map

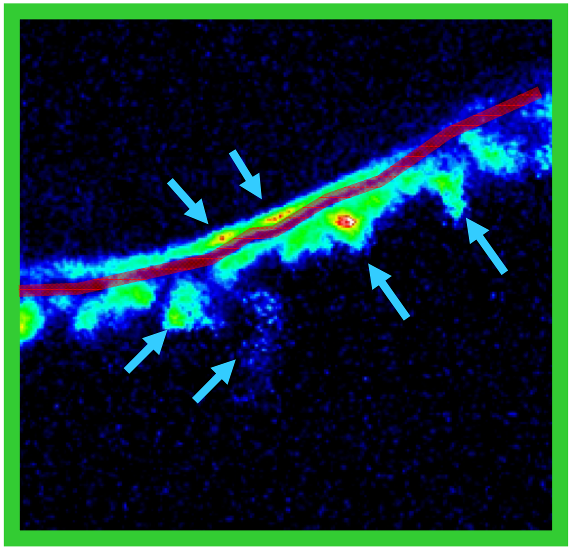

B-Map (corrected)

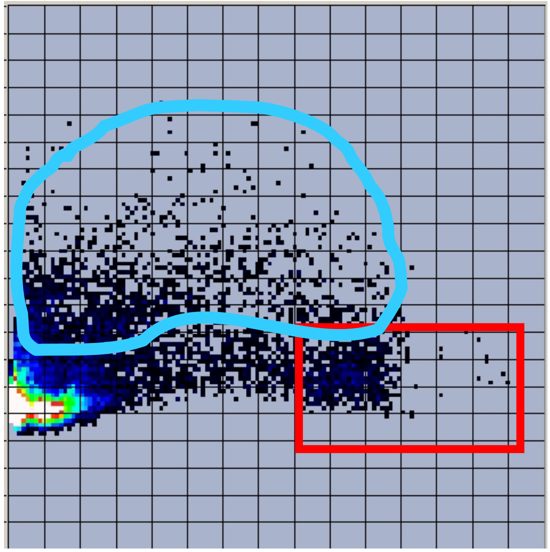
Time sequence (200 frames total):

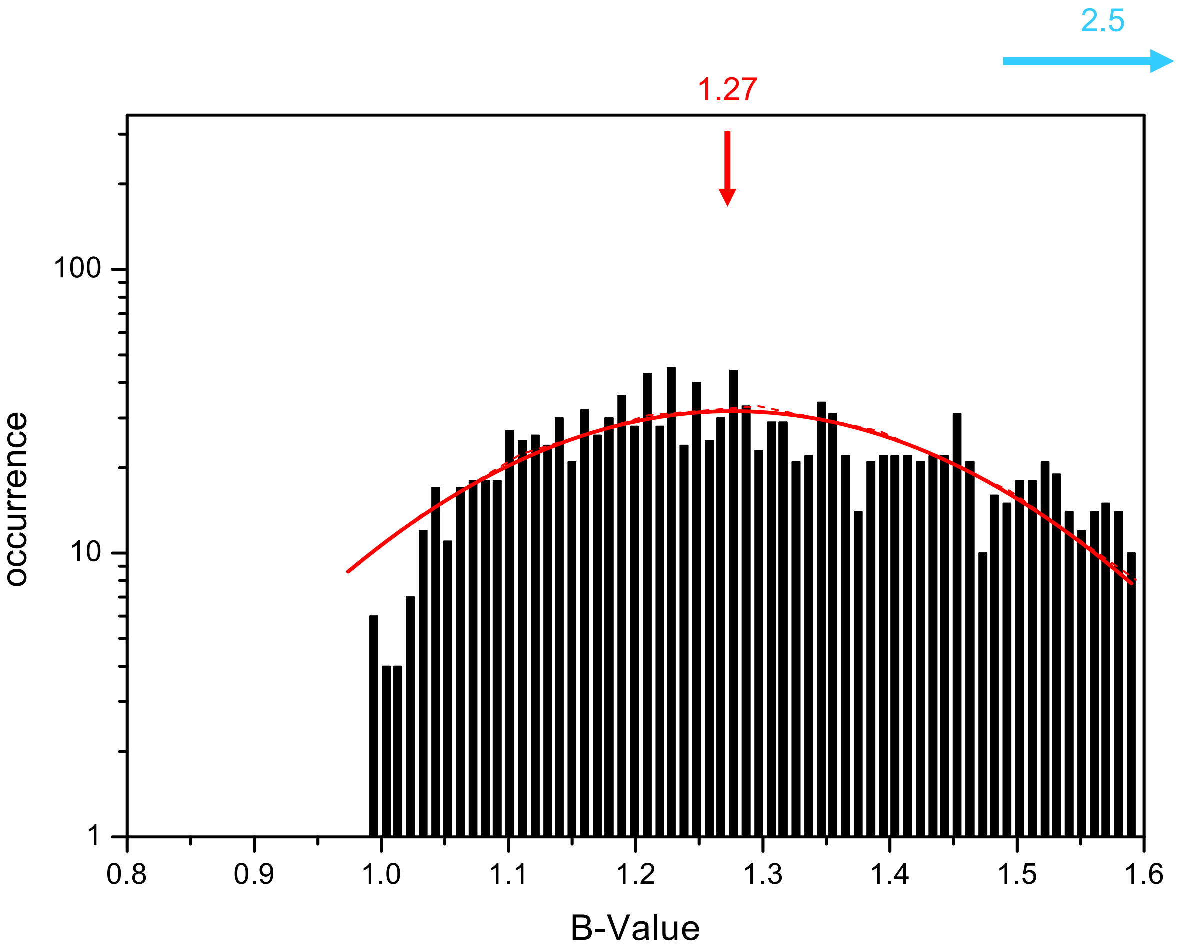
a) Average intensity image is blurred out due to cell motion
b) B-Map shows large distortions caused by membrane slow motion
c) Distortions are minimized using the segment correction
d) Distorted regions are excluded from the histogram analysis

④

Examples



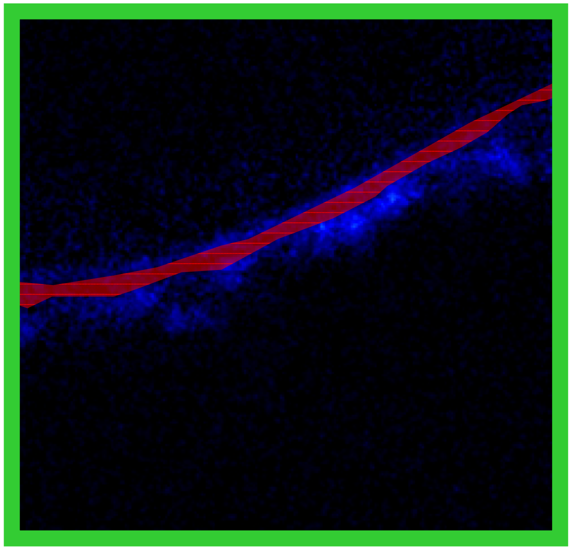


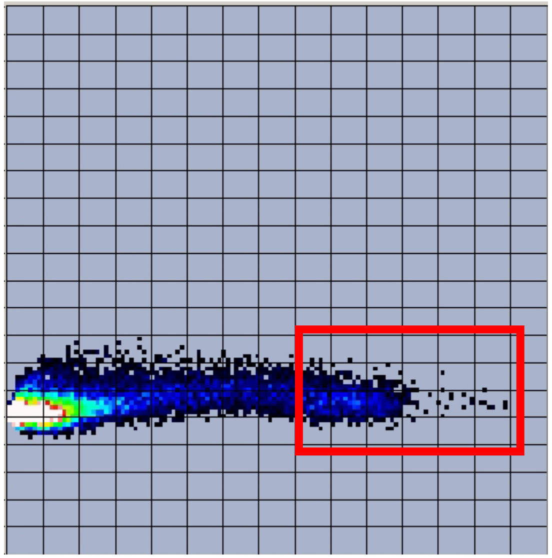


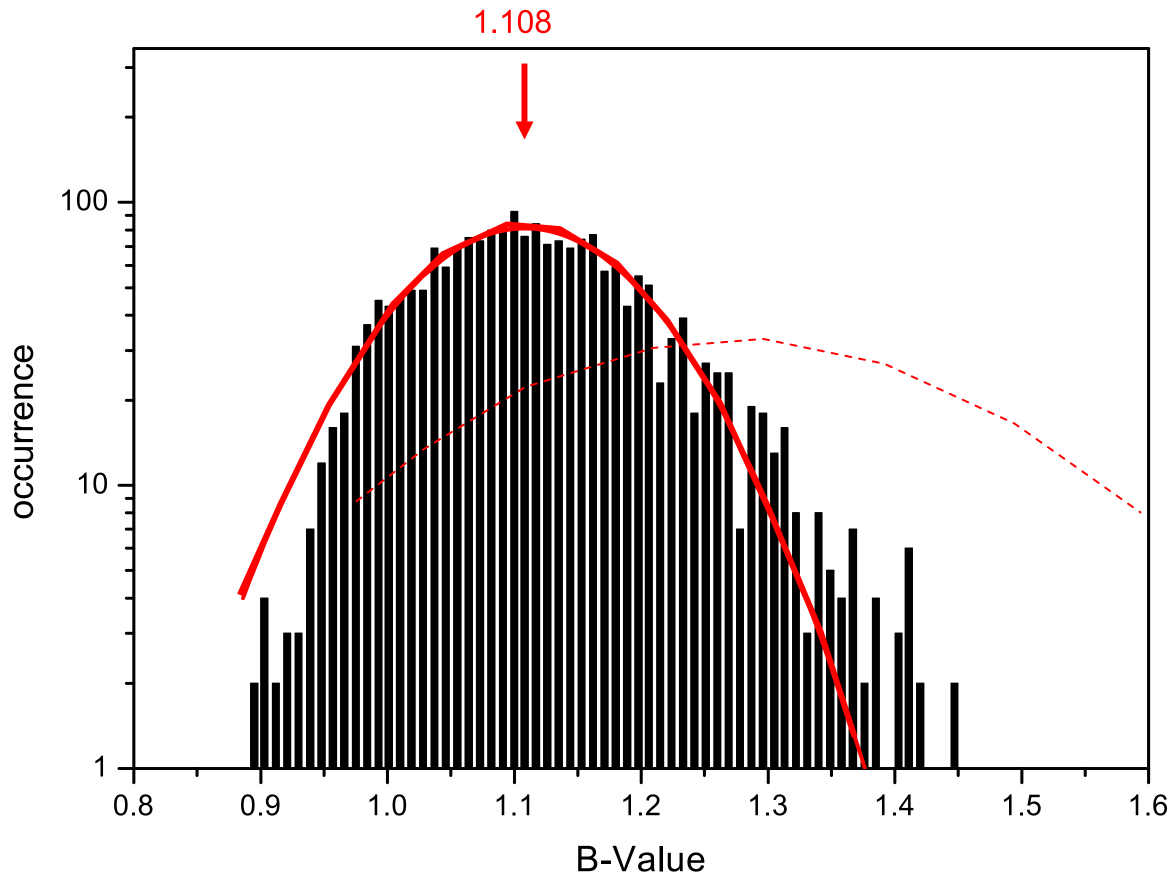
NO SLOW DYNAMICS FILTER

④

Examples

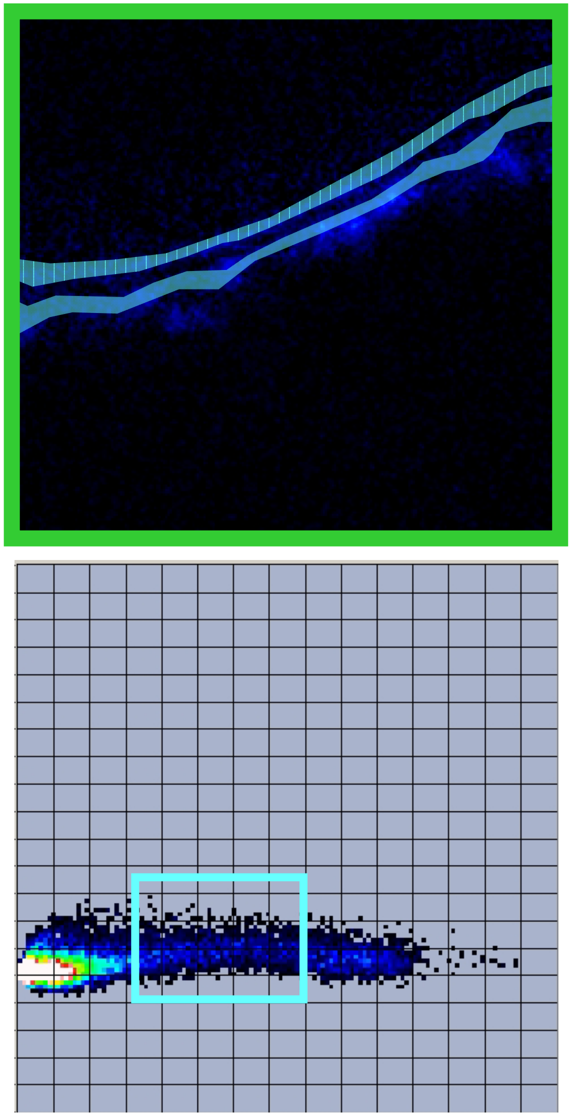


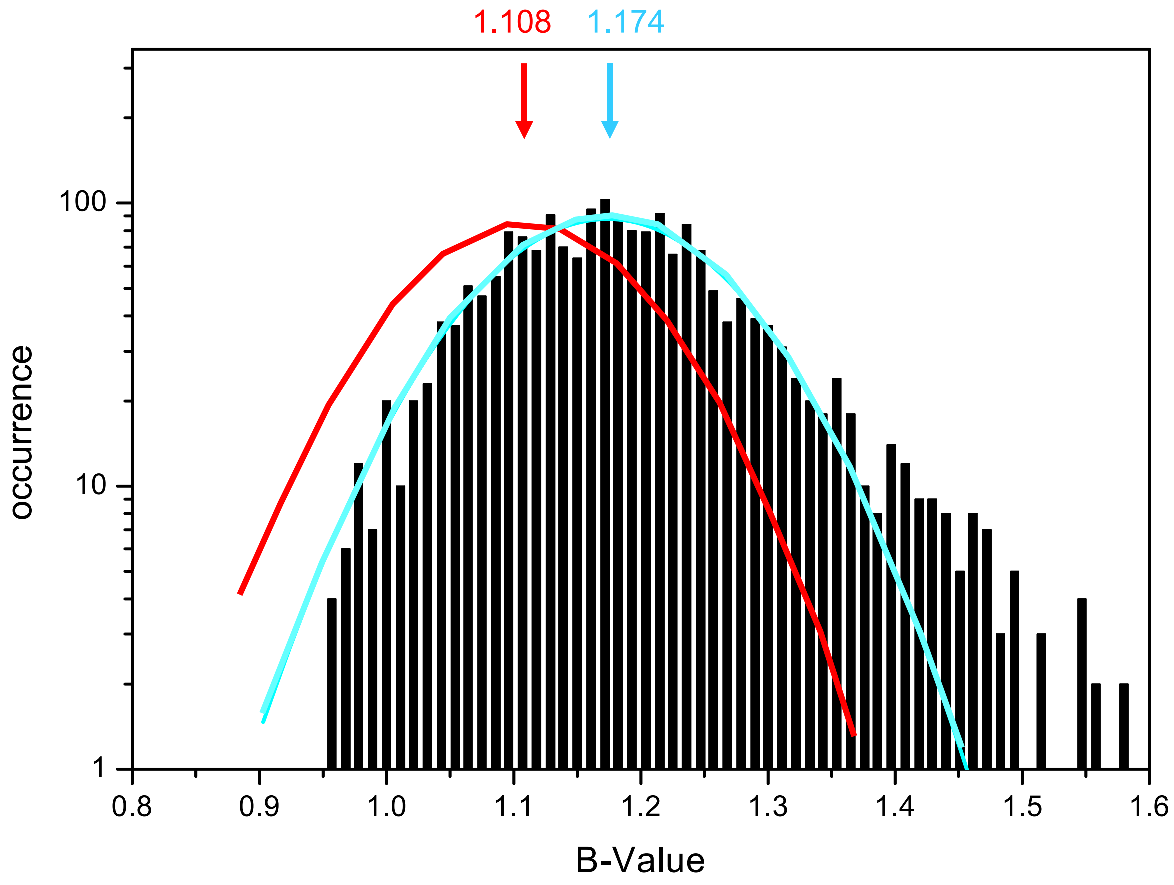




WITH SLOW DYNAMICS FILTER

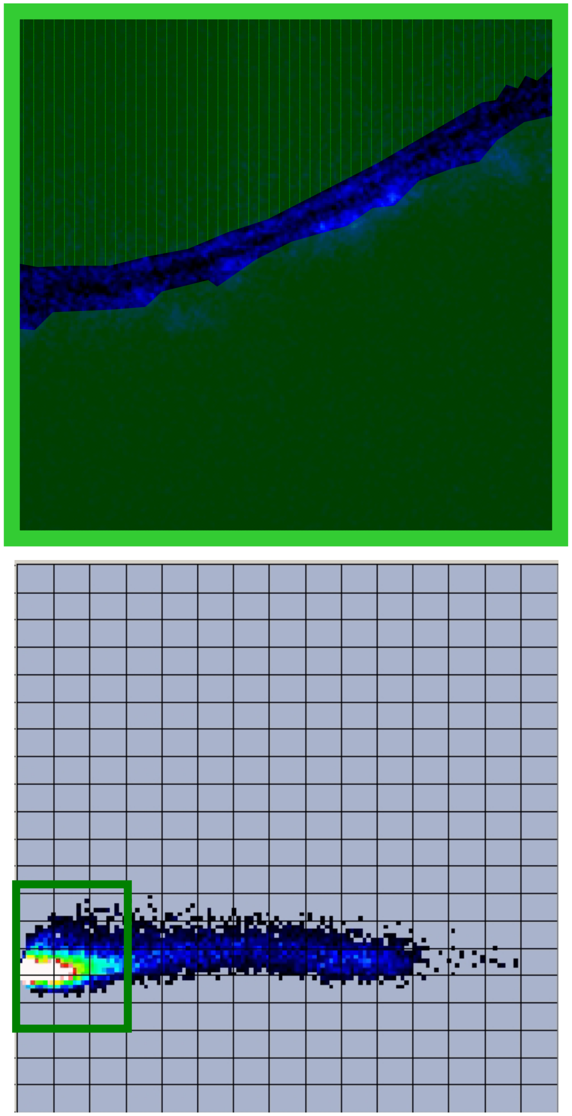
Examples

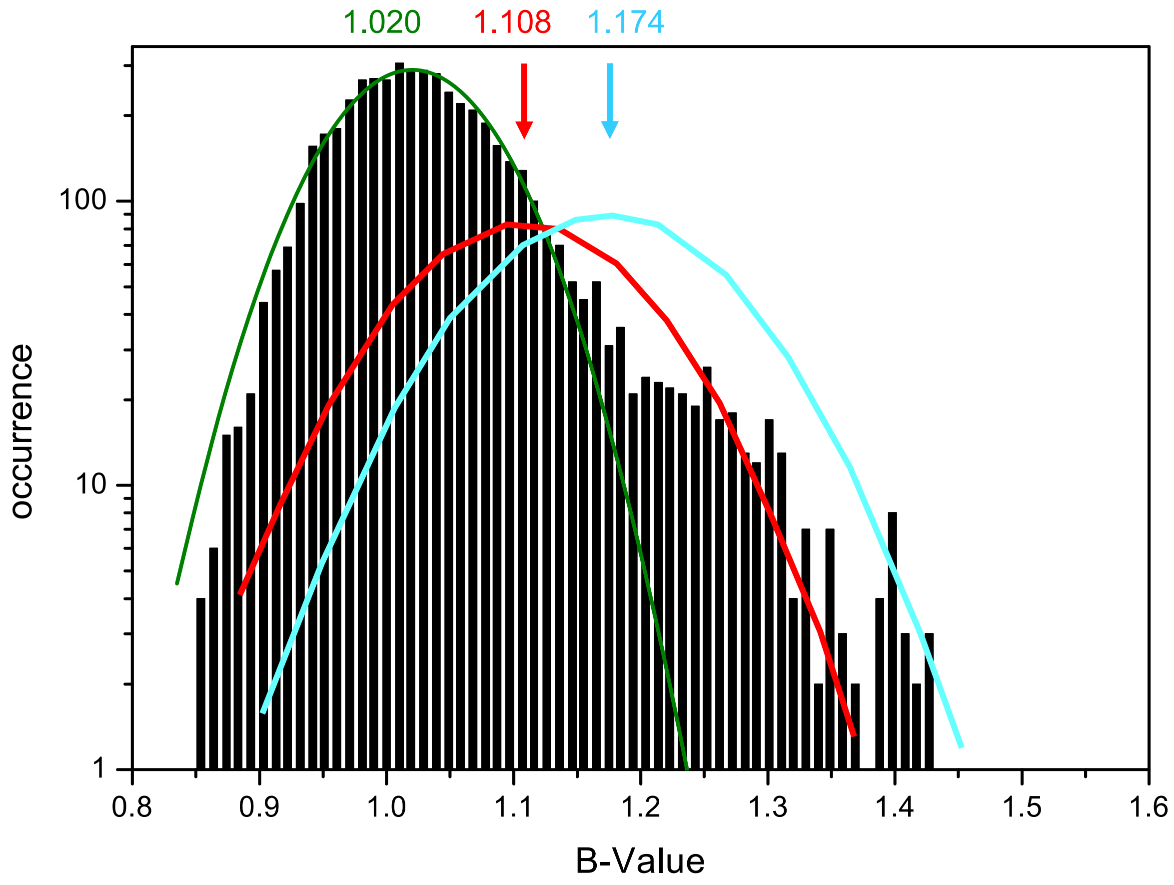




④

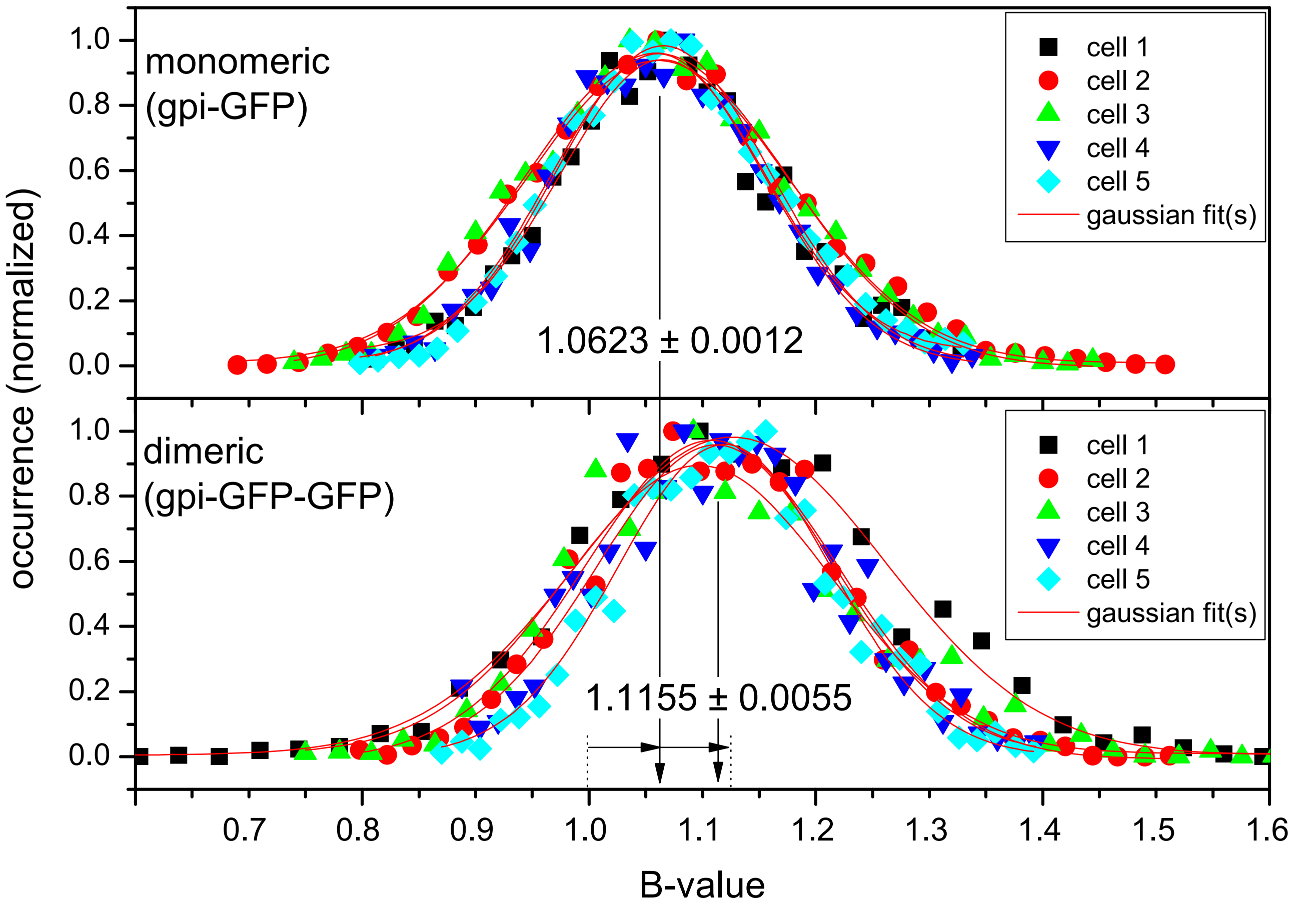
Examples




1.020
1.108
1.174
occurrence
100
10
1
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
B-Value

④

Examples


monomeric
(gpi-GFP)
dimeric
(gpi-GFP-GFP)
1.0623 ± 0.0012
1.1155 ± 0.0055
■ cell 1
● cell 2
▲ cell 3
▼ cell 4
◆ cell 5
gaussian fit(s)
occurrence (normalized)
B-value
0.0
0.2
0.4
0.6
0.8
1.0
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6

⑤

Advantages, Limitations and Future Directions

Advantages:

N&B can be adapted to any image acquisition platform
N&B is straightforward to interpret (images, selection of pixels)
N&B resolves down to monomers versus dimers
N&B does not require complex sample preparation (viz FRET)

Limitations:

N&B is limited to mobile species.
N&B is perturbed by fast blinking dynamics, temporal filtering becomes difficult.
N&B is perturbed by mixed populations, callibration in this case is complex.

Future directions:

Adapted to two-color acquisition (as in FCCS) provides stoichiometry “maps”.
N&B could be adapted to “carpet” like methods (circular scan, tracking…)