Cross-RICS and Cross-N&B
We have expanded the RICS methods to do Cross-Correlation RICS (ccRICS)
The ccRICS approach

The spatial correlation function

\[ G_{ccRICS}(\xi, \psi) = \frac{\langle I_1(x, y)I_2(x + \xi, y + \psi) \rangle}{\langle I_1(x, y) \rangle \langle I_2(x, y) \rangle} - 1 \]

The variables \( \xi \) and \( \psi \) 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/integrin-actin2.jpg
VIN 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.
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.

1. Diffusion
   Few complexes

2. Fast binding
   Different shape
   Smaller than PSF

3. 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 $G_{cc}/AV(G_1,G_2)$

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

Uncorrelated

Correlated

Intensity fluctuations vs. Frames

Intensity fluctuations vs. Frames

Channel 1 vs. Channel 2

Channel 1 vs. Channel 2
Cross N&B Analysis determines stoichiometry

No Cross-Brightness

Positive Cross-Variance

This example is only for ideal systems where the brightness is calibrated for both channels.
The co-variance principle and the derivation of the ccN&B method

\[ \sigma_{cc}^2 = \frac{\sum (G_i - \langle G \rangle)(R_i - \langle R \rangle)}{K} \]

Definition of co-variance. It is the average of product of the fluctuations in the Green and Red channel

\[ N_{cc} = \frac{\langle G \rangle \langle R \rangle}{\sigma_{cc}^2} \]

Definition of the cross-number of molecules. It is the co-variance divided by the product of the intensity in the two channels

\[ B_{cc} = \frac{K}{\langle G \rangle \times \langle R \rangle} \]

\[ N_{cc(fractionofcross)} = \frac{\sum (G_i - \langle G \rangle)(R_i - \langle R \rangle)}{K} \]

\( K \) is the number of frames, \( \sigma^2 \) the variance and \( <> \) indicates average intensity
How to do the Cross-B

1. Calibrate the Molecular Brightness of each respective channel for the monomers. (Calculate the “S” factor if you are using an analog detector)

   - Theoretical Trimer B = 1.48
   - Theoretical Dimer B = 1.32
   - Monomer B = 1.160
   - $\varepsilon = (B-1)/\text{pixel dwell time (sec)} = 16,000\text{cpm (using 10}\mu\text{s/pix}}$

2. Plot all the possible Brightness of Ch1 and Ch2 on a B1 vs B2 plot:

   - Assign these values to the Monomer Brightness for each respective channel.
How to do the Cross-B Cont’

3. After selecting the B monomer values, calculate the Bcc plot. As seen in CH1 and CH2.

In this case the histogram of the Bcc plot is perfectly symmetrical! There is no Cross-variance!

3. If the plots are asymmetrical, you can plot a histogram of the highest most probable complex these molecules form by “probing” the pixels that deviate from the average histogram on non-coincident brightnesses.

“cutting the symmetrical histogram by eye”
To calibrate the system we need to know the brightness of the monomers

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
What is the stoichiometry of these clusters and is this stoichiometry at Focal Adhesions?

Random

1:2
Vinculin-EGFP and Paxillin-mcherry

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.

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

In small adhesions, smaller clusters come off 1:2.

In larger adhesions, large clusters come off 2:4.

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

FAK and Paxillin

Testing for artifacts: FAK mutant does not form complexes

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

(-) treadmilling  (+)

Focal Adhesion


Additional Reading


Acknowledgements

Michelle Digman
Valeria Vetri
LFD

Rick Horwitz
Paul Wiseman
Rooshin Dahal

University of California Irvine
University of Virginia

The cell migration consortium grant: U54 GM064346