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 statistics 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
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
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.
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.
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:

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

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=3.4 µm sampling time: 1) 32 µs/pixel  2) 8 µs/pixel  3) 4 µs/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)
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, $D = 300 \text{ \textmu m}^2/\text{s}$)

Pixels are separated too much relative to PSF
(pixel size $= w_0 = 0.3 \text{ \textmu m}$)

Courtesy of Jay Unruh
RICS: Fits to spatial correlation functions

Olympus Fluoview300 LSM

EGFP in solution

Spatial ACF

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
RICS: G(0) calibrations

100nM mEGFP, 10X, Medium Scan

Fluorescein in 100mM TRIS pH 9

\[ y = 0.8609x + 7.1603 \]

\[ R^2 = 0.9961 \]
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?
Formula used to subtract background:

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

\[
I_{i(x,y)} - \bar{I}_{(x,y)}
\]

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: \( a = \bar{I}_{(x,y)} \)

\[
ICS \left( F_i(x,y) \right) = I_{i(x,y)} - \bar{I}_{(x,y)} + a
\]
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.
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

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
Using RICS to answer biological questions

Simultaneous Cell imaging:
ChoK1 cells expressing Paxillin-EGFP
256x256 pixel (35.5 μm), 32 μs/pixel, 10.4 ms/line, ω₀ = 0.42, total run time 5.2 min
Procedures for processing images

Pixel size = 0.142 um
Pixel time = 32us
Line time = 10.4 ms
Wo (in um) = 0.46
G(0) = 0.038
D = 0.21 um^2/s
Map of paxillin-EGFP diffusion rate

- D = 10.2 μm²/s
- D = 1.09 μm²/s
- D = 0.20 μm²/s
**G(0) and Diffusion Rates Maps of Pax-EGFP**

256x256 pixel (47.1 μm), 8 μs/pixel, 3.1 ms/line, $\omega_0 = 0.36$, total run time = 2.56 min

Fig. 1
Average Intensity image

Fig. 2
G(0) map

Fig. 3
Diffusion map (μm²/s)
Line Scanning Along the Adhesions

Fast Dynamics in the Cytosol

Slow Dynamics in adhesions

D (μm²/s)

3.6 3.9 3.1 0.7

Based on D EGFP cytosol: D = 0.1 μm²/s: MW ~ 3x10^8, D = 0.01 μm²/s: MW ~ 3x10^{11}
Are we measuring diffusion or binding?

Using the Olympus Fluoview™ 300 LSM

For pure “binding equilibria” the function $G(\xi, \psi)$ assumes a different expression.

Fit (black line) of pixel 190 data (red line) of the line scan experiment using:

A) Diffusion equation $D=0.032 \mu m^2/s$

B) Exponential relaxation $\tau=0.63$ s.

The residues (blue lines) of the exponential fit are smaller and less correlated indicating a better fit using the exponential model.
Are the adhesions static? Our data show that multiple dynamic events occur on the order of minutes without disruption of the adhesion.
Conclusions

Table: Techniques and Spatial/Temporal Resolution

<table>
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<th>Techniques</th>
<th>Time Res.</th>
<th>Spatial Res.</th>
<th>Used to Study</th>
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</thead>
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<tr>
<td>FCS</td>
<td>10 μsec</td>
<td>&lt;0.5 μm</td>
<td>Protein aggregates</td>
</tr>
<tr>
<td>Temporal ICM</td>
<td>10-3 sec</td>
<td>~2 μm</td>
<td>Transmembrane proteins</td>
</tr>
<tr>
<td>RICS</td>
<td>μsec-msec</td>
<td>&lt;0.5 μm</td>
<td>Soluble proteins, Binding interactions</td>
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<tr>
<td>Line-RICS</td>
<td>msec</td>
<td>&lt;0.5 μm</td>
<td>Soluble proteins, Binding interactions</td>
</tr>
</tbody>
</table>

Graph: G(τ) vs. τ (s)

Small Molecule
Cytosolic Protein
Transmembrane Protein
Large Protein Aggregates

Line Scan
μsec
msec
T-ICM
Sec to min

Pixel
μsec
msec

RICS

RICS

T-ICM
Sec to min

T-ICM
Sec to min

Graph: G(τ) vs. τ (s)

Small Molecule
Cytosolic Protein
Transmembrane Protein
Large Protein Aggregates

Discussion:

1. **Small Molecules vs. Large Proteins**
   - Small molecules exhibit rapid and direct binding interactions.
   - Large protein aggregates and transmembrane proteins show slower dynamics.

2. **Temporal ICM vs. Line RICS**
   - Temporal ICM is sensitive to protein aggregates and transmembrane proteins.
   - Line RICS is useful for studying soluble proteins and their binding interactions.

3. **Spatial Resolution**
   - Techniques like FCS and RICS can resolve sub-micron structures.
   - Line RICS offers higher temporal resolution but may sacrifice spatial resolution.

4. **Temporal Correlation**
   - The graph illustrates the decay of correlation functions G(τ) for different components.

5. **Applications**
   - **Protein Aggregates**
     - Study of aggregation processes and kinetics.
   - **Transmembrane Proteins**
     - Examination of interactions with the cell membrane.
   - **Soluble Proteins**
     - Analysis of dynamic interactions in solution.
   - **Binding Interactions**
     - Measurement of association and dissociation rates.

Conclusion:

The combination of FCS, Temporal ICM, RICS, and Line RICS provides a comprehensive toolkit for studying protein dynamics, from temporal correlation to spatial resolution, allowing for a detailed understanding of molecular interactions in biological systems.
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