Cross-RICS and Cross N&B

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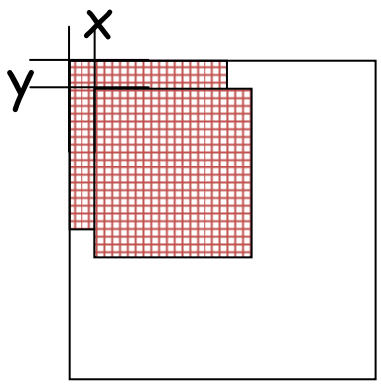
We have expanded the RICS methods to do Cross-Correlation RICS (ccRICS)

The ccRICS approach

The spatial correlation function



$$G_{ccRICS}(\xi,\psi) = \frac{< I_1(x,y) I_2(x+\xi, y+\psi) >}{< I_1(x,y) >< I_2(x,y) >} - 1$$

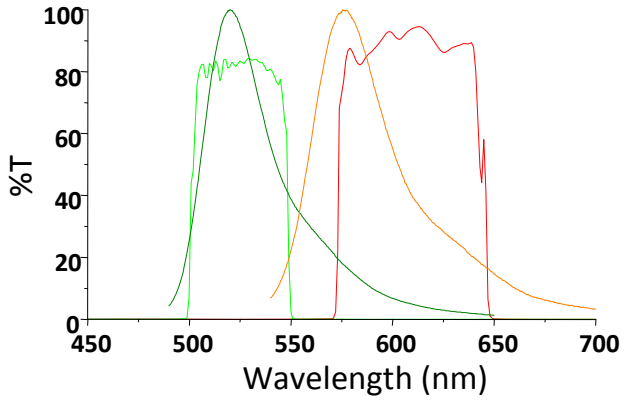
The variables ξ and ψ represent spatial increments in the x and y directions, respectively

The $G_{cc}(0,0)$ value and bleedthrough

$$G_{cc}(0,0) \propto \left[\frac{f_{11}\, f_{12} \langle N_1 \rangle + f_{21}\, f_{22} \langle N_2 \rangle}{f_{11}\, f_{12} \langle N_1 \rangle^2 + (f_{11}\, f_{22} + f_{21}\, f_{12}) \langle N_1 \rangle \langle N_2 \rangle + f_{21}\, f_{22} \langle N_2 \rangle^2} \right]$$

Ch1

$F_1(t) = f_{11}N_1 + f_{21}N_2$



Ch2

$F_2(t) = f_{12}N_1 + f_{22}N_2$

Experimental issues

- The volume of excitation and emission at the two excitation wavelengths must superimpose (we are using the Olympus FV1000 LSCM for these experiments)
- Bleedthrough of the green into the red channel must be small (<5%)
- FRET will strongly decrease the ccRICS signal
- High ratio of labeled to unlabeled molecules are needed (if you have only 10% labeled, in a complex of 1:1, you will only have 1% of the complexes labeled with both proteins)

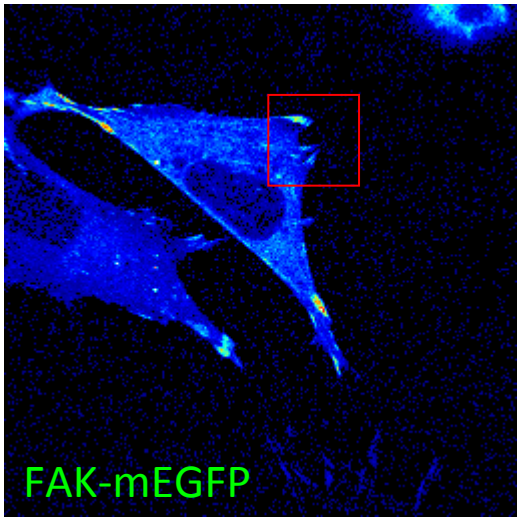
Cells. MEF transfected Vinculin, FAK and paxillin. cDNA were ligated to EGFP or mCherry at the C-terminal end.

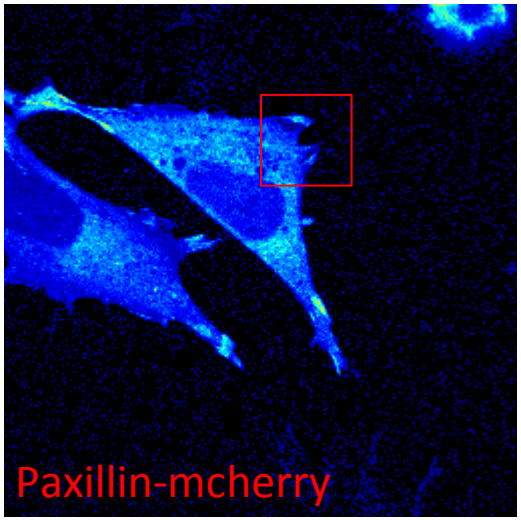
Microscopy. Olympus FV1000 with 60x 1.2NA water objective, 12.5 us/pixel, 256x256 pixels 12.5 μm square, 100 to 200 frames collected for each sample. 1frame/s.

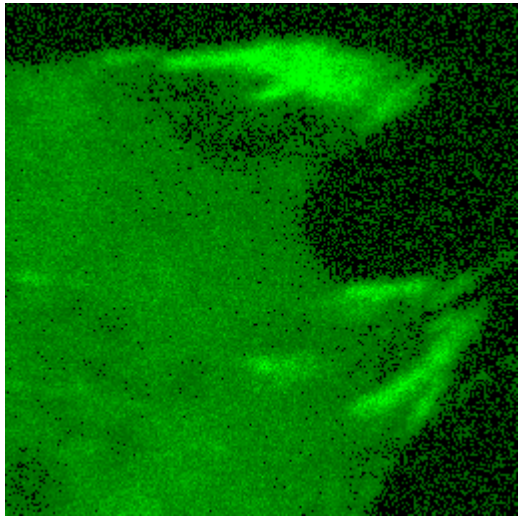
EGFP excitation at 488nm (0.5%) and mCherry at 559nm (adjusted to a max of 1.5%).
Emission filters at 505-540nm and 575-675 nm, for the green and red channels, respectively.

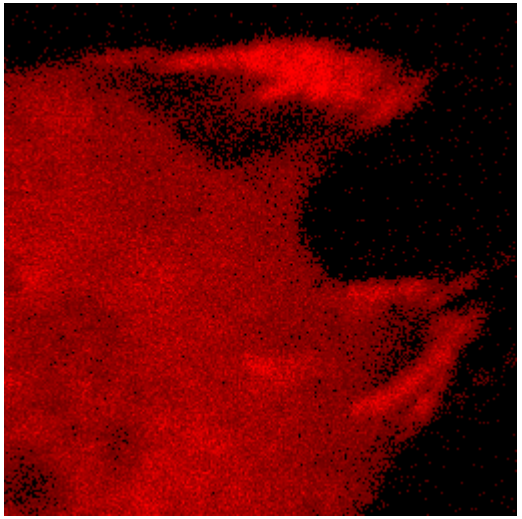
Overlap of the volume of observation was tested by imaging single 100 nm fluorescent beads carrying two colors simultaneously

Does paxillin bind to other proteins before and/or after assembly or disassembly of the focal adhesion?

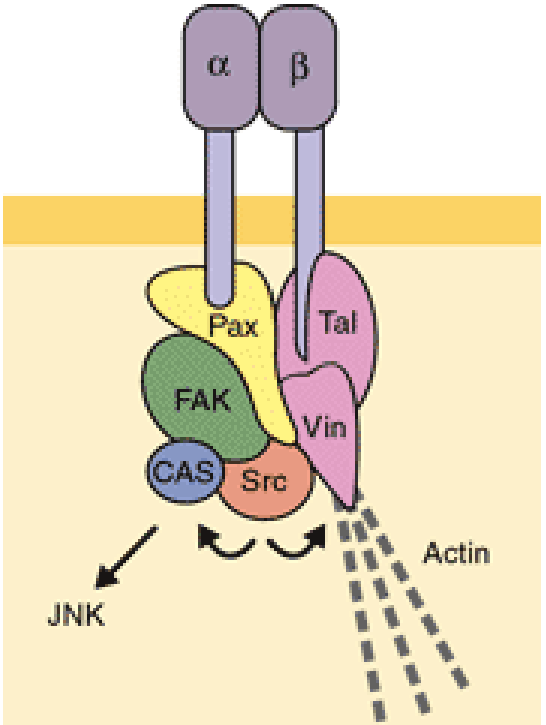






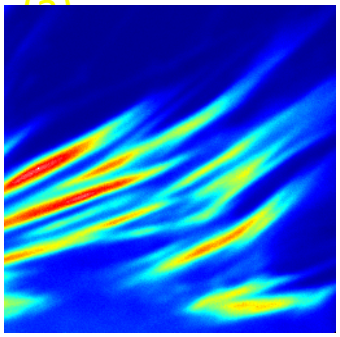


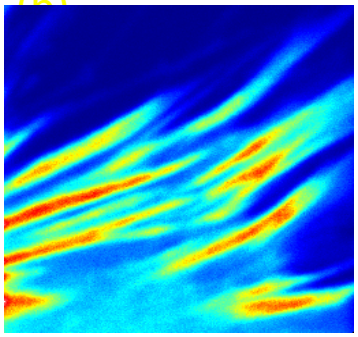
FAK
and
Vinculin

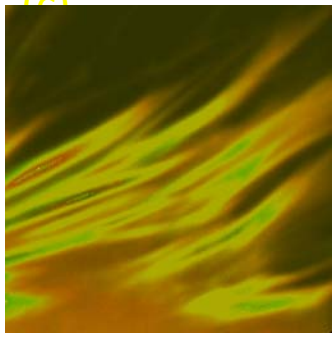


http://student.biology.arizona.edu/honors2001/group08/intro/integrin-actin2.jpg

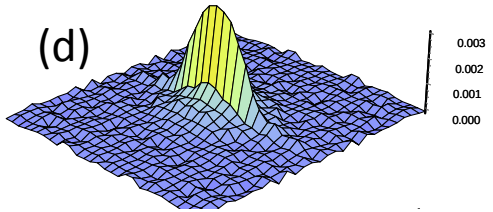
VIN and PAX co localize at adhesions but they are moving independently in the cytoplasm

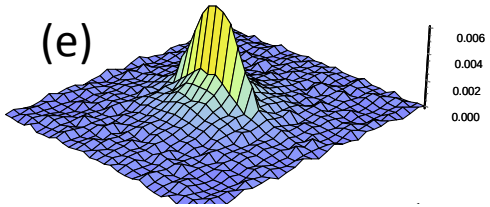


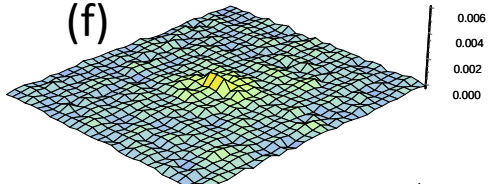




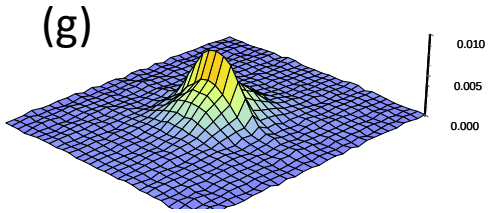
VIN (green)-PAX (red)

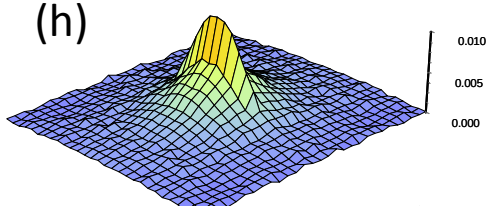


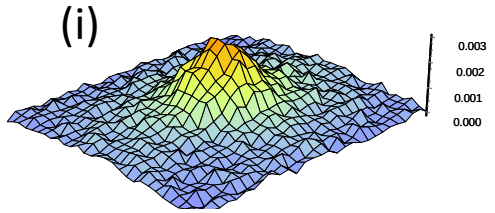




MAV=10s







MAV=40s

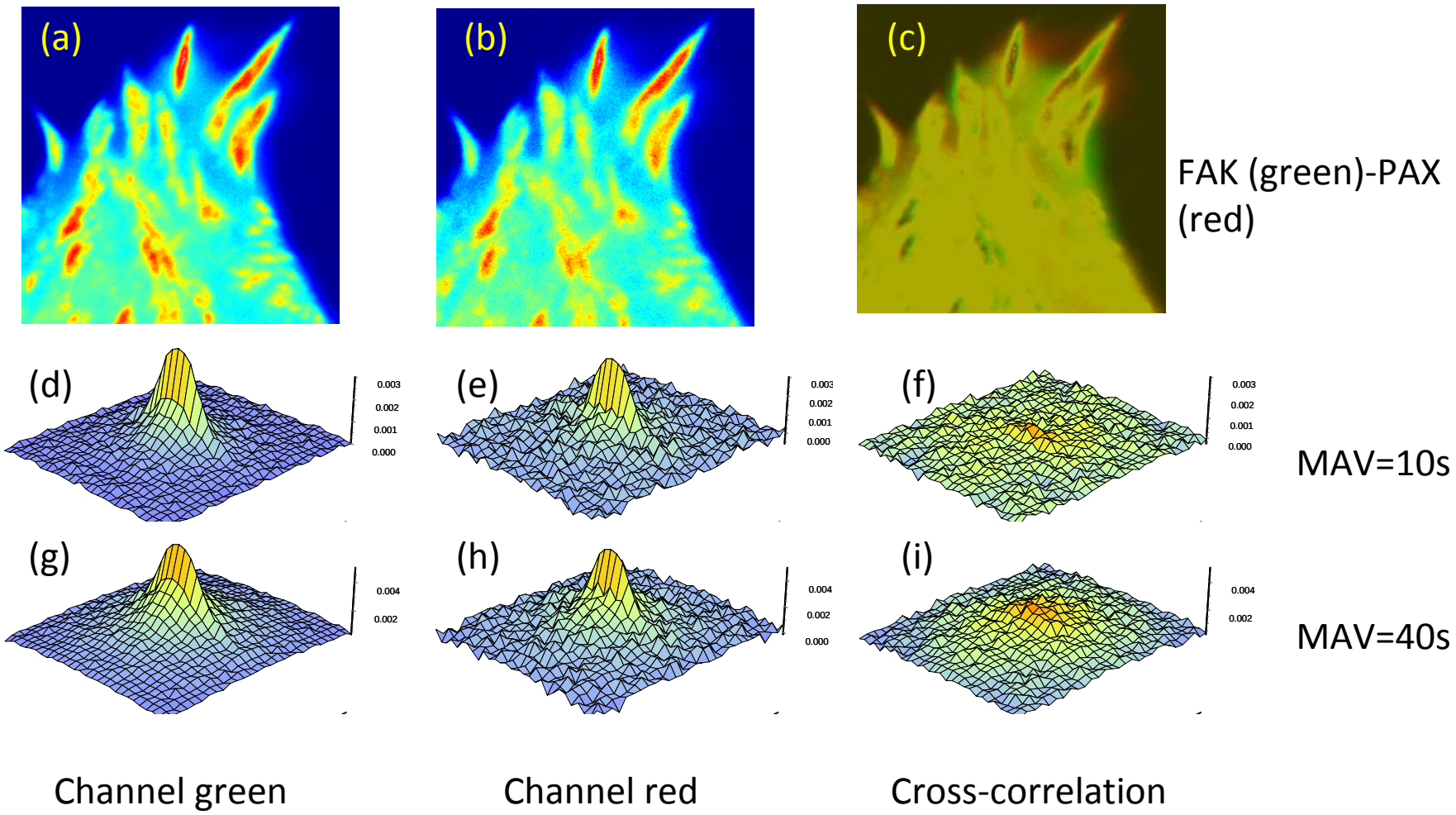
Channel green

Channel red

Cross-correlation

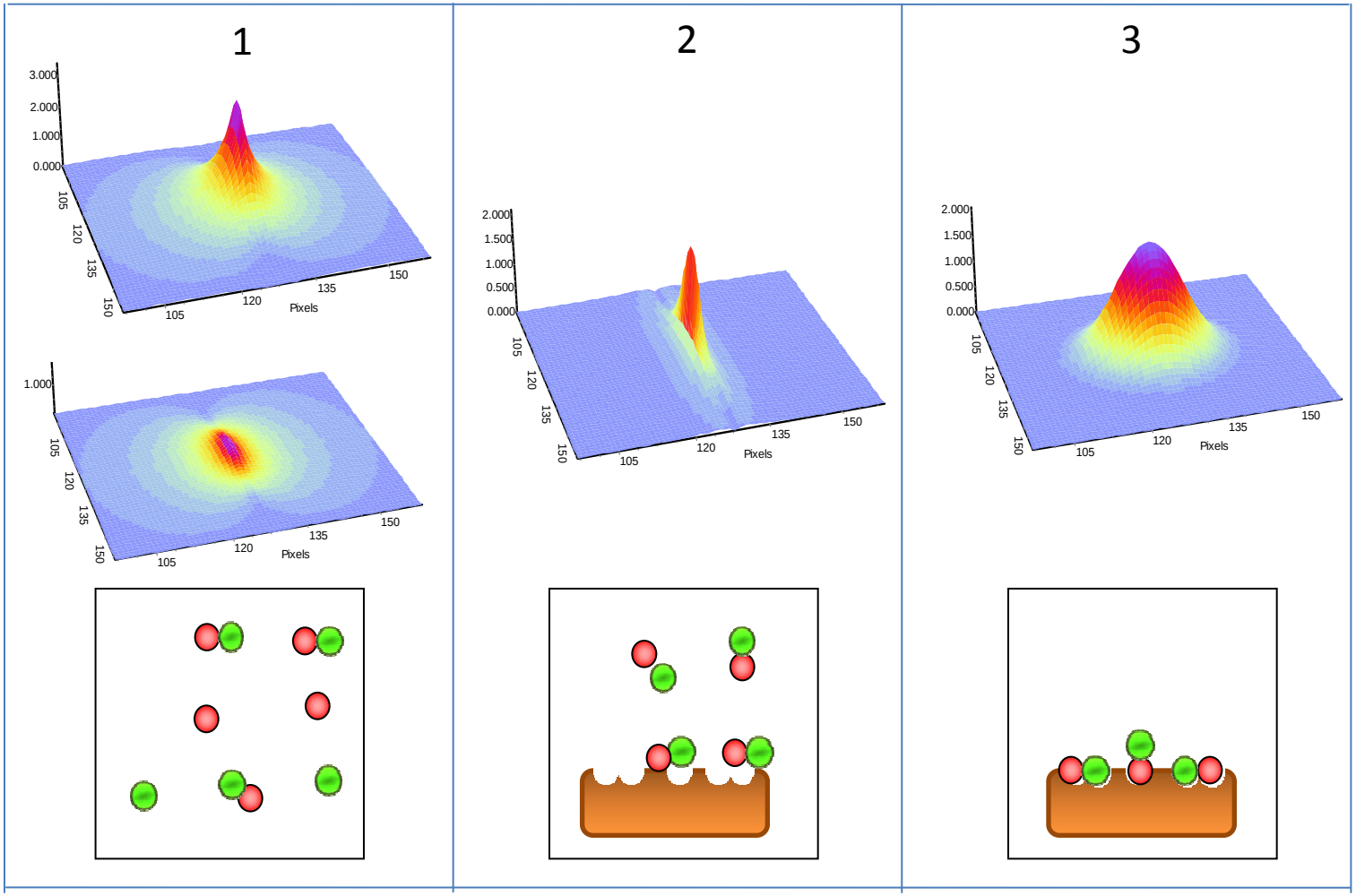
The cross-correlation increases for the slow fluctuations (at MAV=40s). It is round in shape indicating that it is generated at single locations.

FAK and PAX co localize at adhesions but they are moving independently in the cytoplasm



The cross-correlation increases for the slow fluctuations (at MAV=40s). It is round in shape indicating that it is generated at single locations and it is very small.

Schematic representation for the interpretation of the ccRICS experiment. Simulation of binding and diffusion



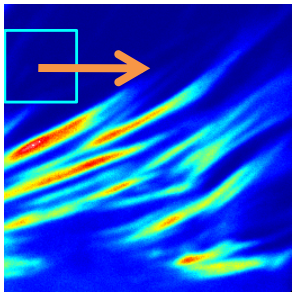
Diffusion
Few complexes

Fast binding
Different shape
Smaller than PSF

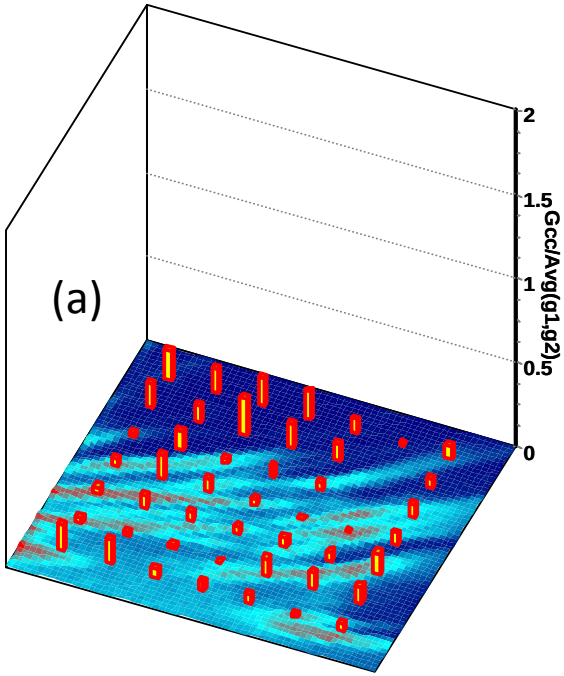
Slow binding
Round shape

Digman, M.A. et al. *Biophys J.* 2009 Jan;96(2):707-16

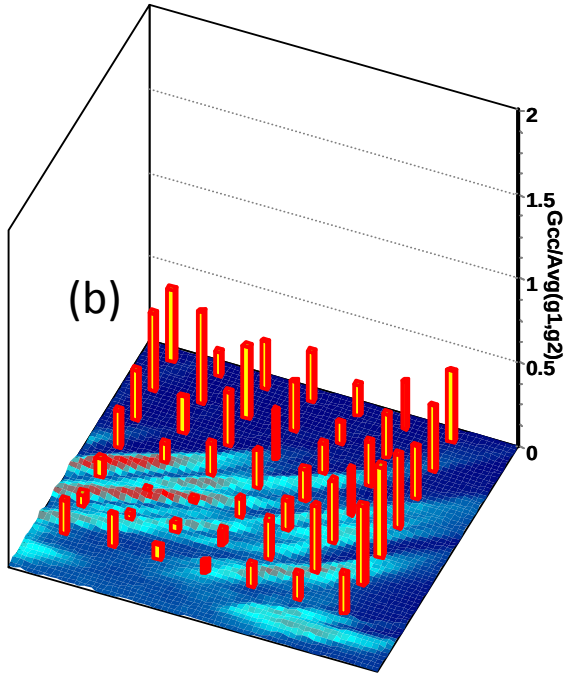
Distribution of fraction of cross-correlation in the cell. Correlation with adhesion disassembling



ccRICS by scanning a region of interest across the image
Calculating the ratio $G_{cc}/AV(G_1,G_2)$



VIN-PAX MAV=10s



VIN-PAX MAV=40s

There is "more" cross-correlation at the locations of adhesion disassembling

Digman, M.A. et al. *Biophys J.* 2009 Jan;96(2):707-16

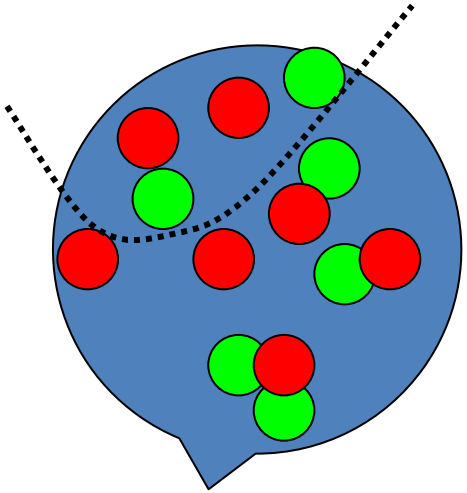
Summary of ccRICS

- We developed a **toolbox** for biophysicists and cell biologists to address common questions regarding the formation of protein complex, their spatial distribution and their stoichiometry

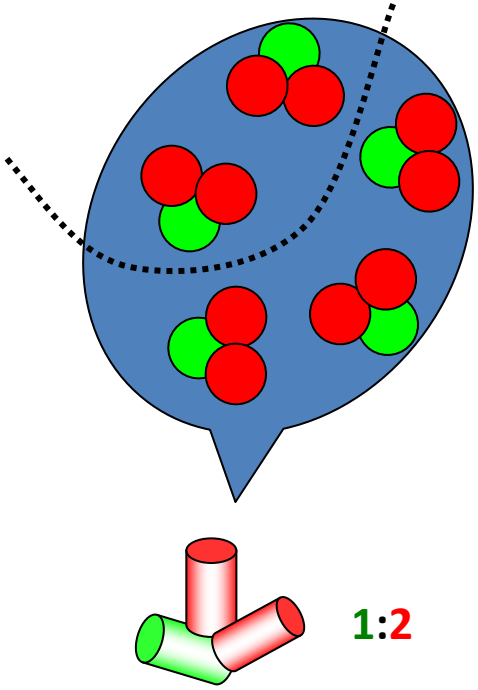
- **ccRICS** is extremely powerful at detecting joint diffusing proteins and in separating diffusion from binding processes

- The Paxillin, vinculin and FAK never crosscorrelate in the cytoplasm before binding to the focal adhesion. We only detect cross correlation due to dissociation of large clusters of proteins.

What is the stoichiometry of these clusters and is this stoichiometry crucial for the biological system?



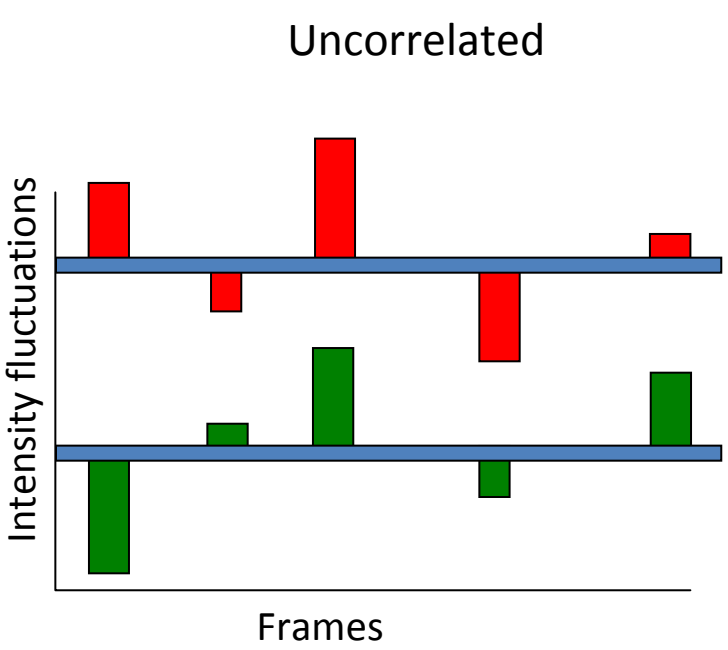
Random

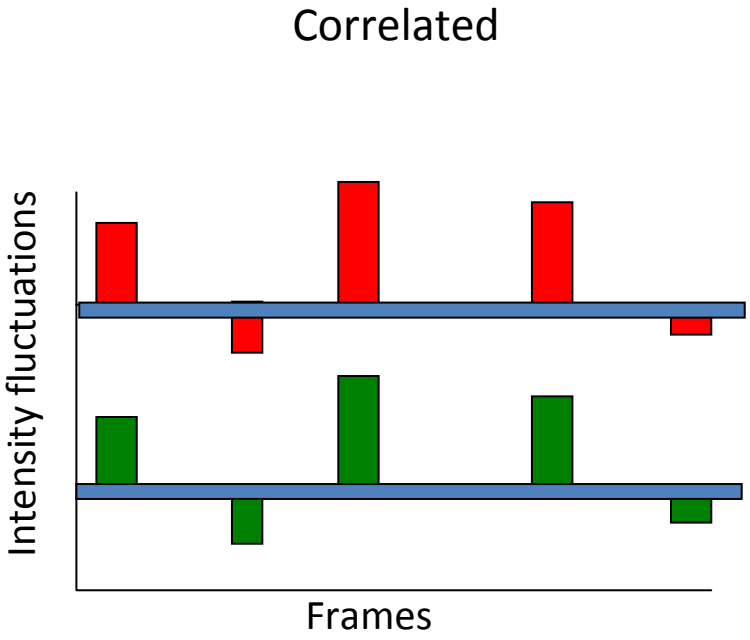


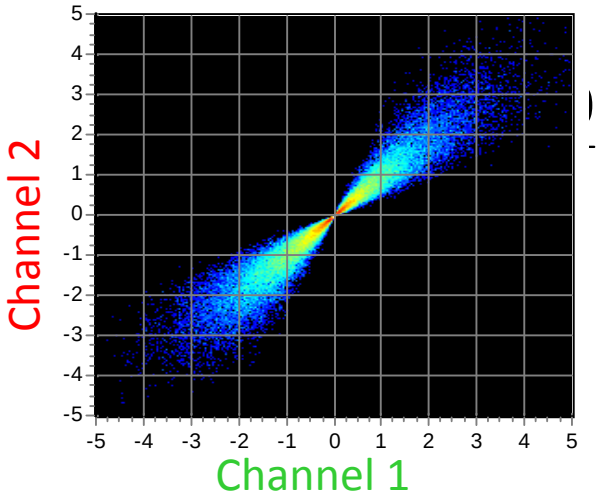
1:2

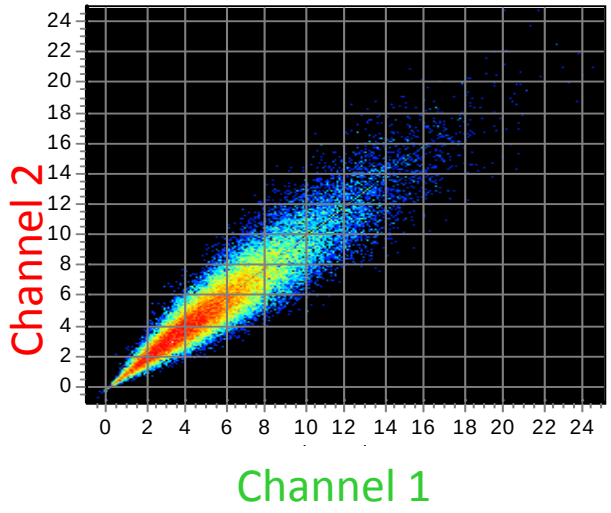
Cross N&B

Conceptual illustration of Cross N&B

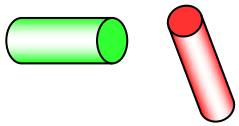






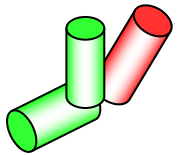


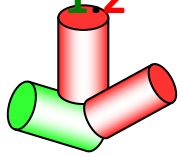
Cross N&B Analysis determines stoichiometry



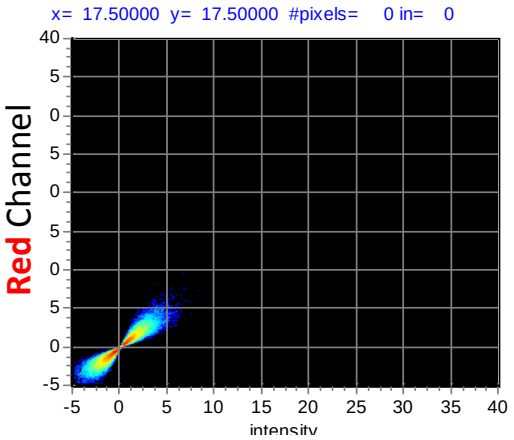
1:1

2:1

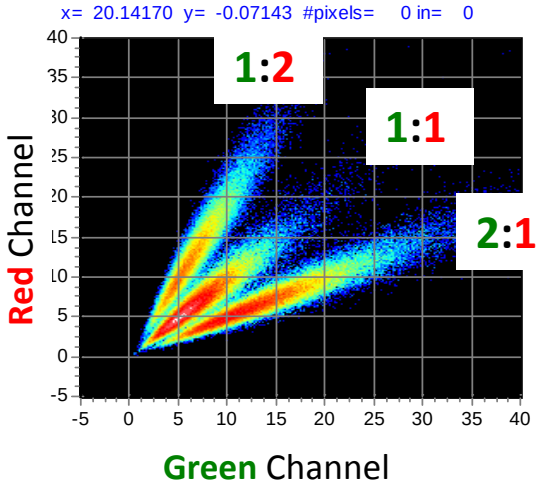




1:2



No Cross-Brightness



Positive Cross-Variance

This example is only for ideal systems where the brightness is calibrated for both channels.

DEFINITIONS

$$I_{xy} = \sum_k i_{xy}/K \qquad \text{for each channel}$$

$$\sigma_{xy} = \sum_k (\mathrm{i}_{xy} - I_{xy})^2/K \qquad \text{for each channel}$$

$$\sigma_{cc} = \sum_k (\mathrm{i}_1 - I_1)(\mathrm{i}_2 - I_2)/K$$

K is the number of frames

$\frac{\sigma_1}{\int_{av}} i_{xy}$ is the intensity at one pixel of each frame

$B_1 = \text{Brightness of channel 1} = \sigma_1 / I_1$

$B_2 = \text{Brightness of channel 2} = \sigma_2 / I_2$

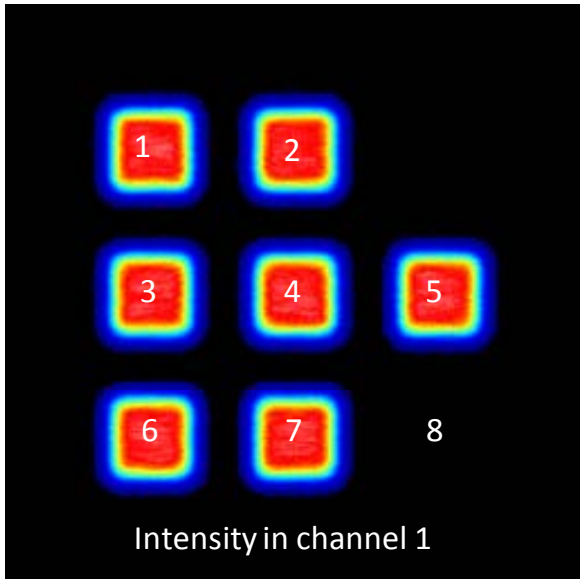
$B_{cc} = Cross - brightness = \sigma_{cc} / (I_1 * I_2)$

$N_1 = \text{Number of particles in Ch 1} = I_1^2 / \sigma_1$

$N_2 = \text{Number of particles in Ch 2} = I_2^2 / \sigma_2$

$N_{cc} = \text{Cross – number fraction} = \sigma_{cc}$

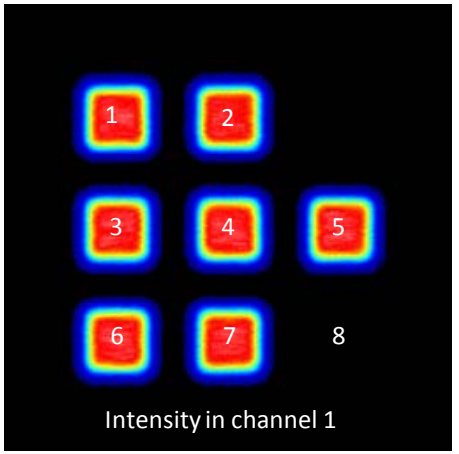
Simulated data for same brightness, but different number of cross-correlated molecules



spot	Channel 1	Channel 2
1	100+0	0
2	90+10	10
3	80+20	20
4	70+30	30
5	60+40	40
6	50+50	50
7	40+60	60
8	0	100

# mo	Br1 (cpsm)	D (um2/s	Rotatio	Br2 (cpsm)	Flow (v/s)	1..6	x-start	y-star
100	1000000	10.000(	10	0	0	1	-64	-64
100	0	10.000(	10	1000000	0	1	64	64
90	1000000	10.000(	10	0	0	1	0	-64
10	1000000	10.000(	10	1000000	0	1	0	-64
80	1000000	10.000(	10	0	0	1	-64	0
20	1000000	10.000(	10	1000000	0	1	-64	0
70	1000000	10.000(	10	0	0	1	0	0
30	1000000	10.000(	10	1000000	0	1	0	0
60	1000000	10.000(	10	0	0	1	64	0
40	1000000	10	10	1000000	0	1	64	0
50	1000000	10	10	0	0	1	-64	64
50	1000000	10	10	1000000	0	1	-64	64
40	1000000	10	10	0	0	1	0	64
60	1000000	10	10	1000000	0	1	0	64

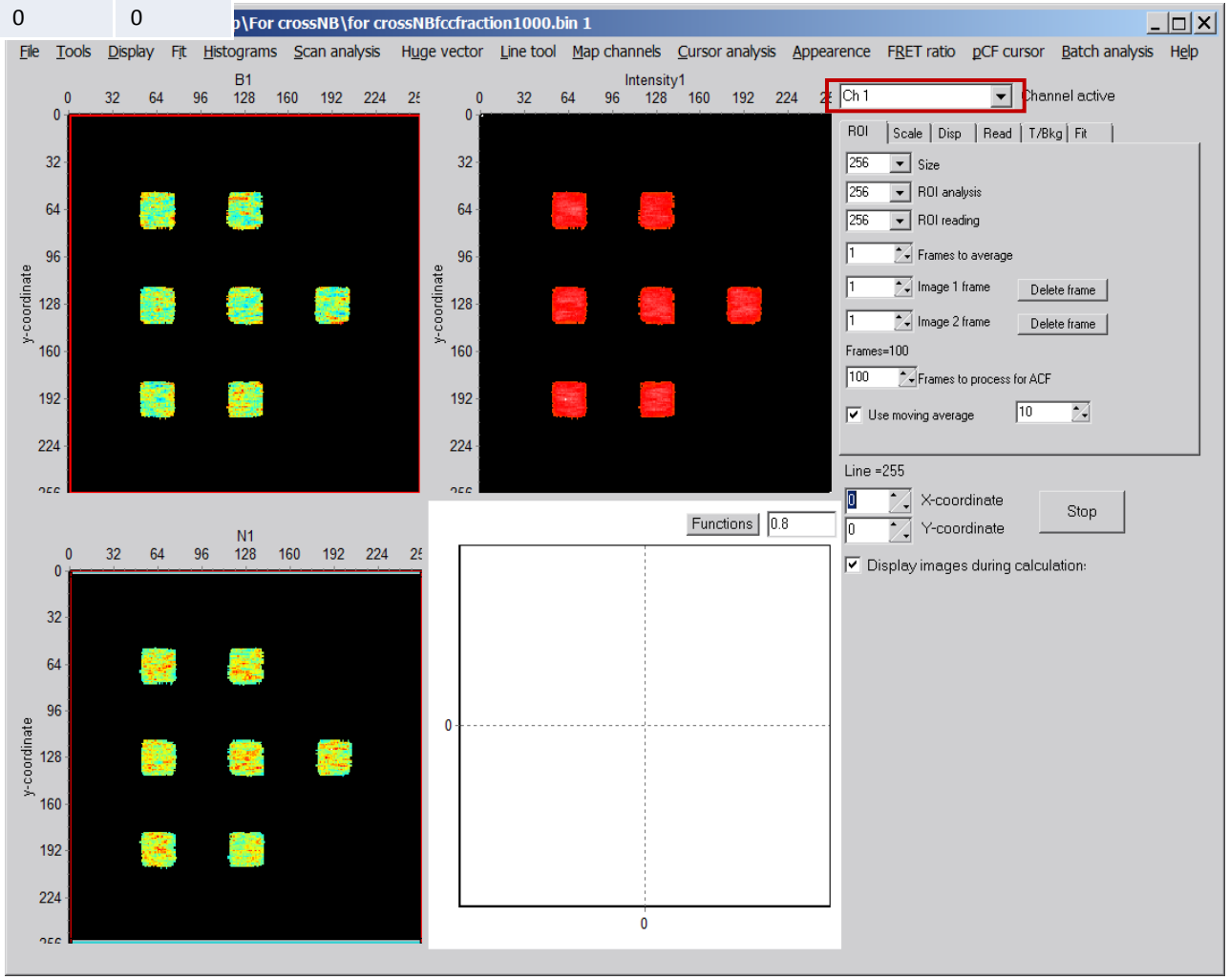
Expected values in the different spots



spot	av1	av2	B1	B2	N1	N2	Ncc	Ncc/N1	Ncc/N2
1	100	0	1	0	100	0	0	0	0
2	100	10	1	1	100	10	10	0.1	1
3	100	20	1	1	100	20	20	0.2	1
4	100	30	1	1	100	30	30	0.3	1
5	100	40	1	1	100	40	40	0.4	1
6	100	50	1	1	100	50	50	0.5	1
7	100	60	1	1	100	60	60	0.6	1
8	0	100	0	1	0	100	0	0	0

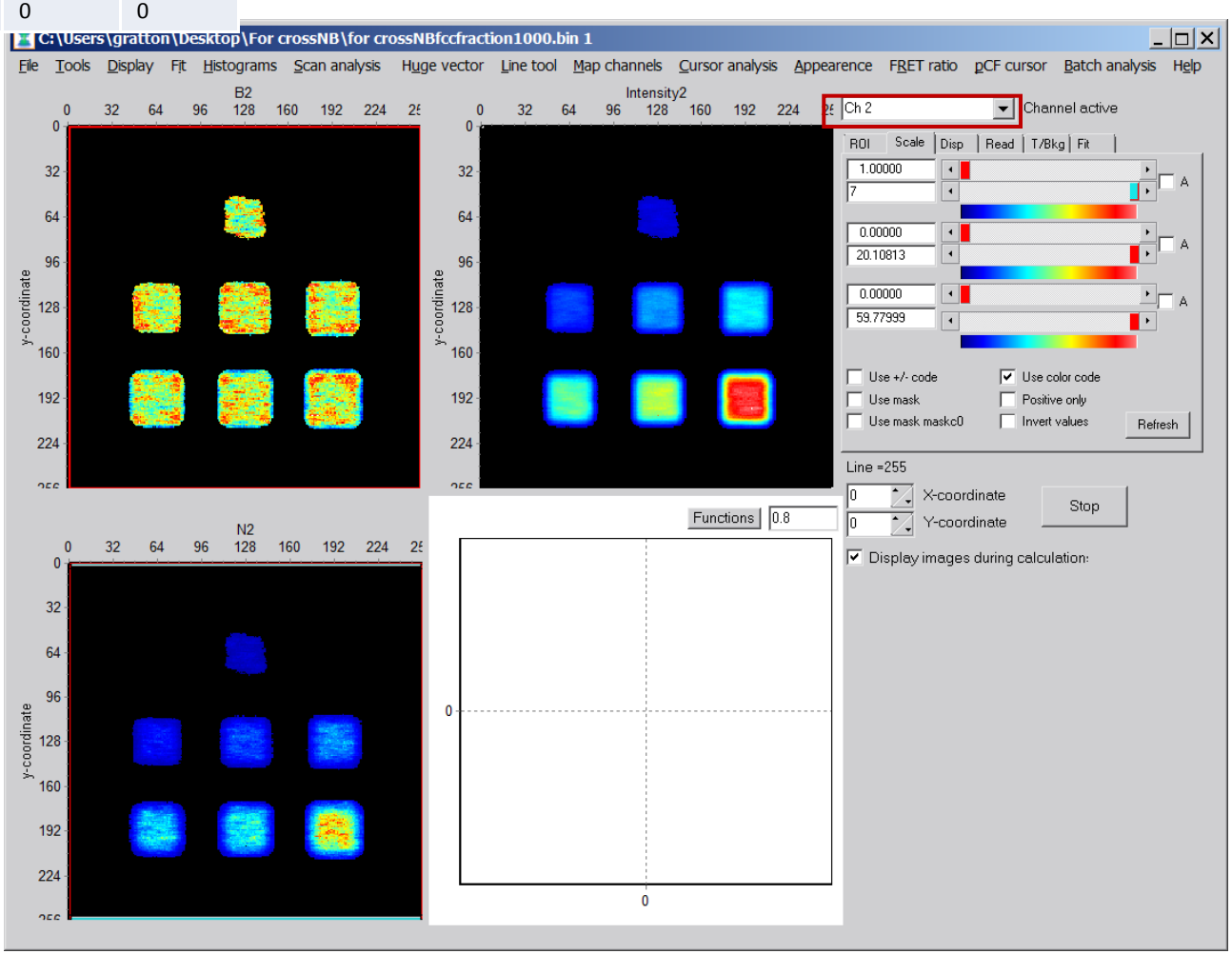
spot	av1	av2	B1	B2	N1	N2	Ncc	Ncc/N1	Ncc/N2
1	100	0	1	0	100	0	0	0	0
2	100	10	1	1	100	10	10	0.1	1
3	100	20	1	1	100	20	20	0.2	1
4	100	30	1	1	100	30	30	0.3	1
5	100	40	1	1	100	40	40	0.4	1
6	100	50	1	1	100	50	50	0.5	1
7	100	60	1	1	100	60	60	0.6	1
8	0	100	0	1	0	100	0	0	0

This is the number and brightness at each pixel for channel 1



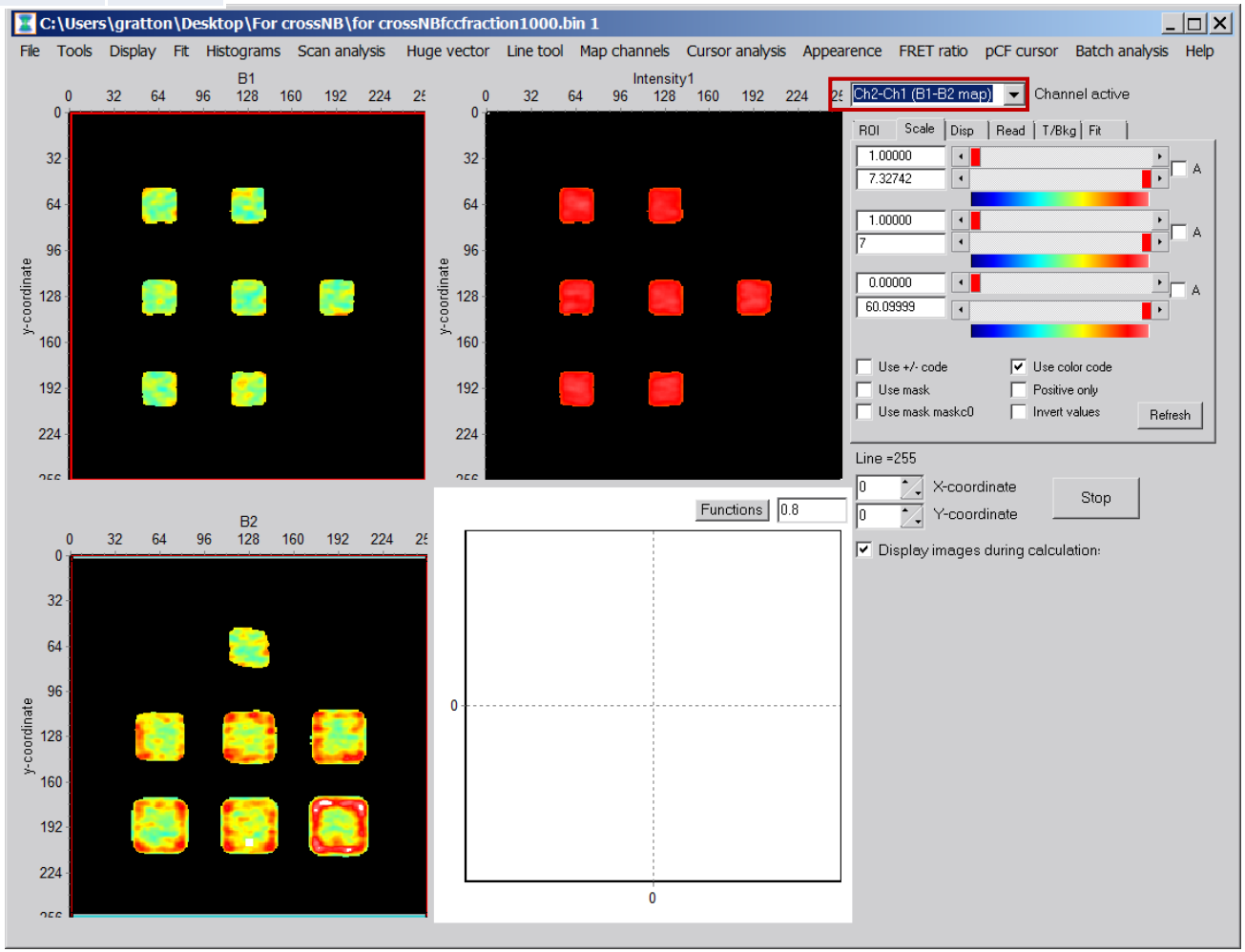
spot	av1	av2	B1	B2	N1	N2	Ncc	Ncc/N1	Ncc/N2
1	100	0	1	0	100	0	0	0	0
2	100	10	1	1	100	10	10	0.1	1
3	100	20	1	1	100	20	20	0.2	1
4	100	30	1	1	100	30	30	0.3	1
5	100	40	1	1	100	40	40	0.4	1
6	100	50	1	1	100	50	50	0.5	1
7	100	60	1	1	100	60	60	0.6	1
8	0	100	0	1	0	100	0	0	0

This is the number and brightness at each pixel for channel 2

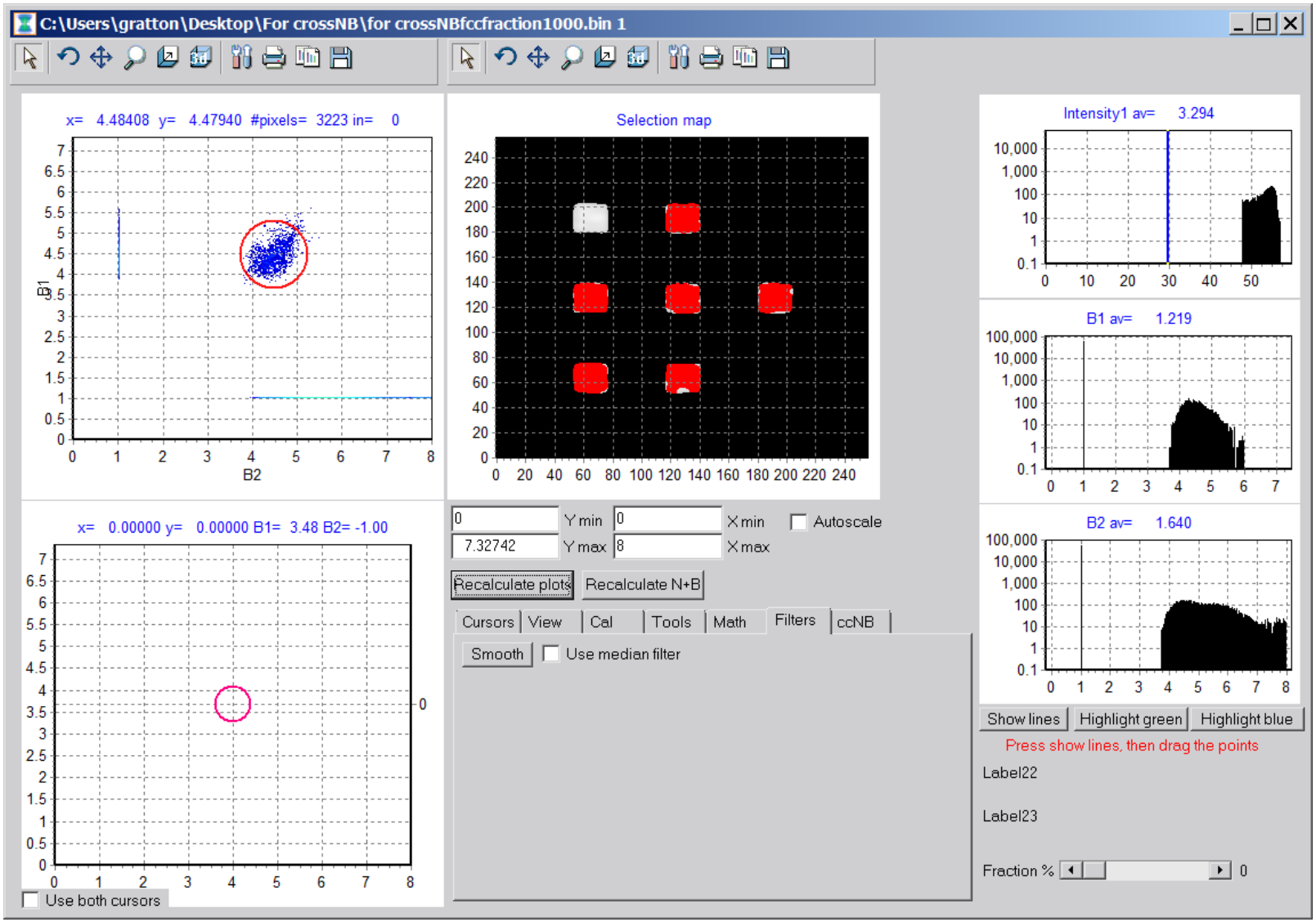


spot	av1	av2	B1	B2	N1	N2	Ncc	Ncc/N1	Ncc/N2
1	100	0	1	0	100	0	0	0	0
2	100	10	1	1	100	10	10	0.1	1
3	100	20	1	1	100	20	20	0.2	1
4	100	30	1	1	100	30	30	0.3	1
5	100	40	1	1	100	40	40	0.4	1
6	100	50	1	1	100	50	50	0.5	1
7	100	60	1	1	100	60	60	0.6	1
8	0	100	0	1	0	100	0	0	0

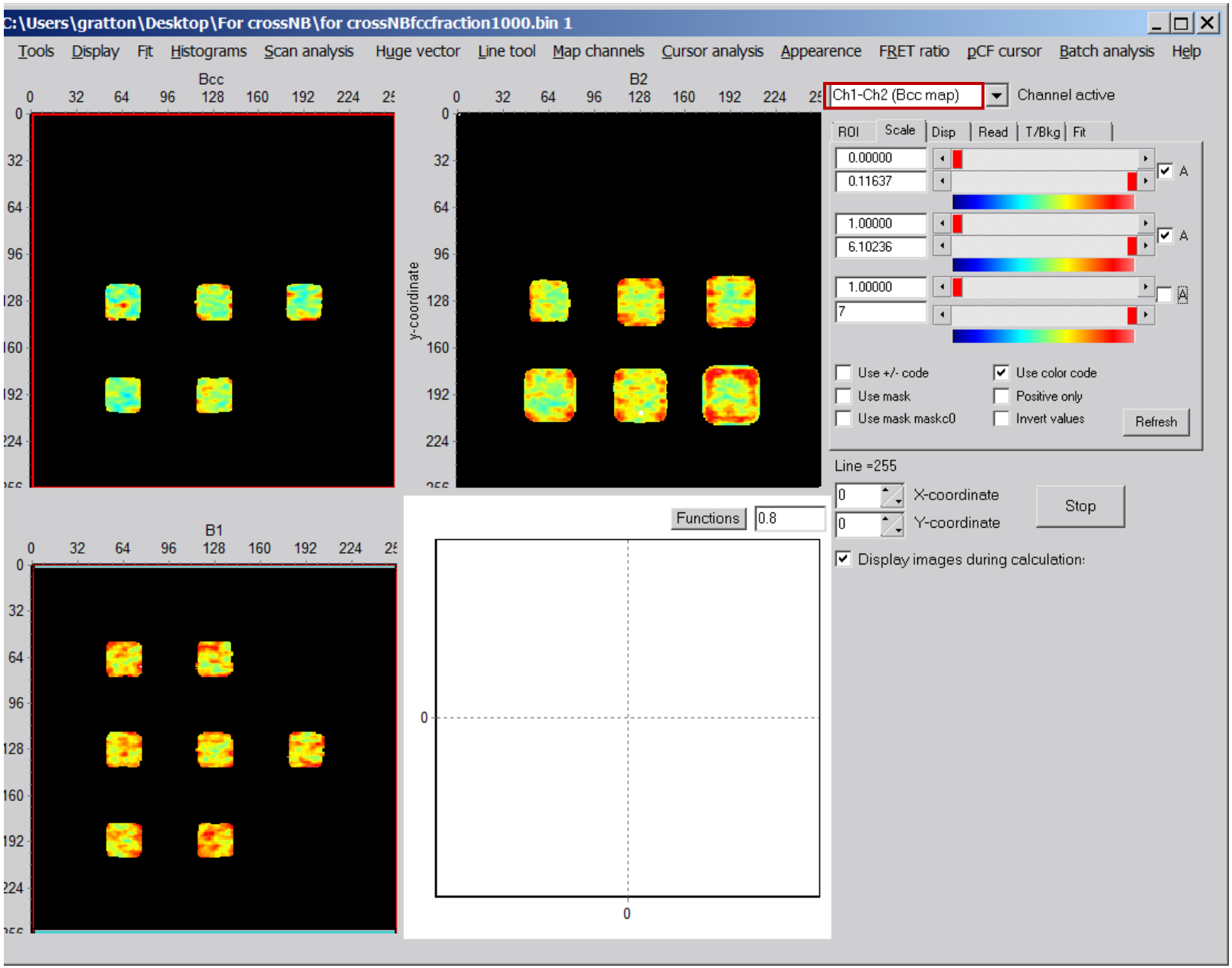
This is the brightness in each channel



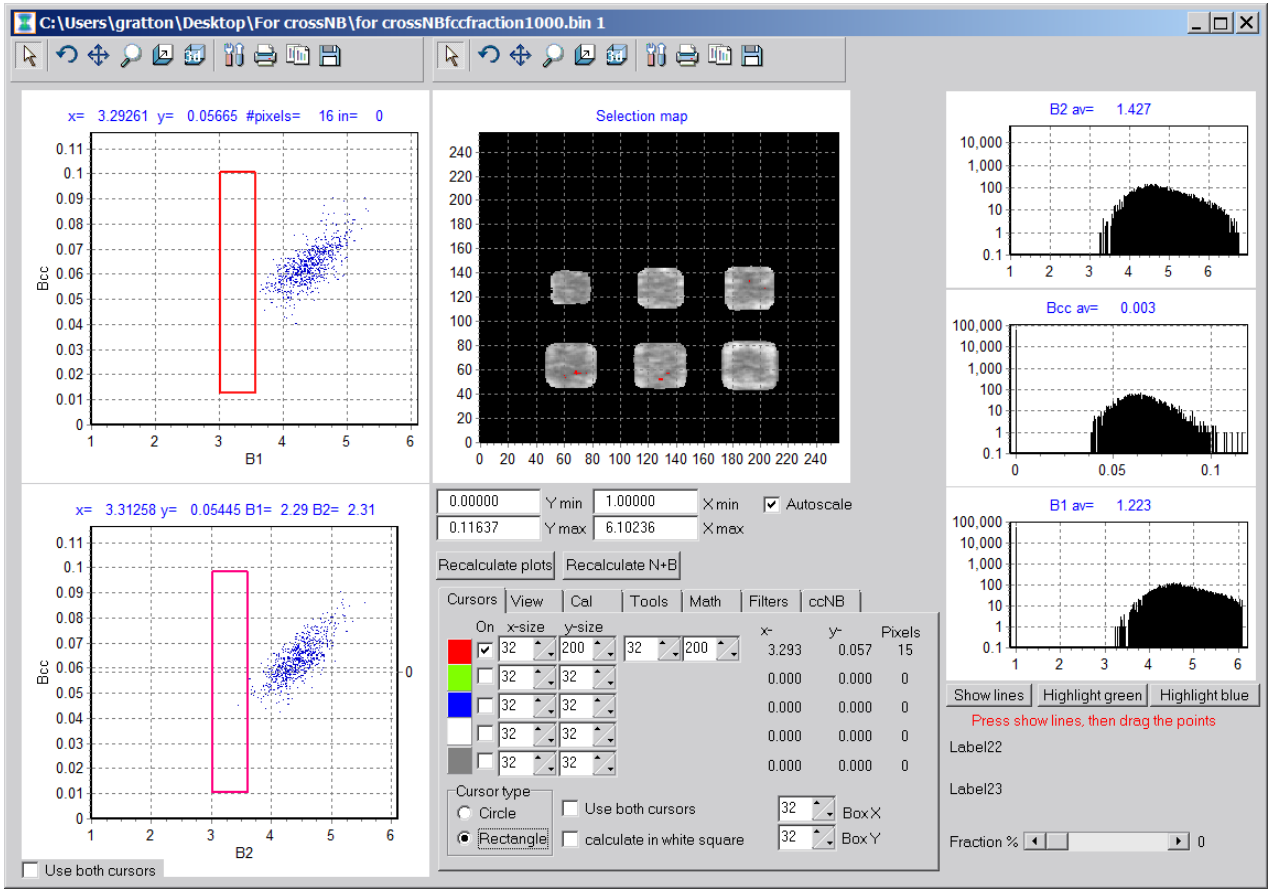
This is the brightness in each channel

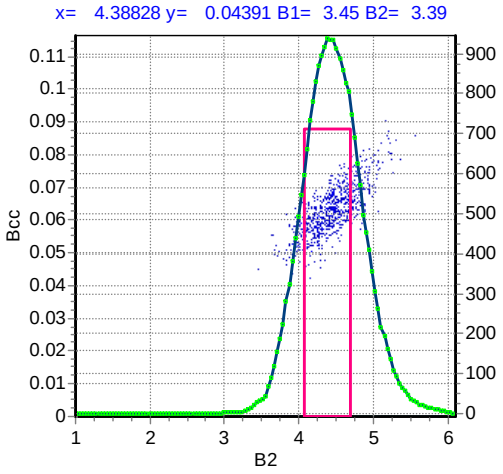


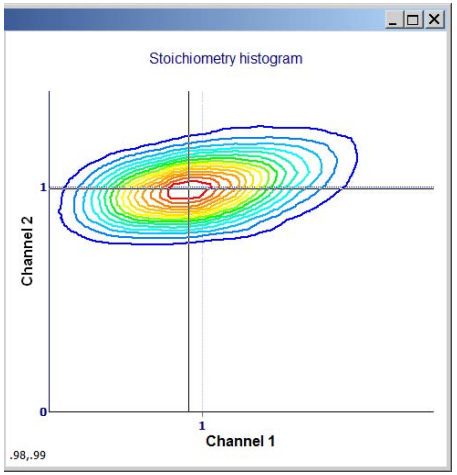
This is the brightness of the cross-correlated molecules



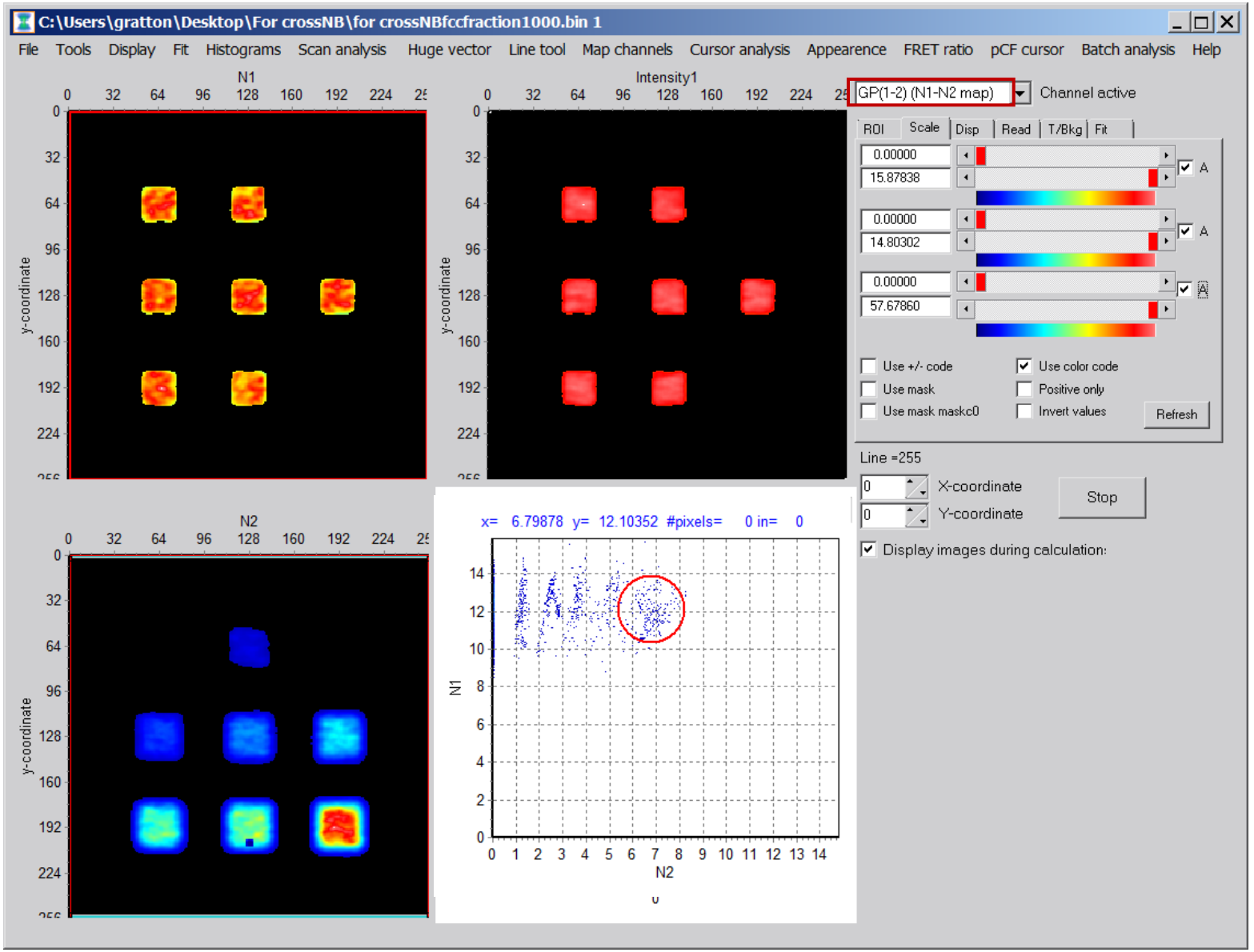
This is the “stoichiometry” of the cross-correlated molecules



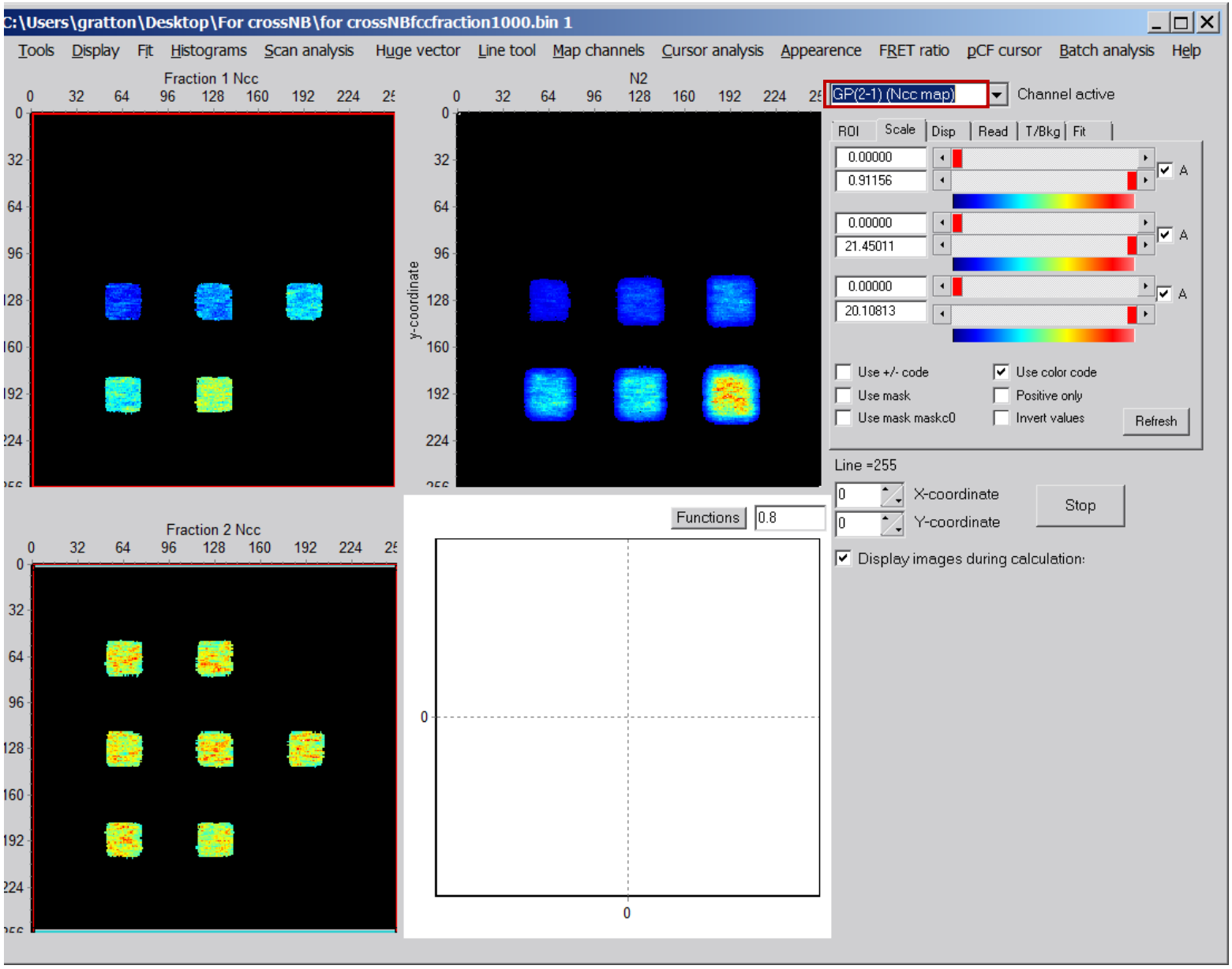




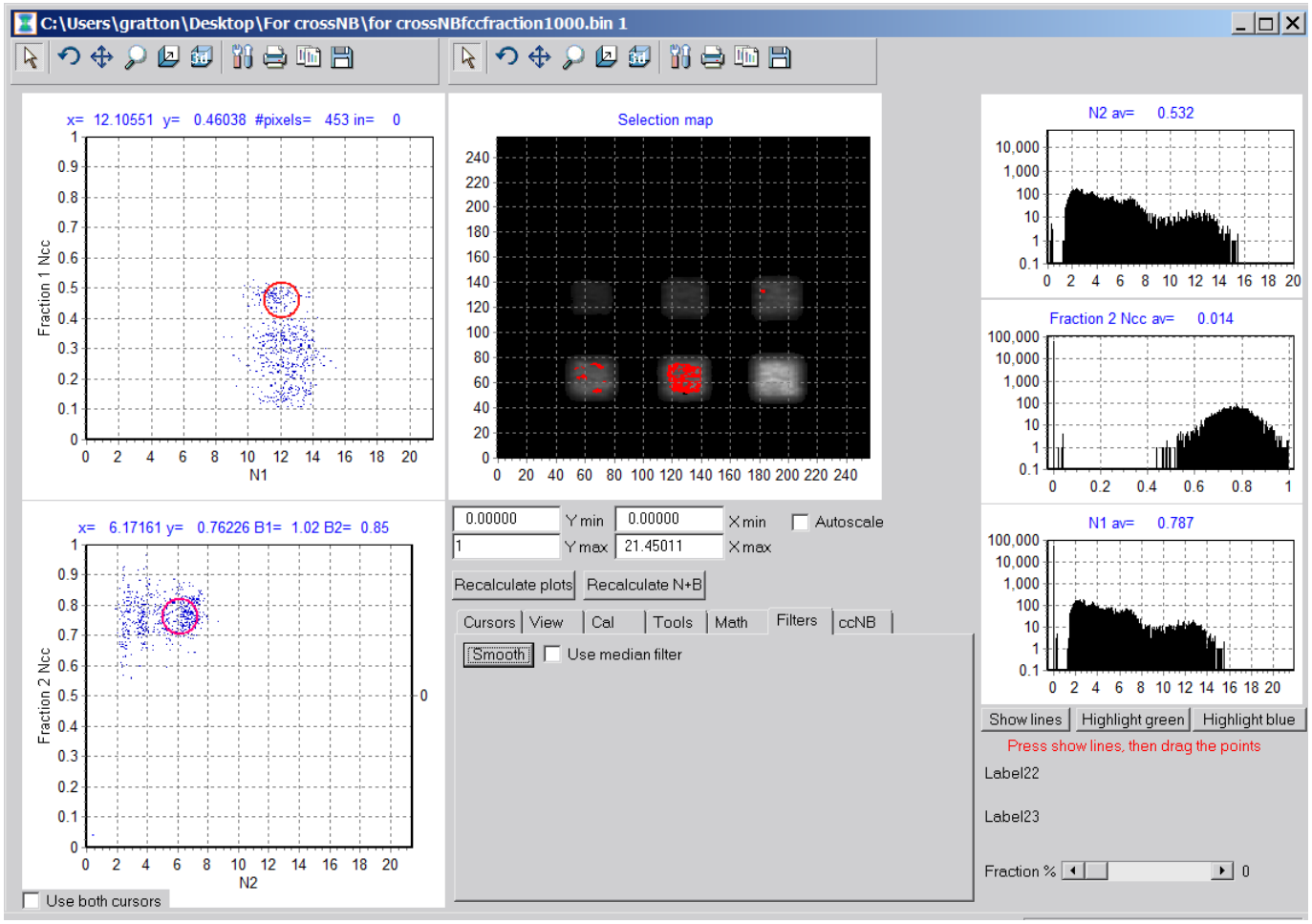
Map of the number of molecules in the two channels. This is not the fraction of cross-correlated molecules



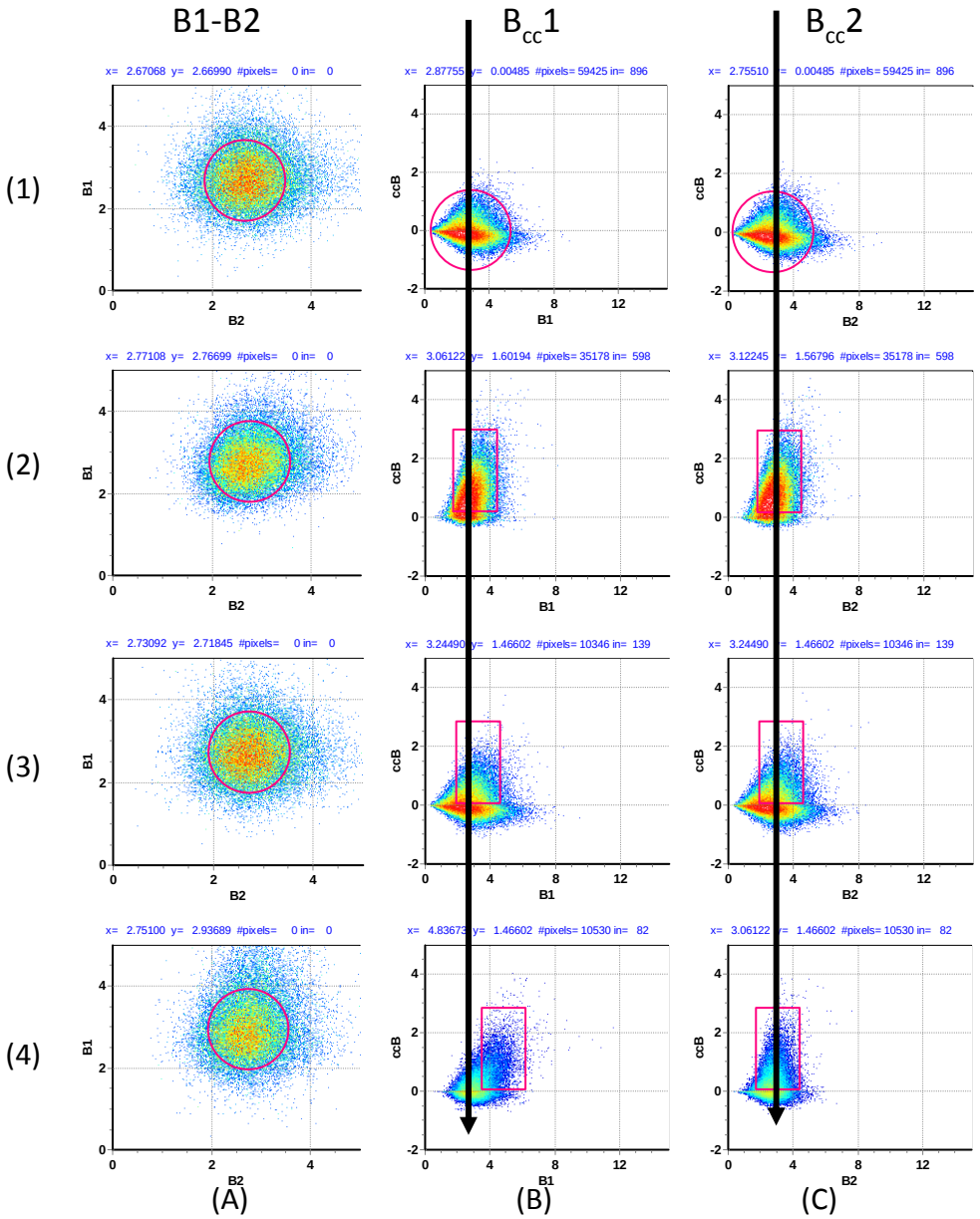
We produce the map of the fraction of cross-correlated molecules



Now we can determine “where” in the image a certain fraction of cross-correlated molecules with different fractions in the two channels can be found



To calibrate the system we need to know the brightness of the monomers



0 % correlated

25 % correlated

5 % correlated

5 % correlated
intensity ratio 2:1

1) calibrate the monomers in both channels The lack of symmetry is due to Poissonian rather than Gaussian distribution of counts

2) Add correlated molecules (still all monomers)

3) At 5% you can still distinguish the positive correlated fluctuations

4) Now we have 2:1 stoichiometry. We have more brightness in B1 but the same in B2

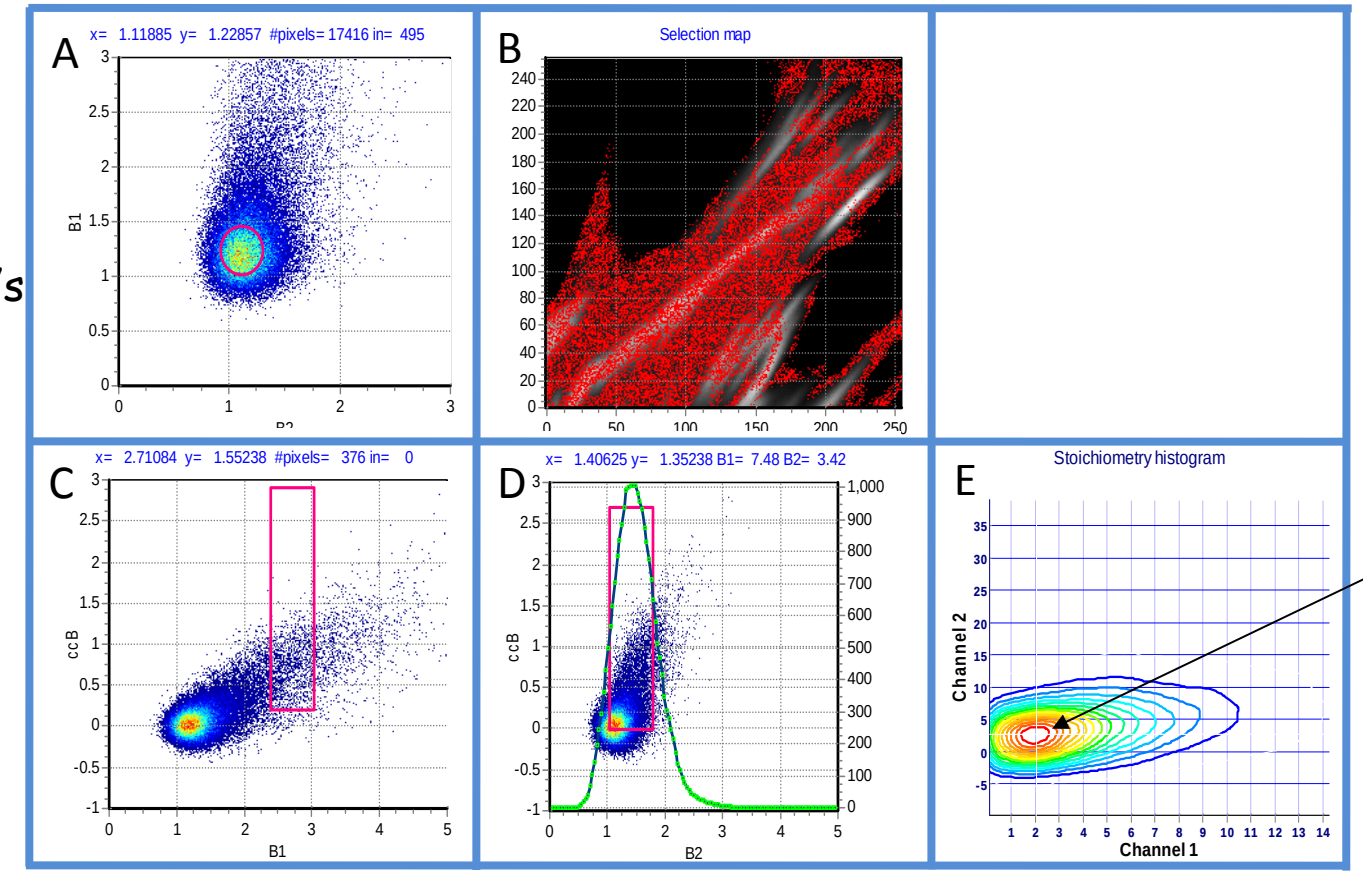
What to look for:

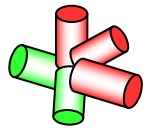
1) First we need to calibrate the monomers
2) We have to see if there is positive cross variance
3) We have to see where the cross variance occurs in respect to the brightness of Ch1 and Ch2

Vinculin-EGFP and Paxillin-mcherry

Monomers
ε=0.118c/d/s

Aggregates

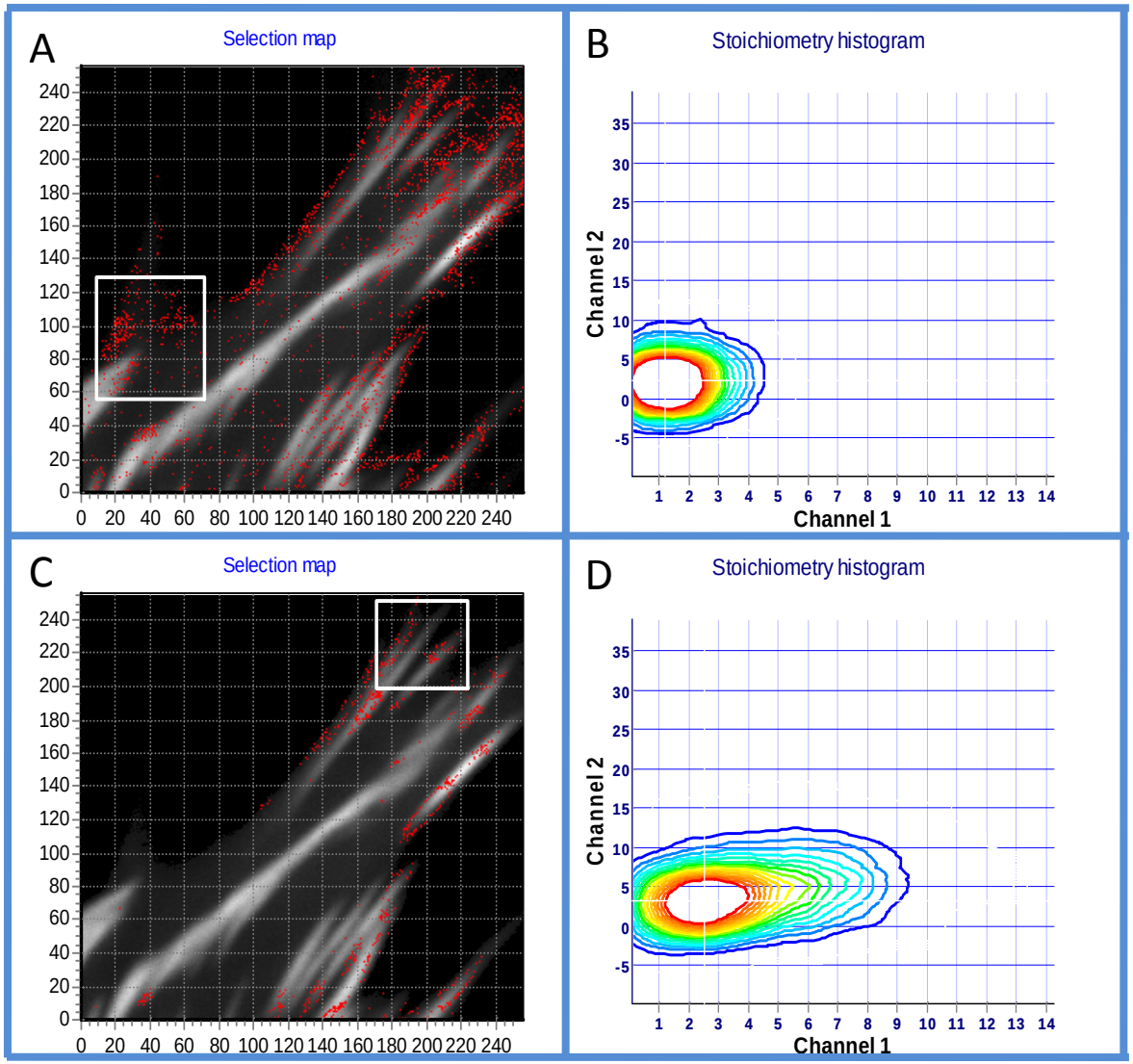




We must find for each value of B1 in one pixel, what is the value of B2 in the same pixel
The fluctuations must be correlated, so we only look at the positive cross-variance

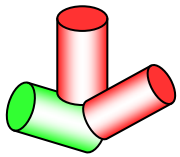
Digman, M.A., et al, *PNAS* 2009

Selecting different regions of the image for vin-pax shows different compositions where large clusters come off at different times



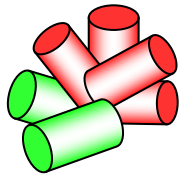
In small adhesions smaller cluster come off

1:2



In larger adhesions large cluster come off

2:4

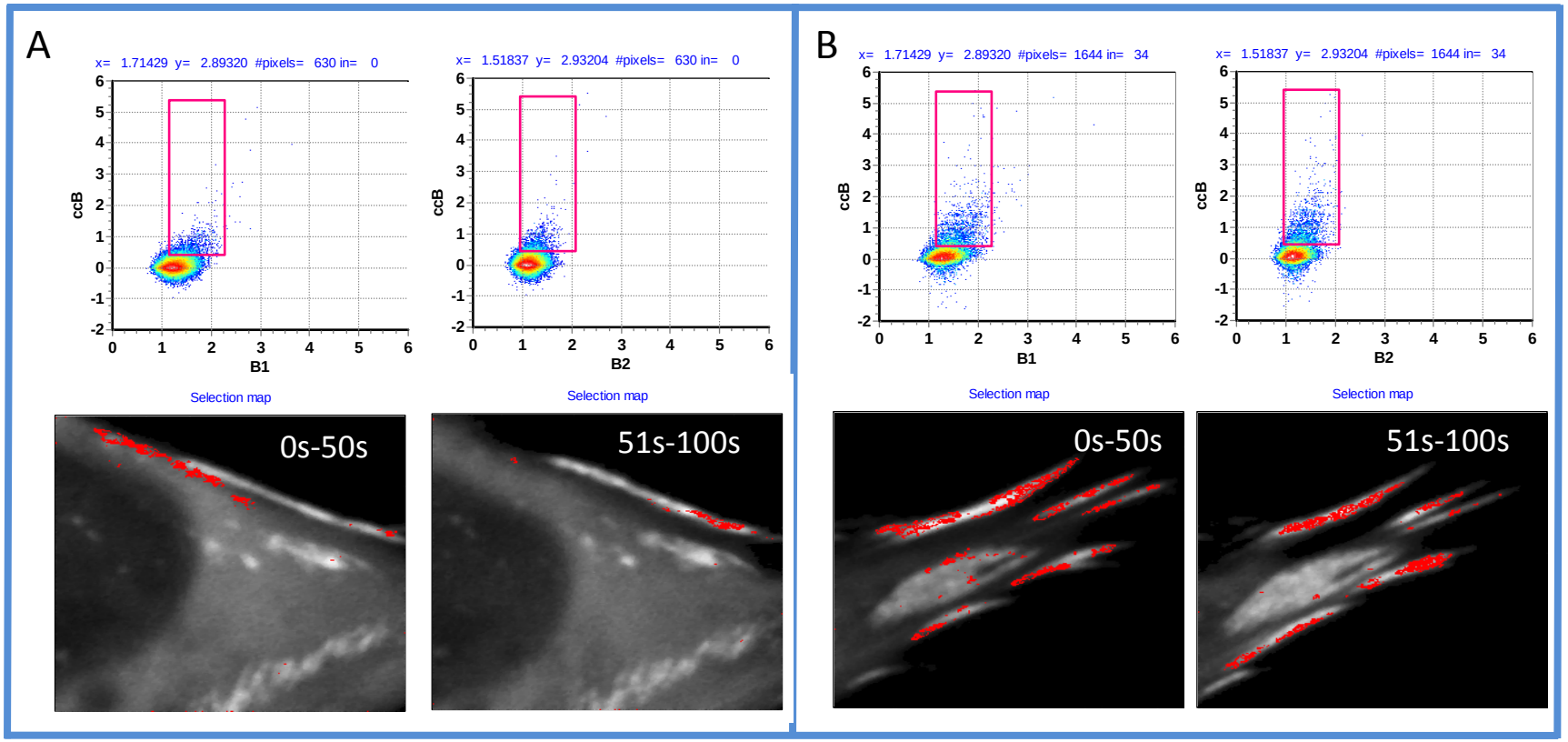


Digman, M.A., et al, *PNAS* ,2009

Cross –correlations occur at specific pixels at the adhesions

FAK-EGFP and Paxillin-mcherry

1. Large Cross variance is only seen at the adhesion
2. Points of large co-variance occur at different regions and different times

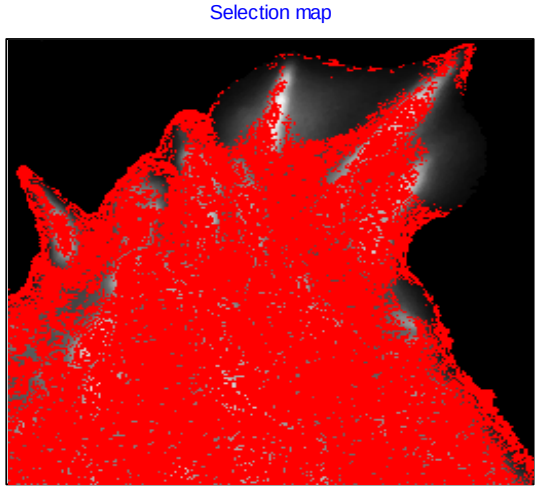


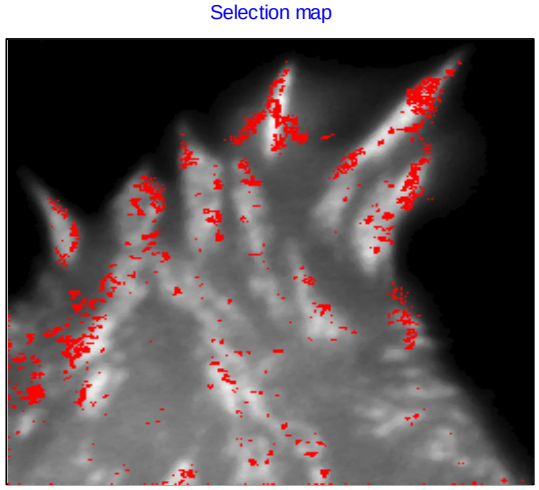
In larger adhesions large cluster come off

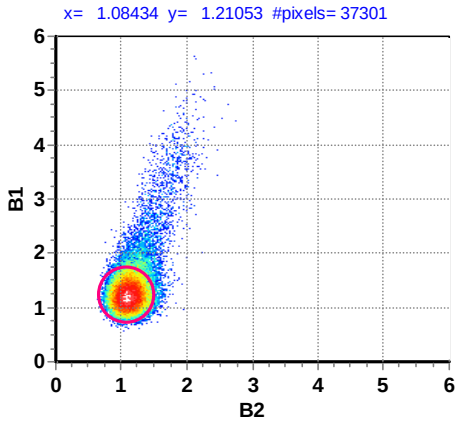
3:6

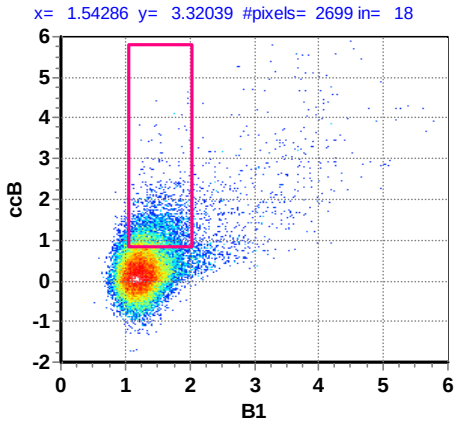
Digman, M.A., et al, *PNAS* ,2009

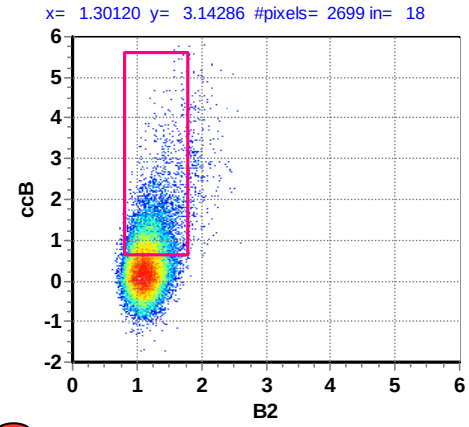
FAK and Paxillin









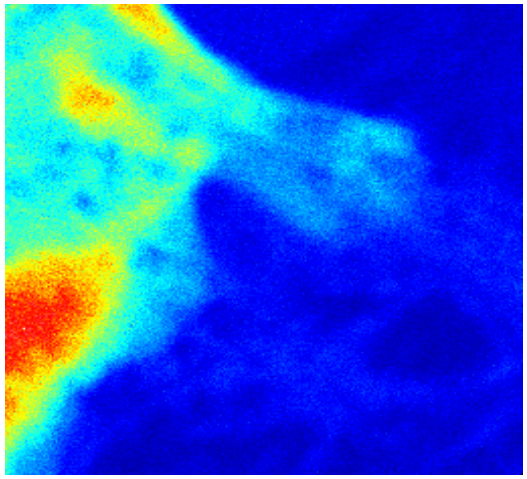


3:4

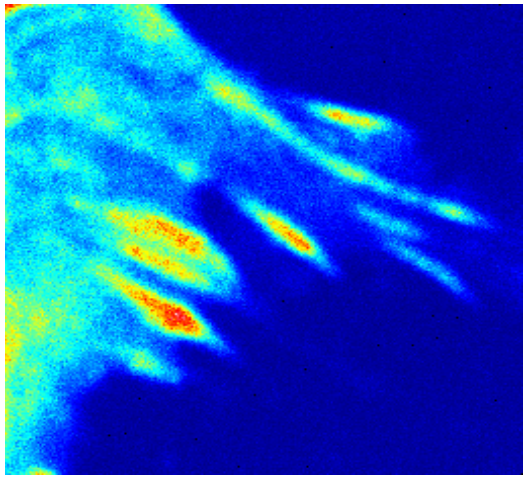
Digman, M.A., et al, *PNAS* ,2009

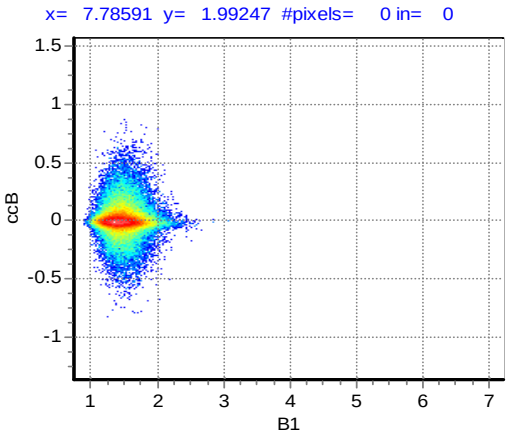
Testing for artifacts: FAK mutant does not form complexes

FAK-EGFP
I937E/I999E



Pax-mCherry

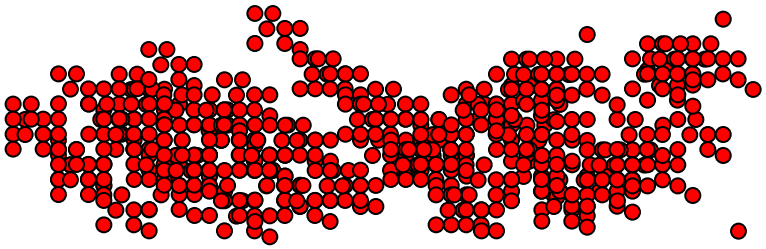


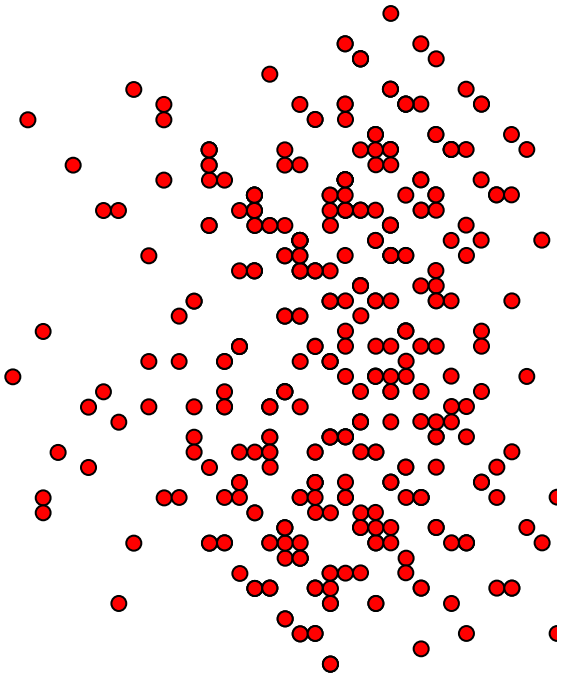


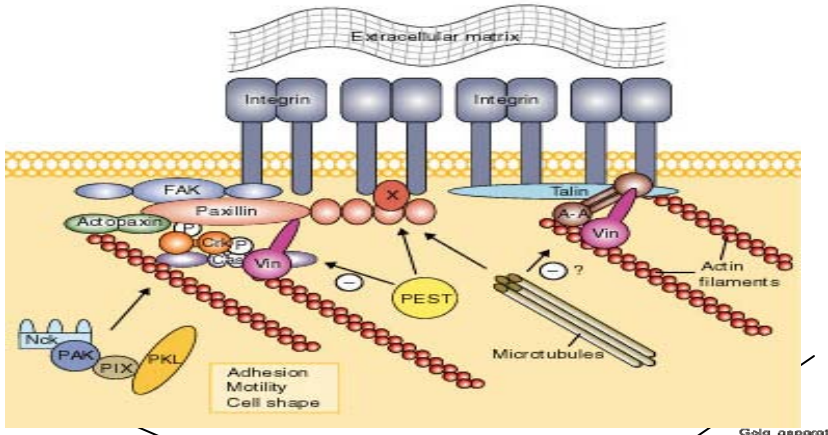
mutFAK-PAX cell shows no cross-correlation although the cell forms adhesion (endogenous FAK?)

Physical motion

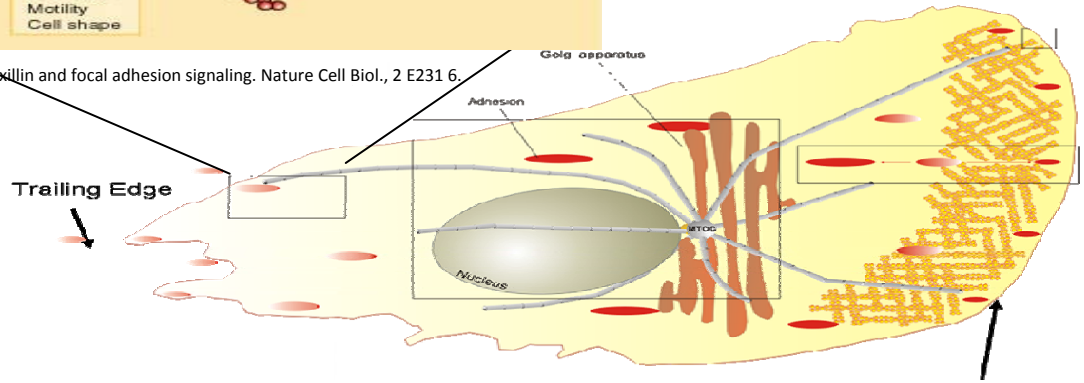
(-) treadmilling (+)







Turner, C.E., (2000). Paxillin and focal adhesion signaling. Nature Cell Biol., 2 E231 6.



Leading Edge

Vicente-Manzanares, M., Webb, D. J., and Horwitz, A. R. (2005) *J. Cell Sci.* **118,** 4917–4919

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].

Acknowledgements

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Cell Migration Consortium