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

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

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 μs/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?
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

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

ccRICKS 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?
Cross N&B
Conceptual illustration of Cross N&B

Uncorrelated

Correlated

Intensity fluctuations

Frames

Intensity fluctuations

Frames

Channel 2

Channel 1

-5 -4 -3 -2 -1 0 1 2 3 4 5

-5 -4 -3 -2 -1 0 1 2 3 4 5

0 2 4 6 8 10 12 14 16 18 20 22 24

0 2 4 6 8 10 12 14 16 18 20 22 24

Channel 1

Channel 2

G

G

R

R

\sum

\sum

Cross variance

Cross variance
Cross N&B Analysis determines stoichiometry

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

1:2

In larger adhesions large cluster 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

Selection map

3:4

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

Focal Adhesion

(-) treading ( +)


Additional Reading


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