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Fluorescence Fluctuation Approaches to the Study of Adhesion and Signaling

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Abstract

Cell–matrix adhesions are large, multimolecular complexes through which cells sense and respond to their environment. They also mediate migration by serving as traction points and signaling centers and allow the cell to modify the surrounding tissue. Due to their fundamental role in cell behavior, adhesions are germane to nearly all major human health pathologies. However, adhesions are extremely complex and dynamic structures that include over 100 known interacting proteins and operate over multiple space (nm– μ m) and time (ms–min) regimes. Fluorescence fluctuation techniques are well suited for studying adhesions. These methods are sensitive over a large spatiotemporal range and provide a wealth of information including molecular transport dynamics, interactions, and stoichiometry from a single time series. Earlier chapters in this volume have provided the theoretical background, instrumentation, and analysis algorithms for these techniques. In this chapter, we discuss their implementation in living cells to study adhesions in migrating cells. Although each technique and application has its own unique instrumentation and analysis requirements, we provide general guidelines for sample preparation, selection of imaging instrumentation, and optimization of data acquisition and analysis parameters. Finally, we review several recent studies that implement these techniques in the study of adhesions.

1. INTRODUCTION

Cells sense and interact with other cells and the extracellular matrix (ECM) through multimolecular assemblies called adhesions. The bidirectional transduction of stimuli through adhesions allows cells, for example, to sense the stiffness of their microenvironment, migrate, and remodel the ECM by proteolysis, contraction, and fibrillogenesis (Geiger, Spatz, & Bershadsky, 2009). Due to their pivotal role in migration and other cell behaviors, adhesions are a focal point in the study of such diverse topics as inflammation, wound healing, tumor progression, embryonic morphogenesis, and tissue engineering and regeneration (Ridley et al., 2003).

Adhesions serve in two general capacities: as a continuous physical connection between the substratum and the actin cytoskeleton, and as signaling centers that initiate and coordinate a complex network of interrelated signal transduction pathways. ECM components bind to integrins (cell adhesion receptors), which span the cell membrane and connect to actin through a system of linked proteins (Vicente-Manzanares, Choi, & Horwitz, 2009). Through this connection, forces from actomyosin contraction and membrane resistance to actin polymerization are transferred to the substratum (Brown et al., 2006). Concurrently, integrin clustering, ligand binding, and the forces exerted on adhesions produce biochemical signals that, among other things, feedback to alter actin polymerization, myosin contractility, and

adhesion (Geiger et al., 2009). Through this loop, physical and chemical stimuli transduced through adhesions modify the cytoskeleton and force development, which in turn feedback to affect adhesion formation and the composition, organization, and function of the cell and its extracellular microenvironment.

While this conceptual framework is well established, the mechanisms through which the physical and biochemical signals are bidirectionally transduced are not well understood. This is due in part to the molecular complexity of adhesions, which can contain over 160 different molecules that participate in over 700 putative interactions, most of which have been primarily identified and characterized by *in vitro* biochemical assays (Zaidel-Bar, Itzkovitz, Ma'ayan, Iyengar, & Geiger, 2007). The components that comprise adhesions serve several general functions; for example, as scaffolds that organize signaling complexes, force-bearing connections, enzymatic de-/activators (e.g., kinases, phosphatases, and proteases), and combinations of these functions. Although recent studies have begun to provide static snapshots of adhesion structure *in situ* (Kanchanawong et al., 2010), we have little knowledge of and few tools to investigate how the functions of adhesion components are organized and function in space and time in living, dynamic cells.

A major challenge to studying adhesions is the large spatiotemporal range that must be spanned. In a “typical” migrating cell, small, diffraction-limited “nascent adhesions” assemble and disassemble within a thin (~2–3 μm) band at the leading edge at rates as fast as tens of seconds (Choi et al., 2008). Under the influence of nonmuscle myosin II, nascent adhesions grow and elongate as they mature initially into focal complexes (~1 μm diameter) and subsequently into focal adhesions and fibrillar adhesions, which can span many micrometers and remain stable for minutes to hours (Geiger et al., 2009). While some adhesions can be static over long time periods, individual adhesion components can exchange on and off the adhesion over a broad timescale: from subsecond to minutes, depending on the adhesion (Goetz, 2009; Lele et al., 2006; Wolfenson, Bershadsky, Henis, & Geiger, 2011). Presumably, these dynamics are intrinsic to the organization and function of the adhesion. Therefore, it is not enough to simply study a single adhesion in a single place and time, since they are in a continuous process of structural and functional change and thus heterogeneous throughout the cell. Understanding the organization and functions of these different adhesions, including their formation and interconversion, requires monitoring potentially short-lived molecular associations in adhesions that are themselves moving and changing size at slower rates and over a larger area. Due to these challenges, we still lack such basic information about adhesions as, for example, the numbers and stoichiometry of components in different adhesions, how adhesions assemble (e.g., whether components exchange as complexes or individual molecules), and when and where signaling complexes form and become active. Ideally, one would like to generate association maps that relate molecular events such as molecular number and clustering, the formation and properties of the integrin–actin linkage, and the specific signals generated by adhesions within the context of large-scale cell processes and ultimately cell movement, itself. However, current mainstream techniques are not well suited for such a multi-scale challenge.

Traditional techniques used to study the interactions and dynamics of adhesion components suffer from limited spatial and/or temporal resolution. The interactions among adhesion components have been characterized primarily by co-immunoprecipitation and fluorescence colocalization. The former provides high temporal but poor spatial resolution and requires an *in vitro* setting; the latter provides *in situ* localization but low spatio-temporal resolution. Fluorescence recovery after photobleaching (FRAP) provides *in situ* information about the exchange rates (apparent affinity) of adhesion components (Wehrle-Haller, 2007; Wolfenson et al., 2011) but is limited to relatively large areas and long timescales (slow-moving objects and slow exchange rates). Fö rster resonance energy transfer (FRET) is used

to study molecular associations in adhesions (e.g., Ballestrem et al., 2006); but it suffers from a low dynamic range: FRET is sensitive to changes in distance between 0.5 and 2 times the Förster radius (i.e., between ~2 and 10 nm) and the magnitude of the differences are small. In adhesions, molecules can be associated indirectly in large complexes (distances >10nm), and therefore may be missed by FRET. Conversely, molecules that are not in common complexes can nevertheless be in juxtaposition and show FRET. Moreover, FRET experiments suffer from artifacts (e.g., multiple donor and acceptor interactions) and thus require extensive controls for every FRET pair studied. Finally, none of these methods can determine the number of molecules of a certain component and stoichiometries within an adhesion.

The need for techniques that can provide data on molecular dynamics and associations in adhesions of living cells has driven the development of a toolbox of fluorescence fluctuation techniques. These methods can derive multiple pieces of information from a single data set and at high spatio-temporal resolution. From a single time series, for example, fluctuation analyses can provide molecular numbers, aggregation states, exchange rates, diffusion and flow rates, and interactions with their stoichiometries. These methods operate over multiple spatiotemporal regimes with high resolution. In this chapter, we discuss how fluorescence fluctuation techniques described elsewhere in this volume are being applied to study adhesions in living cells. Although we limit our discussion to cell-matrix adhesions, the techniques, methods, and considerations outlined in this chapter are generally applicable to other kinds of adhesions as well as the study of other biological processes.

2. A FLUORESCENCE FLUCTUATION TOOLBOX

The machines (e.g., cell protrusion, adhesion, and retraction) and regulatory systems that drive cell migration are dynamic, spatially restricted, and integrated in the migrating cell. They appear to operate using a plethora of transient, localized molecular interactions (Parsons, Horwitz, & Schwartz, 2010; Vicente-Manzanares & Horwitz, 2011). Understanding the molecular machines and interactions underlying cell migration would benefit greatly from high-resolution mapping of the dynamics and associations of adhesion molecules in space and time. In response to this need, a “toolbox” of complementary fluorescence fluctuation techniques has been developed. This toolbox promises to enable the in-depth study of the associations and dynamics that contribute to nearly every aspect of cell migration.

In this section, we introduce the underlying principles of fluorescence fluctuation microscopy beginning with its original development as a single-point temporal correlation approach for solution measurements (fluorescence correlation spectroscopy, FCS). We then describe the recent advances that have extended FCS to migrating cells (image correlation spectroscopy, ICS). The ICS approaches use spatiotemporal correlations from an image time series instead of a single point, thereby extending the scope of the original FCS from a single point to high-resolution cellular maps. Finally, we introduce a variant of intensity variance analysis (number and brightness, N&B) that provides information on molecular aggregation and interactions at pixel resolution.

2.1. Fluorescence correlation spectroscopy

FCS was originally developed to measure diffusion coefficients and chemical rate constants of biomolecules in solution (Magde, Elson, & Webb, 1974). With the development of genetically encoded fluorescence labeling (i.e., fluorescent proteins, FPs) (Miyawaki, 2011; Shaner, Steinbach, & Tsien, 2005), better fluorescence microscopes (Axelrod, 2008), and sensitive fluorescence detectors, this technique and its variants have become established

biophysical approaches for studying biomolecular aggregation, dynamics, and interactions with a broad range of applications both *in vitro* and in cells (Haustein & Schwille, 2007).

FCS is based on measuring fluorescence intensity fluctuations that arise from the transport or movement of fluorophores in and out of an optically defined focal volume (~ 1 fL), that is, the point spread function (PSF) which characterizes the focused laser beam. These fluctuations arise from a number of processes including diffusion, directed movement (flow), and binding and unbinding (exchange) phenomena (Fig. 6.1). In addition to these processes, fluorophore blinking and bleaching also produce intensity fluctuations. Intensity fluctuations are usually analyzed using the autocorrelation function (ACF), the decay rate of which reflects the rates of these fluctuations. Assuming a quasi-equilibrium, the ACF can be modeled and fit to estimate diffusion coefficients, flow speeds, and rate constants (Elson & Magde, 1974; Magde et al., 1974). Fluorescence cross-correlation analysis is also used to quantify molecular interactions through the presence of correlated fluctuations in the intensity of two molecules labeled with different color fluorescent probes (Bacia, Kim, & Schwille, 2006; Berland, 2004).

The amplitude of the ACF can be used to determine the average number of fluctuating units (i.e., individual molecules or complexes) within the beam volume. In most cases, the number of particles follows a Poisson distribution, where the root mean square fluctuation (amplitude of ACF) is inversely proportional to the number of fluctuating units (Fig. 6.1). When the fluctuating units are homogeneous (e.g., all monomers or all dimers), the number of fluctuating units within the observation volume can be accurately determined from the ACF amplitude. When the units are heterogeneous, the ACF gives the average aggregate size. In this case, other fluctuation analysis methods, such as fluorescence intensity distribution analysis (FIDA) or the photon-counting histogram (PCH), can be used to determine the number of molecules in the different size complexes (monomers, dimers, etc.) (Chen, Müller, So, & Gratton, 1999; Chen, Wei, & Müller, 2003; Kask, Palo, Ullmann, & Gall, 1999).

The ability to record fluorescence counts from a single, defined focal volume for a long period of time (1–10 s) is an advantage of FCS. This enables the study of a broad range of processes, from fast photophysical dynamics such as fluorophore blinking (1–10 μ s) to the slower diffusion of molecules in solution or the cytoplasm. The even-slower transport properties of cell-membrane-associated molecules such as lipids and integrins can also be monitored by FCS; however, due to membrane crowding effects and clustering at cellular structures such as cell adhesion sites, reduced characteristic fluctuation times and even immobile populations are often reported. Therefore, the dynamics of adhesions are at or beyond the sensitivity FCS (see Table 6.1). In principle, one can image for a longer period of time to capture these slower dynamics; however, photobleaching, cell toxicity due to continuous laser illumination, and macroscopic membrane fluctuations over time limit its use.

Scanning-FCS—a 2D extension of classical FCS—overcomes some of these limitations (Ruan, Cheng, Levi, Gratton, & Mantulin, 2004). In this approach, multiple points are monitored by moving the observation volume to different points (e.g., a circle) within the sample. Scanning-FCS has been used to study slower membrane protein dynamics (Petrasek et al., 2008; Ries, Yu, Burkhardt, Brand, & Schwille, 2009); however, its applicability to study cell-migration-related processes is limited due to the restricted spatial observation area.

2.2. Image correlation spectroscopy techniques

ICS is an extension of FCS; it was originally developed to measure molecular number densities and aggregation states from individual images (Petersen, Hoddellius, Wiseman,

Seger, & Magnusson, 1993). It now consists of a family of analysis methods that combines spatial and temporal correlations of intensity fluctuations in an image time series and, like FCS, estimates molecular numbers, aggregation states, and dynamics. However, it is capable of capturing a wide range of dynamics that includes the slow transport properties of membrane receptors and adhesion components. ICS can also reveal protein interactions by measuring cross-correlations between intensity fluctuations from pairs of molecules labeled with different color fluorescent markers (Fessart et al., 2007; Wiseman & Petersen, 1999). A major advantage of ICS is that multiple analysis methods can be applied to the same image time series to extract complementary pieces of information (Fig. 6.2). Here, we briefly introduce ICS methods and outline the information each provides. For a more detailed overview of the background theory and implementation of the ICS techniques, refer to other chapters in this volume.

2.2.1 Image correlation spectroscopy—ICS measures spatial correlations within a single confocal image to determine *number densities* and *molecular aggregation states*. The spatial ACF (s-ACF) is fit to a 2D Gaussian function, and the recovered fit amplitude is used to estimate the number density and aggregation state (Petersen et al., 1993). Changes in these parameters can be followed over time by applying the analysis to individual images in an image time series (Wiseman & Petersen, 1999). This approach is best suited for slow moving or stationary proteins since they do not move over the image scan time (~ seconds).

2.2.2 Temporal-image correlation spectroscopy—Temporal-image correlation spectroscopy (TICS) is used to measure *transport coefficients* and *exchange rates* of biomolecules from image time sequences acquired using confocal laser scanning microscopy (CLSM) or camera-based microscopes (Digman, Brown, Horwitz, Mantulin, & Gratton, 2008; Wiseman, Squier, Ellisman, & Wilson, 2000). A temporal ACF (t-ACF) is calculated from region-to-region temporal intensity fluctuations and then fit to appropriate models for diffusion, directed motion, or exchange kinetics. For slow dynamics, for example, membrane receptors, TICS generally requires spatial averaging of temporal intensity fluctuations to improve sensitivity. Similar to ICS, the amplitude of the temporal ACF can also be used to estimate number densities.

2.2.3 Raster-image correlation spectroscopy—Raster-image correlation spectroscopy (RICS) is a complement to TICS and is designed to measure the *rapid diffusion* and *exchange* characteristic of molecules in the cytoplasm (Digman et al., 2005; Digman, Sengupta, et al., 2005). It is based on the temporal information intrinsic to an image acquired using CLSM. The laser scans across in a single line (in *x*-direction) with an ~2–20 μ s pixel-to-pixel scan speed (dwell time) and then moves down (*y*-direction) to scan the next line with ~ millisecond line-to-line scan speed. Fast diffusion and exchange occur on these timescales and will manifest in the spatial autocorrelation function (s-ACF), from which diffusion coefficients and exchange rates are computed. Number densities can also be recovered from the amplitude of the s-ACF.

2.2.4 Spatiotemporal image correlation spectroscopy—Spatiotemporal image correlation spectroscopy (STICS) measures the *velocity of directed movement* from image time series acquired using CLSM or TIRF. Unlike TICS, which averages single-point temporal autocorrelations of intensity fluctuations over a region in a time series, STICS uses combined spatiotemporal autocorrelation to detect directed motion over time (Hebert, Costantino, & Wiseman, 2005). The directed motion manifests as a translocation in the peak of the sp-ACF over time. Temporal tracking of the peak will provide an estimate of the magnitude and direction of flow.

Spatial and temporal mapping of adhesion protein dynamics and interactions can be achieved by applying the image correlation toolbox to different temporal segments of an image time series as well as subregions at different locations within the cell. Small defined regions (e.g., 10×10 pixels) and time segments (~ 10 – 20 frames) provide a more detailed picture of the spatiotemporal coordination of cell migration processes. The size of the spatiotemporal “window” is, however, limited by the need to sample a sufficient number of fluctuations to yield adequate statistics. Computer simulations have tested the limits of these techniques under various experimental acquisition settings (Brown et al., 2008; Costantino, Comeau, Kolin, & Wiseman, 2005; Hebert et al., 2005; Kolin, Costantino, & Wiseman, 2006). In this context, the ICS techniques do not have sufficient spatiotemporal resolution to study molecular interactions and dynamics in diffraction-limited nascent adhesions. The need for greater spatiotemporal resolution drove the development of the N&B variance analysis.

2.3 Number and brightness variance analysis

The N&B analysis is a fluctuation variance method that provides pixel-resolution mapping of number densities and aggregation states of adhesion proteins without sacrificing temporal resolution (i.e., long imaging times) (Digman, Dalal, Horwitz, & Gratton, 2008). N&B analyzes the variance in the intensity fluctuations. In contrast, the correlation approach requires many images to capture the entire spectrum of kinetic information and thereby fit the ACF and is therefore not practical for the rapidly turning over adhesions in migrating cells. In the N&B approach, two parameters are computed independently: N , which is the average number of fluctuating units (monomers or aggregates) and B , the brightness, which is an estimate of the aggregate size. For example, if a molecular complex consists of trimers, the measured B parameter will be three times the value measured for a monomer. Therefore, using appropriate monomer calibration, one can estimate the aggregation size (see Section 3). Furthermore, associations of different adhesion molecules within complexes can be detected by measuring a cross-brightness parameter, B_{cc} (Choi, Zareno, Digman, Gratton, & Horwitz, 2011; Digman, Wiseman, Choi, Horwitz, & Gratton, 2009). Dual-color calibration to monomer species tagged with two different fluorescent probes provides the aggregation state of the individual proteins within the complex (Choi et al., 2011).

3. EXPERIMENTAL IMPLEMENTATION

3.1. Fluorescent labeling

Fluorescence fluctuation techniques in living cells typically rely on ectopically expressed fluorescent proteins (FP) linked to the protein of interest (e.g., mGFP–paxillin). There are numerous resources and guides for selecting FPs for specific applications (Chudakov, Matz, Lukyanov, & Lukyanov, 2010; Shaner et al., 2005). Tables of relevant properties for most common FPs can be found online at the Molecular Probes (Invitrogen, <http://www.invitrogen.com>), Nikon microscopy (<http://www.microscopyu.com/>), Zeiss microscopy (<http://zeiss-campus.magnet.fsu.edu/>), and Olympus microscopy (<http://www.olympusmicro.com/>) Web sites. Here, we present some considerations for choosing a particular FP (or combination of FPs) for fluorescence fluctuation analyses of adhesions.

3.1.1 Spectral properties—Select FPs that are optimally excited by the laser lines available in your system (e.g., mGFP with the 488 nm line) to maximize the signal-to-noise ratio. For cross-correlation analysis in which two different protein types are labeled with different color FPs (“dual-color” experiments), choose FPs that minimize spectral bleedthrough and the potential for FRET. For example, GFP and mKusabira-Orange have large areas of spectral overlap that may contribute to FRET and/or bleedthrough (Fig. 6.3A). Alternatively, we often use mGFP and mCherry for dual-color experiments because they are

spectrally well separated; however, this combination requires a 568-nm laser as mCherry is only weakly excited by the 543-nm HeNe laser usually included on commercial microscope setups (Fig. 6.3B).

3.1.2 Brightness—Fluorescence brightness depends on two intrinsic fluorophore parameters: the extinction coefficient (efficiency of light absorption) and quantum yield (efficiency of light emission). For optimal signal to noise, use FPs with high brightness values.

3.1.3 Photostability—Repeated excitation of FPs can generate photo-oxidation products (free radicals) that can injure living cells (phototoxicity) and ultimately result in permanent loss of fluorescence (photobleaching). Consequently, photostable FPs should be selected. For example, when selecting a red FP, use mCherry instead of DsRed, as it is brighter and more photostable.

3.1.4 Oligomerization—While many FPs (e.g., DsRed) readily oligomerize, most FPs are available in monomeric form or can be made so by mutagenesis (Shaner et al., 2004). Monomeric FPs are required, for example, when quantifying the number density and aggregation state of molecular complexes using fluorescence fluctuation techniques. Transport studies are less sensitive to this, provided the oligomerization of the FP does not impair the function of the protein and thereby bias the recovered data.

In general, most fluorescently tagged proteins are fully functional; however, their biological activity needs to be tested in the context of the process under study. For adhesion molecules, correct localization to adhesions can be verified by immunostaining. Other approaches involve testing whether the genetically tagged construct can properly rescue a knockdown of the endogenous protein and showing that the construct responds to known perturbations, mutations, and/or inhibitors.

3.2. Transfection optimization

The DNA plasmid encoding the FP construct is introduced to cells via transfection. There are three primary transfection methods: cationic lipids, electroporation, and viruses. The last two methods are often used for difficult-to-transfect cell types and are more difficult to implement: electroporation requires additional instrumentation (e.g., Nucleofector from Lonza) and viral transfection requires specialized techniques and can entail working with retroviruses. Lipid-based approaches (Felgner et al., 1987) require no additional equipment and have been successfully used in many cell lines used for migration and adhesion studies.

3.2.1 Lipid-based transfection protocol—The following is a sample procedure for the lipid-based transient transfection of mGFP–paxillin and FAK–mCherry in CHO.K1 cells using Lipofectamine (Invitrogen) (Choi et al., 2011). Detailed protocols and additional reagents can be found on the manufacturer's Web site (www.invitrogen.com). Perform all steps under sterile conditions.

- a. Grow cells to 50–70% confluency in a six-well tissue culture dish (usually seeded the day before transfection).
- b. For each well, fill two sterile 1.5-ml microcentrifuge tubes with 100 μ l of Opti-MEM (Invitrogen, 11058) each.
- c. In the first tube (for each well), add 5 μ l of Lipofectamine reagent (Invitrogen, 18324) for every μ g of DNA used (typically 1 μ g).

- d. In the second tube, add 0.1 µg mGFP–paxillin, 0.1 µg FAK–mCherry, and 0.8 µg pBluescript (“blank” nonexpressing plasmid) to yield 1 µg total DNA
- e. Combine the solution from the Lipofectamine tube with the DNA solution and incubate for 20 min at room temperature.
- f. Meanwhile, rinse cells twice with Opti-MEM, then add 1 ml of Opti-MEM to each dish.
- g. After the incubation period, add 800 µl Opti-MEM to each Lipofectamine-DNA solution (total is now 1 ml), aspirate Opti-MEM from the wells, then add the entire solution dropwise to the wells.
- h. Incubate cells for 3 h in the tissue culture incubator (37 °C, 8.5% CO₂).
- i. Rinse cells twice with PBS, then add normal culture medium.
- j. Cells can be imaged on the following 1–2 days.

3.2.2 Expression level optimization—After transfection, cells typically express multiple copies of the plasmid for a few days and then begin to lose the ones that are not incorporated into chromosomal structures. Cells can be made to stably express constructs by using antibiotic resistance markers on the plasmids or virus-based transfection methods.

Expressing transgenic FP constructs can result in a large, nonphysiological excess of the protein. This can perturb the biology (e.g., mislocalization, alter binding kinetics, and perturb normal structure), mask fluctuations, and increase the fluorescence background as excess protein accumulates in the cytoplasm. Expression levels can be controlled by varying the amount of plasmid DNA and using carrier DNA to ensure high overall efficiency (Webb et al., 2005). There are at least three additional means for controlling expression:

- a. Limit transcription of your construct by using a weak promoter.
- b. Deplete the cells’ endogenous supply of the protein of interest by knocking down or knocking out the corresponding gene.
- c. Generate stably transfected cell lines and/or select for low expressors.

The first method involves simply exchanging the strong promoter used in most expression vectors for a weak one (Watanabe & Mitchison, 2002)—a process that, for most plasmids, can be completed by a straightforward restriction digest and ligation. We have used this modification with great success in our laboratory (Kubow & Horwitz, 2011; Vicente-Manzanares, Newell-Litwa, Bachir, Whitmore, & Horwitz, 2011). When using reduced-expression constructs, larger DNA amounts (0.2–0.4 µg) may be necessary. The second and third approaches are recommended for fluctuation measurements of number densities and aggregation of adhesion proteins. For example, stable cell lines of CHO.B2 expressing α5integrin-mGFP have been used to characterize the aggregation state of integrins in cell adhesions (Wiseman et al., 2004). As CHO.B2 cells lack endogenous α5 integrin expression, the measured numbers from the transfected cells accurately reflect α5 aggregation in adhesions.

3.3. Sample preparation for imaging adhesions in migrating cells

The following is a list of major considerations for preparing samples for imaging adhesions in migrating cells. It is followed by a typical procedure used in our lab for CHO.K1 cells.

3.3.1 Migration promoting conditions—The procedure mentioned below has been optimized to promote migration in CHO.K1 cells. The same basic procedure can be used for

other cell types but may need to be reoptimized to promote migration. Cell-migration speed varies biphasically as a function of adhesion strength, which is itself a function of ligand density, cell adhesiveness (integrin density and avidity), and substrate stiffness (Engler et al., 2004; Lo, Wang, Dembo, & Wang, 2000; Palecek, Loftus, Ginsberg, Lauffenburger, & Horwitz, 1997). Titrate ligand density to obtain optimal migration (Palecek et al., 1997). Moreover, many cell types (e.g., CHO, fibroblasts) will, over time, secrete and organize matrix and/or become highly contractile and stop migrating (~2h); obviously, imaging must be completed before this time. The culture medium will also influence cell migration (see Section 3.3.2).

3.3.2 Cell culture medium for imaging—Most normal cell culture media contain serum, which has a number of factors that affect migration, that is, fibronectin and chemokine molecules such as growth factors. To have a standardized, migration-promoting environment, serum-free medium containing promigratory growth factors (e.g., EGF, PDGF, or IGF) should be used. In addition, select media without phenol red (included as a pH indicator) to decrease background fluorescence. Finally, if the imaging system is not equipped with CO₂ control, the media will need to be formulated to have the correct pH at atmospheric CO₂ levels. The pH of normal media can be stabilized without supplemental CO₂ by adding 10–25 mM HEPES. Our laboratory routinely uses CCM1 (HyClone).

3.3.3 Sample heater—The sample must be maintained at 37°C during imaging. There are basically two ways to accomplish this: encase the entire microscope body in an environmental control chamber, or use a sample holder with a heating element (e.g., DH-35 culture dish heater, Warner Instruments). The first option is expensive, but allows for CO₂ control and is more resistant to temperature fluctuations that result in focus drift. The second option is easier and cheaper to implement but requires a CO₂-independent medium and is more susceptible to temperature fluctuations between the sample and the instrument. An objective heater (e.g., from Biopetech) can help stabilize the focus and local temperature.

3.3.4 Dishes for imaging—We use dishes with a coverglass bottom of the thickness specified by the objective (typically No. 1 or 1.5). Suitable dishes are commercially available (e.g., MatTek dishes; Lab-Tek chambers) or can be made in-house provided they are clean and sterile.

The following is a basic procedure to prepare CHO.K1 cells for imaging adhesions during migration (e.g., Choi et al., 2011).

Day before experiment

1. Transfect CHO.K1 cells with the desired constructs.
2. Incubate glass-bottomed dishes with 2 µg/ml fibronectin in PBS overnight at 5 °C.

Day of the experiment

1. Rinse the fibronectin-coated dishes with PBS.
2. Trypsinize cells, resuspend in CCM1 medium (HyClone), and seed into a rinsed dish.
3. Place the dish in the incubator and allow the cells to attach and spread (~ 20–30 min).
4. Transfer the dish to the microscope and begin imaging. Cells will migrate up to ~ 2 h after plating at which point they slow and become primarily contractile.

3.4. Instrumentation for image-based correlation measurements

The most common commercial instruments used for correlation studies with migrating cells are:

1. TIRF microscope equipped with a CCD camera (or EMCCD for fast dynamic studies and single molecule sensitivity). This setup is used for studying processes at or near adhesions, visualizing diffraction-limited nascent adhesions, and studying fast dynamic process (~ 100–500 ms). It can also be used for dual-color experiments using appropriate filter cubes and laser lines. We use a Dual View adaptor (Photometrics) for simultaneous acquisition of two channels.
2. CLSM (e.g., Olympus FV1000) equipped with multiple laser lines and PMT detectors that can operate in analog or pseudophoton-counting mode. Photon-counting PMTs allow for simplified analysis (see Section 3.4.5) and should be used, if possible. CLSM is usually implemented for easily visualized adhesions under conditions that do not require fast time acquisition. It is also used for protein dynamics and interaction studies in the cytoplasm (e.g., signaling complexes).

The following are some general considerations applicable to either optical configuration.

3.4.1 Laser power—The selection of laser lines depends on the fluorescent probes used. When possible, select lines closest to the absorption maximum of the fluorescent probe. The laser power used for fluorescence excitation should be minimized to avoid photobleaching and phototoxicity while still providing an adequate signal-to-noise ratio. Optimization will also depend on the exposure settings as well as the expression levels of the fluorescently tagged proteins of interest. The extent of photobleaching can be estimated by an average intensity plot over time and should be less than 10%. Adverse cell response to intense light often manifests as blebbing and global cell retraction. Begin with the lowest laser power and exposure settings and increase gradually until a balance is achieved between the aforementioned parameters.

3.4.2 Laser alignment—Since different laser lines have characteristic critical TIRF angles, alignment of the optical fiber should be performed to obtain the optimal TIRF depth of field for a given wavelength. When a combiner is used for multiple laser lines, this process is usually automated and can be controlled via software. Otherwise, for dual-color experiments, an average critical angle for both laser lines is selected through manual alignment of the optical fiber. For CLSMs, laser alignment primarily affects the excitation intensity; but unlike TIRF, misalignment will not result in illumination inhomogeneities—only decreased intensity—and so is less critical.

3.4.3 Objectives—Select high magnification (60 ×, 100 ×), high NA objectives (> 1.2) to maximize resolution, optimize sample illumination intensity and sensitivity, and in the case of systems employing CCDs, enable high spatial sampling frequencies (pixel sizes < 0.1 μm). Ensure that the objective is corrected for chromatic aberrations for the wavelengths used. Live-cell imaging requires that the sample be held at 37 °C. The optimal immersion oil for the objective at 37°C will likely be different than the optimal oil for room temperature. For example, our PlanApo 60 × 1.4 NA objective (Olympus) has an optimal PSF at room temperature when an oil with $n=1.514$ is used; but 37 °C, requires an oil with $n=1.522$. Test your oil by verifying that the PSF is symmetrical under typical experimental conditions (37 °C). The PSF can be viewed by taking high-resolution images of fluorescent beads with a diameter less than the diffraction limit (e.g., 0.1 μm Tetraspeck beads, Invitrogen) (see Cole, Jinadasa, & Brown, 2011 for detailed procedure).

3.4.4 Filter sets—This is primarily a concern for dual-color imaging. Select emission filters to minimize spectral bleedthrough while still ensuring sufficient sensitivity. For example, we use the following emission filter combination for mGFP and mCherry on the Olympus FV1000: SDM560 dichroic mirror; DM505–525; and DM590–650 band pass filters (Fig. 6.3D). In contrast to a CLSM, the TIRF configuration uses only one detector, usually a CCD camera (Cascade 512B or QuantEM 512SC, Photometrics). Therefore, for simultaneous dual-color imaging on a TIRF microscope, we use a polychroic mirror (Z488/568rpc), a dual-pass emission filter (Z488/568m) (Chroma Technology; Fig. 6.3C), and a Dual-View simultaneous imaging system (Photometrics) to project the two channels onto separate areas of the chip. Alignment of the Dual View is critical to ensure accurate pixel overlap for both channels and should be checked over the course of the experiment as temperature or mechanical fluctuations can result in misalignment of the system. A protocol for the Dual-View alignment is found online (http://www.photometrics.com/support/pdfs/manuals/dv2_alignment.pdf).

3.4.5 Photon detectors—If possible, photon-counting detectors should be used for correlation measurements in lieu of analog detectors such as PMTs and CCDs. The shot noise of photon-counting detectors has a larger dynamic range and follows a Poisson distribution, which simplifies the fluctuation analysis. The pseudophoton counting mode of the Olympus FV1000 has been used successfully in lieu of true photon-counting PMTs (Choi et al., 2011; Digman & Gratton, 2009). However, not all commercial microscopes are equipped with photon counting detectors; therefore, additional theory has been developed for the N&B technique to account for the dark current of analog PMTs and CCDs (Dalal, Digman, Horwitz, Vetri, & Gratton, 2008; Unruh & Gratton, 2008). A crucial consideration for fluctuation techniques is the stability and sensitivity of the detectors over time. This can be tested by collecting a blank image time series (no light) and plotting a histogram of the digital counts for individual images within the series. The same time series can be used to determine the detector parameters used in the N&B analysis (see below). Correlation techniques like ICS, STICS, and TICS are less sensitive to these variations over time and do not require any detector parameter corrections in the analysis. To avoid camera gain saturation over time, use moderate gain settings. For QEM:512SC, we use $3 \times$ multiplication gain and 500–700 EM gain settings. Exact settings will depend on the camera. PMT detectors should also be operated at moderate settings: 600–700 V with no multiplication gain and no offset. The latter two parameters are usually manipulated to improve image quality and should be avoided as they affect fluctuation analyses.

3.5. General considerations for image series acquisition

All the fluorescence fluctuation analyses mentioned in this chapter operate on individual images or image time series. The exact acquisition protocol, correlation analysis, and data fitting routines will vary depending on the technique (e.g., see Chapter 10 and others in this volume). In this section, we outline general considerations for selecting acquisition settings.

3.5.1 Sampling rate—The sampling rate is critical for all fluorescence fluctuation techniques. It depends on the image exposure (TIRF-CCD) or scan (line scan and rescan time; CLSM) time and the user-defined delay between frames. The delay time between frames is limited by the camera readout time (~ milliseconds) and the confocal scan time (~ seconds), both of which scale with image size (number of pixels). The choice of these parameters will vary depending on the characteristic timescale of the dynamic process under study (e.g., molecular exchange, diffusion, and flow), the type of adhesion, and the particular adhesion molecule. For molecular aggregation and interaction studies of cell adhesion proteins like paxillin, a 100–200 ms camera exposure time with no delay time between frames (stream acquisition) has been used for N&B analysis (Choi et al., 2011). For

retrograde flow studies of paxillin, images are acquired every few seconds (Brown et al., 2006). As the dynamics of adhesion proteins depend on the location (cytoplasm vs. adhesion) and type of adhesion (focal vs. nascent), the sampling time should be tailored accordingly. FRAP measurements provide an initial estimate of this parameter and can be used as a guideline. FRAP also identifies immobile populations which are commonly observed for adhesion molecules. Even though immobile populations do not contribute intensity fluctuations to the autocorrelation analysis, they do contribute to the total intensity and thereby can bias the correlation analysis. Immobile population removal methods were developed to account for these effects (see below). An alternative is to collect image time series over a range of timescales that span an order of magnitude (if possible) to determine the correct ranges. Sampling too slow or too fast relative to the characteristic timescale of the fluctuation will produce no temporal or spatial correlation function (for TICS, STICS, and RICS) and indicates that the selected sampling time is inappropriate. For variance analysis, sampling at the wrong timescale will simply bias the measured values.

3.5.2 Number of sampled fluctuations—The number of sampled fluctuations is also critical for fluctuation analyses. For N&B, this will depend on the total number of frames in a time series. For example, for analyses of paxillin dynamics and interactions in nascent adhesions, a typical time series will consist of 2000–4000 frames acquired with a 100 ms exposure time using streaming acquisition (i.e., no delay between frames) (Choi et al., 2011). We then divide the series into 200–500 frame segments for analysis. For spatiotemporal correlation approaches like TICS and STICS, the required number of frames will also depend on the size of the analyzed area, as increases in spatial average produce better statistics. Velocity mapping retrograde flow of adhesion proteins in adhesions has been reported from areas as small as 16^2 pixels in time series consisting of a few hundred images, acquired every 2–5 s (Hebert et al., 2005). In the case of image correlation analysis (ICS, RICS), the size of the image and the pixel resolution are important parameters for determining the number of sampled fluctuations. Typically, 32^2 – 64^2 pixel regions are used with a pixel size of $\sim 1/3$ the beam radius size. Small, defined regions ($<10 \times 10$ pixels) and time segments (~ 10 – 20 frames) provide higher spatial resolution. However, one should be cautious when selecting small ranges as the accuracy of the parameters recovered by fluctuation correlation techniques depends on the number of sampled fluctuations. For example, smaller areas used for enhanced spatial resolution will require longer sampling times to achieve similar sampling statistics. Computer simulations have tested the limits of these techniques under various experimental acquisition settings and guide implementing the analyses in this toolbox (Brown et al., 2008; Costantino et al., 2005; Hebert et al., 2005; Kolin et al., 2006).

3.6. Post-acquisition image processing

Standard microscope imaging software (e.g., Metamorph, Olympus FluoView) typically saves images as 12- or 16-bit tiff files. An ImageJ plugin for ICS and ICCS is available through the Stower's Institute Web site (<http://research.stowers.org/imagejplugins/>). For complete fluctuation correlation toolbox analysis, the Globals Software for Images (SimFCS) is also available through the Laboratory for Fluorescence Dynamics free of charge for a trial period. Custom codes for selected correlation techniques have also been written in programming platforms such as Matlab and are available through the Cell Migration Consortium Web site (<http://www.cellmigration.org/>). For all correlation techniques, image time series should be processed to account for the following issues.

3.6.1 Background intensity—Background noise affects the accuracy of number density, interaction fraction, and aggregation estimates. This can be addressed by subtracting the average background intensity in each image. The average background intensity can be

estimated from a selected adjacent background region in each image in the time series to account for any variations over time.

3.6.2 Analog detector—To account for analog detector (PMT, CCD) parameters like dark current and digital conversion on variance analysis approaches such as N&B, blank (no light) image time series are acquired under the same detector settings used for imaging the protein(s) of interest. A histogram count of the digital counts is generated from the image time series and used to estimate: (1) the Offset parameter (dark current in absence of photons) and (2) the *S*-factor (photon–analog current conversion factor). The Offset factor is estimated from a Gaussian fit of the digital counts distribution. This parameter is then subtracted from the image time series. The apparent brightness parameter *B* of a background region is then used to estimate the *S*-factor. *S*-values that yield a *B* distribution centered around “one” are used for the analysis. Refer to Dalal et al. (2008) and Unruh and Gratton (2008) for the theoretical framework and a detailed discussion of analog detector effects on N&B analysis.

3.6.3 Photobleaching and macroscopic fluctuations—Photobleaching and macroscopic fluctuations due to cell edge protrusion or slow movement of adhesion structures bias correlation analyses by introducing artifactual fluctuations. Theoretical models have been developed to account for photobleaching in temporal autocorrelation analyses (Kolin et al., 2006). A generalized approach applicable to most fluctuation techniques is to use a moving average filter on an image time series (Digman, Brown, et al., 2005). This eliminates low frequency fluctuations while maintaining the high-frequency molecular fluctuations. The cutoff frequency will depend on the length of the moving average window, which will vary for different data sets depending on the kinetics of the process under study. Typically, we have used a 20–40 s filter for cell migration related studies.

3.6.4 Immobile populations—Immobile molecular complexes are commonly observed in adhesions. These structures mask intensity fluctuations and thereby affect correlation analysis. Hebert et al., 2005 implemented an immobile filtering algorithm to eliminate intensity contributions in an image time series from static structures. In this algorithm, the image series is averaged over time to yield an “average image.” As immobile structures will contribute constant fluorescence intensity at a given location over time, subtraction of the “average image” from each image in the series will eliminate the immobile population contributions (Hebert et al., 2005). Moving average filters (see Section 3.6.3) can also eliminate contributions from immobile populations.

3.7. Control samples

In addition to labeling your protein of interest(s), control constructs appropriate for the fluorescence fluctuation technique should be produced.

3.7.1 Bleedthrough control—For cross-correlation measurements, cells expressing only one fluorescent construct (e.g., either mGFP–paxillin or mCherry–FAK) are used to measure the extent of spectral bleedthrough or indirect excitation under the same experimental conditions used for the actual experiment (e.g., laser intensity, camera exposure time or confocal scan speed, detector gain, and filter combinations). For example, to measure the bleedthrough of mGFP into the mCherry detector channel (Fig. 6.3B), cells expressing mGFP–paxillin are excited using the 488 nm Ar ion laser line, and the fluorescence signal is collected in both the mGFP and mCherry detection channels. The ratio of the average background-corrected signal in the mCherry channel to the average background-corrected signal in the mGFP channel is a measure of the bleedthrough and should usually be $\leq 10\%$.

3.7.2 Positive and negative controls for cross-correlation—For cross-correlation studies, it is useful to have positive and negative controls. For example, in our cross-correlation studies of adhesions using mGFP–paxillin and mCherry–FAK (Choi et al., 2011), we created a dual-color paxillin (mGFP–paxillin–mCherry) and membrane targeted versions of mGFP and mCherry (GAP–mGFP, GAP–mCherry). The dual-color paxillin, which did not show detectable FRET, served as a positive control for maximal cross-correlation. The membrane-localized GAP–mGFP and GAP–mCherry (GAP-43; growth-associated protein; Moriyoshi, Richards, Akazawa, O’Leary, & Nakanishi, 1996) were coexpressed as negative controls, as they do not interact and diffuse freely in the membrane.

3.7.3 Fluorescence intensity calibration controls—To determine the aggregation state (stoichiometry) of individual adhesion proteins in a complex using N&B analysis or ICS, controls of fluorescently tagged monomer species are needed (e.g., the membrane targeted versions mentioned above). Control samples are imaged under the same experimental conditions as the protein(s) of interest (e.g., laser intensity, camera exposure time or confocal scan speed, detector gain, and filter combinations). The following is a basic protocol for measuring aggregation states from:

a. TIRF-based N&B measurements.

- Collect ~ 1024–2048 images of GAP using stream acquisition settings and 50–100 ms camera exposure time (Cascade 512B or QuantEM:512SC, Photometrics).
- Calculate the average apparent brightness parameter B_{GAP} from a selected region of a cell expressing GAP–mGFP or/and GAP–mCherry (Choi et al., 2011).
- Calculate the apparent brightness parameter B_{protein} for the protein(s) of interest at selected adhesion locations and over the desired time segments (~ 20–40 s).
- For single-channel experiments, the aggregation state of a protein at a given pixel location is the ratio of $(B_{\text{protein}} - 1)/(B_{\text{GAP}} - 1)$.
- For dual-channel experiments, select the pixel locations that indicate the presence of complexes (positive Bcc value) to estimate the aggregation state of each protein in the complex. For example, use GAP–mGFP and GAP–mCherry for mGFP–paxillin and FAK–mCherry calibration, respectively. For more analysis details, refer to Choi et al. (2011) and Digman, Wiseman, Choi, Horwitz, and Gratton (2009).

b. ICS measurements.

- Collect confocal images (512^2 or 1024^2 pixels) of GAP using 8 μs pixel dwell time and an optical zoom ($\sim 4\text{--}6\times$) that yields $\sim 0.1\ \mu\text{m}$ pixel resolution.
- Calculate the spatial ACF of a cell region expressing GAP–mGFP and fit the x -axis component of the spatial ACF to a 1D Gaussian function. From the fit amplitude (G_0) and the beam area ($\pi\omega^2$; ω , beam radius), calculate the number of particles (NP) per micron ($1/(G_0 \cdot \pi\omega^2)$).
- Calculate the cluster density (CD) from the fluorescence average intensity ($\langle I \rangle$) and the NP per micron ($CD = \langle I \rangle / \text{NP}$). For GAP–mGFP, CD should be proportional to the fluorophore parameters under the given imaging parameters.

- Calculate CD for the protein of interest in a selected cell region. Use CD_{GAP} as a normalization factor to estimate the protein degree of aggregation. For more analysis details, refer to Wiseman et al. (2004) and references therein.

4. APPLICATIONS

Fluorescence fluctuation techniques have only recently been applied to adhesion and migration. So far the applications have focused on the dynamics and interactions of adhesion proteins and have provided critical information on the mechanisms of adhesion formation, signaling, and turnover. Here, we outline some of these applications:

1. Mapping integrin dynamics and aggregation in migrating cells (Wiseman et al., 2004).
2. Differential retrograde flow dynamics of adhesion components in the integrin–actin linkage (the molecular clutch model) (Brown et al., 2006).
3. Association of adhesion components in complexes occurs at adhesion sites (Paxillin–FAK (Choi et al., 2011) and Paxillin–Vinculin (Digman, Wiseman, Choi, Horwitz, & Gratton, 2009)).

4.1. Mapping of integrin aggregation and dynamics in migrating cells

Integrin receptors are critical components of adhesions, as they provide the mechanical link between the substratum and the actin cytoskeleton. Integrin clustering at adhesion sites reinforces this link and provides a hub for recruitment of numerous adaptor and signaling molecules (Vicente-Manzanares et al., 2009). Wiseman et al. (2004) used ICS to map the number density and aggregation state (number of molecules per aggregate) of integrins. In this study, image time series of CHO.B2 cells stably expressing the mGFP- $\alpha 5$ integrin subunit and plated on fibronectin-coated coverslips were acquired on a CLSM. Different areas (or adhesions) within the cells were selected from individual images in the time series for spatial autocorrelation analysis. The average number density and aggregation states were estimated from the amplitude of the spatial autocorrelation function. CHO.K1 cells expressing GAP-GFP (GFP with a membrane-targeting sequence) were used as a monomeric control. The analyses revealed that integrins are clustered throughout the cells—even at locations away from visible adhesions (with 3–4 molecules per cluster) where no visible structural organization was discerned. At the sites of large adhesions, the integrin number and aggregation increased by approximately four- to five-fold. These parameters changed as adhesions disassembled. The authors also used TICS and cross-TICS on selected regions of cells to characterize the dynamics of $\alpha 5\beta 1$ integrin and α -actinin in and away from adhesion sites. Temporal image correlation functions were fit to 2D diffusion, 2D flow, or combined 2D diffusion and flow (for single or two populations) equations to estimate relevant transport parameters and their respective populations. Immobile fractions were also estimated from the fits (Fig. 6.4A). The cross-TICS functions revealed associations between integrin and α -actinin in molecular complexes at adhesion sites and throughout the cell; interactions were not observed for integrin and paxillin (Fig. 6.4B). Similar maps of the dynamics and percent populations of the interacting integrin- α -actinin fractions were also presented.

4.2. Retrograde flow of adhesion components in the integrin–actin linkage

Adhesions are sites of traction where the forces arising from membrane resistance to actin polymerization and myosin-mediated contraction are transmitted to the substratum. In the absence of a firm linkage between actin and the substratum, these forces lead to retrograde flow of actin (opposite the direction of polymerization) as new actin monomers are added or

the filaments are contracted and moved toward the cell center. The presence of actin retrograde flow suggests that this linkage might not always be efficient and points to the presence of a molecular “clutch” with varying efficiency (Mitchison & Kirschner, 1988; Ponti, Machacek, Gupton, Waterman-Storer, & Danuser, 2004). Brown et al. (2006) used spatiotemporal image correlation spectroscopy to address this issue. Single-channel spatiotemporal ACFs were calculated from image time series of cells coexpressing RFP-actin and GFP-tagged variants of paxillin, $\alpha 5$ -integrin, talin, FAK, vinculin, and α -actinin. The magnitude and direction of flow of the individual molecules were estimated from the temporal displacement of the peak of the spatial autocorrelation. An example of a spatial map of the flow of paxillin-GFP and RFP-actin is presented in Fig. 6.5A. The relative magnitude and direction of the velocity vectors for the various adhesion components relative to actin revealed a differential correlation between certain adhesion and actin retrograde velocities (Fig. 6.5B). The nature of the correlation was cell-type dependent: highly contractile cells, like MEF or 3T3 cells, showed a stronger coupling between adhesion molecules and actin, whereas CHO cells, which are less contractile, showed reduced coupling (Fig. 6.5B). Thus, it appears that the forces on adhesions are modulated by the efficiency of the integrin-actin linkage to retrograde forces. As the force on adhesions feeds back to dictate adhesion size, organization, and signaling, this clutch has major ramifications for the cell.

4.3. Association of adhesion components in complexes occurs at adhesion sites

The fluorescence fluctuation toolbox has been used to characterize the interactions between focal adhesion kinase (FAK) and paxillin, which constitute a key signaling complex that regulates cell migration (Choi et al., 2011). Paxillin and FAK organize and control signals that regulate protrusion and adhesion formation/disassembly through phosphorylation (FAK) and scaffolding functions (FAK and paxillin). As scaffolds, they organize, regulate, and generate signals that impinge on Rho GTPases, which in turn control actin polymerization and contraction (Deakin & Turner, 2008; Schaller, 2010). Paxillin and FAK are regulated by several kinases, including Src, a tyrosine kinase (Schaller & Parsons, 1995; Zaidel-Bar, Milo, Kam, & Geiger, 2007). Choi et al. (2011) investigated the role of tyrosine phosphorylation on two sites in paxillin on paxillin-FAK interactions in adhesions. Using ccN&B analysis of TIRF image time series of CHO cells expressing mGFP-paxillin and FAK-mCherry, they showed that wild-type paxillin and FAK exchange as molecular complexes in newly forming (nascent) adhesions. They also quantified the size and relative stoichiometry of the complex and showed that phosphomimetic mutants of paxillin (Y31E-Y118E) increased its size. In contrast, the non-phosphorylatable mutant of paxillin (Y31F-Y118F) resulted in few detectable paxillin-FAK complexes; those that existed had a reduced complex size (Fig. 6.6). In addition, using ccRICS analysis on CLSM image time series, they showed that paxillin-FAK interactions were not detected in the cytoplasm outside the immediate vicinity of adhesions, indicating that their association occurs once they are in adhesion sites. This confirms and extends earlier correlation studies by Digman, Wiseman, Choi, Horwitz, and Gratton (2009) that showed little, if any, vinculin-paxillin or paxillin-FAK association in the cytoplasm.

Digman et al. also investigated whether molecular complexes mediate the formation or disassembly of adhesions. Using temporal correlation analysis (FCS, scanning FCS, TIRF-TICS) and fluorescence variance analysis (PCH, ccN&B), they studied paxillin dynamics and interactions with FAK and vinculin. They observed heterogeneity in the dynamics and aggregation state of paxillin in different adhesions and in regions across the cell. Whereas diffusion is the main mode of transport for adhesion molecules in the cytoplasm, exchange (binding-unbinding) kinetics with a broad range of rates (0.1 – 10 s^{-1}) dominate in the vicinity of adhesions (Fig. 6.7) (Digman, Wiseman, Choi, Horwitz, & Gratton, 2009). They

observed large clusters and complexes exchanging relatively slowly in the vicinity of the disassembling regions of adhesions, whereas small aggregates (largely monomers) were observed exchanging rapidly in assembling adhesions (Fig. 6.8) (Digman, Wiseman, Horwitz, & Gratton, 2009). This suggests that adhesion assembly involves the addition of monomer units or small aggregates, whereas adhesion disassembly results from the dissociation of large clusters or complexes.

5. CONCLUSION

Until recently, methodological limitations have hindered the study of the molecular-scale interactions and dynamics of the molecules that comprise adhesions in living cells. Fluorescence fluctuation techniques have recently been developed that potentiate these studies in a way that senses fluctuations down to the level of single molecules, provides spatial maps across the entire cell, and enables analysis of multiple pieces of information with a single data set. Two major themes are critical in the implementation of these methods. First, when working with biological specimens, there are a number of potential artifacts that arise as one attempts to increase the signal-to-noise ratio: increasing excitation power increases photobleaching/phototoxicity; increasing FP construct expression results in higher background fluorescence and may also alter the biology. Second, the timescale of the process under study is critical: optimizing the sampling rate imaging times will have a profound effect on the detection of fluctuations. While the experimental implementation of most fluorescence fluctuation techniques is relatively straightforward and uses commercially available instrumentation, the subsequent analysis requires care.

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Figure 6.1.

Principles underlying fluorescence correlation spectroscopy (FCS). (A) Examples of dynamic processes (diffusion, flow, and exchange) that give rise to fluorescence intensity fluctuations in a focal volume. (B) Fluorescence intensity fluctuations over time. (C) Autocorrelation analysis of the intensity fluctuations quantifies the transport processes underlying the fluorescence fluctuations and the particle number densities and aggregation states.

Figure 6.2.

Schematic illustration of the fluorescence fluctuation toolbox. (A) CLSM dual-color fluorescence images of a cell expressing two proteins, each tagged with a different color fluorescent probe visualized in channels 1 and 2 (CH1 and CH2). In a confocal setup, fluorescence excitation is achieved as a laser beam is scanned across a sample with microsecond pixel dwell time and millisecond line-scan time. Image correlation spectroscopy (ICS) and raster-image correlation spectroscopy (RICS) are implemented on CLSM images (or selected regions in an image) to measure molecular numbers and fast dynamics using spatial autocorrelation analysis. (B) Image time series acquired using either CLSM or TIRFM are used for temporal image correlation spectroscopy (TICS) and spatiotemporal image correlation analysis (STICS) analysis to study transport dynamics (diffusion, flow) and exchange kinetics. Number density information can also be recovered from TICS analysis. Single pixel variance analysis of fluorescence fluctuation in the time series provides high-resolution mapping of number densities and stoichiometry of molecular aggregates (N&B). All of these techniques can be applied to two fluorescence detection channels to detect and quantify molecular interactions in complexes.

Figure 6.3.

(A) Absorbance and emission spectra of EGFP (green) and mKusabira-Orange (mKO; orange). Spectral overlaps potentially leading to FRET (black outlined area) or signal bleedthrough (gray area) are indicated. (B) Absorbance and emission spectra of EGFP (green) and mCherry (red) with spectral overlaps indicated as in (A). Note that the red-shifted mCherry spectra have less overlap with EGFP but also require a long-wavelength laser (568 nm) for optimal excitation. (C) Emission spectra of EGFP and mCherry, overlaid with the transmission spectra of the polychroic mirror (black) and emission filter (gray) used for TIRF microscopy. The two channels are further separated and projected onto different areas of a CCD chip using a Dual-View adapter (spectra of these mirrors not shown). (D) Emission spectra of EGFP and mCherry, overlaid with the transmission spectra of the dichroic mirror (black) and band-pass filters for the EGFP (dark gray) and mCherry (light gray) channels used in confocal microscopy. Notch filters at specific wavelengths remove reflected light from the lasers (spectra not shown).

Figure 6.4.

Spatial map of the dynamics of (A) $\alpha 5$ integrin-GFP and (B) α -actinin GFP in CHO.B2 (A) and CHO.K1 (B) cells and plated on fibronectin. Image time series consisting of 100 images were captured every 5 s using (A) CLSM and (B) two-photon microscopy. Scale bars are 5 μ m in (A) and 10 μ m in (B). Highlighted regions (32^2 – 128^2 pixels) were selected for TICS analysis. The circles represent the root mean square average diffusion distance from the center of the circle for a 10-min period; it is derived from the average diffusion coefficient for each region. The vectors represent the mean translation distance and direction over a 10-min period based on the measured velocities for regions exhibiting flowing integrin populations. The colored bars depict the proportion of immobile (green), flowing (yellow),

and diffusing (cyan) integrin or α -actinin within each region. (C) Cross-TICS reveals associations between α_5 integrin–CFP and α -actinin–YFP in molecular complexes. Similar associations were absent in the case of α_5 integrin–CFP and paxillin–YFP. The presented single-channel autocorrelation and cross-correlation functions are calculated from CHO.K1 image time series expressing the fluorescently labeled constructs and captured using two-photon microscopy. *Figure and caption reproduced from Wiseman et al. (2004).*

Figure 6.5.

Retrograde flow of adhesion proteins. (A) Velocity maps for paxillin-EGFP and mRFP-Actin coexpressed in NIH3T3 fibroblasts plated on fibronectin. The image time series consisted of 121 images captured every 5 s using TIRF. Scale bar is 5 μm . Velocity scale arrow is 1 $\mu\text{m}/\text{min}^{-1}$. (B) Comparison of the plots of the median relative magnitude and directional correlation coefficients of various adhesion proteins relative to actin in CHO cells and 3T3 cells. *Figure and caption reproduced from Brown et al. (2006).*

A

Paxillin (Y31E–Y118E) – FAK

B

Paxillin (Y31F–Y118F) – FAK

Figure 6.6.

Cross-number and -brightness (ccN&B) analysis of Paxillin–FAK interactions in nascent adhesions (Choi et al., 2011). Highlighted pixels (red) indicate the presence of complexes and are selected for positive cross-brightness (Bcc) parameter values as indicated in the highlighted box in the histogram plots of pixels with a given Bcc value and corresponding B1 or B2 for each channel, respectively. For pixels that show complexes, the B1 and B2 values will reflect the size of stoichiometry of the individual species in the complex. More molecular complexes are detected for the phosphomimetic (Y31E, Y118E) versus the non-phosphorylatable (Y31F, Y118F) mutants of paxillin. TIRF image time series of CHO.K1 cells expressing paxillin-GFP and mCherry–FAK were acquired with 100 ms exposure time using stream acquisition for ~3.5 min. *Figure and caption reproduced from Choi et al. (2011).*

Figure 6.7.

Temporal pixel-autocorrelation analysis of CHO cells expression paxillin-EGFP. (A) Average fluorescence intensity of the image time series. Spatial mapping of the (B) amplitude (G_0) of the temporal autocorrelation function and (C) exchange rate constants (s^{-1}) obtained from the fits of the single pixel autocorrelation functions to an exponential decay function. Red and green pixels correspond to rates >1 and $<1 s^{-1}$, respectively. (D) Histogram plot of the rate constants obtained from the single pixel temporal autocorrelation analysis. *Figure and caption reproduced from Digman, Brown, Horwitz, Mantulin, and Gratton (2008).*

Figure 6.8.

Cross-number and brightness (ccN&B) analysis of paxillin–vinculin reveals the presence of (A) larger molecular aggregates between adhesions and smaller molecular aggregates in adhesions (B) as indicated by the B1 and B2 values observed for pixels that indicate the presence of molecular complexes. CLSM image time series of MEF cells expressing vinculin–GFP and paxillin–mCherry were acquired every 1.15 s for ~4 min. *Figure and caption reproduced from Digman, Wiseman, Choi, Horwitz, and Gratton (2009).*

Table 6.1

Characteristic timescales for biological processes and the fluorescence fluctuation techniques used to study them

Dynamic process	Characteristic timescale	Fluctuation correlation technique
EGFP fluorescence blinking	0.1 μ s–0.1 ms	FCS
EGFP diffusion in solution	~ 0.05–0.1 ms	FCS-sFCS-RICS
EGFP diffusion in cytoplasm	0.5–1 ms	FCS-RICS-sFCS
Lipid diffusion in cell membrane	0.01–1 s	FCS-sFCS-TICS-RICS
Protein receptor diffusion in cell membrane	1–10 s	sFCS-TICS
Retrograde flow of adhesion and cytoskeletal molecules	~ 1–100 s	STICS
Adhesion molecules exchange rates	500 ms to ~min	TICS-RICS