Raster Image Correlation Spectroscopy
RICS

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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.

How does it work?
Raster Scanning

Pixel time
Start here

Line time + retracing time

LSM image contains time structure.
Situation 1: slow diffusion

Situation 2: fast diffusion

Using Temporal Information Hidden in the Raster-Scan Image: The RICS Approach
### How is the Spatial Correlation Calculated?

This operation has to be repeated for all possible combinations of pixel and line shifts:

<table>
<thead>
<tr>
<th>$\xi$</th>
<th>$\psi$</th>
<th>Pixel and Line Shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>$\tau = \tau_p$</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>$\tau = 2 \cdot \tau_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\vdots$</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>$\tau = \tau_l$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\vdots$</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>$\tau = 2 \cdot \tau_p + \tau_l$</td>
</tr>
</tbody>
</table>
How is the Spatial Correlation Calculated?

Operation:

\[
\begin{align*}
(0,0) & \quad \text{Image 1} \\
(0,256) & \quad \text{Matrix 1} \\
(x,0+y) & \quad \text{Image 1 shifted} \\
(0,256) & \quad \text{Matrix 2}
\end{align*}
\]

Shift in the x and y direction
\[
(0,0 \times 0,0) + (0,1 \times 0,1) + (0,2 \times 0,2) \ldots (0,127 \times 0,127) \\
+ (1,0 \times 1,0) + (1,1 \times 1,1) + (1,2 \times 1,2) \ldots (1,127 \times 1,127) \\
+ \ldots
\]

One number is obtained for each pixel delay normalized 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 **pixel sequence**, it is **contiguous** in space.
  - If we consider the **time sequence**, it is **not continuous** in time.

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

\[
G_{RICS}(\xi, \psi) = \frac{< I(x, y)I(x + \xi, y + \psi) >}{< I(x, y) >^2} - 1
\]

- 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 use the “RICS concept” we **must account for the relationship between time and position of the scanning laser beam**.
The RICS Approach for Diffusion

• The dynamic at a point is independent on the scanning motion \( S \) 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 value 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:

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

- 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

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, 128 x 128 pixels, 8 µs/px, pixel size 30 nm

Horizontal ACF

![Horizontal ACF graph with data points for different D values (D = 100, 10, 1, 0.1).](image)

Vertical ACF

![Vertical ACF graph with data points for different D values (D = 100, 10, 1, 0.1).](image)

Done using SimFCS.

Brown et al, JMI, 2007
Scan Speeds ($\mu$s/pixel):

- 4 $\mu$s for fast molecules ($D > 100 \, \mu m^2/s$).
- 8 – 32 $\mu$s for slower molecules ($D = 1 \, \mu m^2/s – 100 \, \mu m^2/s$).
- 32 – 100 $\mu$s 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 $\leq$ 300nm).

Molecular Concentrations

Same conditions as conventional FCS methods.
Common Mistakes in RICS

Scanning speed too slow (100 $\mu$s/pixel, $D = 300 \, \mu$m$^2$/s)

Pixels are separated too much compared to the PSF (pixel size = $w_0 = 0.3 \, \mu$m)
RICS: Fits to Spatial Correlation Functions

Olympus Fluoview300 LSM

EGFP in solution

Spatial ACF

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

Fit to Spatial ACF

\[ D = 105 \pm 10 \, \mu m^2/s \]

Digman et al. Biophys. J., 2005
What ROI Size to Use? How Many Frames to Acquire?

### 100nM mEGFP

- **Graph 1:**
  - Y-axis: \( g(0) \) average
  - X-axis: ROI size
  - Data points for 500 frames, 100 frames, 75 frames, 50 frames, 25 frames, 10 frames, and 1 frame.

- **Graph 2:**
  - Y-axis: \( D (\mu m^2/s) \)
  - X-axis: Number of Frames Analyzed
  - Data points for 100 frames, 50 frames, 10 frames, and 5 frames.

*Brown et al, JMI, 2007*
Obtaining Concentrations From RICS

Fluorescein in 100 mM TRIS pH 9

\[ y = 0.86x + 7.1 \]

\[ R^2 = 0.996 \]

Brown et al, JMI, 2007
How Do 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 disappears 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.
• Average intensity of each pixel on the overall stack: \( \overline{I(x, y)} \)

• The intensity of each pixel minus the average intensity from entire stack for each pixel:

\[
I_i(x, y) - \overline{I(x, y)}
\]

• However, this yields negative values. A scalar must be added: 

\[
a = \overline{I}
\]

\[
RICS(F_i(x, y)) \quad \text{where} \quad F_i(x, y) = I_i(x, y) - \overline{I(x, y)} + a
\]
Subtraction of a Moving Average

Problem: Bleaching during image acquisition

![Graph showing intensity over frames with start and end of analysis marked.](image)
Moving Average Operation on Frames:

Frame #5

Average between 1-10

Matrix 1 - Matrix 2 = A scalar average is then added

Operation is repeated for frame #6 - average between frames 2-11
frame #7 - average between frames 3-12
...

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 = 3.15 ms
w₀ = 0.35 μm

G₁(0) = 0.0062
D₁ = 7.4 μm²/s
G₂(0) = 0.00023
D₂ = 0.54 μm²/s
Bkg = -0.0012
Cell Migration

Cell migration is important for:

- Embryonic development
- Wound healing
- Immune responses
- Cancer metastasis

Lack of regulation of cell migration may lead to:

- Vascular disease
- Congenital brain defects
- Chronic inflammatory disease
- Tumor formation
- Metastasis

MEF cells expressing the Raichu biosensor 1 frame/30s. Total run time: 48 min.
Cell Migration and Paxillin Function


Confocal Cell Imaging: CHO-K1 Cells Expressing Paxillin-EGFP

256 x 256 pixel (35.5 µm), 32 µs/pixel, 10.4 ms/line, $\omega_0 = 0.42$, total run time 5.2 min.
Map of paxillin-EGFP diffusion rate

D = 10.2 \mu m^2/s
D = 1.09 \mu m^2/s
D = 0.20 \mu m^2/s
RICS analysis using auto-scan of a small area

Spatial maps of the apparent diffusion coefficient of paxillin across the cell.

With diffraction limited microscopy, the spatial resolution is limited to ~2 µm.
Optical Resolution

\[ |\vec{k}_0| = \frac{2\pi}{\lambda} \]

\[ \delta k_r = \frac{2}{\lambda} \]

\[ \delta r_{\text{min}} = \frac{\lambda}{2 \cdot n \cdot \sin \alpha} \]

lateral ~200 nm

axial ~500 nm
STED Principle

STED relies on fluorophore switching:

dark

\[ k_{\text{on}} \]

\[ k_{\text{off}} \]

bright
**STED Principle**

**Stimulated emission:**

Excitation:

Spontaneous emission:

Stimulated emission:

- $k_{on}$
- $k_{off}$
- $S_0$
- $S_1$
STED Principle

- Excitation
- Depletion
- Effective excitation

\[ \lambda_{\text{exc}} \quad \lambda_{\text{STED}} \]

200 nm

STED power
STED Setup

excitation 640 nm
STED Principle

Excitation at 640 nm

STED at 760 nm

Intensity vs. distance (nm)

$\lambda/4$

$\pi$
\[
\delta r_{\text{min}} = \frac{\lambda}{2 \cdot n \cdot \sin \alpha} \cdot \frac{1}{s}
\]

\[
s = \sqrt{1 + \frac{I_{\text{STED}}}{I_{\text{sat}}}}
\]
β-Tubulin-Alexa594 in Fixed HeLa Cells

Application of STED to RICS

confocal

STED

membrane

Tunable observation volume

STED power

RICS

5 µm scan

correlation

G(ξ, ψ)
Lipid bilayer labeled with Atto647N-DPPE:

Hedde et al., Nature Commun. 4 (2013) 2093
Atto647N-DPPE in the plasma membrane of XTC cells:

Hedde et al., Nature Commun. 4 (2013) 2093
Atto647N-DPPE in the plasma membrane of XTC cells:

Hedde et al., Nature Commun. 4 (2013) 2093
Conclusions

Temporal-ICM

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