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Fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy

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Abstract

This article focuses on methods based on fluctuation correlation spectroscopy to determine the formation of protein complexes in living cells. We present the principles of the fluctuation method applied to cells. We discuss the novelty and the promises of this approach. The emphasis is in the discussion of the underlying statistical assumptions of the image correlation spectroscopy analysis rather than in reviewing applications of the method. Although one example of the application of the fluctuation method is given, this article also contains simulations that are better suited to illustrate and support the basic assumptions of the method.

INTRODUCTION

Cellular phenomena are characterized by vast regulatory networks acting on the complex machines that drive various cellular processes. Protein complexes are a key feature of both the regulatory pathways and machinery. The complexity and ubiquity of these interactions are seen in the interaction networks, or interactomes, that are being developed using 2-hybrid, mass spectrometry, informatics, and other global systems approaches. While it is likely that most of the interactions found in various interactomes do in fact occur, the existence of only a few have been demonstrated in living cells. Furthermore, it is likely that many are transient or compartmentalized in the cell. Thus, there is a need for robust methods for demonstrating the existence of complexes and their spatial location in living cells, and thereby creating functional interactomes in living cells executing cellular processes.

In this article, we outline recently developed fluorescence microscope-based methods aimed at detecting molecular complexes in living cells. These methods require fluorescent labeling of the macromolecules of interest, which are now widely accessible due to the development of genetically encoded fluorescent proteins like green fluorescent protein (GFP) and its colored variants. In these fluorescence-based methods, complexes are detected by analysis of the fluorescence emitted by the two fluorescent proteins in two detection channels of the microscope.

Foster resonance energy transfer (FRET) is the most popular method for detecting complexes in live cells. This method senses the formation of a complex in which the donor and acceptor molecules reside within a distance of 1 to 10 nm.^{1,2} Although this method is extremely powerful and can even be used at a single molecule level, it is prone to a series of artifacts due to cellular autofluorescence.^{3,4} Furthermore, FRET is not easy to interpret when molecular complexes contain more than two proteins, and requires careful engineering of donor and acceptor positions to ensure appropriate proximity on interaction.

Colocalization is another approach that is commonly used in cell biology. However, colocalization only establishes that two molecules are at a distance within the point spread function (PSF) of the microscope used. The PSF is typically in the 200 nm range for confocal microscopy and could be reduced to about 50 nm using super resolution microscopy such as the STED technique.^{5,6} Nevertheless, the colocalization method is widely used to infer that two fluorescent molecules are part of the same structure. A common variant of this method is based on following the colocalization of two (or more) proteins in time. For each time frame, the colocalization is verified. This approach only establishes that two molecular species participate to the same underlying structure rather than showing that the two proteins reside in the same “molecular” complex, which is the information inferred in the reactome and of interest to systems biology.

Cross-correlation fluctuation correlation spectroscopy (ccFCS) techniques is a powerful method for demonstrating that two proteins reside in a molecular complex.^{7–16} The method is based on the temporal analysis of fluorescent amplitude fluctuations; if these fluctuations occur simultaneously in two channels, the proteins must be moving together in a complex. Earlier versions of this method relied either on the analysis of fluctuations within a single, focused beam or on the analysis by spatial intensity fluctuations. A new methodology, cross-correlation raster scanning imaging correlation spectroscopy (ccRICS) combines the spatial and temporal correlation under one mathematical framework, thereby provides a powerful new tool for characterizing molecular complexes.^{17,18} In this article, we discuss the various cross-correlation techniques, spatial and temporal, and the ccRICS technique from the conceptual and mathematical viewpoint.

There are two important parts of the fluctuations that must be considered, that is, the temporal part (duration of the fluctuation) and the amplitude part (the magnitude of the changes in fluorescence intensity). Although this paper focuses on the temporal part, there is additional information on the amplitude part, which can be used to extract the number of molecules of two species that comprise the complex. Since fluctuations can be measured in different parts of the cell, it is possible to produce maps of molecular interactions in cells and in tissues and follow their evolution with time.

The cross-correlations’ methods described in this article are only some of the possible ways through which fluctuations in time and space can be analyzed. There are other ways (not described here) that can be used to extract information about molecular interactions,¹⁹ and we anticipate that we will see further developments in the image correlation techniques.

One emerging area of research in systems biology is the description of reactions and feedback mechanism in cells and protein networks. In these approaches, the temporal part is emphasized, although there is a need to account for the spatial distribution of the protein complexes. It is the mapping of the interactions that should interest system biologists. These methods are ideally suited for high throughput screens that require measurements of interactions.

The appeal of the fluctuation techniques applied to the cellular interior is in their intrinsic sensitivity to single molecule interactions, which is the basic component of computational methods in system biology. Using the fluctuation approach, the statistics of the interactions at small number of molecules can directly be proved. Recent advance in the fluctuation techniques to provide directly spatial and temporal correlation of fluctuation in cells could be used to prove the basic assumptions of the system biology approach. For example, the correlation techniques can distinguish between physical processes, such as diffusion and binding including weak binding that is at the origin of the spatial distribution of proteins in a cell. These and many more are the type of questions in systems biology that may be

addressed through fluctuation correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) when the methods are used in the imaging mode. We believe that the exploitation of spatiotemporal correlations to describe the reactions occurring in the cell interior is just at the beginning. The extension of the fluctuation methods to the analysis of the entire images produces high-resolution maps of molecular interactions that could form the basis for testing various computational models.

MACROSCOPIC CORRELATIONS AND MOLECULAR COMPLEXES: DEPENDENCIES ON TIME SCALE

Fluctuations can arise from diffusion of molecular complexes, in and out of the excitation volume, or from changes in intensity due to accumulation of reactants in a given region of the cell. For example, two proteins can accumulate in the nucleus. If we measure the average fluorescence from the nucleus in two channels, we will observe a simultaneous increase of the intensity. On the time scale of several seconds or minutes, the fluctuation in the average intensity in both detection channels will be correlated. In this specific example, the cross-correlation is due to a macroscopic event (the transport of proteins into the nucleus) rather than to formation of a molecular complex, a molecular correlation. There are approaches that we can utilize to separate macroscopic from molecular correlations. One is based on the spatial size and another on the time scale of the correlation. For the spatial scale of the correlation, methods based on segmentation of the pixels of an image can identify the locations where complexes can form. The segmentation approach is based on establishing a rule to select valid pixels of an image either manually or automatically.²⁰ For example, we could select pixels in an image that have intensity in one channel above a given threshold and then compare the intensities of the same pixel with the other channel. If there is statistical correlation between the intensities of the two pixels, we can conclude that there is a common underlying source for this correlation. This correlation could also be maintained in time as different frames are analyzed. Of course, there could be several physical origins for this colocalization or spatial cross-correlation of the pixel intensities. Since the segmentation approach depends on the algorithm used for the classification of the pixels, the results depend on these criteria. The spatial cross-correlation method can only be used with very slow events, e.g. those occurring on the cell membrane, but not for macromolecular complexes diffusing in the nucleus or the cytoplasm.

IMAGE CORRELATION SPECTROSCOPY

N.O. Petersen and coworkers^{21–23} introduced an elegant approach that can partially circumvent problems related to the (arbitrary) choice of the criteria for segmentation. In this approach, a rigorous statistical analysis, based on the calculation of spatial correlation functions, is used to establish the size and composition of membrane molecular aggregates of size comparable or larger than the PSF. The spatial autocorrelation function is defined in Eq. 1,

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

where $I(x, y)$ is the intensity at pixel x and y and the brackets indicate the average over all values of x and y . The symbols ξ and ψ indicate a shift of the image (in pixels) in the x and y direction, respectively. If aggregates are randomly distributed spatially, the shape of the spatial correlation is the convolution of the average size of the aggregates and of the PSF of the microscope.

Examples of the spatial autocorrelation function are shown in Figure 1 for various sizes of aggregates. These examples are for aggregates of different sizes at random locations in a plane that simulates the biological membrane. The aggregates are not moving, and a single frame is used. Details of the simulation are given in the figure caption.

In a homogeneous population, the value of the spatial correlation function at spatial shift (0,0) is proportional to the inverse of the number of particles. The term (0,0) of the spatial correlation contains the shot noise. This term produces a spike in the spatial correlation. Generally, it is eliminated from the analysis or is estimated by extrapolating the correlation function. This inverse relationship with the number of particles is very convenient because it provides a rapid and robust method to count particles (Figure 2(a)). In the presence of aggregates of different sizes, the weighting of different populations depends on the square of their fractional contribution to the fluorescence intensity. This is due to the normalization factor and the mathematical form of the autocorrelation function.

Figure 1 shows that the spatial autocorrelation function is a direct representation of the average size of the aggregates. If the aggregates are larger or comparable to the size of the PSF, a simple fit of the autocorrelation function, using a Gaussian function, provides an evaluation of the aggregate size if the system is relatively homogeneous (in size). If there is a distribution of aggregate sizes, a more sophisticated fit must be used using either a sum of discrete Gaussian components or a continuous distribution of Gaussian components.

The idea underlying the spatial correlation method is that the average size and number of the aggregates can be obtained without measuring each of the aggregates. It works because the random location of the aggregates in the membrane results in a null average value of the aggregate-to-aggregate correlations giving an autocorrelation function that contains the information on the average size of aggregates and not of their distribution in the membrane.

The spatial cross-correlation between frames acquired in two different spectral channels in the microscope has properties similar to the autocorrelation. The mathematical definition is given below.

$$G_{cc}(\xi, \psi) = \frac{\langle I_1(x, y) I_2(x + \xi, y + \psi) \rangle}{\langle I_1(x, y) \rangle \langle I_2(x, y) \rangle} - 1 \quad (2)$$

where the indexes 1 and 2 indicate the two detector channels in the microscope used to simultaneously acquire the two different colored images. Given the normalization, the cross-correlation is always less than the maximal autocorrelation (that from the channel with larger correlation). In contrast to autocorrelation, the point at shift (0,0) in the cross-correlation function is not affected by detector shot noise, since the noise of the two detectors is uncorrelated. Instead, the cross-correlation between two channels is affected by the bleedthrough, if any, of the signal from one protein into the channel for the other protein (color). In the absence of bleedthrough, cross-correlation methods will have a very high sensitivity, theoretically limited only by the S/N of the measurement. Unfortunately, for all practical situations in microscopy there is spectral cross-talk between the two channels producing a cross-correlation signal even in the absence of a complex. It is then important to evaluate the amount of this spurious cross-correlation signal in the absence of true correlation. If channel 1 corresponds to “green” fluorescence and 2 to the “red” fluorescence, then the red channel can be “contaminated” by the green fluorescence due to the long red emission tail of most organic dyes and fluorescent proteins. Assuming that the fluorescence intensity in channel 2 is due to the fluorescence of the true red emission plus a

small fraction of the green emission, the cross-correlation at shift (0,0) for *totally uncorrelated particles* is given by the following expression:

$$G_{cc}(0,0) \propto \left[\frac{f_{12}}{f_{12}N_1 + f_{22}N_2} \right] \quad (3)$$

Where f_{22} is the brightness of the red species as seen in the red channel and f_{12} is the brightness of the green species as seen by the red channel. In this formula, we neglected the leakage of the red channel into the green channel, and we have not included a constant factor due to the profile of illumination (the γ factor in FCS). N_1 and N_2 represent the number of green and red molecules in the excitation volume, respectively. According to the formula above, in the presence of bleedthrough, the cross-correlation is equal to the autocorrelation ($1/N_1$) in the absence of the red molecules ($N_2 = 0$). The case $N_1 = 0$ must be evaluated separately since in this case the cross-correlation will be zero. The bleedthrough factors that depend on the choice of filters used and on the particular fluorophore must be carefully evaluated to avoid interpretation artifacts. To evaluate these factors, we should prepare a solution at a given concentration of green molecules and determine the fluorescence of this sample in the red channel. We should also prepare a solution containing the same concentration of red molecules and measure the fluorescence of this sample in the red channel. Since the concentrations are the same, the relative contributions of the two samples in the red channel can be used to evaluate these factors. Typically, the bleedthrough factors are in the order of few percent, and they can be minimized by increasing the excitation power in the red channel. After accounting for bleedthrough effects, the existence of cross-correlation is a signature that both molecular species are present at the same location.

TEMPORAL CROSS-CORRELATIONS

Single point FCS autocorrelation and cross-correlation (ccFCS) have been around for some time.^{16,24} The method consists of measuring at a single point in the sample the fluorescence intensity in one or two channels as a function of time. From these data the time autocorrelation function can be computed from:

$$G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} - 1 \quad (4)$$

where τ represent a time delay and the average, indicated by the brackets, is calculated over the entire time sequence. In the classical temporal cross-correlation, the intensity fluctuations at two channels are measured at high frequency, generally in kHz to MHz range. It is argued that if both channels fluctuate simultaneously in this time scale, the underlying cause of the fluctuation must be at the molecular level. Therefore the ccFCS technique, also called dynamic colocalization, is the preferred method to detect molecular interactions. This technique can be applied to each location in a cell, and it detects rapid fluctuations, unlike the image correlation spectroscopy (ICS) methods, which only see slow fluctuations. In principle, ccFCS is only sensitive to the fluctuations due to molecular complexes carrying both molecular species. In practice, the ccFCS method must also be calibrated for the bleedthrough between channels and is strongly affected by the presence of FRET, if it exists. While this method readily shows cross correlation, deriving quantitative information is more challenging. Assuming that we have calibrated the bleedthrough factors, the amount of molecular species in each color can only be determined if we assume that there is only one kind of complex of known stoichiometry. Figure 2 shows the % of cross-correlation as a function of the % of complexes in the presence of 10% bleedthrough and

assumes that there are only two types of molecular species with a 1:1 stoichiometry. Due to bleedthrough, the cross-correlation is not decreasing accordingly to the amount of double-labeled species, preventing the accurate determination of very low concentration of complexes.

For this article, an important discussion of the autocorrelation or cross-correlation method relates to the “single molecule” interpretation of the correlation function. In the case of independently diffusing particles in solution, the appearance of a non-zero autocorrelation function is due to the permanence of the “same” particle in the volume of observation. At very long delay time τ , the autocorrelation function becomes zero because the transit of one molecule is not correlated with the transit of another molecule. At very short time delays (shorter than the time it takes for a molecule to transit across the PSF), the value of the autocorrelation function is proportional to the inverse of the number of particles in the illumination volume.

When the fluorescence fluctuations are recorded in a cell, the above argument regarding the measurement of the “same molecule” depends on the time scale of the event. For example, let us assume that there is a fixed site in the cell where fluorescent molecules can bind. If the site can bind only one molecule and all sites in the cell are independent of each other, the fluctuations from this specific site will correlate for a time that is dependent on the sum of the “on” and “off” time for a single molecule to bind. On a longer time scale, we will measure an average intensity for that pixel. If the affinity at this binding site is modulated at low frequency, we could have another part of the correlation function, which is dependent on the slow modulation of the binding affinity. Clearly, the autocorrelation (or cross-correlation) arising from this slower process is not due to single molecule events. Also, if neighbor sites are not independent of each other, the autocorrelation (and cross-correlation) could be due to the binding of several molecules. From this discussion, it appears that it is critical to measure fluctuations in the cell on the time scale of molecular diffusion (which is sub-millisecond in the cytoplasm and sub-second in membranes) to properly identify molecular events.

SPATIOTEMPORAL CORRELATIONS AND CROSS-CORRELATIONS

Recently, spatiotemporal image correlation spectroscopy (STICS) method was developed by Hebert et al. based on the cross-correlation of subsequent images in an image stack.²⁵ This method is designed to detect velocities of directed movement. Mathematically, the STICS operation is described by the following correlation function

$$G_{\text{STICS}}(\xi, \psi, \tau) = \frac{\langle I_1(x, y, t) I_1(x + \xi, y + \psi, t + \tau) \rangle}{\langle I_1(x, y, t) \rangle \langle I_1(x, y, t) \rangle} - 1 \quad (5)$$

where τ indicates that the correlation is calculated between different frames of the stack with a delay τ among them and the brackets indicate averaging over the spatial coordinates and over the time. This is identical to the ccICS for images described above except that the two images are now delayed, one with respect to the other. We could calculate several correlation functions, one for each time delay τ .

If there is a change in the image as a function of time, the cross-correlation achieved by the STICS operation provides an unbiased and robust statistical method to analyze these changes. This method is particularly useful to detect systematic changes in an image as a function of time due, for example, to flow. Since this method relies on the changes between two subsequent images of a temporal stack, the temporal resolution of the method is

determined by the frame time. STICS is essentially a colocalization method with a robust statistical analysis. Therefore, due to the slow frame-to-frame time, this method does not provide directly information on molecular complexes, in the same way as the other ICS methods.

RICS CROSS-CORRELATIONS

The raster image correlation spectroscopy (RICS) method, calculates correlations between pixels of the same image.^{17,18} In a raster scanned image, there is a direct relationship between the pixel position and the time when a pixel is measured. In a typical laser scanning confocal microscope, the time delay between the measurements of the intensity of adjacent pixels along the scan line is in the microsecond time scale and among adjacent pixels in two subsequent lines of an image is in the millisecond range. Since the RICS function contains information in the micro-millisecond time scale, it can detect fast molecular events. The proper time scale and the oversampling to be used when collecting data for RICS was previously discussed.²⁶ When used in the cross-correlation mode, the ccRICS method is sensitive to the formation of molecular complexes.

The definition of the RICS correlation function is identical to Eqs. 1 and 2 already introduced in the context of the ICS methods. What is different is the way the image is acquired using the raster scan method. The RICS function is always symmetric since it is an autocorrelation function, but the ccRICS function is non-symmetric. Figure 3 shows the RICS function of the same sample acquired in a raster scan mode or the snapshot acquired with a camera in which all pixels are measured at the same time. The camera can operate with a fast shutter or in an integration mode. With the fast shutter, the motion of the particles appears frozen and therefore will give a symmetric spatial correlation. In the integration mode, the image is still symmetric in all directions. The correlations due to molecular motions are lost in the camera mode of data acquisition, while in the raster scan mode they survive.

EXAMPLE OF A DATA STACK ANALYZED USING THE VARIOUS CROSS-CORRELATION METHODS

In this section, we show how the different image fluctuations techniques here discussed provide different kinds of information. The sample consists of a mouse embryo fibroblast coexpressing vinculin-EGFP, measured in the green channel and paxillin-mCherry emitting in the red channel. The bleedthrough of the green channel into the red for this sample and these conditions is less than 5%. The excitation for EGFP was from an argon ion laser emitting at 488 nm, at a nominal power of less than 10 μ W measured at the sample. The red excitation was from a 559-nm laser also with a nominal power of less the 10 μ W. The microscope used is an Olympus FV1000 with a 60 $\times$ water objective NA = 1.2. The zoom was set to have an image of 12.8 μ m on each side, and the image was acquired in the 256 $\times$ 256 format with a pixel dwell time of 12.5 μ s, line time of 4.35 ms and a frame time of about 1 s. Figure 4(a) and (b) show the image of the cell in the two channels. The bright, band-like diagonal structures are adhesions. The adhesions are apparently moving in the 7 o'clock direction. The biological question is whether or not vinculin and paxillin form a complex in the cytoplasm, and whether they bind to the adhesions independently or as a complex. Comparison of Figure 4(a) and (b) shows colocalization of the two proteins at the adhesions. The adhesions show an apparent coordinated movement, or sliding; this is due to the binding of proteins at the leading edge (at 7 o'clock) and disassembling at the opposite edge (at 1 o'clock). However, this apparent sliding of the adhesion is macroscopic and does not reflect the movement of the individual proteins that constitute the adhesion.

The RICS autocorrelation (Figure 4(c) and (d)) for the two channels nicely shows that the proteins diffuse in the cytoplasm by the strong difference between the widths of the RICS function along the two image axes. However, the ccRICS is essentially flat (Figure 4(e)) except for a small bleedthrough, indicating that the proteins do not diffuse as preassembled molecular complexes.

The STICS autocorrelation functions (not shown) as well as the ccSTICS show a large diagonal structure (Figure 5(a)–(f)). The STICS correlations at different time delays show a coordinate macroscopic motion of the entire adhesion, although we have shown from the ccRICS analysis that there are no molecular complexes. In this case, the spatial and temporal (slow) cross-correlation is due to macroscopic events (the building and disassembling of the entire adhesion).

The ICS autocorrelation or cross-correlation only gives information on the shape and average size of the adhesions. This function is identical to the STICS at zero delay time (Figure 4(a)).

DISCUSSION OF POSSIBLE SCENARIOS OF MOLECULAR INTERACTIONS

One important discussion about the value of the cross-correlation methods is in regard to the quantitative evaluation of molecular interactions in terms of dissociation constants and stoichiometry of the interactions. Fluctuation methods have been originally developed to study chemical reactions and there is a relatively large literature describing application of fluctuation spectroscopy in solution to obtain equilibrium constant and stoichiometry of the complexes. In this article, the focus is on the application of the cross-correlation methods to the cell interior. In this case, the meaning of chemical equilibrium and the description of the chemical reactions in simple terms are not obvious. For example, in the application illustrated in the previous section, the interaction between vinculin and paxillin has been studied in solution, but in the cell the interaction seems to occur at the scaffold represented by the focal adhesions. In fact, at the concentrations found in the cytoplasm, the two proteins do not form a complex, yet both bind to the adhesion as part of a larger structure. This interaction through the scaffold is more complex than the solution case and the simple description of the reaction in solution is not applicable. Another important distinction is in regard to the nature of the process responsible for the transport of molecules from one location in the cell to another. In solution, this process is mainly determined by free diffusion. In a cell, the transport is influenced by a series of weak interaction with many partners, either part of immobile cellular structures or floating around in large aggregates. It is notable that spatiotemporal cross-correlation methods can distinguish between these cases as recently discussed in the context of the RICS technique.²⁷ Directed transport and incorporation of proteins in vesicles can affect the spatiotemporal correlation function in a predictable way. Also, the binding of protein to large structures can provide additional spatial correlations that can be used to establish the existence of these processes. Therefore, different biological relevant scenarios can be studied using the various cross-correlation techniques described in this article.

CONCLUSION

The existence of molecular complexes in cells can be established using the simultaneous intensity fluctuations that arise from the movement of molecular complexes as seen in two channels of the microscope. A fast, microsecond, time scale is necessary for detecting complexes in the cytoplasm. However, the time scale for events at the membrane is much slower, often in the sub-second. No matter what the time scale is, the measurement method must have a time resolution that is faster than the process that produces the fluctuations.

Methods based on cross-correlation fluctuation spectroscopy unequivocally establish the presence of a molecular complex based on the physical principle that rapid simultaneous intensity fluctuations in two channels must be due to the motion (diffusion) of the molecular complex. Among the methods based on this principle, we described in this article the single point time correlation (ccFCS) and the RICS method (ccRICS). The additional reading material explains how maps of diffusion and interactions can be obtained. The spatial resolution of the RICS method is limited by the fact that the motion of molecules must be measured over a certain area. The size of this area depends on how fast the molecules are moving. For molecules freely diffusing in a cell, this area has a size in the order of a micron. This size can be reduced when the diffusion of the macromolecular complex is slow.

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References

1. Clegg RM. FRET tells us about proximities, distances, orientations and dynamic properties. *J Biotechnol.* 2002; 82:177–179. [PubMed: 11999688]
2. Jares-Erijman EA, Jovin TM. Imaging molecular interactions in living cells by FRET microscopy. *Curr Opin Chem Biol.* 2006; 10:409–416. [PubMed: 16949332]
3. Chen Y, Periasamy A. Localization of protein-protein interactions in live cells using confocal and spectral imaging FRET microscopy. *Indian J Exp Biol.* 2007; 45:48–57. [PubMed: 17249327]
4. Chen Y, Mills JD, Periasamy A. Protein localization in living cells and tissues using FRET and FLIM. *Differentiation.* 2003; 71:528–541. [PubMed: 14686950]
5. Willig KI, Harke B, Medda R, Hell SW. STED microscopy with continuous wave beams. *Nat Methods.* 2007; 4:915–918. [PubMed: 17952088]
6. Willig KI, Rizzoli SO, Westphal V, Jahn R, Hell SW. STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature.* 2006; 440:935–939. [PubMed: 16612384]
7. Bacia K, Kim SA, Schwille P. Fluorescence cross-correlation spectroscopy in living cells. *Nat Methods.* 2006; 3:83–89. [PubMed: 16432516]
8. Bacia K, Majoul IV, Schwille P. Probing the endocytic pathway in live cells using dual-color fluorescence cross-correlation analysis. *Biophys J.* 2002; 83:1184–1193. [PubMed: 12124298]
9. Bacia K, Schwille P. A dynamic view of cellular processes by in vivo fluorescence auto- and cross-correlation spectroscopy. *Methods.* 2003; 29:74–85. [PubMed: 12543073]
10. Bacia K, Schwille P. Practical guidelines for dual-color fluorescence cross-correlation spectroscopy. *Nat Protoc.* 2007; 2:2842–2856. [PubMed: 18007619]
11. Kettling U, Koltermann A, Schwille P, Eigen M. Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy. *Proc Natl Acad Sci U S A.* 1998; 95:1416–1420. [PubMed: 9465029]
12. Kim SA, Heinze KG, Bacia K, Waxham MN, Schwille P. Two-photon cross-correlation analysis of intracellular reactions with variable stoichiometry. *Biophys J.* 2005; 88:4319–4336. [PubMed: 15792970]
13. Kim SA, Heinze KG, Waxham MN, Schwille P. Intracellular calmodulin availability accessed with two-photon cross-correlation. *Proc Natl Acad Sci U S A.* 2004; 101:105–110. [PubMed: 14695888]
14. Kim SA, Schwille P. Intracellular applications of fluorescence correlation spectroscopy: prospects for neuroscience. *Curr Opin Neurobiol.* 2003; 13:583–590. [PubMed: 14630222]
15. Kohl T, Haustein E, Schwille P. Determining protease activity in vivo by fluorescence cross-correlation analysis. *Biophys J.* 2005; 89:2770–2782. [PubMed: 16055538]

16. Schwille P, Bieschke J, Oehlenschläger F. Kinetic investigations by fluorescence correlation spectroscopy: the analytical and diagnostic potential of diffusion studies. *Biophys Chem.* 1997; 66:211–228. [PubMed: 9362560]
17. Digman MA, et al. Measuring fast dynamics in solutions and cells with a laser scanning microscope. *Biophys J.* 2005; 89:1317–1327. [PubMed: 15908582]
18. Digman MA, et al. Fluctuation correlation spectroscopy with a laser-scanning microscope: exploiting the hidden time structure. *Biophys J.* 2005; 88:L33–L36. [PubMed: 15792971]
19. Kolin DL, Ronis D, Wiseman PW. k-Space image correlation spectroscopy: a method for accurate transport measurements independent of fluorophore photophysics. *Biophys J.* 2006; 91:3061–3075. [PubMed: 16861272]
20. Das R, Hammond S, Holowka D, Baird B. Real-time cross-correlation image analysis of early events in IgE receptor signaling. *Biophys J.* 2008; 94:4996–5008. [PubMed: 18326662]
21. Rocheleau JV, Wiseman PW, Petersen NO. Isolation of bright aggregate fluctuations in a multipopulation image correlation spectroscopy system using intensity subtraction. *Biophys J.* 2003; 84:4011–4022. [PubMed: 12770905]
22. Wiseman PW, Petersen NO. Image correlation spectroscopy. II. Optimization for ultrasensitive detection of preexisting platelet-derived growth factor-beta receptor oligomers on intact cells. *Biophys J.* 1999; 76:963–977. [PubMed: 9916027]
23. Brown CM, Petersen NO. An image correlation analysis of the distribution of clathrin associated adaptor protein (AP-2) at the plasma membrane. *J Cell Sci.* 1998; 111:271–281. [PubMed: 9405317]
24. Schwille P, Meyer-Almes FJ, Rigler R. Dual-color fluorescence cross-correlation spectroscopy for multi-component diffusional analysis in solution. *Biophys J.* 1997; 72:1878–1886. [PubMed: 9083691]
25. Hebert B, Costantino S, Wiseman PW. Spatiotemporal image correlation spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO cells. *Biophys J.* 2005; 88:3601–3614. [PubMed: 15722439]
26. Brown CM, et al. Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope. *J Microsc.* 2008; 229:78–91. [PubMed: 18173647]
27. Digman MA, Gratton E. Analysis of diffusion and binding in cells using the RICS approach. *Microsc Res Tech.* 2008; 648(1–2):23–31.

FURTHER READING

28. Brown CM, Dalal RB, Hebert B, Digman MA, Horwitz AR, Gratton E. Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope. *J Microsc.* 2008; 229:78–91. [PubMed: 18173647]
29. Digman MA, Brown CM, Horwitz AR, Mantulin WW, Gratton E. Paxillin dynamics measured during adhesion assembly and disassembly by correlation spectroscopy. *Biophys J.* 2008; 94:2819–2831. [PubMed: 17993500]
30. Elson EL. Fluorescence correlation spectroscopy measures molecular transport in cells. *Traffic.* 2001; 2:789–796. [PubMed: 11733045]
31. Elson EL. Quick tour of fluorescence correlation spectroscopy from its inception. *J Biomed Opt.* 2004; 9:857–864. [PubMed: 15447006]
32. Elson EL, Webb WW. Concentration correlation spectroscopy: a new biophysical probe based on occupation number fluctuations. *Annu Rev Biophys Bioeng.* 1975; 4:311–334. [PubMed: 1098559]
33. Magde D, Elson EL, Webb WW. Fluorescence correlation spectroscopy. II. An experimental realization. *Biopolymers.* 1974; 13:29–61. [PubMed: 4818131]

FIGURE 1.

Image correlation spectroscopy (ICS): simulations of aggregates of different sizes. (a) 50 nm, (b) 100 nm, (c) 400 nm, (d) 800 nm, (e) 1600 nm, and (f) combination of 50 nm and 1600 nm particles. For the simulation, the point spread function (PSF) was 300 nm, pixel size 50 nm, the image was 256×256 pixels and 100 particles were used.

FIGURE 2.

Simulations: (a) $G(0,0)$ is proportional to the inverse of the number of particles N . (b) Fraction of cross-correlation as a function of the fraction of double-labeled particles at 10% bleedthrough.

FIGURE 3.

Differences between (a) raster scan and (b) camera snapshot acquisition mode. (c) and (d) are the correlation functions for (a) and (b) images. Parameters for the simulation were identical to these described for Figure 1. The diffusion coefficient of the particles was $10 \mu\text{m}^2/\text{s}$ and the pixel dwell time was $15.2 \mu\text{s}$.

FIGURE 4.

(a) Vinculin-EGFP and (b) Paxillin-mCherry in MEF cell. (c) and (d) RICS autocorrelation functions and (e) Cross-correlation. There is no appreciable cross-correlation. The small amplitude is the bleedthrough.

FIGURE 5.

Vinculin-EGFP and paxillin-mCherry in MEF cells. The entire adhesion is moving in macroscopic concerted apparent motion. The spatiotemporal image correlation spectroscopy (STICS) correlation functions are at delays (a) 0 s, (b) 4 s, (C) 8 s, (D) 12 s, (E) 16 s and (F) 20 s.