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 different characteristics of the detector (analog filter) must be accounted for
- RICS provides an intrinsic method to quantify the immobile fraction
- It provides a powerful method to distinguish diffusion from binding

**How does it work?**
Raster Scanning

- Pixel time
- Line time + retracing time

Start here
Temporal information hidden in the raster-scan image: the RICS approach

Situation 1: slow diffusion

Situation 2: fast diffusion

Spatial correlation vs. Pixel

- Slower diffusion
- Faster diffusion
How is the spatial correlation done?

**Operation:**

In the x direction

PLUS In the y direction

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

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.

FRAME #1

FRAME #100
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 \( \xi \) and \( \psi \) 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 fast diffusions the amplitude decreases as a function of time and the width of the Gaussian increases as a function of time
RICS: space and time relationships

At any position, the ACF due to diffusion takes the familiar form:

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

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

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

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

\[ D = 0.1 \, \mu m^2/s \] (membrane proteins)

\[ D = 5.0 \, \mu m^2/s \] (40 nm beads)

\[ D = 90 \, \mu m^2/s \] (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   \( D > 100\mu\text{m}^2/\text{s} \)
  - 8 - 32μs for slower molecules   \( D = 1 \mu\text{m}^2/\text{s} - 100\mu\text{m}^2/\text{s} \)
  - 32 - 100μs for slower molecules   \( D = 0.1 \mu\text{m}^2/\text{s} - 10\mu\text{m}^2/\text{s} \)

- **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 um²/s)

Pixels are separated too much relative to PSF
(pixel size = \( w_0 = 0.3 \) um)

Courtesy of Jay Unruh
RICS: Fits to spatial correlation functions
Olympus Fluoview 300 LSM

EGFP in solution

Spatial ACF

Fit to Spatial ACF

D = 105 ± 10 \( \mu \text{m}^2/\text{s} \)

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

100nM mEGFP

**g(0) average**

- 256 frames
- 100 frames
- 75 frames
- 50 frames
- 25 frames
- 10 frames
- 1 frame

**ROI size**

- 256x256
- 128x128
- 64x64
- 32x32

**D (μm²/s)**

- 100 frames
- 50 frames
- 10 frames
- 5 frames

Brown et al, JMI, 2007
Fluorescein in 100mM TRIS pH 9

y = 0.8609x + 7.1603

$R^2 = 0.9961$

Obtaining concentration from RICS

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?
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: $I(x, y)$

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 $F_i(x, y) = I_i(x, y) - \bar{I}(x, y) + a$
Average of the “sea of molecules” only

Average of the image including the immobile part

Immobile feature

Intensity before removal

Intensity after removal

Intensity before removal

line
Moving average
Subtraction of moving average

Start the analysis

End of the analysis

Describe how many frames to use for subtracting moving average
Example of the Removal of Immobile Structures and Slow Moving Features

What is left after removal

Spatial ACF
No removal

Spatial ACF
With removal

Fit using 3-D diffusion formula

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pixel size</td>
<td>0.092 μm</td>
</tr>
<tr>
<td>Pixel time</td>
<td>8 μs</td>
</tr>
<tr>
<td>Line time</td>
<td>3.152 ms</td>
</tr>
<tr>
<td>Wo</td>
<td>0.35 μm</td>
</tr>
</tbody>
</table>

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

FCS
Temporal ICM
RICS
RICs
Line-RICS

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Time Res.</th>
<th>Spatial Res.</th>
<th>Used to Study</th>
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</thead>
<tbody>
<tr>
<td>FCS</td>
<td>Temporal correlation</td>
<td>0.5 μm</td>
<td>Protein aggregates, Transmembrane proteins</td>
</tr>
<tr>
<td>Temporal ICM</td>
<td>μsec-msec</td>
<td>~2 μm</td>
<td>Soluble proteins, Binding interactions</td>
</tr>
<tr>
<td>RICS</td>
<td>μsec-msec</td>
<td>&lt;0.5 μm</td>
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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