The RICS method: Measurement of fast dynamics in cells with the laser scanning microscope

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Abstract

Single point fluctuation correlation spectroscopy (FCS) is an established technique to study diffusion and chemical equilibria in solution. It has limitations when applied to the cell interior. A major difficulty is that the movements of the entire cell or of cellular components are difficult to separate and filter out from the fast dynamics of the molecules moving in the cell. It is the study of these fast dynamics that helps us in understanding molecular interactions. Scanning FCS, in which the laser beam is moved in a circular orbit, provides the fluctuation amplitude and dynamics at many points simultaneously. It can be
used to infer cell movement, but has limitations in the time scales accessible. Image correlation spectroscopy, an alternative technique in which the entire field of view is analyzed at once, has the potential to provide detailed maps of the dynamics in a cell, but it suffers from limitations imposed by the relatively slow frame rate. We have developed an approach that exploits the intrinsic time structure of a confocal raster scan image to determine spatiotemporal correlations at several time scales simultaneously. Using this approach, termed RICS (Raster-scan Image Correlation Spectroscopy), we can distinguish diffusion of molecules in the cellular interior, binding processes and cellular and organelle movement. Here we present the basis of RICS, the mathematical framework for extracting quantitative information and examples of applications to the dynamics of focal adhesions in live cells. Finally, we show that raster-scan images obtained using a commercial laser confocal microscope are of sufficient quality to extract diffusion constants of small molecules in solutions and in cells. A map of diffusion constants and rates of binding can also be generated.

**Introduction**

As we advance our knowledge about cellular components and structures, the need arises for methods that can detect molecular interactions across the entire cell. These interactions are manifested by assembling and disassembling of structures, transport and control of the level of expression of proteins in different parts of the cell. In addition, ion concentrations fluctuate due to cellular activity. All these processes occur in a wide range of time scales. Fluorescence microscopy is particularly useful for the study of the dynamics of the cellular interior. The appeal of fluorescence microscopy is in the molecular sensitivity and the very high time and spatial resolution that this technique can provide as well as in the non-invasive nature of the method. Recent advances in optical design and light detectors have pushed the limit of sensitivity to the level of single molecule observation inside cells. The introduction of new optical methods such as laser scanning confocal microscopy and multiphoton microscopy has resulted in rapid advances in our understanding of biological processes. A common challenge of all methods aimed at the study of cellular interior is in the nature of the optical signal. The fluorescence emitted at one pixel rapidly fluctuates due changes in concentration of fluorescent molecules at any given location and to molecular interactions. These rapid fluctuations are intrinsic to the signal and they carry the information about the molecular dynamics at a given location of the cell. These fluctuations superimpose on the intrinsic noise from the physical processes of light emission and detector statistics. The brighter the probe, the less the effect of the detector noise and the statistics of photon emission on the signal.
As a consequence of the fluctuating nature of the signal, during the past decade a number of methods were developed to analyze systems made of few molecules [1-19]. The methods were originally developed for the study of reaction kinetics in homogeneous systems and they were gradually adapted to the study of cells. In contrast to homogeneous solutions, the study of the cellular interior requires the acquisition of fluctuation data at several points of the sample; ideally over the entire cell.

In this contribution, we review some of the current statistical methods used to measure the dynamics at several points in the cell and we describe the newest methodologies in this field. In particular, we describe the RICS (raster-scan image correlation spectroscopy) technique because of the potential wide application of this technique in the cellular biology community. This technique can be implemented in commercial confocal microscopes that are commonly available in many cellular biology laboratories.

A closer look at existing techniques

Measurements of fast dynamics in the cellular interior started with the work of Berland et al in 1995 [20] when fluctuation correlation spectroscopy (FCS) was used to measure the diffusion of molecules in the cellular interior. Since then, many researchers applied FCS to study a variety of cellular

![Conventional FCS](image)

**Time resolution: μsec-msec**

**Figure 1.** Single point FCS has very high time and spatial resolution but only measures one point in the cell at a time. Processes such as the motion of the entire cell, the passage in the field of observation of large cellular components such as vesicles complicates the interpretation of the results.
systems [2-19]. The availability of fluorescent proteins also contributed to the rapid advance of the field [9]. A common result of all these studies was that the diffusion of small proteins in the cell was slower than the diffusion of the same molecule in buffer by approximately a factor of 3 to 5. The slower diffusion was attributed to an increase of viscosity in the cell’s interior with respect to the buffer condition. However, other interpretations of this general result were not excluded. For example, weak non-specific interactions of the small molecule with the surrounding cellular matrix could also cause restrictions on the rate of motion. It was also observed that different locations in the cell displayed different diffusion rates for the same molecule [21]. In some cases, the rate of diffusion changed by factors of 2 to 5, suggesting the change in internal viscosity alone could not explain the decrease of the diffusion [21]. Furthermore, other processes such as motion of the entire cell and local bleaching complicated the interpretation of the results.

**Single-point FCS**

FCS was first described in the 1970's and was used to characterize chemical reactions using fluorescent molecules in solution. It is a non-perturbing method used to gain molecular information to study particle diffusion, chemical kinetics, hybridization reactions and molecular aggregation [22-24]. In a typical FCS measurement, a laser beam is focused to a diffraction limited spot into a cuvette containing fluorescent molecules (Figure 2) and the fluctuations in the fluorescence intensity as molecules undergo Brownian motion moving in and out of the focused laser beam are recorded [25,26]. Fluorescence fluctuations can originate from the diffusion of the fluorescent molecules through the observing volume (sub-femtoliters), or from changes of fluorescence quantum yield of the fluorophore due to chemical reactions. Fluorescence intensity fluctuations can be mathematically analyzed and two important values can be derived: (i) the number of molecules in the excitation volume and (ii) the characteristic time of the fluctuation. In the case if molecules freely diffusing in the excitation volume, this characteristic time can be related to the diffusion coefficient [27]. The changes in the diffusion coefficient of a particle in solution reflect the change of its size (or shape) or the viscosity of the solution. Changes in the number of particles detected in the excitation volume suggest particle association or dissociation. [20,21,28].

The mathematical basis for the analysis of the fluorescence fluctuation is based on the calculation of correlation functions (Figure 2). The shape of the autocorrelation function provides dynamic information about the molecules while the magnitude of the function at zero time lag gives the average concentration of molecules in the laser beam volume, N [17,24].
Figure 2. The fluctuation of particles diffusing in and out of the excitation volume are detected and recorded as Intensity (counts) vs time. The autocorrelation function is described in the upper equation in the figure, where \( N(t) \) is the fluorescence intensity at time \( t \), \( \tau \) is the time lag, and the angle brackets denote a time average. At \( G(0) \) the autocorrelation function is inversely proportional to the average number of molecules in the excitation volume. As \( \tau \) approaches infinity, for a stochastic, stationary system, the numerator in the autocorrelation function approaches the product of the averages, and therefore \( G(\tau) \) approaches zero.

In Figure 3 we show a typical measurement of the diffusion of enhanced Green Fluorescence Protein (EGFP) in a point in the cell obtained using the FCS technique described above.

**Scanning FCS**

FCS measurements at several points simultaneously can be obtained by rapidly scanning the beam in a circular motion. This approach allows the simultaneous measurements of fast (points along the circle) and slow (same point at each pass of the laser beam) diffusing molecules while retaining spatial information [20,29-31]. By moving the beam, this approach minimizes photobleaching, allows the monitoring of several sub volumes in parallel and cancels the shot noise in the first peak of the autocorrelation function.

When the beam is scanned in a circular path with a specific radius, the intensity distribution of the laser beam is time and position dependent. The autocorrelation function of the intensity fluctuations can be calculated from each column (Figure 4).
Figure 3. Two-photon excitation images of stable CHO cells expressing EGFP. Autocorrelation function at a point in the cell yields a diffusion coefficient of 19.3 \( \mu m^2/s \).

Figure 4. Scanning FCS measures the fluctuation at a millisecond time resolution over a relatively large area. As the laser beam scans in a circle with a given radius, the data from each point (or sub volume) in the circle are collected. The fluctuations from one scanning period are recorded as a row of data for that one cycle. Upon the second scanning period, the data is transposed beneath the first row and so on with the subsequent data points. Data in a column reflect the fluctuations in time at that location in the circular scan.

Need for spatial resolution and fast data acquisition

Although scanning FCS (or line FCS) allows collection of fluctuation data at many points quasi-simultaneously, the limitation of the mechanical scanner technology limits the sampling time at the same location to few milliseconds.
Figure 5. CHO cell expressing Paxillin-EGFP in a focal adhesion. Each horizontal line in the FCS carpet corresponds to fluorescence along the orbit. Each vertical line of the carpet corresponds to fluorescence intensity at the same volume element.

Temporal ICS

Time resolution: sec-min

Figure 6. ICS monitors temporal fluctuations at every point in a stack of 2-D images. The calculation of the autocorrelation of the intensity fluctuation at the same pixel can be used to measure very slow diffusion (Frame rates in the sub-second range).
Small proteins in solutions have typical correlation times in the order of a fraction of millisecond. Therefore, when the beam returns to the same position along the orbit (or the line), the particle is gone. Also, using the orbit or line scan method it is still difficult to assess global or local cellular movements. Ideally, we will like to have sampling times (at the same location) in the 10-100 μs range while maintaining the pixel resolution. About a decade ago, Petersen et al proposed a method to analyze fluorescence intensity fluctuations in cells using spatial correlation functions (32). Image Correlation Spectroscopy (ICS) was originally conceived to extract spatial correlation from images to obtain parameters such as the number of molecular aggregates in a membrane and their typical sizes.

One distinct advantage of ICS is that the data are collected at the highest spatial resolution possible. Later, the method was extended to measure fluctuations occurring at the same pixel but with poor time resolution (on the second time scale), since the same volume is sampled only once per frame [33]. At this point in history, complementary methods (FCS and ICS) were available to detect fluorescence fluctuations in cell. Single point FCS provides the best time resolution, but has no spatial information. The ICS method has the best spatial resolution, but very poor time resolution. Scanning FCS is a hybrid with intermediate time and spatial resolution. However, being able to collect the image of the entire cell is crucial for data interpretation in terms of separating the contribution of the immobile or slowly mobile fluorescent structures in the cell from the fast moving components.

**The rics approach**

In 2005 we introduced a new approach to fluctuation spectroscopy that has the potential of bridging the gap between the points FCS and the ICS approach [34]. This new approach (RICS) is based on the observation that images obtained with a laser scanning confocal microscope, contain both spatial and temporal information due to fast movement of the beam across the sample. The RICS approach has the advantage of providing a stack of images from which cell movements and other slow dynamics are readily detected. By exploiting the relatively fast pixel sampling rate, the RICS approach can provide information on fast diffusing molecules. Other major benefits of the new technique include use of commercial laser scanning microscopes (either one or two photon systems), with analog detection, as well as with photon counting systems, although the different statistics of the two kinds of detectors must be accounted for. The new technique provides an intrinsic method to quantify the immobile fraction. RICS provides a powerful method to distinguish diffusion from binding or other processes. How does it work?
2-D Spatial correlations

Images obtained with a laser scanning microscope contain temporal information because they are recorded sequentially pixel after pixel, as opposed to a camera snapshot. In the raster scan confocal image, the spatial coordinate is also related to time. Therefore, the spatial correlation function can be used to obtain information about time correlations if we assign a time to each pixel corresponding to the time sequence of the raster scan motion. The RICS method consists of obtaining time correlation by calculating spatial correlations. The 2-D spatial correlation function is calculated as illustrated in Figure 7 and related formula.

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

**Figure 7.** Schematic illustration of the spatial correlation approach. Spatial correlation is obtained by multiplying each point of an image by the image translated by a given shift \( \xi, \psi \) both in the x and y directions as described by the equation.

The rics approach to diffusion

As particles diffuse, after some time there is a finite probability of finding a particle relatively far from its original point. The diffusion process can be described by the following function

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

(1)

As time increases, the width of the Gaussian function describing the diffusion process increases, while the amplitude of the function decreases. This function
must be convoluted with the function describing the laser beam profile (PSF) to obtain the changes in fluorescence as a function of time which is a characteristic of the diffusion process. The resulting equations are reported below.

\[
G(\xi, \psi) = \frac{\gamma}{N} \left(1 + \frac{4D(\tau_{\rho} \xi + \tau_{\psi} \psi)}{w_{0}^2} \right)^{-1} \left(1 + \frac{4D(\tau_{\rho} \xi + \tau_{\psi} \psi)}{w_{0}^2} \right)^{-\frac{1}{2}}
\]  

(2)

\[
S(\xi, \psi) = \exp \left\{ \frac{1}{2} \left[ \left( \frac{2\xi \delta r}{w_{0}} \right)^2 + \left( \frac{2\psi \delta r}{w_{0}} \right)^2 \right] \right\} 
\left(1 + \frac{4D(\tau_{\rho} \xi + \tau_{\psi} \psi)}{w_{0}^2} \right)^{-1}
\]  

(3)

\(\tau_{\rho}\) and \(\tau_{\psi}\) indicate the pixel time and the line time, respectively. \(\delta r\) is the pixel size. For \(D=0\) the spatial correlation gives the PSF (point spread function), with an amplitude equal to \(1/N\). As \(D\) increases, the correlation (\(G\) term) becomes narrower and the width of the \(S\) term increases.

The total correlation function is given by the following product

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

(4)

The correlation due to the scanner movement is independent on the diffusion motion of the molecule.

**Fit of spatial correlation functions**

Once experimental data have been obtained and the spatial correlation functions have been calculated, one can fit the data using the expression for the 2-D spatial correlation functions in terms of the parameters \(N\) and \(D\), i.e., the number of molecules and the diffusion coefficients (Eq. 2-4). Figure 8 shows the result for a simulation of random diffusion for particles with sizes corresponding to fluorescent beads of 40 nm radius and to EGFP.

Due to motion, the image of EGFP is made of streaks which give a 2-D spatial correlation very elongated along the horizontal axis in the figure. Instead, particles of larger size, do not move appreciably during image acquisition and they appear relatively round, although the spatial correlation shows that the particles images are flatter in the vertical direction. The fit of the spatial correlation function to the theoretical expressions (2-4) is relatively good and recovers the value of the diffusion used for the simulation.
Figure 8. Simulation of diffusion of particles the size of 40 nm beads (slow diffusing particle) and of a molecule the size of EGFP (fast diffusing particle). In the image panel, EGFP molecules appear as streaks due to the rapid molecular motion during the time of image acquisition. Instead the image for the beads gives relatively round objects. In the spatial correlation motion panel, the EGFP spatial correlation gives a very narrow (in the vertical direction) function due to rapid motion. For the 40 nm beads, the 2-D spatial correlation is not perfectly round, revealing the motion of the particle. The fit panel shows the theoretical function according to equations 2-4 and the residues (upper surface).

The following figure shows data obtained with an Olympus Fluoview300 LSM of freely diffusing EGFP proteins in buffer. Although it is difficult to see the streak shapes in the image due to poor S/N, the 2-D correlation function recovers the expected elongated shape of the function. The fit to the theoretical equations provides the value of the diffusion coefficient of EGFP, in the range previously reported by several authors [21].

We have shown that for solutions, the raster scan images contain the information about the molecular motion of fast diffusing particles and that the S/N ratio of a commercial LSM is sufficient to recover diffusion coefficients in a wide range. However, to recover the dynamics of molecules in the cellular environment, we need to be able to distinguish in the 2-D spatial correlation function between the spatial correlations due to the immobile features of the image and those due to motion of molecules or particles.
Figure 9. RICS analysis of EGFP in solution. The image stack was obtained with an Olympus Fluoview 300 LSM. The spatial autocorrelation function shows the characteristic correlation along the horizontal direction due to the motion of the molecules. The recovered value of the diffusion coefficient according to equations 2-4 is in accord with literature values.

Removal of the immobile fraction

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. We have observed that even if the bright immobile fraction represents more than 95% of the total signal, the algorithm for subtraction of the immobile fraction still recovers the residual correlation due to the motion of particles in the presence of the very bright image features. In practice, the algorithm calculates the average image using the entire image stack and then subtracts this average image from every image of the stack. However, after this operation is performed, the average intensity at each pixel is zero. The formula for the calculation of the spatial autocorrelation function (see formula in the panel of figure 7) contains the average intensity in the denominator. To avoid the division by zero, we add to each pixel of the image a quantity which is equal to the average intensity of the entire stack. Note that the correction for the immobile fraction is based on the properties of the shot noise. While the shot noise is uncorrelated in time for instruments with photon counting detection, this is not true for analog systems; not even in the first order approximation. For analog detection systems the shot noise is time (and space) correlated due to the finite response of the detector amplifier. In practice, the analog response gives correlation only for a few points (in time). This region of the autocorrelation function is disregarded from the analysis.
To demonstrate the behavior of the immobile subtraction algorithm, we performed simulations in which we can add or remove bright immobile particles in the presence of dim fast diffusing particles (Figure 10). When the image contains bright immobile particles the correlation function mostly reflects the shape of the immobile bright particles (Figure 10, central upper panel). After application of the immobile subtraction algorithm, the RICS analysis only shows the fast diffusing molecules (Figure 10, right upper panel). Then simulations are performed without the bright immobile particles. The RICS analysis recovers an autocorrelation function which is identical to the correlation function obtained after application of the correction algorithm in the presence of the bright particles (Figure 10, lower right panel).

![Original Image, Spatial Correlation, Corrected Image: With and Without Immobile Fraction](image)

**Figure 10.** Simulations of the motion of dim fast moving particles in the presence (upper panels) and in the absence (lower panels) of bright immobile particles. The immobile fraction subtraction algorithm gives the same spatial correlation function as that obtained when the immobile fraction is absent. Compare the corrected upper right panel with the spatial correlation function of the lower right panel.

In summary, we have shown that the RICS technique is capable of measuring dynamic processes from the μsec-to very long time scales. Anyone with a commercially available laser confocal microscope can implement RICS. Immobile structures can be filtered out and fast fluctuations can be detected. RICS has high spatial and temporal resolution. By calculating spatial correlations, the intrinsic time information of the raster scan image can be exploited to obtain information on a very fast time scale, as well as slow time scales, limited only by the total time of data acquisition. The range of these dynamic rates covers a wide range of processes from quasi immobile
molecules to cytosolic diffusion (0.01-20 μm²/s). The S/N ratio is very good and other types of processes and interactions are also measured, such as binding of molecules to receptors or other large structures.

**Mapping fast cellular dynamics in cells**

In this section, we show an application of the RICS method for the measurement of the diffusion coefficient of Paxillin-EGFP in CHO cells (Figure 11). Paxillin is an adaptor molecule involved in the formation of focal adhesions. The relatively bright structures visible in areas 5 and 6 of the cell are the focal adhesions. In the rest of the cell, paxillin is relatively free to diffuse. We have collected a stack of images (100 frames at 1.5 frames per second) that we used to determine the local diffusion of paxillin in different parts of the cell. Regions 1-4 are in the cytoplasm. The RICS analysis, after subtraction of the immobile fraction, shows similarly shaped 2-D spatial correlation functions. The fit using the theoretical equations recovers diffusion coefficients in the range 9-15 μm²/s. In areas 5 and 6, the analysis reveals that

![Figure 11](image_url)

**Figure 11.** CHO cells expressing Paxillin-EGFP. Several areas of the cell, in the cytoplasm and in the protruding parts have been selected. The RICS analysis shows a definitive difference of the mobility of the paxillin molecules in the different areas. The upper right panel shows a partial map of the values of the diffusion coefficient recovered (the height of the red bar corresponds to the value of D).
the diffusion of paxillin is slower. This effect can be due to the presence of the focal adhesion in these areas that partially inhibit the motion of paxillin. Note also that in areas 5 and 6, the cell geometry is such that paxillin cannot diffuse very far in the horizontal direction of the image.

In these experiments, the cell was also slowly retracting. The immobile subtraction algorithm was modified to use a moving average of the image stack rather than the average of the entire stack. The moving average method is sufficient to correct for the slow motion of the cell.

Conclusions

Fast dynamics in cells can be measured by exploiting spatio-temporal correlations intrinsic to images obtained with a laser scanning confocal microscope. These instruments are common in most cell biology laboratories. The RICS method is a mathematical analysis performed on a stack of sequential images. The raster scan image contains information on a wide range of time scales. The pixel residence time is generally on the order of microseconds, the line time is in the millisecond range and the frame time is in the second range [34]. All these time scales are revealed by spatial correlations, since the position of the laser beam and the time the data is acquired are related by the raster scan motion. We presented the mathematical framework for extraction of this information for the case in which molecule are free to diffuse. For other processes such as binding of molecules to immobile structures, different mathematical models must be employed. The RICS approach provides a convenient method to simultaneously monitor the entire cell so that the cell motion can be recognized and accounted for. The RICS approach allows us to perform FCS experiments even in the presence of slowly moving and immobile structures. More importantly, the contribution of immobile features to the image can be subtracted so that the motion of molecules or small particles can be revealed. The presence of fast mobile particles, which is in every confocal image of live cells, is generally disregarded. The RICS method recovers this information which is of crucial importance to determine molecular interactions in cells.

We have shown one application of the RICS method for the simultaneous determination of diffusion rates of Paxillin-EGFP in a cell. In this example, the fast moving paxillin molecules in the cytoplasm were detected and the decrease of the diffusion in the proximity of focal adhesion was revealed.

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