Raster Image Correlation Spectroscopy RICS
We can have a combination of very high time resolution with sufficient spatial resolution.

**Major benefits of RICS:**

- It can be done with **commercial laser scanning microscopes** (either one or two photon systems)

- It can be done with **analog detection**, as well as with photon counting systems, although the characteristic of the detector must be accounted for (time correlations at very short times due to the analog filter)

- RICS provides an intrinsic method to separate the immobile fraction

- It provides a powerful method to distinguish diffusion from binding
Raster Scanning

Pixel time
Start here

Line time + retracing time
Temporal information hidden in the raster-scan image: the RICS approach

Situation 1: slow diffusion

Situation 2: fast diffusion

Spatial correlation

Pixel

Slower diffusion
Faster diffusion
How is the spatial correlation done?

**Operation:**

In the x direction

PLUS In the y direction

\[
(0,0 \times 0,0) + (0,1 \times 0,1) + (0,2 \times 0,2) ... (0,127 \times 0,127) \\
+ (1,0 \times 1,0) + (1,1 \times 1,1) + (1,2 \times 1,2) ... (1,127 \times 1,127)
\]

One number is obtained for x and y and is divided by the average intensity squared.
How to use a stack of images?

Spatially correlate each frame individually then take the average of all the frames.
The RICS approach: 2-D spatial correlations

In a raster-scan image, points are measured at different positions and at different times simultaneously.

If we consider the time sequence, it is not continuous in time.
If we consider the pixel sequence, it is contiguous in space.

In the RICS approach we calculate the 2-D spatial correlation function (similarly to the ICS method of Petersen and Wiseman).

\[
G_{RICS}(\xi, \psi) = \frac{\langle I(x, y)I(x+\xi, y+\psi) \rangle}{\langle I(x, y) \rangle^2}
\]

The variables \(x\) and \(y\) represent spatial increments in the \(x\) and \(y\) directions, respectively.

2-D spatial correlation can be computed very efficiently using FFT methods.

To introduce the “RICS concept” we must account for the relationship between time and position of the scanning laser beam.
The RICS approach for diffusion

The dynamics at a point is independent on the scanning motion of the laser beam

\[ G_{RICS}(\xi, \psi) = S(\xi, \psi) \times G(\xi, \psi) \]

Consider now the process of diffusion. The diffusion kernel can be described by the following expression

\[ P(r, t) = \frac{1}{(4\pi Dt)^{3/2}} \exp\left(-\frac{r^2}{4Dt}\right) \]

There are two parts:
(1) the temporal term,
(2) the spatial Gaussian term

For any diffusion the amplitude decreases as a function of time and the width of the Gaussian increases as a function of time.
At any position, the ACF due to diffusion takes the familiar form:

\[
G(\xi, \psi) = \frac{\gamma}{N} \left(1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{w_0^2}\right)^{-1} \left(1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{w_z^2}\right)^{-1/2}
\]

\(t_p\) and \(t_l\) indicate the pixel time and the line time. The correlation due to the scanner movement is:

\[
S(\xi, \psi) = \exp \left(-\frac{4D(\tau_p \xi + \tau_l \psi)}{(1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{w_0^2})}\right)
\]

Where \(\delta r\) is the pixel size. For \(D=0\) the spatial correlation gives the autocorrelation of the PSF, with an amplitude equal to \(\gamma/N\). As \(D\) increases, the correlation (G term) becomes narrower and the width of the S term increases.

Digman et al. Biophys. J., 2005
RICS Simulations of three different diffusion rates:

Box size = 3.4 µm sampling time: 1) 32 µs/pixel  2) 8 µs/pixel  3) 4 µs/pixel

- **D = 0.1 µm²/s**
  - (membrane proteins)

- **D = 5.0 µm²/s**
  - (40 nm beads)

- **D = 90 µm²/s**
  - (EGFP)
Horizontal and Vertical fits:
Simulations of beads 300 frames, 128x128 pixels, 8μs/pix, size of pixels=30nm

Horizontal ACF

Vertical ACF

In SIMFCS

Brown et al, JMI, 2007
How to Setup the Laser Scanning Confocal Microscope

- **Scan Speeds (μs/pixel):**
  - 4μs for fast molecules \( D > 100 \mu m^2/s \)
  - 8 - 32μs for slower molecules \( D = 1 \mu m^2/s - 100 \mu m^2/s \)
  - 32-100μs for slower molecules \( D = 0.1 \mu m^2/s - 10 \mu m^2/s \)

- **Pixel Size:**
  - 3-4x smaller than the Point Spread Function (PSF ≈ 300nm)

- **Molecular Concentrations**
  - Same conditions as conventional FCS methods
Common Errors in RICS

Scanning Too Slow
(100 us/pixel, D = 300 μm²/s)

Pixels are separated too much relative to PSF
(pixel size = w₀ = 0.3 μm)

Courtesy of Jay Unruh
RICS: Fits to spatial correlation functions
Olympus Fluoview300 LSM

EGFP in solution

Spatia ACFS

128x128, 4 μs/pixel, 5.4 ms/line, 0.023 μm/pixel

Fit to Spatial ACF

D = 105 ± 10 μm²/s

Digman et al. Biophys. J., 2005
What ROI size to use? How many frames to acquire?

100nM mEGFP

**g(0) average**

<table>
<thead>
<tr>
<th>ROI size</th>
<th>256</th>
<th>128</th>
<th>64</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 frames</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 frame</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D (μm²/s)**

<table>
<thead>
<tr>
<th>Number of Frames Analyzed</th>
<th>100</th>
<th>50</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>256x256</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128x128</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64x64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32x32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Brown et al, JMI, 2007
Obtaining concentration from RICS

Fluorescein in 100mM TRIS pH 9

\[ y = 0.86x + 7.1 \]

\[ R^2 = 0.9961 \]

Brown et al, JMI, 2007
How we go from solutions to cells?

In cells we have an immobile fraction

The 2-D-spatial correlation of an image containing immobile features has a very strong correlation pattern

We need to separate this immobile fraction from the mobile part before calculating the transform

How is this achieved?
Does noise from the detectors correlate?

In a “truly immobile” bright region, the intensity fluctuates according to the Poisson distribution due to shot noise.

The time correlation of the shot noise is zero, except at time zero.

The spatial correlation of the intensity at any two pixels due to shot noise is zero, even if the two points are within the PSF.

If we subtract the average intensity and disregard the zero time-space point, the immobile bright region totally disappear from the correlation function.

**Attention!!!!**

This is not true for analog detection, not even in the first order approximation. For analog detection the shot noise is time (and space) correlated.
Formula used to subtract background:

Average intensity of each pixel on the overall stack: $\bar{I}(x, y)$

The intensity of each pixel minus the average intensity from entire stack for each pixel: However, this yields negative values.

A scalar must be added: $a = \bar{I}$

$ICS(F_i(x, y))$ where $F_i(x, y) = I_i(x, y) - \bar{I}(x, y) + a$
How to subtract immobile features from images?
Immobile feature

Average of the image including the immobile part

Average of the “sea of molecules” only

Intensity before removal

Intensity after removal
Subtraction of moving average

Start the analysis

End of the analysis

Frames

Intensity

0 10 20 30 40 50 60
Moving average operation on frames:

Frame #5

Matrix 1

- Average between 1-10

Matrix 2

A scalar average is then added

Operation is repeated for frame #6 - average between 2-11
frame #7 - average between 3-12
Example of the Removal of Immobile Structures and Slow Moving Features

Spatial ACF
No removal

Spatial ACF
With removal

What is left after removal

Fit using 3-D diffusion formula

Pixel size = 0.092 μm
Pixel time = 8 μs
Line time = 3.152 ms
Wo = 0.35 μm

G1(0) = 0.0062
D1 = 7.4 μm²/s
G2(0) = 0.00023
D2 = 0.54 μm²/s
Bkgd = -0.00115
Conclusions

Techniques | Time Res. | Spatial Res. | Used to Study
--- | --- | --- | ---
Temporal-ICM | sec | <0.5 μm | Protein aggregates, Transmembrane proteins
RICS | μsec-msec | ~2 μm | Soluble proteins, Binding interactions
Line-RICS | msec | <0.5 μm | Soluble proteins, Binding interactions

G(τ) vs. τ (s) graph showing different protein types: Cytosolic Protein, Transmembrane Protein, Large Protein Aggregates, Small Molecule.
Summary

- Measures dynamic rates from the μsec–msec time scale
- Anyone with a commercially available instrument can use it
- Immobile structures can be filtered out and fast fluctuations can be detected
- RICS has high spatial and temporal resolution
- The range of these dynamic rates covers a wide range from immobile to cytosolic diffusions (0.2–12um2/s)
- Other types of processes and interactions are also measured
- Line scanning is essential for determination of binding process and complements the RICS analysis