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Analysis of focal adhesion turnover: A quantitative live cell imaging example

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Abstract

Recent advances in optical and fluorescent protein technology have rapidly raised expectations in cell biology, allowing quantitative insights into dynamic intracellular processes like never before. However, quantitative live cell imaging comes with many challenges including how best to translate dynamic microscopy data into numerical outputs that can be used to make meaningful comparisons rather than relying on representative data sets. Here we use analysis of focal adhesion turnover dynamics as a straight-forward specific example on how to image, measure and analyze intracellular dynamics, but we believe this outlines a thought process and can provide guidance on how to understand dynamic microscopy data of other intracellular structures.

1. Introduction to focal adhesion dynamics

Cell migration is essential for development, tissue remodeling and wound healing, and requires complex rearrangements of intracellular macromolecular structures. Quantitative analysis of the molecular processes that drive cell migration are essential for our understanding of how cell migration is deregulated in pathological states, such as in cancer metastasis, for example. In addition to the coordination of signaling pathways to control polarity and cytoskeleton rearrangements, cell migration requires force generation that relies on the coordinated remodeling of interactions with the extracellular matrix (ECM). These interactions are mediated by integrin-based focal adhesions (FAs) that were first described in the 1970's by interference reflection microscopy (Heath & Dunn, 1978), and recent superresolution microscopy demonstrate the complex multilayered architecture of FA plaques (Kanchanawong et al., 2010). Despite recent controversy of FAs being a tissue culture artifact of cells growing on stiff, flat surfaces, cells clearly utilize FAs in physiological 3D environments during migration along ECM fibers (Kubow & Horwitz, 2011; Gierke & Wittmann, 2012) although FA-independent, amoeboid modes of cell migration exist. The FA life cycle involves formation of integrin-mediated, nascent adhesions near the cell's leading edge, which either rapidly turn over or connect to the actin

cytoskeleton (Parsons, Horwitz, & Schwartz, 2010; Stehbens & Wittmann, 2012). Actomyosin-mediated pulling forces allow a subset of these nascent FAs to grow and mature, and provide forward traction forces. However, in order for cells to productively move forward, FAs also have to release and disassemble underneath the cell body and in the rear of the cell. Spatial and temporal control of turnover of these mature FAs is important as they provide a counterbalance to forward traction forces, and regulated FA disassembly is required for forward translocation of the cell body.

The FA binding kinetics of specific proteins can be analyzed by fluorescence recovery after photobleaching (FRAP) in which fluorescently tagged FA components are photobleached and the rate by which fluorescence returns to the bleached area is monitored (Lele, Pendse, Kumar, Salanga, Karavitis, & Ingber, 2006; Pasapera, Schneider, Rericha, Schlaepfer, & Waterman, 2010). FRAP data contain information on how rapidly specific FA-associated proteins exchange with the soluble cytoplasmic pool. While this may influence FA turnover, it is not a priori directly related to turnover of the FA structure. For example, typical FA lifetimes are in the order of tens of minutes while turnover of most FA-bound proteins is in the order of seconds. Thus, during the life of an individual FA, bound proteins dissociate and reassociate many times. This is also the case for many other intracellular assembly and disassembly processes, and it is crucial to not confuse these two types of dynamics: the lifetime of the underlying structure versus the binding kinetics of individual molecules. A macroscopic analogy is, for example, the lifetime of an ants' colony compared with the time an individual ant spends in the colony, which obviously cannot be used to make any conclusions about the growth or stability of the colony.

In contrast, analysis of intensity changes of fluorescently tagged FA components over time can be used to determine assembly and disassembly rates of the FA structure, and thus to quantitatively test how FA dynamics and therefore cell migration are controlled. A landmark paper by Webb et al. first utilized this method by linear regression of a semilogarithmic plot of fluorescence intensity as a function of time (Webb et al., 2004). This approach assumes that both assembly and disassembly follow exponential kinetics, which is likely not a good model for the assembly phase (see below). In contrast, we find that direct curve fitting of the fluorescence intensity profiles with appropriate functions provides more robust results, and more completely describes FA turnover dynamics (Meenderink, Ryzhova, Donato, Gochberg, Kaverina, & Hanks, 2010). However, further improvements in live cell imaging technology show that FA dynamics can be complicated, and more complex FA dynamics such as sliding, splitting and merging will require different analysis approaches. In this chapter, we describe a step-by-step procedure of how we image, measure, and analyze FA turnover in migrating cells, which can also be used as a general guideline of important points to consider when attempting dynamics analysis of any fluorescent structure.

2. Focal adhesion turnover analysis

2.1 Sample Preparation

In the example discussed here, we analyse FA turnover dynamics at the edge of a migrating HaCaT keratinocyte epithelial cell sheet. We grow these cells on fibronectin-coated coverslips and generate an experimental cell sheet edge by removing half of the cell

monolayer (Stehbens, Pemble, Murrow, & Wittmann, 2012). Coverslips are then mounted in sealed aluminium slide chambers (see Chapter 5) and returned to the tissue culture incubator overnight to allow cells to recover and to polarize. Because polarity is largely determined by cell-cell contacts in the cell monolayer, epithelial sheet migration is highly directional. Depending on the experimental question, different cell types and/or different imaging chambers can be used, and the method adapted accordingly. For example, mesenchymal cell types such as fibroblasts and melanocytes tend to migrate as individual cells and may require less time to recover after wounding, and FA shape, dynamics and morphology are cell-type dependent.

In general, reproducibility of the results will depend to a large extent on reproducible cell culture conditions, and care should be taken at every step to ensure sample preparation of the highest quality. Cells should be at low passage, and not cultured for too long. Different matrices such as fibronectin, laminin, or collagen can influence cell migration and FA morphology because different integrins have different ECM specificities. It is also recommended that surfaces for migration are clean, and we routinely acid wash or detergent-sonicate coverslips before ECM coating and plating cells.

A large number of FA-associated proteins including talin, vinculin, paxillin, zyxin and others have been tagged with fluorescent proteins (FPs) and used to image FA dynamics. Although transient transfection of FP-tagged constructs can be used, we believe it is well worth the initial investment of time to generate stable cell lines expressing the FP-tagged protein of interest to be able to reproducibly image large numbers of cells within one sample. For this reason, we routinely use lentivirus-mediated gene transduction to produce stable cell lines. Sorting the population for the desired FP-expression level by FACS reduces the variability between cells which becomes important when the sample size is reduced to cells along a wound edge, ensuring adequate cell numbers within each experiment. Care needs to be taken when expressing FP-tagged proteins as overexpression can introduce artifacts affecting the dynamics of the process under investigation. For example, overexpression of many FA proteins results in FA stabilization. In our hands, lentivirus expression is the method of choice as FP-tagged FA proteins express evenly at close to endogenous levels, which in a stable cell line can be verified by immunoblotting, in addition to comparing FA morphology to endogenous protein immunofluorescence. Of note, lentiviral packaging limitations need to be taken into consideration when designing an experiment, and some FA proteins, such as α -actinin, are quite large and approach the upper limitations of lentiviral packaging capacity.

In our system, we find that stable, low-level expression of paxillin-mCherry is a reliable reporter of FA dynamics (Fig. 1) (Hu, Ji, Applegate, Danuser, & Waterman-Storer, 2007). However, it is important to note that different FA markers may report different phases of the FA turnover cycle and choice will be determined by cell system and experimental question. For example, paxillin is recruited to both nascent and mature adhesions, while other components such as vinculin or zyxin are thought to associate with FAs only at later maturation stages.

2.2 Imaging

As with any live cell imaging experiment, care should be taken to ensure a physiological environment for the observed cells. Light exposure and associated photobleaching and photodamage that will inhibit cell migration should be limited as much as possible (see Chapter 5). Because FA turnover is a comparably slow process and requires time-lapse recordings of several hours at high magnification, this imaging benefits significantly from a high precision, linear-encoded motorized stage for imaging multiple fields of view, and an autofocus system in order to allow for parallel acquisition of many cells in a single experiment (Chapter 5).

We routinely acquire 3-hour time-lapse sequences at 90–180 second intervals of typically 10–20 different stage positions. While these time-lapse settings work well for relatively slow FA turnover in our experimental system, it is important to determine the right compromise of time-lapse intervals and duration with respect to the process under investigation (Chapter 1). For example, too long intervals between images may not provide sufficient data points for reliable analysis, and dynamics are missed. In contrast, too short intervals will limit the total duration of imaging at useful signal-to-noise ratio due to photobleaching, and in our case greatly reduce the number of FAs for which both assembly and disassembly can be observed. mCherry-paxillin expressing cells are imaged by spinning disk confocal microscopy using a Nikon 60x 1.49 N.A. CFI Apochromat TIRF objective and a cooled Interline CCD camera with 6.45 μm x 6.45 μm pixels (see (Stehbens et al., 2012) for a more detailed description of our spinning disk microscope setup). 60x is recommended because it provides sufficient resolution to image FA dynamics and allows more light collection per pixel compared with a higher magnification. Typical exposure settings using a 100 mW 561 nm solid-state excitation laser are 4–8 mW light power at the objective front lens and exposure times of 200–400 ms. We use a 568 nm longpass emission filter to collect as much of the mCherry emission spectrum as possible. Although this is certainly a subjective criterion and depends on the type of light source used for epifluorescence, to limit paxillin-mCherry overexpression effects we aim to image cells that are barely visible through the eyepiece. It is also important to note that paxillin-mCherry and many other FA-associated proteins have a quite substantial soluble cytoplasmic pool, which makes it near impossible to discern FAs at low expression levels by epifluorescence illumination by eye, and cells will appear mostly as a faint fluorescent glow. Thus, in setting up the multi-point experiment we minimize direct viewing and instead take quick snapshots at reduced excitation light intensity to select and focus appropriate cells to minimize light exposure and photobleaching before starting time-lapse acquisition. Never use any ‘live view’ function on a sample that is to be used for quantitative imaging purposes. Finally, all microscope settings should be recorded for later reference (ideally this is automatically done by the imaging software), and most importantly, all settings that alter image intensities (i.e. camera gain, exposure time, light power etc) or spatial and temporal sampling (i.e. camera binning, magnification, time-lapse intervals) must be kept constant to ensure reproducibility in comparing different conditions.

2.3 Image Analysis

Fully computerized image analysis methods are becoming more commonly used within the cell biology community. For example, a Matlab-based software package that segments FAs and extracts dynamics from TIRF image sequences has recently been published (Berginski, Vitriol, Hahn, & Gomez, 2011). Whilst the continued development of such tools is advantageous, users should be aware of the potential pitfalls associated with such software. To the end user, computational image analysis tools often carry the risk of being a black box, and the systematic and random error introduced by high-throughput image analysis essentially remains unknown. It can be extremely difficult to validate computational image analysis in specific experimental conditions, and to assess how exactly a software algorithm has generated a set of numbers. This is especially true for commercial solutions for which the underlying code is not available. We would argue that image analysis tools should not be used for scientific purposes if they cannot be fully understood by the user. In addition, one should remain aware that human-written software inevitably contains coding errors that may affect analysis outcome. It is also important to remember that any analysis can only be as good as the input data, and that results obtained from noisy, out-of-focus, and unevenly illuminated images can be essentially meaningless. Finally, free parameters of the software, such as for example segmentation thresholds, often are not robust and may have to be optimized for specific sets of input images, which may not be trivial.

Here, we describe how we analyse FA dynamics using readily available image analysis software and subsequent data analysis in Microsoft Excel. While this is certainly not the most high throughput method, it stays close to the data, and can also be used as a guideline on how to analyze the dynamics of other intracellular structures. Most importantly, quantification of fluorescence intensity should only be done on original microscope data that have not been altered by non-linear intensity transformations. Image data must not be compressed or downsampled and the original bit depth should be used, ensuring data is not lost at any point during analysis.

We use image analysis tools in Nikon NIS Elements AR (v4.22) software to measure the FA-associated intensity of paxillin-mCherry as a function of time although other microscopy software suites such as Metamorph, ImageJ or Fiji can certainly be used. A region of interest (ROI) is drawn around a FA to determine the average FA-associated paxillin-mCherry fluorescence, $I_{FA}(t)$ (Fig. 1). In order to fully capture FA turnover dynamics and obtain meaningful lifetime measurements, it is important that the entire FA turnover cycle can be observed in the dataset, i.e. FAs selected for analysis should be absent both in the beginning and at the end of the time-lapse sequence. However, if only assembly or disassembly rates are being measured, for example as a result of pharmacological treatment, this may not be necessary. The ROI defining $I_{FA}(t)$ should be drawn closely around the FA at its maximum size to minimize the background correction error. If the number of background pixels is large compared with the number of pixels encompassing the signal of interest, the average intensity will decrease substantially and noise becomes dominant. FAs will often significantly change size and location during the image sequence, and it may thus be necessary to adjust size and position of the ROI during the time-lapse series to obtain accurate FA intensity measurements. For example, especially under conditions that inhibit

FA disassembly, we have observed significant sliding of FAs as well as splitting and merging of FAs, which complicates the analysis. Not all software packages will support ROI editing in a time-lapse sequence, but in NIS Elements this can be done using the function 'Edit ROIs in time', which generates a trajectory of changing ROIs through the time-lapse sequence. We then duplicate the FA-associated ROI and place it next to the FA for background correction, and measure the average fluorescence intensity in each frame. Measurements in this region, $I_{BKG}(t)$, include both cytoplasmic background fluorescence and the camera offset (Fig. 1). In NIS Elements, this is done in the 'Time Measurements' dialog. It is important to determine $I_{BKG}(t)$ in each time frame (not only the first) as the local background over time will fluctuate due to cell migration across the adhesion site and associated cell thickness changes. In addition, one should verify that no other fluorescent structure (i.e. another FA) wanders into the background region, which would result in artificially high background values. This generates two fluorescence intensity profiles, one of the FA and one of the background ROI over time, which allows for background correction at each point within the adhesion lifecycle. Data are then exported to Microsoft Excel for further analysis.

2.4 Data Analysis

FA turnover can be separated into three different phases: Assembly, during which the FA increases in size and intensity; disassembly, during which FA intensity decreases; and the time in between during which FA intensity may fluctuate, but on average the FA neither assembles nor disassembles. We use this simple model to describe FA turnover and determine assembly and disassembly rate constants by curve fitting of the different phases. Before curve fitting, a background-corrected intensity profile is calculated, and a three-frame running average can be used to smoothen frame-to-frame intensity variations to better determine the transition points between FA turnover phases:

$$I(t) = \frac{1}{3} \sum_{n=t-1}^{t+1} (I_{FA}(n) - I_{BKG}(n))$$

Even though photobleaching occurs at the same rate throughout the image, it is important to remember that background subtraction does not accurately correct for photobleaching. The absolute amount of photobleaching depends on fluorescence intensity (i.e. 10% of a 100 grey level background value is not the same as 10% of a 500 grey level signal), and photobleaching correction thus requires normalization of the total image fluorescence to a reference value (i.e. intensity in the first frame; see Chapter 1). However, generating a good photobleaching curve from long time-lapse experiments is difficult as cells will migrate in and out of the field of view making it impossible to accurately measure fluorescence intensity in the whole image as a function of time. We are not measuring absolute fluorescence, and the degree of photobleaching is small compared with fluorescence intensity changes associated with FA turnover. Thus, photobleaching introduces only a small error into FA turnover measurements, and we therefore do not typically correct for signal decrease due to photobleaching.

Once FA disassembly is initiated, it is reasonable to assume that dissociation of paxillin-mCherry molecules from the FA mostly depends on the amount of paxillin-mCherry still bound to the FAs. Thus, like radioactive decay, FA disassembly is expected to follow a single exponential decay with the rate constant k_d in which a is the offset of the exponential function along the time axis, and f_0 the intensity at $t=a$:

$$I_{disassembly}(t) = f_0 e^{-k_d(t-a)}$$

The assembly phase is more complicated, and without precise knowledge of the underlying molecular mechanism it is difficult to design an accurate mathematical model. However, for descriptive purposes, as previously described we found that a sigmoid, logistic function as a model for self-limiting growth fits the FA assembly phase well (Meenderink et al., 2010) in which k_a is the rate constant, f_{max} is the maximum fluorescence intensity, and $t_{1/2}$ the time at half maximum:

$$I_{assembly}(t) = \frac{f_{max}}{1 + e^{-k_a(t-t_{1/2})}}$$

In both equations, the rate constants k describe the steepness of the curve, i.e. smaller k corresponds to slower assembly or disassembly. This relatively simple model has worked well for us to describe FA dynamics in migrating epithelial cell sheets during which FAs assemble and disassemble in a very coordinated manner. However, depending on the conditions and cell type, FA turnover can be more complex, i.e. incomplete disassembly and reassembly or FA merging and splitting that are not described well by this model. Curve fitting can be done in many software packages. However, Microsoft Excel, a program that is commonly installed on most computers, includes a ‘Solver’ add-in that is a powerful optimization engine that can be used to calculate a least-square curve fit of virtually any function. The ‘Solver’ add-in is not automatically installed, but can be found in the Microsoft Excel options tab and easily added. The basic outline of how to set up an excel worksheet to run a curve fit is shown in Fig. 2. Briefly, three columns are set up that contain the data (D10:D35 for the Assembly phase in Fig. 2), a calculated fit (E10:E35) based on initial values of the free parameters (E3:E5) and the independent variable (in this case time in Column A), and the residual, i.e. the difference between observed value and calculated fit for each time point (F10:F35). A dedicated cell (F4) contains the sum of squares of the residuals:

$$\chi^2 = \sum_{t=1}^n (I(t) - I_{fit}(t))^2$$

Minimization of χ^2 by altering the free parameters of the fitting function will determine the best fit of the data. This is done by running the ‘Solver’ with the ‘Objective’ cell set to F4 ‘By Changing Variable Cells’ E3:E5 using the ‘GRG Nonlinear’ engine. Whether the fit has worked can be easily determined graphically (Fig. 2). If the fit fails, it likely indicates conversion of the optimization algorithm to a local minimum, a common problem in non-

linear curve fitting. This can usually be corrected by adjusting the initial parameters closer to the expected values before re-running the ‘Solver’.

Finally, based on $t_{1/2}$ of the logistic curve fit we can define a FA lifetime, t_{life} (Fig. 2), as the time during which paxillin-mCherry fluorescence intensity remains above the half maximum, which can be calculated from the parameters determined in the assembly and disassembly curve fits:

$$t_{life} = a - \frac{\ln\left(\frac{f_{max}}{2f_0}\right)}{k_d} - t_{1/2}$$

It is important to note that the values for disassembly rate constant and lifetime are interrelated and depend on how well the FA disassembly phase is fitted. A steeper disassembly fit will result in increased apparent lifetime. Thus, in case of a disassembly defect, both values are affected in opposite ways, which makes the method quite robust in detecting differences between conditions even if it is difficult to accurately determine the start of the disassembly phase.

Fig. 3 shows the outcome of FA turnover analysis of a moderately large number of FAs in mCherry-paxillin expressing, migrating HaCaT keratinocytes. As expected, the results for all three calculated FA turnover parameters are relatively broadly distributed demonstrating the variability of stochastic intracellular dynamics. Further analysis of the distributions shows that only the FA assembly rate is normally distributed, disassembly rates and lifetimes are not. In fact, a Poisson distribution may be expected to better describe the FA lifetime distribution as the time at which a FA disassembles is randomly distributed. In any case, it is important to remember that many parameters derived from intracellular dynamic processes are not normal distributed, and thus statistic testing for differences should not be done by using t-tests or ANOVA that assume normal distribution of the underlying data. Instead non-parametric statistics such as Kruskal-Wallis analysis of variance should be used. For the same reason, it is best practice to show non-parametric representations of data such as box-and-whisker plots in figures rather than bar graphs indicating only mean and standard error (Spitzer, Wildenhain, Rappsilber, & Tyers, 2014). Finally, it is important to remember that a statistically significant difference does not necessarily imply a biologically meaningful effect.

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Figure 1. Images from a 3 hour spinning disk confocal time-lapse sequence of mCherry-paxillin expressing migrating HaCaT keratinocytes. The bottom panels show example FA and background ROIs used for analysis of FA turnover.

Figure 2.

Example spreadsheet layout to use the Microsoft Excel ‘Solver’ for least square curve fitting of FA turnover dynamics, which can be adapted to fit any non-linear function. Columns D–F contain the data for the assembly, logistic fit and G–I for the disassembly, exponential fit. Boxes indicate spreadsheet formulas. The transitions between phases at which curve fits should begin or end are visually determined from a plot of the running average of the intensity data.

Figure 3.

Statistical analysis of FA assembly and disassembly rates and lifetimes from $n=135$ FA intensity profiles in ~ 20 migrating HaCaT keratinocytes. Normality plots on the right show the results of Shapiro-Wilk tests indicating a normal data distribution only for FA assembly rates.