Raster Image Correlation Spectroscopy
RICS

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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

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
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.
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).

$$G_{RICS}(\xi,\psi) = \frac{\langle I(x,y)I(x+\xi,y+\psi) \rangle}{\langle I(x,y) \rangle^2} - 1$$

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

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

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.

**FAST**

**SLOW**
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:

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

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

In 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~300nm)

Molecular Concentrations

- Same conditions as conventional FCS methods
Common Errors in RICS

Scanning Too Slow
(100 us/pixel, $D = 300 \text{ um}^2/\text{s}$)

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

Courtesy of Jay Unruh
EGFP in solution

Spatial ACF

DICM: Fits to spatial correlation functions

Olympus Fluoview300 LSM

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

D = 105 ± 10 µm²/s

Fit to Spatial ACF

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

100nM mEGFP

D (µm²/s)

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?
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: \( I(x, y) \)

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

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: \( a = \overline{I} \)

\[ ICS(F_i(x, y)) \quad \text{where} \quad F_i(x, y) = I_i(x, y) - \overline{I(x, y)} + a \]
How to subtract immobile features from images?
Average of the "sea of molecules" only

Average of the image including the immobile part

Immobile feature

Intensity before removal

Intensity after removal
Subtraction of moving average

Start the analysis

End of the analysis
Moving average operation on frames:

Frame #5

Matrix 1 - Matrix 2 =

Average between 1-10

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

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
Conclusions

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Summary of RICS

- Measures dynamic rates from the μsec-sec 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 (0.001-300um²/s)
- Other types of processes and interactions are also measured
- Line scanning is essential for determination of binding process and complements the RICS analysis
The Number & Molecular Brightness (N&B) Method

Laboratory for Fluorescence Dynamics
University of California, Irvine
Existing Methods to determine protein concentration and aggregation of proteins in cells

1. Calibration of the free fluorophore based on intensity

However, it doesn’t give you the size distribution. Only concentration is given.

If “free” EGFP at 10nM gave 30,000 counts/sec then the conclusion would be that:

- A = 10nM
- B = 30nM

Average intensity of MEF cells expressing Paxillin-EGFP

INTENSITY
31,250 counts/sec
93,750 counts/sec
This method is very sensitive to detect the formation of pairs.
3. Image correlation Spectroscopy (ICS)

However, the events must be slow >1sec (no movement during one frame) and the aggregates must be large.

Petersen and Wiseman: Biophys J. 1999
The Number and Brightness (N&B) analysis

Purpose: Provide a pixel resolution map of molecular number and aggregation in cells

Method: First and second moment of the fluorescence intensity distribution at each pixel

Source: Raster scanned image obtained with laser scanning microscopes TIRF with fast cameras Spinning disk confocal microscope

Output: The N and B maps, B vs intensity 2D histogram

Tools: Cursor selection of pixel with similar brightness Quantitative analysis of center and std dev of the e and n distribution Tools for calibration of analog detectors

Tutorials: mathematical background, data import, analysis examples (our web site)
How to distinguish pixels with many dim molecules from pixels with few bright molecules?

- Given two series of equal average, the larger is the variance, the less molecules contribute to the average. The ratio of the square of the average intensity ($<k>^2$) to the variance ($\sigma^2$) is proportional to the average number of particles $<N>$.

\[
G(0) = \frac{\sigma^2}{<k>^2} = \frac{1}{N}
\]

* Originally developed by Qian and Elson (1990) for solution measurements.
Calculating protein aggregates from images

This analysis provides a map of \( <N> \) and brightness (B) for every pixel in the image. The units of brightness are related to the pixel dwell time and they are “counts/dwell time/molecule”.

\[
<k> = \frac{\sum k_i}{K}
\]

\[
\sigma^2 = \frac{\sum (k_i - <k>)^2}{K}
\]

\[
B = \frac{<k>}{<N>} = \frac{\sigma^2}{<k>}
\]

\[
<N> = \frac{<k>^2}{\sigma^2}
\]

\( \sigma^2 \) = Variance
\( <k>\) = Average counts
\( N \) = Apparent number of molecules
\( B \) = Apparent molecular brightness
\( K \) = # of frames analyzed
To increase the apparent brightness we could increase the dwell time, since the brightness is measured in counts/dwell time/molecule.

Increasing the dwell time decreases the amplitude of the fluctuation.
What contributes to the variance?

Variance due to particle number fluctuations

$$\sigma_n^2 = \varepsilon^2 n$$

Variance due to detector shot noise

$$\sigma_d^2 = \varepsilon n$$

The measured variance contains two terms, the variance due to the particle number fluctuation and the variance due to the detector count statistics. These two terms have different dependence on the number of molecules. We can invert the equations and obtain $n$ and $\varepsilon$.

Both depend on the intrinsic brightness and the number of molecules. $n$ is the true number of molecules, and $\varepsilon$ is the true molecular brightness.
How to Calculate \( n \) and \( \varepsilon \)

\[
B = \frac{\sigma^2}{\langle k \rangle} = \frac{\sigma_n^2}{\langle k \rangle} + \frac{\sigma_d^2}{\langle k \rangle} = \frac{\varepsilon^2 n}{\varepsilon n} + \frac{\sigma_d^2}{\langle k \rangle} = \varepsilon + 1
\]

This ratio identifies pixels of different brightness due to mobile particles.

The “true” number of molecules \( n \) and the “true” molecular brightness for mobile particles can be obtained from

\[
n = \frac{\langle k \rangle^2}{\sigma^2 - \langle k \rangle}
\]

\[
\varepsilon = \frac{\sigma^2 - \langle k \rangle}{\langle k \rangle}
\]

If there are regions of immobile particles, \( n \) cannot be calculated because for the immobile fraction the variance is \( = \langle k \rangle \). For this reason, several plots are offered to help the operator to identify regions of mobile and immobile particles. Particularly useful is the plot of \( N \) vs \( B \).
Quadratic dependence of the variance on particle brightness
20nM EGFP in solution as a function of laser power

\[ y = 4.4321x^2 + 0.9438x \]

\[ \sigma_n^2 = \varepsilon^2 n \]

2-photon excitation using photon counting detection
If we change the laser power, a plot of the ratio variance/intensity vs intensity can distinguish the mobile from immobile fraction. The two curves are for different pixel integration times.
The effect of the immobile part: with photon counting detectors

Fluorescent beads in a sea of 100nM Fluorescein.
Brightness and number of molecules can be measured independently.

- **2D histogram Brightness vs laser intensity**
  - Brightness (counts/s/molecule) vs Laser intensity
  - Color scale: 0 to 2

- **Brightness vs concentration**
  - Brightness (counts/s/molecule) vs Concentration (nM)
  - Concentration range: 0 to 200 nM

- **Number of particles vs concentration**
  - Number of particles (n) vs Concentration (nM)
  - Concentration range: 0 to 200 nM

**EGFP in solution**

- Brightness and number of molecules can be measured independently.
What are the parameters for analog systems?
Additional considerations with analog detection systems:

- digital levels are recorded (instead of photon counts)
- an offset is typically present
- additional detector variance at low currents

\[
S = \frac{<N>}{\sigma_0^2}
\]

If we fix the PMT settings (voltage and gain), then \(S\) and \(\sigma_0^2\) should not change.
Detector characterization
Analog detector response (dark current)
Detector characterization
Photon counting detector response (dark current)
Solution experiments: using analog detectors

Recovery of $n$ and $\varepsilon$ in the analog system for 20nM EGFP in solution

In the analog system, the recovery of relative values is good, for absolute values the calibration is more problematic. The best obtained so far is within a factor of 2.
EGFP in CHO-k1 (1-Photon LSM)
homogenous Brightness & heterogeneous Number of Molecules
Summary of N&B

- N&B distinguishes between number of molecules and molecular brightness in the same pixel.

- The acquisition for the N&B can be done with a commercial Laser Scanning Microscope (LSM) and the same data used for RICS can be used to map N and B.

- The Immobile fraction can be separated since it has a Brightness value =1.

- The N&B analysis of paxillin at adhesions shows large aggregates of protein during disassembly.


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