Mapping Molecular Interactions and Transport in Cell Membranes by Image Correlation Spectroscopy

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1. INTRODUCTION

Protein-protein interactions control the majority of events at the plasma membrane, such as signal transduction, endocytosis, attachment and locomotion, to name a few. It is evident from the theme of this book that measurement of intermolecular interactions in cellular systems is critical to our understanding of mechanisms of many cellular processes. It is equally evident that determining the nature of the interactions and their dynamics is challenging and requires multiple approaches that take advantage of fundamental physical and chemical tools to inform the biochemistry and the cell biology (Webb et al. 2003). In this chapter we present a family of image processing tools that are complementary to the Fluorescence

Resonance Energy Transfer measurements of intermolecular interactions and discuss their strengths and weaknesses.

1.1. Molecular Interactions

Intermolecular interactions of proteins in a cell membrane can take many forms characterized by the energetics, the dynamics and the length scale over which the interactions take place.

Strong interactions will form complexes that withstand solubilization and interrogation by biochemical techniques such as immunoprecipitation and chromatography or electrophoresis. The presence of these complexes can be ascertained without ambiguity, but it is virtually impossible to provide quantitative estimates of the number of such interactions within a complex or the fraction of a particular species that is involved. *Weak interactions* can also lead to complexes, particularly if there are many such interactions but these cannot withstand isolation procedures. They must be measured directly in the membrane in the living cell.

Persistent interactions will last for long periods of time because the kinetics of dissociation is slow. These will usually also correspond to the strong interactions and

they can therefore be detected following isolation procedures. However, *transient interactions* will last for short periods of time only since they are governed by rapid exchange between the free and complexed forms of the molecules. During isolation, the equilibrium may shift so that these interactions are best determined in situ.

Whether intermolecular interactions are *strong*, *weak*, *persistent or transient*, measurement in living cells is a benefit, particularly if it is possible to determine the stoichiometry, kinetics and spatial distribution of the interactions and hence to get insight into the mechanisms underlying the formation of functional protein complexes.

1.2. Organization of complexes, clusters and aggregates

Intermolecular interactions can lead to formation of assemblies of molecules that have properties distinct from their surroundings. There are a number of terms that have been used to describe such assemblies in cell membranes and these include complexes, clusters, aggregates and domains. For the purpose of this chapter, we will use the following definitions:

Complex: an assembly of similar or dissimilar molecules created by specific intermolecular

interactions that determine the stoichiometry and structure.

Cluster: a complex of proteins or lipids or both that persists for a period of time long enough to be detected as a unit by microscopic techniques.

Aggregate: a complex of identical molecules (or a cluster in which only a single component is detected or studied).

Association complex: a complex of different molecules.

Domain: a region of the membrane that is structurally and functionally distinct from the rest of the membrane. There are both lipid and protein domains.

Large complexes have dimensions an order of magnitude or more greater than the dimensions of the individual proteins in the complex, yet they may be smaller than the resolution of most optical tools used for their detection (~ 300 nm). *Small complexes* contain only a few of the proteins of interest and each would be in molecular contact with the others.

Fluorescence Resonance Energy Transfer (FRET) is an excellent tool for measuring intermolecular interactions among pairs of molecules in small complexes and aggregates in particular since the distance between pairs of molecules

needs to be relatively small (~10 nm or less). In large complexes and domains, the density of proteins may be high, but the distance between the proteins of interest may be greater than the optimal energy transfer distance. In those circumstances, FRET will fail to detect the interactions. FRET is also a very sensitive tool for measurement of the distances of the interactions among a small number of fluorescent molecules (or sections of molecules), but because of the sensitivity to distance and orientation, interpretation of FRET is difficult when there are many chromophores, as in large complexes or aggregates. This makes it difficult to assess the number of proteins participating in the formation of the complex. Further, it can be difficult to prepare and introduce good probes and the absence of energy transfer does not always preclude that proteins are indeed interacting. Nevertheless, FRET imaging is showing tremendous potential for mapping the nature and extent of intermolecular interactions across the surface of living cells. We are hoping that Image correlation spectroscopy, as described herein, will complement FRET imaging by providing a means to measure protein interactions in larger complexes even when these proteins may not be in close proximity or bound directly to one another.

2. FLUCTUATION SPECTROSCOPY

Fluctuation spectroscopy refers to a number of techniques in which the variation in a signal is measured and analyzed to obtain information about the average properties of the system (Magde et al, 1972). Examples include noise analysis in electronics, quasielastic light scattering in liquids and fluorescence correlation spectroscopy in solutions. In each case, a signal is monitored with time and the temporal fluctuations from the mean value are analyzed by spectral analysis, autocorrelation of the function with itself or cross-correlation of the function with a reference signal. The outcome is information about the dynamic properties of the system at a characteristic length scale (defined by the measurement tool) and in some cases information about the number of entities that give rise to the fluctuations in the signal.

In the early 1970's, Magde, Elson and Webb (Magde et al. 1974) introduced Fluorescence Correlation Spectroscopy (FCS) as a tool to measure the diffusion of proteins in solution and in cell membranes. They demonstrated that the autocorrelation function, g(J), of the relative fluctuation in intensity of fluorescence, (i(t) - <i(t)>)/<i(t)>, would

have an amplitude determined by the average number, <N>, of molecules in the observation volume and that it would decay at a rate, F(**J**), determined by the dynamics of these molecules (Equation 1):

$$g(\tau) = \frac{\left\langle \left(i(t) - \langle i(t)\rangle\right)\left(i(t+\tau) - \langle i(t)\rangle\right)\right\rangle}{\langle i(t)\rangle^2} = \frac{1}{\langle N\rangle} F(\tau) \qquad (1)$$

where the angular brackets indicate an average over the time course of the observation. The decay function, F(**J**), depends on the details of the dynamics and on the geometry of illumination and detection of the fluorescence in the volume being observed. For example, if the molecules are diffusing in two dimensions and they are illuminated with a focused laser beam in the TEM_{00} mode of characteristic width, w, then the decay function is $F(\mathbf{J}) = 1/(1+\mathbf{J}/\mathbf{J}_D)$, where $\mathbf{J}_D = w^2/4D$ represents the characteristic diffusion time in two dimension for a diffusion coefficient of D. Correspondingly, if the molecules are flowing through the same laser beam at a velocity v, then the decay function is $F(\mathbf{J}) = \exp(-(\mathbf{J}/\mathbf{J}_F)^2)$, where $\mathbf{J}_F = w/v$ (Magde et al. 1978).

In recent years, FCS has reemerged as a major tool for studying intermolecular interactions in solutions at low concentrations (Rigler and Elson, 2001). In fact, commercial systems are now available from several microscope manufacturers and suppliers. The application of

FCS to work in cellular systems is more difficult, but progress is being made (Elson, 2001; Thompson et al., 2002). Still, applications to studies of intermolecular interactions in membranes have been hampered by the fact that the fluctuations are inherently slow since the diffusion and kinetics is slow (diffusion coefficients in membranes tend to be at least two orders of magnitude smaller than in solution leading to characteristic fluctuation times of seconds to minutes).

In 1984, Petersen introduced the concept of Scanning Fluorescence Correlation Spectroscopy (S-FCS) (Petersen, 1984) as a means of imposing a flow on the sample at a known velocity and thereby capturing the amplitude as a means of estimating the number of molecules on a cell surface. Subsequently, he demonstrated (Petersen, 1986) that the amplitude of the correlation function could be used to estimate the density of clusters of proteins on the surface as well as the number of molecules per cluster. While the approach did not provide information about the natural dynamics (diffusion or flow) it was argued that this could be obtained by complementary fluorescence photobleaching experiments. The Scanning FCS experiment was the first approach to obtain quantitative information about the state of aggregation of proteins in cell

membranes and was applied successfully to studies of several protein systems in fixed cells (Petersen et al., 1986; St. Pierre and Petersen, 1990; 1992). However, the measurements were slow and cumbersome and required high precision scanners that could move the sample over 100 :m distances with tens of nm precision (Petersen and McConnaughey, 1981).

3. IMAGE CORRELATION AND IMAGE CROSS CORRELATION SPECTROSCOPY

Image Correlation Spectroscopy (ICS) was introduced (Petersen et al. 1993) as a two-dimensional implementation of the Scanning Fluorescence Correlation Spectroscopy approach to take advantage of the rapid image acquisitions provided by laser scanning confocal microscope systems developed in the late 1980's. Rather than scanning the sample, the laser beam is moving linearly across the stationary sample in the x-direction and this is repeated at different y-positions to generate a two-dimensional image. At each position in the image (defined as a pixel) the intensity of fluorescence is measured as a photon count, i(x,y), reflecting the number of molecules that are excited within the beam area when the beam is centered at

that x,y-position. The image represents a large number of samples of fluorescence across the surface as compared to a large number of samples of fluorescence measured in a single position as a function of time. For an ergodic system, the two types of measurements will provide the same estimate of the average properties of the system, such as the average number of molecules in the illumination area by the laser beam. Image Correlation Spectroscopy has now been demonstrated to be a powerful tool to measure, quantitatively, the average cluster density of receptor molecules on a cell surface. It is currently the best demonstration of the application of the general concept of Spatial Fluorescence Correlation Spectroscopy (Petersen, 2001).

3.1 Acquisition of Data

Figure 1 illustrates the critical elements of data acquisition for ICS measurements. An image is collected from a section of the membrane of an adherent cell under conditions where the image (in our work 512x512 pixels) covers a small area of the surface (in our work 15.5x15.5 :m) (Figure 1A). Under these conditions, the nominal resolution is ~30 nm per pixel while the actual resolution is on the order of 300 nm (the width of the laser beam at

the focal plane). Thus the data is sampled at about 10-fold higher resolution – it is oversampled. This suggests that all clusters or domains that are smaller than about 300 nm will appear as spots with a 300 nm radius (Figure 1B). Correspondingly, a trace of the intensity as a function of distance (Figure 1 C) will show intensity variations that are close to Gaussian in shape and with a width determined by the laser beam intensity profile. The amplitude of each of these peaks will depend on the number of molecules present in the cluster or domain being detected.

Figure 2A is an image of the distribution of epidermal growth factor receptors on A431 cells. There are a number of bright spots, each representing a cluster of proteins. The spots have the same dimensions, but differ in brightness, indicating that the clusters are all smaller than the beam size and that they contain different numbers of receptors. The receptor distribution is not uniform.

3.2. Image Correlation Spectroscopy (ICS)

Image Correlation Spectroscopy involves calculating the autocorrelation function of a digital image, such as a confocal image in which the intensity is given by i(x,y) at

the pixel located at positions x and y in the image (Equation 2).

$$g(\xi,\eta) = \frac{\left\langle \left(i(x,y) - \left\langle i(x,y)\right\rangle\right)\left(i(x+\xi,y+\eta) - \left\langle i(x,y)\right\rangle\right)\right\rangle}{\left\langle i(x,y)\right\rangle^2} \quad (2)$$

This two-dimensional autocorrelation function, g(>,0), will decay in the spatial lag coordinates, > and 0, as a two-dimensional Gaussian function (reflecting the beam shape, Equation 3) with a width corresponding to the width of the laser beam, w. The correlation function for the image in Figure 2A is depicted in Figure 2B. The front quadrant in Figure 2B shows the fit to the expected function (Equation 3) with three adjustable variables: the amplitude, g(0,0), the width, w, and the baseline offset, g_0:

$$g(\xi,\eta) = g(0,0)\, e^{-(\xi^2+\eta^2)/w^2} + g_0 \quad (3)$$

The amplitude of the autocorrelation function has the same interpretation as in FCS and thus in the first instance, g(0,0) = 1/<N>. However, when the system consists of a mixture of clusters with a distribution of the number of monomers in each cluster, as seems to be the case in Figure 2A, the interpretation is less obvious. Magde et al (1978) demonstrated that for a mixture of molecules with different extinction coefficients, g, quantum yield, Q, and concentration C, the amplitude of the correlation function is given by Equation 4.

$$g(0,0) = \frac{1}{A} \frac{\sum_{j=1}^{N} (\varepsilon_j Q_j)^2 \overline{C}_j}{\left(\sum_{j=1}^{N} (\varepsilon_j Q_j) \overline{C}_j \right)^2} \qquad (4)$$

Later it was shown (Petersen 1986; 1993; 2001; Wiseman and Petersen, 1999) that for a single distribution, f(j), with a mean, :, and a variance, F^2, the amplitude of the correlation function can be rewritten as Equation (5)

$$g(0,0) = \frac{1}{\overline{N}_c} \frac{\sum_{j=1}^{n} j^2 f(j)}{\left(\sum_{j=1}^{n} j f(j) \right)^2} = \frac{1}{\overline{N}_m} \left[\frac{\mu^2 + \sigma^2}{\mu} \right] \quad , \qquad (5)$$

where N_c is the number of clusters, N_m is the number of monomers and the bar indicates an average over all of the image. It is clear from Equation 5 that if the mean of the distribution is significantly greater than the variance then the amplitude of the autocorrelation function can be approximated as the inverse of the average number of clusters observed in the observation area.

$$g(0,0) \approx \frac{1}{\overline{N}_m}(\mu) \approx \frac{1}{\overline{N}_c} \qquad (6)$$

Accordingly, we have defined two useful parameters: the cluster density, CD, and the degree of aggregation, DA:

$$CD = \frac{1}{g(0,0)\, \pi w^2} \approx \frac{\overline{N}_c}{\mu m} \qquad (7)$$

$$DA = \langle i(x,y) \rangle g(0,0) \approx c\overline{N}_m \frac{1}{\overline{N}_m}(\mu) = c\mu = c\frac{\overline{N}_m}{\overline{N}_c} \qquad (8)$$

where the constant c is an instrumental parameter that relates the measured intensity in a particular pixel, i(x,y), to the number of monomers, N_m, in the beam area at that location.

Equations 2-8 illustrate that the autocorrelation function (Figure 2B) of a confocal image (Figure 2A) can serve to provide a measure of the average number of clusters per unit area and the average number of monomers per cluster in that area of the cell. The key to the success of ICS is that it is relatively simple to make measurement of many images on many cells in a population, calculate the autocorrelation function of each image, estimate the CD and DA values and average these for the population of cells. More importantly, these measurements are quite sensitive and thus provide good quantitative estimates of the desired parameters. Figure 3 illustrates the sensitivity for a pair of images in which the average number of molecules per unit area is the same (the average intensities are 15.9 and 16.0 respectively) while the degree of aggregation differs by a factor of four (DA = 1.50 for Figure 3A and 0.36 for Figure 3B). The latter difference is a direct reflection

of the difference in the amplitude of the correlation functions (0.07 in Figure 3C and 0.014 in Figure 3D).

3.3 Image Cross Correlation Spectroscopy (ICCS)

ICS provides excellent estimates of the average number of clusters and the degree of aggregation of a particular molecule on the cell surface from a set of images of the distribution of that molecule. To obtain information about the interaction between different molecules, we must collect separate images for each of the molecules on the same areas of the cell and calculate the cross correlation functions.

Figure 4 illustrates the principle. Two images are collected from the identical region of the same cell. Cross multiplication of the intensities of corresponding pixels in the two images will yield a positive contribution to the cross correlation function only if there are spatially coincident fluctuations in the two intensities. The spatially coincident fluctuations will arise if the two molecular species are located in the same region of the cell.

The most general cross correlation function is calculated for a pair of images represented by i(x,y;t) and j(x,y;t), where the time parameter indicates that the two images could be collected at different times, as illustrated in Equation 9:

$$g_{ijt}(\chi,\xi;\tau) = \frac{\left\langle \left(i(x,y;t) - \langle i(x,y;t)\rangle\right)\left(j(x+\chi,y+\xi;t+\tau) - \langle j(x,y;t)\rangle\right)\right\rangle}{\langle i(x,y;t)\rangle\langle j(x,y;t)\rangle} \qquad (9)$$

It has been shown (Brown et al, 1999; Srivastava and Petersen, 1996; 1998; Srivastava et al., 1998) that this cross correlation function will decay in the spatial parameters as a Gaussian function and in the time domain as the F(**J**) described in Equation 3. The amplitude of the cross-correlation function at zero lag distances and lag time, g(0,0;0), can be approximated (Schwille et al., 1997, Petersen, 2001) by

$$g_{ijt}(0,0;0) = \lim_{\chi\to 0,\xi\to 0;\tau\to 0} g_{ijt}(\chi,\xi;\tau) = \frac{\overline{N}_{ijt}}{\left(\overline{N}_{it}+\overline{N}_{ijt}\right)\left(\overline{N}_{jt}+\overline{N}_{ijt}\right)} \qquad (10)$$

where N_{it} is the number of clusters with species i only, N_{jt} is the number of clusters with species j only and N_{ijt} is the number of clusters with both species i and j present. Thus, the sums (N_{it}+N_{ijt}) and (N_{jt}+N_{ijt}) represent the total number of clusters containing species i and j, respectively. Each of these can be measured directly from

the amplitudes of the corresponding autocorrelation functions as given by Equation 6. Equation 10 was derived for pairs of individual molecules in solution (Schwille et al., 1997), but it has been applied with reasonable success to clusters of molecules (Brown et al., 1999) and beads in solution (Wiseman et al., 2000). We believe that Equation 10 will remain a reasonable approximation as long as the distribution of sizes of clusters is reasonably narrow (as required for Equation 6).

Figure 5 is an example of two images collected for two proteins labeled with different antibodies. The image in Figure 5A yields the autocorrelation function in Figure 5C from which the density of clusters of molecule A and its degree of aggregation can be inferred (Equations 7 and 8). Correspondingly, the image in Figure 5B yields the autocorrelation function in Figure 5D from which the density of clusters of molecule B and its degree of aggregation can be inferred. The cross correlation function for the two images is shown in Figure 5E from which the number of clusters with both molecule A and B can be determined. Combining Equations 6 and 10 yields the average density of clusters with both molecules present

$$CD_{ij} = \frac{g_{ijt}(0,0;0)}{g_{it}(0,0;0)\, g_{jt}(0,0;0)\, \pi w_i^2} = \frac{\overline{N}_{ijt}}{\pi w_i^2} \qquad (11)$$

Thus measurement of two images (Figures 5A and 5B) provides the data to calculate the amplitudes of the two autocorrelation functions (Figures 5C and 5D) and the cross correlation function (Figure 5E) which combine in Equation 11 to give the density of mixed clusters.

For convenience, we have defined (Equation 12) a pair of fractions that provide estimates of what proportion of clusters that contain one molecule also contains the other. Crudely, this can be thought of as the fraction of co-localization: the fraction of one molecule that is co-located with the other and visa versa.

$$F(i\,|\,j) = \frac{CD_{ijt}}{CD_{it}} = \frac{g_{ijt}(0,0;0)}{g_{jt}(0,0;0)\, \pi w_j^2} \quad \text{and} \quad F(j\,|\,i) = \frac{CD_{ijt}}{CD_{jt}} = \frac{g_{ijt}(0,0;0)}{g_{it}(0,0;0)\, \pi w_i^2} \qquad (12)$$

For example, F(i|j) is the ratio of all the clusters that contain both species i and j to all the clusters that contain i (whether alone or not). If all of species i is co-located with j, then this fraction is expected to be one. Note that even if all of species i is co-located with species j, there is no requirement that all of species j be completely co-located with species i. The two fractions in

Equation 12 are therefore not necessarily directly related to each other.

It is possible to make reasonable control experiments that demonstrate the validity of the approximations inherent in the above analysis and to get an estimate of how accurately the ICCS experiments can be. First, as a positive control one may label the same molecule simultaneously with two different probes. In that case, one expects complete co-localization and the fractions in Equation 12 should both be close to one. Second, as a negative control one may cross correlate images collected from different cells measured under similar conditions. In this case, one expects no co-localization and the fractions in Equation 12 should both approach zero.

Table I illustrates one example of the estimate of the co-localization of pairs of receptors (Petersen et al., 1998). The positive control experiment, in which transferrin receptors (Tf-R) were labeled simultaneously with antibodies that have green fluorescent probes (Tf-R-G1) and red fluorescent probes (Tf-R-R1), shows that the two fractions are close to one (within ~10%). Correspondingly, the negative control experiment, in which the images from green fluorescent probes (Tf-R-G1) were randomly cross correlated with images from red fluorescent

probes (Tf-R-R2), shows that these fractions are close to zero (within a few percent). This suggests that the ICCS experiments can provide estimates with an accuracy approaching 10%. The actual experiments show that there is no co-localization between transferrin receptors (Tf-R) and epidermal growth factor receptors (EGF-R) wheras there is strong co-localization between transferrin receptors (Tf-R) and platelet derived growth factor receptors (PDGF-R). Approximately 80% of the transferrin receptors are located in clusters that also contain the PDGF receptor, suggesting that about 20% of the transferrin receptors are not associated with PDGF receptors. Correspondingly, about 70% of the PDGF receptors are in clusters that also contain the transferrin receptor, suggesting that about 30% are not associated with transferrin receptors. There are therefore at least two populations of each receptor type – those in common clusters and those in separate clusters. The table also shows (CD_{ij}) that there are about four to five clusters with both receptors per square micrometer. Whether these clusters in turn represent the coated pits in cells (Brown et al., 1999) remains to be determined.

4. DYNAMIC IMAGE CORRELATION SPECTROSCOPY

The ICS experiments provide quantitative estimates of receptor cluster distributions and the numbers of monomers in a cluster. The ICCS experiments provide quantitative estimates of the extent of co-localization of pairs of molecules in clusters or domains. In both measurements, the dynamic information is lost. It is, however, possible to collect images in multiple spectral ranges as a function of time, which provides the opportunity to capture some of the dynamic processes within the time scale defined by the speed of image acquisition. This leads to three distinct applications of Dynamic Image Correlation Spectroscopy (DICS)[1]: ICS measurements as a function of time, ICCS measurements as a function of time and calculation of cross-correlation functions of images separated in time.

4.1 Time Dependence of ICS and ICCS experiments

The simplest approach to the dynamics is to calculate the time dependence of the cluster density, CD(t), and the degree of aggregation, DA(t), from the autocorrelation function amplitudes of each of the images in the time series. In principle, this can lead to measurements of the

kinetics of assembly into clusters or disassembly of clusters into monomers on the surface. Figure 6 illustrates how the temporal variation of the density of clusters and the degree of aggregation can be used to follow the disassembly of a5-integrin receptors in CHO B2 cells on the time scale of minutes. As is evident from the images of the cells shown at four selected times (Figures 6A-D) there are a number of bright adhesion complexes that disappear in Region 1. The average degree of aggregation (Figure 6E) deceases about four-fold over a period of approximately 200 seconds, while there is no change in the state of aggregation in the neighboring area (Region 2). Correspondingly, the cluster density (Figure 6F) increases by a corresponding factor over similar time scale, although it appears that there is a delay between the decrease in the degree of aggregation and the increase in the number of clusters. It is evident that the number and the size of the receptor clusters vary on the minute time scale for this system and that the DICS measurements provide a convenient tool to obtain quantitative kinetic data for rather complex systems. To date, there are only a few examples of this type of analysis (Petersen et al., 1986; Nohe and Petersen, 2002; 2004) but it can provide useful

[1] The term Temporal ICCS has also been used to describe these experiments (CMB).

insight into the mechanisms whereby protein or lipid domains assemble or disappear. For example, the dispersal of viral lipids occurs more rapidly than the dispersal of viral proteins during the fusion of a virus to a cell membrane suggesting that there are intermediate states where the lipids can transfer from the virus to the cell membrane, but the proteins cannot (Rocheleau and Petersen, 2001).

It is also possible to monitor the cross-correlation function as a function of time to obtain the kinetics of association or dissociation of different molecules in the clusters or domains. So far, this type of experiment has not been explored systematically, even though it can provide important insight into the mechanism of formation of clusters and domains in the membrane.

4.2 Dynamic Image Cross Correlation Spectroscopy

The general expression for the cross correlation function (Equation 9) contains both the space and time coordinates. If the two images, i(x,y;t) and j(x,y;t) are collected for the same molecules from the same area of the same cell but at different times, then the cross correlation function will measure the extent to which the correlation of clusters with themselves is disappearing because of the

movement of the clusters. This is illustrated in Figure 7. A series of images were collected for EGF-Receptors over time from the same region of a single A431 cell (Figure 7A). All the images were created with a red look-up table so that each of the fluorescent clusters appears red. Then the first image, corresponding to t=0, was created using a green look-up table and superimposed on each of the other images. Figure 7B shows the result when the first image (t=0) is superimposed on itself. As expected, there is complete 'co-localization' of all of the clusters (all of the spots are yellow since the red and the green images are identical). Figures 7C to 7F show the result when the first image (t=0) is superimposed with images collected at t = 24, 128, 160 and 400 seconds. It is evident that as the time separation between the first and the subsequent image increases, the extent of co-localization decreases (the number of yellow spots decrease) and at 400 seconds there is no co-localization at all. The lateral movement of the clusters causes a loss of correlation of the clusters with them selves. We expect the amplitude of the cross correlation functions for pairs of images separated in time will decrease correspondingly.

For a series of images collected from the same region as a function of time, we may express the time variation of the amplitude of the cross correlation function as

$$g_x(0,0,\tau) = \lim_{\chi\to 0,\xi\to 0} \frac{\left\langle \left(i(x,y) - \langle i(x,y)\rangle\right)\left(i(x+\chi, y+\xi; t+\tau) - \langle i(x,y)\rangle\right)\right\rangle}{\langle i(x,y)\rangle^2} = \frac{1}{\overline{N}_i} F(\tau) \quad (13)$$

where the decay function, F(J), has the same functional form as that in Equation 1 (Srivastava and Petersen, 1996; Wiseman et al., 2000). In effect, this experiment is an ensemble averaged FCS experiment in which the dynamics of all of the regions of the surface of the cell are measured simultaneously. Figure 8 shows an example of the decay of the amplitude of the cross correlation functions as a function of the time separation between images. The solid line represents a fit to the data according to

$$g(0,0,\tau) = \frac{g(0,0;0)}{1+\frac{\tau}{\tau_d}} + g_0 \quad (14)$$

which suggests that the dynamic process is a two-dimensional diffusion process with a diffusion coefficient $D \sim 10^{-11}$ cm^2 s^{-1}. This is consistent with the changes in position of the clusters illustrated in Figures 7B-7F. This analysis provided information about the dynamics of proteins within the cell membrane. If it is extended to the cross-correlation spectroscopy domain with two different proteins, the analysis will provide dynamic

information that can arise only from co-movement of proteins within a common complex (Wiseman et al., 2000).

5. DISCUSSION

The systematic analysis of confocal images described above provides a set of tools to analyze quantitatively the extent of formation of clusters, the extent of association between different molecules and the dynamics of this interaction. While these approaches are relatively new, there are several applications emerging (Boyd et al., 2002; Brown et al 1999, Brown and Petersen, 1998; 1999; Fire et al., 1997; Nohe and Petersen, 2002; 2004; Nohe et al., 2003; Rocheleau et al, 2003) and it appears that these approaches can provide new and useful information.

The strengths of the Image Correlation Spectroscopy approaches are that the measurements are relatively easy with modern confocal imaging tools, that the data analysis is straightforward and that it is possible to make many measurements on a large number of cells in a population. The individual measurements are quantitative and it is simple to obtain good statistics for comparison of changes that may occur as a result of perturbing the cell system.

The greatest weaknesses of Image Correlation Spectroscopy are that one must carry out significant numbers of controls and the data must be corrected for systematic effects (Wiseman and Petersen, 1999). Because the measurements are simple, it is easy to collect apparently meaningful data so vigilance is also required to ensure that the results are physically meaningful.

The Image Cross Correlation Spectroscopy measurements have great potential for delivering quantitative information about the extent to which pairs of membrane molecules interact in clusters or domains on the cell surface. In principle, ICS and ICCS will capture all of the molecular interactions that persist on the timescale of the measurement, which is the time needed to capture a particular cluster in the image. For a typical laser scanning confocal microscope, this corresponds to microseconds in the x-direction and milliseconds in the y-direction. These tools should therefore measure strong as well as weak interactions and persistent as well as transient interactions.

It is important to recognize that the correlation analysis only requires that the molecules being measured be organized in some fashion on the surface. They need not be in molecular contact as long as they are part of the same

complex. Thus, without other evidence from immunoprecipitation or FRET measurements, for example, it is impossible to determine the nature of the intermolecular interactions. For example, all of the components in a domain such as a coated pit will be seen to co-localize whether they are in fact interacting directly with each other or only with a common component, such as an adaptor protein. However, interactions between molecules that are part of a common protein complex but not in molecular contact would be undetectable without the ICCS approach. These types of interactions could be very central to understanding the mechanism of formation and disassembly of protein complexes within the cell such as coated pits, focal adhesions and tight junctions which in turn may lead to significant new insight into mechanisms underlying activation of specific signal transduction pathways.

To further advance the utility of both ICS, ICCS or FRET, we propose that it may be possible to perform the correlation analysis on FRET images. If properly collected, the FRET image should represent the fluorescence intensity arising from the acceptor only, which should in turn derive only from those clusters or domains in which the donor-acceptor interactions are within the appropriate distance. The ICS analysis will then yield an estimate of

the number of clusters on the surface in which there are direct intermolecular interactions. Comparison with the regular ICS could then reveal the fraction of clusters in which this direct interaction occurs. We are currently working on FRET-ICS experiments, but have so far seen very few systems in which the FRET is sufficiently strong to make the ICS analysis robust. Perhaps this is an indication that for the receptors we have studied so far, the molecular contact between the receptors in these clusters and domains is relatively rare.

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Figure Captions

Table 1: Illustration of co-localization of receptors on a cell surface. The number of pairs of images is given by N. The "%fit" refers to the percentage of the cross-correlated images that provide a fit at the origin of the cross-correlation function and hence have meaningful amplitudes. The other parameters are explained in the text (Data courtesy of Mamta Srivastava).

Figure 1: Schematic representation of the image acquisition for Image Correlation Spectroscopy

Figure 2: Example of a confocal microscope image (A) and the corresponding autocorrelation function (B)

Figure 3: Illustration of the sensitivity of ICS to the state of aggregation

Figure 4 (color): Schematic representation of the cross correlation function calculation: Spatially coincident fluctuations will give positive results in the cross multiplications inherent in the cross correlation function calculation.

Figure 5 (color): Illustration of the image cross-correlation spectroscopy principle.

Figure 6: A CHO B2 cell expressing a5-integrin-GFP. Images A-D show sample images from a time lapse movie. In region 1 a row of adhesions disassembles while in region 2 there is no significant change in the overall protein distribution. Panel E and F show how the degree of aggregation (E) decreases and the cluster density (F) increases as the adhesion complex disassembles (filled circles). Note that there is little change in the DA for region 2 and no really systematic change in the CD over the same time interval (open triangles). It should be noted that the DA and the CD can become somewhat misleading when there are large organized protein structures such as in region 1 but can still be used to follow the overall changes in protein aggregation.

Figure 7 (color): Illustration of the movement of clusters relative to their original positions

Figure 8: Decay of the amplitude of the cross correlation function between images collected at different times