Brightness and number cross-variance

Purpose: analyze data in the NB page for cross-variance. Data must be in channel 1 and channel 2.

Definitions

B1  Brightness of channel 1  \( \text{var1/av1} \)
B2  Brightness of channel 2  \( \text{var2/av2} \)
Bcc Cross-brightness variance  \( \text{cross-variance/(av1*av2)} \)
N1  Number of particles channel 1  \( \sqrt{\text{av1}/\text{var1}} \)
N2  Number of particles channel 2  \( \sqrt{\text{av2}/\text{var2}} \)
Ncc Fraction of cross number  \( \text{cross-variance} \)

Where the sum is over the same pixel in each frame

\( \text{av}=\text{sum}\text{(intensity)}/K, \text{ for each channel} \)
\( \text{var}=\text{sum}[\text{sqr(intensity-average)]}/K, \text{ for each channel} \)
\( \text{cross-variance}=\text{sum}[(\text{intensity1-av1)}*(\text{intensity2-av2})]/K \)

K is the number of frames and intensity is the intensity at one pixel of each frame

For this tutorial we are using simulated data obtained by the T-Zone simulation in which the image is divided in spots with different ratio of cross-correlated molecules according to the following table. You can open these files in the RICS page using the file menu entry “open bin (all files)”. Below is the table of the molecules in the various spots of the image according to the simulation.
All molecules have the same brightness. Channel 1 has constant intensity but variable number of cross-correlated molecules. Channel 2 has a variable number of molecules and all of them correlate with a fraction of the molecules in channel 1. The page of SimFCS from the simulation screen is shown above.

Below is the table of the expected intensities, relative brightness and number of molecules for both channels for this simulation as well as the cross-correlated molecules.

<table>
<thead>
<tr>
<th>spot</th>
<th>av1</th>
<th>av2</th>
<th>B1</th>
<th>B2</th>
<th>N1</th>
<th>N2</th>
<th>Ncc</th>
<th>Ncc/N1</th>
<th>Ncc/N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>10</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>20</td>
<td>0.2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>30</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>30</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>40</td>
<td>0.4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>50</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>50</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>60</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>60</td>
<td>0.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

After reading the files (or performing the simulation), in the tools menu select N&B, no detrend and then select channel 1 in the channel selector (shown in red in the figure below) and then analyze channel 2. You should obtain the following images. Note that the scales for channel 2 have been adjusted to account for one pixel with a very large brightness. Also note that the image in the bottom left shows the number of molecules, rather than the intensity as in the previous edition of SimFCS. I have also used a threshold for channel 1 of 48 counts and 4 counts for channel 2. This is due of some particles that get “trapped” in the simulation at the borders of the T-zone.

![Image of simulation screen with analyzed images]
As expected, for channel 1 each spot has the same brightness and the same number of molecules. Note that spot 8 is absent in channel 1, since it only contains molecules detected in channel 2.

Channel 2 has the same brightness for all spots but different number of molecules. Spot 1 is absent in channel 2 since has molecules only detected in channel 1.

So far you have performed to classical NB analysis for the two channels separately. However, the plot of the number of molecules is new in this edition of SimFCS.

**Cross-Brightness analysis**

First we used the selector for the B1-B2 plot to compare the average brightness in both channels. This plot does not provide the cross-variance between molecules.
Note that the scales are not in autoscale. I also used the smooth filter once to better resolve the various spots. In the N&B page you should see the following:

In the upper left you have the B1-B2 plot. Since the brightness in both channels is the same, you have a similar center and distribution for both channels. Note the lines at B2=0 and variable B1 and at B1=0 and variable B2. These lines represent the few particles that get trapped during the simulation. Since they don’t move they have a B=1. Since the selection map is the intensity of channel 1, you cannot select spot 8 since it is only visible in channel 2. Also note the histograms in the right side of the screen. Apparently the average does not correspond to what you see. This is because all pixels, including these with zero value are averaged.

Place the cursor at the center of the image as to select most of the point. Right click and assign this spot to the “monomer” which in this case is the average relative brightness in both channels.
For this simulation all molecules either cross-correlated or not have the same brightness. There is only one molecular species that is cross-correlated and carrying both colors.

After this calibration of the “monomers” select Bcc map in the channel selector and recalculate NB.

In the main RICS page you have the map of Bcc and the two maps of B1 and B2. Note the scales, which are not in autoscale. Note that all the maps in this screen have equal brightness since all particles have the same brightness for this simulation.

The brightness map of B1 or B2 does not distinguish if the molecules carry a fluorophore emitting in both channels. Only the cross-correlation between pixels in the sequence that are bright at the same time (in the same frame in the same pixel) will provide the cross-correlated brightness signal. To perform this analysis, we scan the map to find the correlations. This is done by selecting a given range of Bcc (positive values) and then exploring the B1 plot for the pixels in the B2 plot that have a positive cross-correlation. In the NB page, set up the cursors as shown below.
Check the “use both cursors” flag. You can manually move the cursors and you will observe that for some combinations of the two cursor positions you will have pixel highlighted in the image. To find automatically for each position of B1, what is the position of B2 that highlights more pixels, go to the ccNB page and press “scan Bcc or Ncc ++”. You will see a green line in the left bottom plot.

This line indicates the amount of cross-variance obtained for the selected position of B1. To scan all possible values of B1 press the button “Scan B1-B2 or N1-N2”. The program generates a contour plot for the combination of brightness that has the highest cross-variance. In this case the distribution is narrow and centered around 1:1 since all complexes are identical and have the same brightness in both channels.
The number of coincident pixels is maximal for a brightness ratio of 1:1, which is the expected value for this simulation.

Comment: note that the scan of the coincidences is done using a long cursor in the Bcc axis. Therefore, the dependence of the Bcc on the intensity that arises because of the normalization has no effect in this analysis.

**Ncc analysis**

In the channel selector, select the N1-N2 map and recalculate NB.
In the N1-N2 map of the NB screen place the cursor in the position equal to the maximum number of molecules in the channel that has maximum molecules. This is the value that will be used for calibrating the fraction of cross-correlating molecules.

We can see that N1=12 and N2=6.5 is about the maximum molecules number in both channels, respectively. So we place the cursor at 6.5,12 and the fractions of cross-correlated molecules will be calculated normalized to the maximum number of molecules in both channels. Right click and assign this value to N1-N2.

Now select the Ncc map in the channel selector and recalculate NB.
Change the scales and change the shape and size of the cursor and place the cursors as indicated in the figure above. I also used the smoothing button once to better differentiate the Ncc in the spots.

The scan N1-N2 button in the ccNB page (cross-stoichiometry plot) tell us that cross-variance can be found at N1 in the region 9-13, which in the stoichiometry plot is calibrated to be approximately 0.8 (this value depends on the calibration). To find in the image the locations of these combinations you must use two cursors simultaneously.

For example, the combination of cursors above (large fraction for N1 and large fraction for N2) selects spot 7. By moving the cursor in the Ncc-N2 plot, you can select all the spots, namely where the specific combination of the number of particles with cross-variance are located.