

CHAPTER 3

A systems microscopy approach towards understanding tumor cell migration: quantitative multiparameter evaluation of cellular and molecular dynamics

Running title: Adhesion dynamics: visualization and quantification

Sylvia Le Dévédec¹, Kuan Yan², Hans de Bont¹, Veerander Ghotra¹, Hoa Truong¹, Erik Danen¹, Fons Verbeek² and Bob van de Water¹

¹ *Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands*

² *Imaging & BioInformatics, Leiden Institute of Advanced Computer Science, Leiden University, Leiden, the Netherlands.*

Conditionally accepted for publication in *Cell. and Mol. Life Sci.*, January 2010

ABSTRACT

Cell migration is essential for embryonic development and a number of processes in the adult, including inflammatory response, wound healing, angiogenesis and cancer metastasis. Especially, invasion of cancer cells in the surrounding tissue is a crucial step that requires increased cell motility. Cell migration is a well orchestrated process that involves the continuous formation and disassembly of matrix adhesions. Those cell-matrix adhesions are structural anchor points that interact with the extra-cellular matrix and also participate in adhesion-dependent signaling. Although those processes are very important for understanding cancer metastasis, still little is known about the molecular mechanisms that regulate adhesion dynamics during tumor cell migration. In this review we provide an overview of recent imaging strategies together with quantitative image analysis that can be implemented to understand adhesion dynamics and signaling in relation to tumor cell migration. This dynamic cell imaging together with multiparametric image analysis applied to relevant models will help understanding the molecular mechanisms that control cell migration and in this way advances the discovery of candidate genes that can be targeted for inhibition of metastasis.

1. Introduction

Cell migration, both single and collective, is a highly integrated multistep process that is essential in embryonic morphogenesis; tissue homeostasis and immune surveillance. While collective migration requires the movement of cohesive group¹, the single migrating cell is highly polarized with complex regulatory pathways that are spatiotemporally controlled². Migration contributes to several important pathological processes, including cancer progression and metastasis formation. Metastasis, dissemination of malignant tumors to a distant organ, is the major cause of cancer mortality. Tumor cell motility is the hallmark of invasion and is an essential step in metastasis.

Cell migration can be seen as a cyclic process. The initial response of a cell to a chemotactic signal is to polarize and extend protrusions in the direction of movement. These protrusions are usually driven by actin polymerization, and are stabilized by adhering to the extracellular matrix (ECM). These adhesions serve as traction sites for migration as the cell moves forward on top of them, and they are disassembled at the cell rear, allowing it to detach. This process depends on the cell type and environment. Matrix adhesion turnover is clearly visible in slow-moving cells such as fibroblasts or epithelial cells which show large protrusions, and is less visible in fast-moving cells such as neutrophils or cancer cells, which display small protrusions with a fast turnover². Interestingly, the movement of cell sheets shows some features of single-cell migration; however, the polarization extends over the entire sheet¹. In addition to typical matrix adhesions typically referred to as focal adhesions, cells can form another variety of adhesive structures which are podosomes and invadopodia also termed as podosome-type adhesions (PTAs)³. Those unique actin-rich adhesion structures are typically associated with sites of proteolytic degradation of the extracellular matrix components. Matrix degradation localized at podosomes or invadopodia is thought to contribute to cellular invasiveness in physiological and pathological situations. Cell types that form podosomes include monocytic, endothelial and smooth muscle cells, whereas invadopodia have been mostly observed in carcinoma cells⁴⁻⁶. In this review, we will focus on the study of focal adhesions in relation to tumor cell migration, however most of what we will discuss here is also relevant for the study of PTAs, and especially invadopodia^{7,8}.

Although focal adhesion dynamics is very important for understanding cell migration behaviour, still little is known about the molecular mechanisms that regulate adhesion dynamics during tumor cell migration. Until recently, advances in microscopic imaging technology, fluorescent reporter reagents and multiparametric image analysis have enhanced our understanding of cell migration and adhesion dynamics. In this review, we will focus only on single cell migration and discuss the current and emerging imaging technologies that can be implemented to study adhesion dynamics in migrating cells. Tables 1 and 2 are recapitulative of all the different techniques to study respectively protein

dynamics and modes of tumor migration and can be used as guidelines. We will also discuss the different multiparametric image analysis tools that can be applied after image acquisition to generate more detailed and reliable cellular and molecular measurements. Finally, studying the fundamental mechanisms underlying cell migration will help discovering new interesting targets for inhibition of tumor invasion.

1.1 Adhesion proteins

Matrix adhesion complexes are cytoplasmic structures that have been originally identified by electron microscopy⁹ or by interference reflection microscopy¹⁰. They are the closest site of contact between the cell and the underlying substratum. Integrins are the principal cell surface adhesion receptors mediating cell-matrix adhesions¹¹. Integrins are heterodimeric receptors that directly bind extracellular matrix molecules and couple them to the actin cytoskeleton (for reviews, see^{12,13}). Integrin cytoplasmic domains form multi-molecular complexes with proteins involved in cell adhesion signalling and with adaptors that provide a connection to the cytoskeleton¹¹. In the last years, considerable information has been accumulated on the molecular composition of matrix adhesions. Approximately 150 proteins, which form the so-called 'integrin adhesome', have been to date retrieved to be part of the matrix adhesions including kinases, phosphatases and structural proteins (for review see¹⁴). Even a larger number of over 600 proteins are defined to be involved in the spatiotemporal regulation of matrix adhesions (van Roosmalen et al., unpublished data). Upon attachment, integrins will cluster and promote local recruitment of structural proteins like vinculin, paxillin, talin, α -actinin and tensin, and signaling molecules including tyrosine kinases such as focal adhesion kinase (FAK), serine/threonine kinases and various adapter proteins. The molecular complexity of cell-matrix adhesions enables them to fulfill their dual role as modulators of both mechanical cell anchorage and transmembrane signaling¹². Integrins are not the unique transmembrane receptors that have been described to control and regulate adhesion signalling; syndecans can also bind directly to the ECM while growth factors such as EGFR can crosstalk with the integrins to regulate the recruitment of cytoplasmic proteins to adhesion sites (for review see¹⁵).

1.2 Adhesion types in 2D and 3D and cell behavior

Matrix adhesions are diverse in molecular composition, size and shape. Their distribution over the whole cell body is also heterogeneous and depends on cell type and environment. Typically adherent cultured cells on two-dimensional substratum *in vitro* show a large number of matrix adhesions ranging in size from less than 1 μm^2 to several μm^2 . Detailed analysis based on morphology, molecular composition and method of formation of mainly fibroblasts and

epithelial cells allowed those adhesions to be classified in three different classes (Fig. 1A). The most common forms of integrin-mediated cell-matrix adhesions in cultured cells are focal adhesions (FA), fibrillar adhesions (FB) and focal complexes (FC)^{12,13}. Focal adhesions are oval structures, usually a few μm^2 in area, and are associated with the termini of actin stress fibers. Fibrillar adhesions, which are derived from FAs, are elongated contact sites, associated with fibronectin fibrils. Focal complexes are small, dot-like adhesions that are mainly found at the cell edge and apparently nucleate FA formation (reviewed by¹²). Based on intensity based segmentation, matrix adhesions can be analyzed using fluorescence microscopy images obtained after immunofluorescence staining of cells for focal adhesion marker such as vinculin or paxillin. Indeed, the morphological and intensity parameters of adhesions can be quantified by analyzing images following waterline image segmentation (¹⁶ and Yan et al., manuscript in preparation) (Fig. 1B). This segmentation defines adhesions as "objects" (Fig. 1B-c), based on their fluorescence intensity and size range (Fig. 1B-d). The image analysis provides various features such as size (Fig. 1B-e), elongation, mean fluorescence-signal intensity and cell localization with respect to the nucleus which can be used for further clustering (Fig. 1B-f). Furthermore, matrix adhesions are heterogeneous in their molecular composition which can be studied using fluorescence ratio imaging. For example, FC do not contain zyxin whereas FB do not contain $\beta 3$ integrin or phosphorylated FAK¹⁷ but is highly enriched with tensin¹³. In general, quantitative multicolor fluorescence imaging can be used to unravel the molecular complexity of the various types of adhesions¹⁸. Matrix adhesion complexity depends also on the environmental stimuli. FAs are mechanosensors that sense the matrix physical properties whether it is rigid or soft and whether it is two- or three dimensional¹⁹. Distinctive 3D-matrix adhesions were described for the first time when cells were cultured in cell-derived 3D matrices: they appear like fibrillar adhesions also enriched in tensin but also rich in tyrosine phosphorylated proteins²⁰. Epithelial cells stably expressing the reporter for tyrosine phosphorylation GFP-dSH2²¹ show large peripheral and ventral focal adhesions on a rigid substrate whereas on a soft substrate, the adhesions become smaller, dot-like and only localized in the periphery of the cell (Fig. 1C). In addition, the GFP-dSH2 construct localized in a fibrillar pattern alongside the elongated cell protrusions, in a 3D collagen matrix (Fig. 1C-c). In conclusion, matrix adhesion complexity and diversity can be studied with static immunofluorescent images, but those sites are also very dynamic during cell migration in 2D and 3D environments.

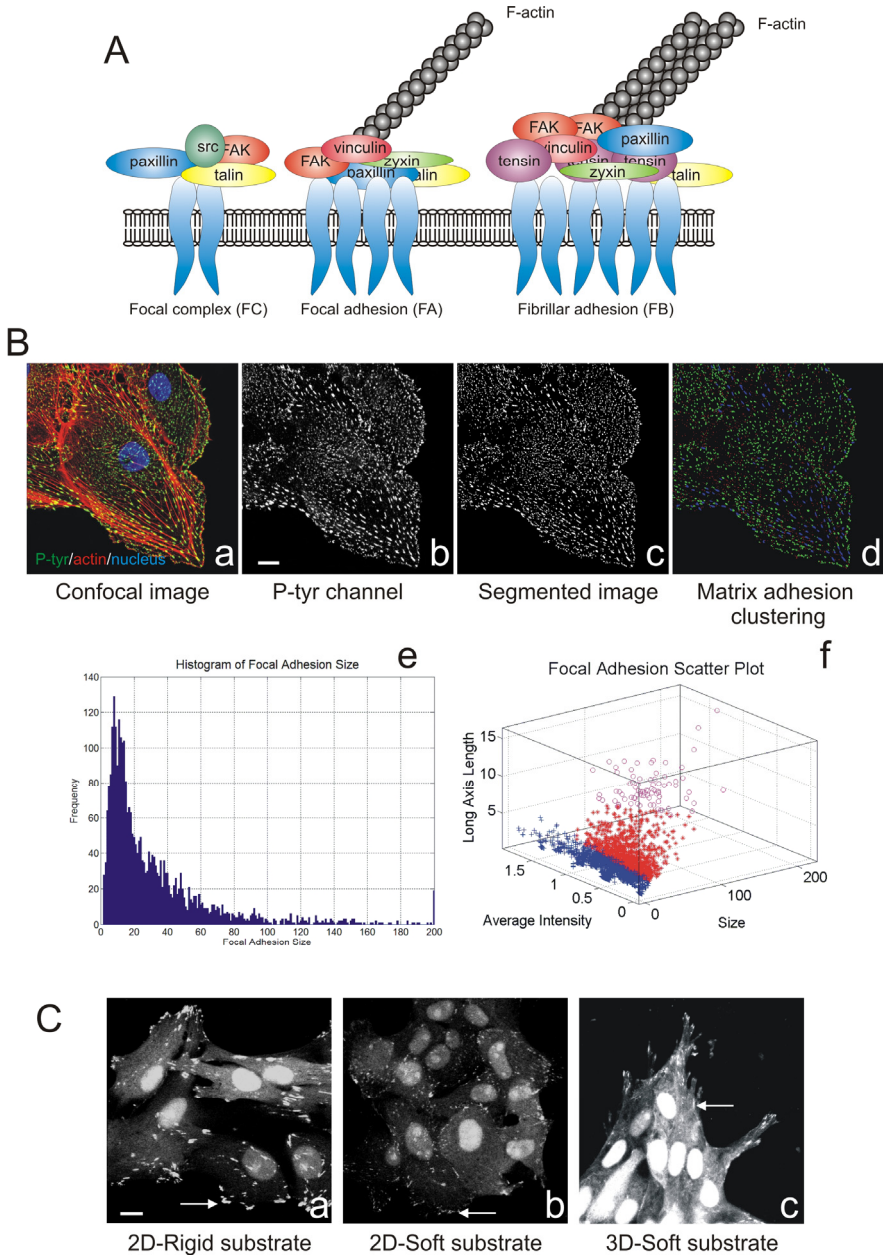


Figure 1: Matrix adhesions diversity and composition. (A) Schematic view of the three classes of matrix adhesions found in adherent cells *in vitro*. (B) Image analysis of matrix adhesions. Confocal picture of epithelial cell stained for Hoechst (blue), P-Tyr (green) and F-actin (red) (a) scale bar is 10 μ m. Confocal picture of focal adhesions only (b). Matrix adhesions segmentation (c) and clustering according to size (d). Distribution of the matrix adhesions according to their size (e) and clustering according to matrix adhesions intensity and length (f). (C) Matrix adhesions differ in size and shape according to their environment: in 2D rigid versus soft and in 3D; scale bar is 10 μ m.

1.3 Adhesion turnover during cell migration

Cell migration is an integrated process that requires the coordinated regulation of various structural and signaling molecules, including distinct kinases and phosphatases²². Cell migration requires the establishment of cell polarity to create a leading edge and a trailing edge. The leading edge undergoes membrane protrusive activities driven by actin polymerization that establish new matrix contacts, whereas at the trailing edge cell adhesions are disassembled to promote retraction of the cell rear and forward cell movement. The rate of cell migration can be limited by the rate of rear retraction, and thus the dynamic formation and disassembly of cell-matrix adhesions are critical to cell migration².

Formation of adhesions. The mechanism by which adhesions assemble in migrating cells is still under investigation. Some cells, particularly rapidly migrating ones such as leukocytes, have few visible integrin clusters, and thus very small submicroscopic adhesions are probably important for their migration. In other cells, small adhesions known as focal complexes can be observed at the leading edge. Formation of these adhesions depends on Rac- and Cdc42-GTPases, and these adhesions stabilize the lamellipodium by mediating attachment to the ECM, thereby contributing to efficient migration. However, cells with large integrin clusters ("focal adhesions") are tightly adherent and are typically either non-migratory or move very slowly. The assembly of focal adhesions involves Rho-GTPase as well as myosin-induced contractility. During their formation, some protein components enter adhesions with similar kinetics, which suggests that they exist in preformed cytoplasmic complexes²². However, other components enter adhesions with very distinct kinetics, which is consistent with a model in which a regulatory event initiates the serial addition of different proteins. Paxillin, for example, is present in nascent adhesions, whereas α -actinin appears more prominently in "older" adhesions²².

Adhesion disassembly at the front and the rear. Adhesion disassembly is observed both at the leading edge, where it accompanies the formation of new protrusions, and at the cell rear, where it promotes tail retraction. At the front of migrating cells, adhesions at the base of a protrusion disassemble as new adhesions form at the leading edge (for review see²²). However, some adhesions persist and mature into larger, more stable structures. Little is known about adhesion disassembly versus maturation; however, targeting of microtubules has been implicated as one factor that promotes adhesion disassembly (for review see²³). Both protein kinases and phosphatases also appear to be central to the regulation of adhesion turnover and stability²⁴. For example, cells lacking the tyrosine kinases FAK or Src have more and larger adhesions and migrate poorly²⁵. The interaction of FAK with Src and the adapter proteins Cas and Crk, which in turn activate Rac-specific GEFs, appears to regulate adhesion turnover. Adhesion turnover in migrating cells is also regulated by a complex of Rac-associated proteins^{26,27}. In conclusion, functional studies that will systematically

determine the role of individual proteins involved in cell-matrix adhesions using dynamic imaging of the protein localization, kinetics and interactions will shed a more complete light on the mechanisms of cell migration.

1.4 Cell migration/matrix adhesion and cancer

Metastasis is the most frequent cause of death for patients with cancer. Tumor cell motility is the hallmark of invasion and is an initial step in metastasis. In order to metastasize, cancer cells must first detach from the primary tumor, migrate, invade through tissues, and attach to a second site. Invasive carcinoma cells acquire a migratory phenotype associated with increased expression of several genes involved in cell motility such as matrix adhesion associated genes. Recent data have provided evidence for a requirement for certain focal adhesion protein expression (e.g. integrin, FAK/Pyk2, paxillin, ILK, Ezrin) in metastatic dissemination. Focal adhesion kinase (FAK) is one of the most studied protein which role in cancer has long been characterized. FAK expression and activity are enhanced in metastatic tumors of diverse sort²⁸⁻³⁰. FAK is considered as a promising gene candidate and inhibitors of FAK are currently in clinical trial (see review^{31,32}). Similar to FAK, increased expression of paxillin is observed in breast carcinoma³³. Therefore, studying the motility mechanisms used by cancer cells would clarify some of the key events influencing metastasis in cancer. In addition, identification of the molecular pathways that play a role in cancer cell motility will provide new diagnostic approaches and targets for the treatment of metastatic cancer.

2. Studying migration and adhesion dynamics in living cells

Recent advances in fluorescence probes and microscopy technologies have provided powerful approaches that present advantages over the traditional biochemical approaches. In particular, the newly developed recombinant fluorescent proteins (FPs) and genetically encoded biosensors are useful tools for imaging protein distribution, dynamics and interactions in live cells with high temporal and spatial resolutions enabling the elucidation of molecular mechanisms behind adhesion turnover responsible for the cell migration³⁴⁻³⁸.

2.1 Phase-contrast/DIC imaging:

In general, laboratories use wide field fluorescence imaging (also known as epi-fluorescent) equipped with a basic CCD camera. Time-lapse phase-contrast microscopy is easy, not photo-toxic to cells and can be very useful to record cell migration under the condition that appropriate software to track the moving cells is available. Using this imaging technique, it has been shown that increased ECM density and substrate rigidity regulate the scattering of MDCK cells³⁹.

Differential interference contrast (DIC or Nomarski) imaging is a modification of phase-contrast microscopy imaging that provides detailed information about the cell shape and structure. It can be successfully used to follow up migrating cells, quantify the cell movement and study in detailed protrusions and lamellipodia formation (Fig. 2A). Although automated tracking of individual cells is possible with DIC, due to high signal to noise ratio, it remains difficult to analyze the cell features. Fluorescence imaging is an answer to that problem.

2.2 Fluorescence Live cell imaging

2.2.1 Cell migration: wide field

Wide field fluorescence microscopes equipped with the appropriate excitation and emission filters and sensitive CCD camera are of great use for studying cell migration and also cell structure. This fluorescence technique is fast and sensitive enough to detect GFP stably expressing migrating cells. The dynamic imaging of 2D cell migration includes various assays such as directed cell migration (chemotaxis and woundhealing) and random cell migration⁴⁰. The dynamic imaging of migrating cells which implies tracking of fast object needs high temporal resolution. The fast image acquisition is mostly possible on an epifluorescence microscope equipped with sensitive CCD camera. Indeed, to track fast moving objects, the interval between two sequential frames should be short enough to ensure an overlap of the moving object between frame t and frame $t+1$. Using cell-lines stably expressing GFP together with advanced image analysis tools allow the simultaneous detection of multiple cellular characteristics that define phenotypic response such as EGF response in rat mammary carcinoma cell-line (Fig. 2B). Application of advanced bioinformatics and statistical methods to multiparametric image data generates non-biased phenotypic fingerprint that describes the effect of genetic or pharmacological manipulation (Fig. 2C). The extension of multiparametric image analysis to live cell studies can reveal further mechanistic insight (Yan K et al., manuscript in preparation). Epifluorescence microscopy provides high speed image acquisition necessary to follow fast moving cells but also to visualize adhesion dynamics. The quantification of adhesion dynamics has been described first by Webb and colleagues⁴¹ using epi-fluorescence microscopy. Adhesion assembly and disassembly rate constants can be calculated by measuring the incorporation or loss of fluorescence (e.g. GFP-paxillin). Assembly will provide an increase in fluorescence intensity whereas disassembly will lead to a loss in intensity. Intensity values at various time points are plotted on a semi-logarithmic scale representing fluorescent intensity ratios over time. These ratios are calculated using the formula $\ln(I/I_0)$ for assembly and $\ln(I_0/I)$ for disassembly (where I_0 is the initial fluorescent intensity and I is the fluorescent intensity at the indicated time). Rate constants can then be calculated from the slope of the resulting line of best fit. The study conducted by Webb et al., revealed for the first time that

fibroblast with a knockout of FAK, Src and p130Cas decreased the disassembly rate constant of $1.2 \times 10^{-1} \text{ min}^{-1}$ in wild type cells to between 6×10^{-3} and $9 \times 10^{-3} \text{ min}^{-1}$ in knock out cells⁴¹.

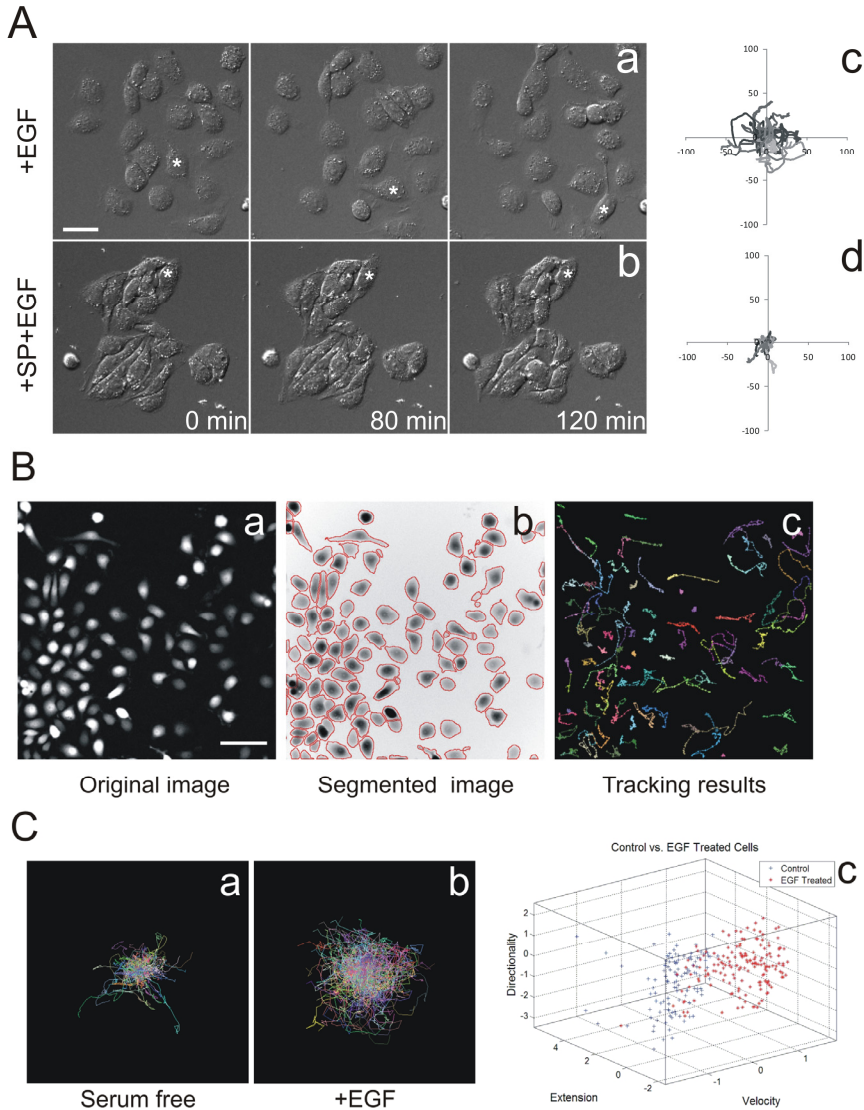


Figure 2: Imaging and analysis of single cell migration. (A) DIC imaging of EGF-induced migration of rat carcinoma MTLn3 cells treated with (a) or without (b) JNK inhibitor (SP600125) for 2 hours; scale bar is 20 μm . (c-d) Cell tracks of both time lapses. (B) Epifluorescence imaging and analysis of migrating MTLn3 cells ectopically expressing GFP. Epifluorescent pictures (a) are waterline based segmented (b) and cells are consequently tracked (c); scale bar is 50 μm . (C) Individual cell tracks of MTLn3 stimulated (a) or not by EGF (b) and clustering analysis of both treatments based on directionality, extension and velocity (c).

2.2.2 *Confocal Laser Scanning microscopy (CLSM)*

While epifluorescence microscopy provides high time resolution which is required to visualize fast processes, confocal microscopy offers several advantages over conventional optical microscopy, including controlled depth of field, the elimination of image degrading out-of-focus information, and the ability to collect serial optical sections from thick specimens. It provides high spatial resolution but still many physiological processes and events take place faster than they can be captured by most CLSMs, which have image acquisition rates typically in the order of one frame per second. CLSMs using acousto-optical devices and a slit for scanning are faster than the galvanometer-driven point scanning systems, and are more practical for physiological studies. These faster designs combine good spatial resolution with good temporal resolution, which may be 30 frames per second at full screen resolution, or near video rate. The slower point scanning microscope systems can achieve the best temporal resolution only by scanning a much reduced area on the specimen. If full spatial resolution is required, the frames must be collected less frequently, losing some temporal resolution. The confocal systems using disk scanning or oscillating mirror scanning methods are also capable of imaging fast physiological or other transient events. Nowadays, new developments in confocal microscopy provide both high-speed and high-resolution imaging to capture intracellular biological processes. A resonant confocal scan head dramatically improves time, spatial and spectral resolutions and allows high-speed imaging up to 230 frames per second (512 × 64 pixels) which make it an ideal technique for bleaching and protein kinetic experiments (photoactivation and photobleaching, see below). A major drawback of confocal microscopy is photo bleaching and photo damage from the illuminating laser beam that can be cumulative over multiple scans. So the exposure to the beam should be kept to the minimum necessary to acquire the image. Another recent development is Controlled Light Exposure Microscopy (CLEM) which should be ideal for live imaging as it helps to reduce photobleaching and phototoxicity; the two main limitations in live-cell confocal microscopy⁴². In CLEM illumination excitation light is reduced using two strategies. The first is based on the principle that if there is no signal, then no illumination is required (for example, when imaging the background). The second detects whether there is sufficient signal to acquire an image. If so, illumination is stopped. But confocal microscopy is definitely the best to visualize in living tissue but this will be further discussed in the review.

2.2.3 *Total internal reflection fluorescence (TIRF) microscopy*

It has long been recognized that TIRF microscopy could potentially become a powerful tool in answering a number of biological questions, and although utilized for over 20 years, the technique has not received a considerable amount

of attention until recently. Cell-substrate contacts of human skin fibroblasts, labeled with fluorescent lipids, were investigated by TIRF microscopy in the early 1980s. One key advance in the field of dynamic imaging of matrix adhesions is the recent commercial development of TIRF microscopy. Although, total internal reflection microscopy was long ago already described (see review⁴³), its wide commercialization is just recent and a major advance for the matrix adhesion field. Elimination of background fluorescence from outside the focal plane can dramatically improve the signal-to-noise ratio, and consequently, the spatial resolution of the features or events of interest. TIRF microscopy exploits the unique properties of an induced evanescent wave or field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices. In practice, the most commonly utilized interface in the application of TIRF microscopy is the contact area between a specimen and a glass coverslip or tissue culture container. In a typical experimental setup, fluorophores located in the vicinity of the glass-liquid or plastic-liquid surface can be excited by the evanescent field, provided they have potential electronic transitions at energies within or very near the wavelength bandwidth of the illuminating beam. Because of the exponential fall-off of evanescent field intensity, the excitation of fluorophores is restricted to a region that is typically less than 100 nanometers in thickness. By comparison, this optical section thickness is approximately one-tenth that produced by confocal fluorescence microscopy techniques. Because excitation of fluorophores in the bulk of the specimen is avoided, confining the secondary fluorescence emission to a very thin region, a much higher signal-to-noise ratio is achieved compared to conventional wide-field epifluorescence illumination. This enhanced signal level makes it possible to detect single-molecule fluorescence by the TIRF microscopy method. In Fig. 3A, we show the improvement of TIRF imaging above wide-field and confocal microscopy by eliminating out of focus background fluorescence. Another example of the application of high speed and resolution TIRF imaging of focal adhesion turnover is shown in Fig. 3B. The matrix adhesion turnover can be consequently quantified with multiparametric analysis (Fig. 3C) (Yan K et al., manuscript in preparation).

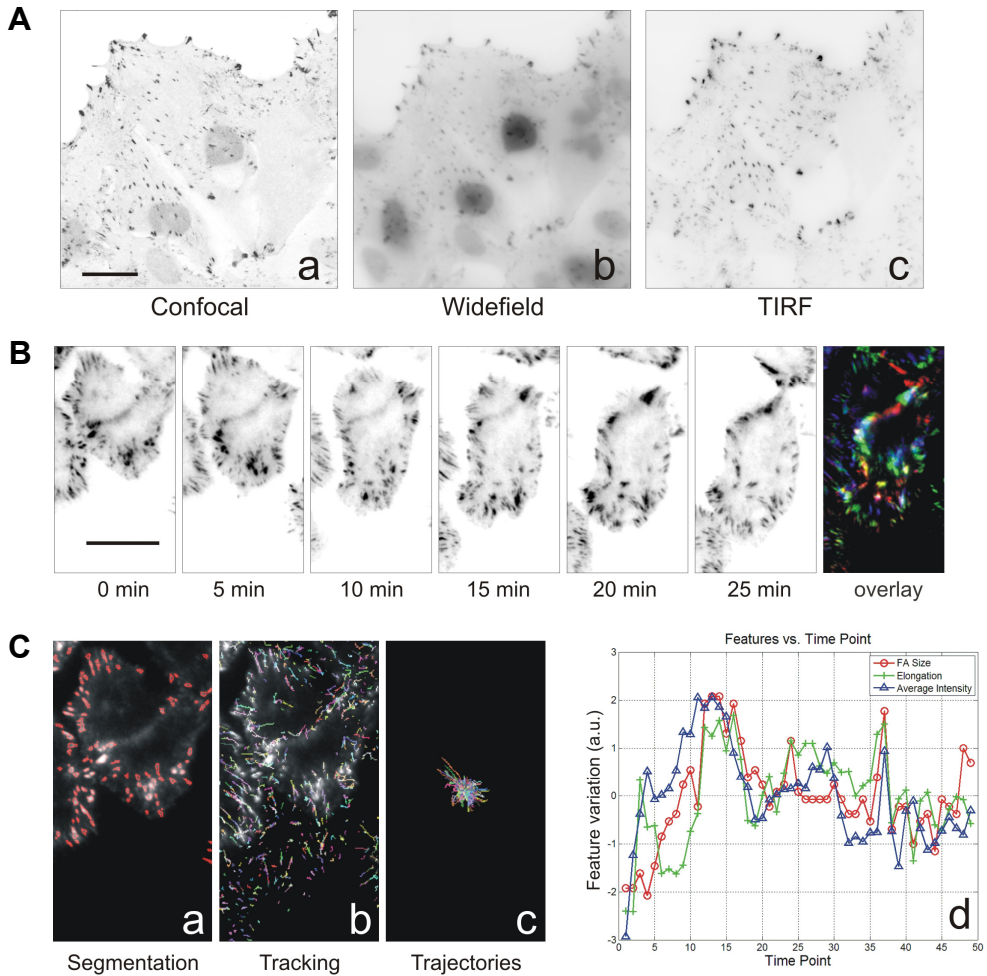


Figure 3: Imaging adhesions by confocal, widefield and TIRF microscopy. (A) Z-scan series of the same renal epithelial LLC-PK1 cell overexpressing the reporter construct GFP-dSH2 performed with confocal (a), widefield (b) and TIRF microscopy (c), scale bar is 20 μm . Note the advantage of TIRF microscopy for visualizing matrix adhesions. (B) Analysis of matrix adhesions dynamics with TIRF microscopy. Time lapse of a migrating MTLn3 cell expressing GFP-paxillin and overlay of the different frames to illustrate the focal adhesion turnover; scale bar is 10 μm . Note the fast turnover of matrix adhesions in these cells. (C) Multiparametric analysis of matrix adhesion dynamics. (a) matrix adhesion segmentation, (b) tracking of individual matrix adhesions, (c) plot of all individual matrix adhesion trajectories and lifetime, (d) example of possible plot of different features (FA size, elongation and intensity) of an individual matrix adhesion over the time. The different features are normalized so that the data distribution is scaled to 1 and the average of all features are shifted to zero. A FA size of "-2" indicates that the FA size in this frame is smaller than its average size by 2 in the normalized feature space.

3. Studying protein dynamics and interactions in adhesion

While imaging matrix adhesion proteins using fluorescence microscopy provides information about spatial localization, it does not allow direct measurement of protein movement. The goal of imaging matrix adhesions in migrating cells is to determine their function, the interaction of the different adhesome components, and the mechanisms that regulate adhesion formation and disassembly. To be able to quantify and model protein dynamics in adhesions, many quantitative measurements are needed: interaction between adhesion components, k_{on} and k_{off} of individual components and complexes, diffusion values, protein concentration in both the cytosol and the focal adhesions. The challenge is to develop methods for measuring these parameters during adhesion formation and disassembly in migrating cells. These measurements should be obtained at high spatial and temporal resolution.

3.1 Photo-activation, Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP)

Many fluorescent proteins (FPs) have been designed by mutagenesis to change their fluorescence intensity (photoactivation) or color (photoconversion) upon illumination by lights with specific wavelength, intensity and duration (see review³⁷). Those FPs can be grouped into three classes: (a) irreversible changes in color upon illumination which includes photoactivatable (PA)-GFP⁴⁴ that can be photoactivated with UV illumination and emits green fluorescence; (b) irreversible change in color from green to red upon UV illumination which includes Kaede⁴⁵ and Dendra⁴⁶; and (c) reversible change in intensity/color upon UV illumination such as Dronpa⁴⁷ which is a very valuable marker for photoconversion studies and fast cellular processes. Until now, mainly PA-GFP has been successfully used to investigate actin dynamics⁴⁸, protein adhesion movement⁴⁹ and trafficking of integrin receptors⁵⁰. Those photoswitchable fluorophores are valuable probes than can be used in live cells to monitor with high resolution diffusion, trafficking and stability of protein targets.

Fluorescence recovery after photobleaching is a common used technique to measure protein kinetics. In a typical FRAP experiment, a small defined region within a larger volume (for instance cell nucleus or a focal adhesion) is shortly illuminated at high laser intensity (Fig. 4A)^{51,52}. Immediately after the bleach-pulse the majority of the GFP-tagged proteins within the region irreversibly have lost their fluorescent properties: they are photobleached. In a situation where all GFP-tagged proteins are mobile, proteins from outside will diffuse into the bleached region resulting in the increase of the fluorescence signal in the region until it reaches equal signal intensity as outside the bleached region. In contrast, if permanently immobile proteins are present, there will be no movement of GFP-tagged protein in the bleached region resulting in an incomplete

redistribution of the fluorescence signal. Transient immobilization as was observed for most adhesion proteins, results in a delayed, secondary fluorescence redistribution in the bleached region. In summary, FRAP experiments provide information on protein mobility parameters: diffusion coefficient, immobile fraction and residence time. Several variants of FRAP have been developed including spot-FRAP, strip-FRAP, FLIP (Fluorescence loss in photobleaching), combined FLIP-FRAP. Spot-FRAP is based on photobleaching of a small spot within a focal adhesion (Fig. 4B), whereas in a strip-FRAP, a narrow strip spanning the cytoplasm in between focal adhesions is bleached. FRAP is widely and successfully used in adhesion biology. Several FRAP studies have addressed the dynamic properties of numerous adhesion properties⁵³⁻⁶⁴.

In a FLIP experiment, the loss of fluorescence in a region or structure far from the bleached region is monitored. FRAP and FLIP can also be combined (FLIP-FRAP): two regions are monitored simultaneously after bleaching only one of them. FLIP-FRAP is specifically useful to determine the residence time of proteins inside structures such as focal adhesions. In our lab, we developed successfully combined a FLIP-FRAP bleaching procedure so that in one experiment we can analyze all the focal adhesions distributed over the whole cell body (Le Dévédec et al., submitted). Together with Monte Carlo simulation, we observed that FAK and paxillin had equal diffusion rate but differential residence time that is related to adhesion size and strength. With this technique, we are able to extract mobility parameters of adhesion proteins as well as a mapping of the protein kinetics according to focal adhesion size, type and localization in the cell (Fig. 4B).

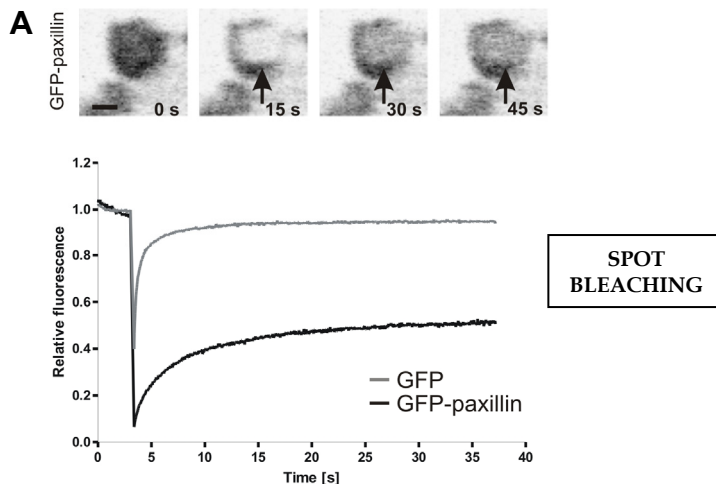


Figure 4A: Studying dynamics of matrix adhesion associated proteins by FRAP analysis. Time lapse of a typical spot bleaching experiment. A region of interest within a focal adhesion is defined, bleached with a high power laser intensity and subsequently followed over the time until fluorescence intensity reached a steady state. Fluorescence redistribution over the time is plotted. Scale bar is 1 μm .

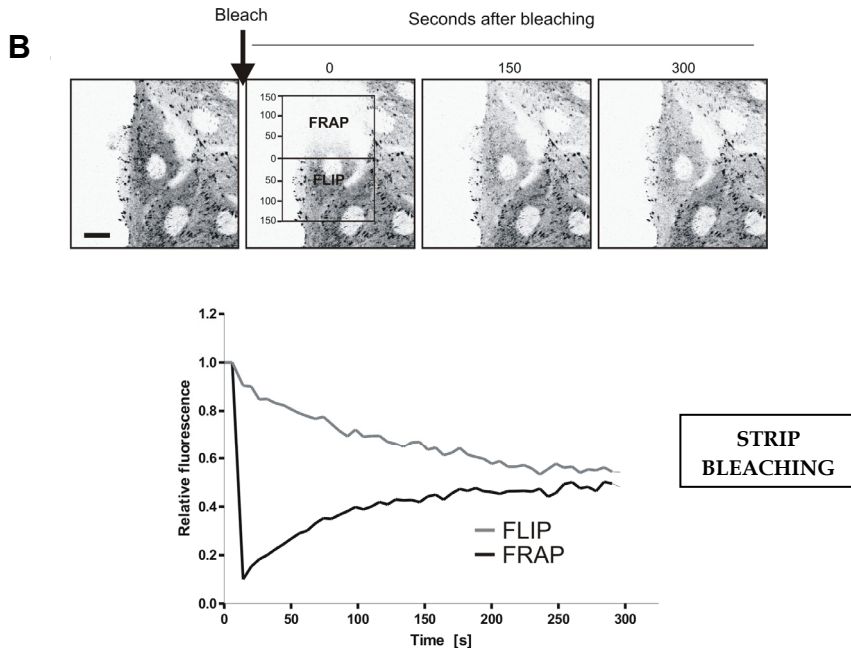


Figure 4B: Studying dynamics of matrix adhesion associated proteins by FRAP analysis. Combined FLIP-FRAP experiment is performed over the whole cell which allows analysis of several adhesions in the same time. Average loss in fluorescence and redistribution of fluorescence are plotted over the time. Scale bar is 10 μm .

3.2 Fluorescence speckle microscopy (FSM)

Another fluorescence technique that detects protein dynamics, turnover and interaction is a method called fluorescence speckle microscopy (FSM) that uses a very low concentration of fluorescent subunits, conventional wide-field fluorescence light microscopy and digital imaging with a low-noise, cooled charged coupled device (CCD) camera⁶⁵. In FSM, the fraction of fluorescently labeled molecules in the cell, relative to the level of endogenous unlabeled molecules, has to be very low (typically 0.5% or less). Labeled and unlabeled molecular subunits stochastically co-assemble into structures, giving a random and sparse distribution of fluorescent subunits with a 'speckled' appearance in high-resolution fluorescence images. The low level of fluorescent subunits reduces background fluorescence. Translation of the fluorescent speckle distribution indicates movement of structures whereas changes in speckle intensity and pattern reveal assembly dynamics and subunit turnover. Keys to successful FSM are the ability to image diffraction limited regions ($\sim 0.25 \mu\text{m}$) containing few (2-10) fluorophores and the capacity to inhibit photobleaching which is only possible with sensitive imaging system that includes a low noise/high quantum efficiency camera. Extensive characterization of actin

dynamics using FSM has revealed two spatially, kinetically and kinematically distinct actin networks; with the local expansion of the lamella network being a source of persistent cell protrusion^{66,67}. There is also evidence that the actin network is dynamically coupled to adhesions⁶⁸. A recent study using FSM did measure the coupling of focal adhesion proteins to actin filament. Very diverse behaviors of the seven GFP-tagged focal adhesion associated proteins were found: integrins show a much slower motion than FAK, talin and α -actinin⁶⁹. Their FSM analysis of the dynamic interactions between matrix adhesion components and F-actin in living cells revealed that there is a hierarchy of motion from fast to slow, from actin-binding proteins to adhesion proteins within matrix adhesions and to integrins. Those FSM-mediated molecular measurements provided considerable knowledge on the mechanism behind matrix adhesion dynamics.

3.3 Fluorescence correlation spectroscopy (FCS) and variants

FCS analyses concentration fluctuations as a function of time to determine kinetic parameters, molecular associations and concentrations. This technique requires laser excitation of small focal volume and measure fluctuations in fluorescence intensity over many time intervals. Single or cross-correlation analysis (in the case of two different fluorophores) is applied in local areas across time course to determine rates of diffusion, degree of aggregation, number of fluorescent entities and flow velocities. When two different fluorescent proteins are used, the cross correlation function provides estimates of their fractional association and rates of co-transport. FCS is used most widely to study molecules in solution; only few groups have applied FCS to analyze adhesion protein movements in intact living cells. A variant of FCS, image correlation spectroscopy (ICS) was implemented by Wiseman and coworkers⁷⁰ and allowed them to investigate the distribution, dynamics and interactions of $\alpha 5$ -integrin, and α -actinin in the context of the formation and disassembly of adhesions during cell migration. Integrins are clustered throughout the cell and in nascent adhesions get 1,4 times more concentrated and 4,5 times more clustered and less mobile than in surrounding regions. Although FCS has a high temporal resolution (microseconds) but low spatial resolution, ICS has a low temporal resolution (seconds) but a high spatial resolution. A new analysis method, termed raster imaging correlation spectroscopy (RICS) can be applied on any confocal microscope⁷¹ and bridges the timescales of FCS and ICS, and provides spatially resolved dynamic information such as the diffusion and binding of paxillin-GFP stably expressed in CHOK1 cells⁷² and of FAK-GFP in MEFs⁷³. Another variant of FCS, a general velocity-mapping technique termed spatio-temporal image correlation spectroscopy (STICS) has been described and provided new insight into the protein mobilities within the focal adhesions: while integrins were mostly immobile, paxillin and FAK immobile fractions were equal (74%), and

actin was more rapidly diffusing (24%)⁷⁴. Finally, a recent study that combined different fluorescence fluctuation approaches demonstrated that paxillin-GFP shows heterogeneous dynamic within the cell⁷⁵. In the cytoplasm, paxillin is uniformly distributed and diffuses freely as a monomer. Near adhesions, paxillin binds to protein partners and so its dynamics is reduced. These dynamic were different from assembling to disassembling adhesion regions, even within a single adhesion⁷⁵. The implementation of complementary fluctuation methods will provide new data on the dynamics of protein adhesions during cell migration.

3.4 Fluorescence resonance energy transfer (FRET)

A powerful imaging method to study protein-protein interactions in living cells is fluorescence resonance energy transfer (FRET)^{76,77}. FRET is the non radiative transfer of energy from a donor fluorophore in excited state to a nearby acceptor fluorophore to allow energy transfer if within only 10 nm. Because this distance is in the range of protein sizes, FRET can also be used to study conformational changes of proteins tagged with a FRET donor and FRET acceptor. The most frequently used FRET methods are sensitized emission, ratio imaging, acceptor photobleaching FRET⁷⁸ but the latter is not appropriate for studying rapid changes of protein interactions over time. The sensitized emission approach detects the emission of the acceptor fluorophore (often Cyan Fluorescent Protein, CFP) while the donor fluorophore (often Yellow Fluorescent protein, YFP) is excited. Although still widely used, sensitized emission requires careful data processing⁷⁹ and due to signal-to-noise ratio can be poorly sensitive. Cross talk and bleed through from one fluorophore to another makes the analysis highly dependent on control measurements of cells in which only one of the two fluorophores is present. An alternative approach to determine FRET is acceptor/donor ratio imaging (e.g. YFP/CFP) where both donor and acceptor emission are detected simultaneously when excited at the excitation wavelength of the donor. However, this method can be only applied when donor and acceptor are equally expressed in a cell system which is always the case when using FRET biosensors. In the study of adhesions, few FRET biosensors have been designed to monitor in live cells the activity of a number of kinases, e.g. Src and FAK^{80,81} and GTPases, e.g. Rho, Rac and Cdc42⁸²⁻⁸⁵. A fourth method to detect FRET is based on the reduced lifetime of excited donor molecules when they are in the proximity of acceptors^{86,87}. This technique is considerably more sensitive and accurate than intensity based methods, but is slower and requires specific detector. Consequently, this has limited the application of FLIM in live cell studies. However improvements in microscope design detector technology have reduced the time for data acquisition. When correctly applied, FRET is a useful tool for investigating the molecular mechanisms that regulate integrin-mediated signaling in migrating cells^{76,80,85,88}.

4. Optical imaging towards understanding tumor cell migration and intravasation

Of course studying and understanding adhesion dynamics in cells migrating onto a rigid 2D substrate still does not reflect the *in vivo* situation. The biological relevance of focal adhesions was initially questioned, since equivalent structures to these prominent 2D adhesion structures were not easily observed in most tissues. However focal adhesions have been found at point of high fluid shear stress in blood vessels⁸⁹ and in proximal tubular cell-ECM contact sites in kidney tissue⁹⁰. Imaging migration and adhesions in 3D culture systems *in vitro* and *in vivo* is still in development. Despite the recent advances in dynamic imaging, a number of technical challenges remain to be overcome to allow functional and biochemical study of tumor invasion mechanism in 3D ECM substrates and *in vivo*.

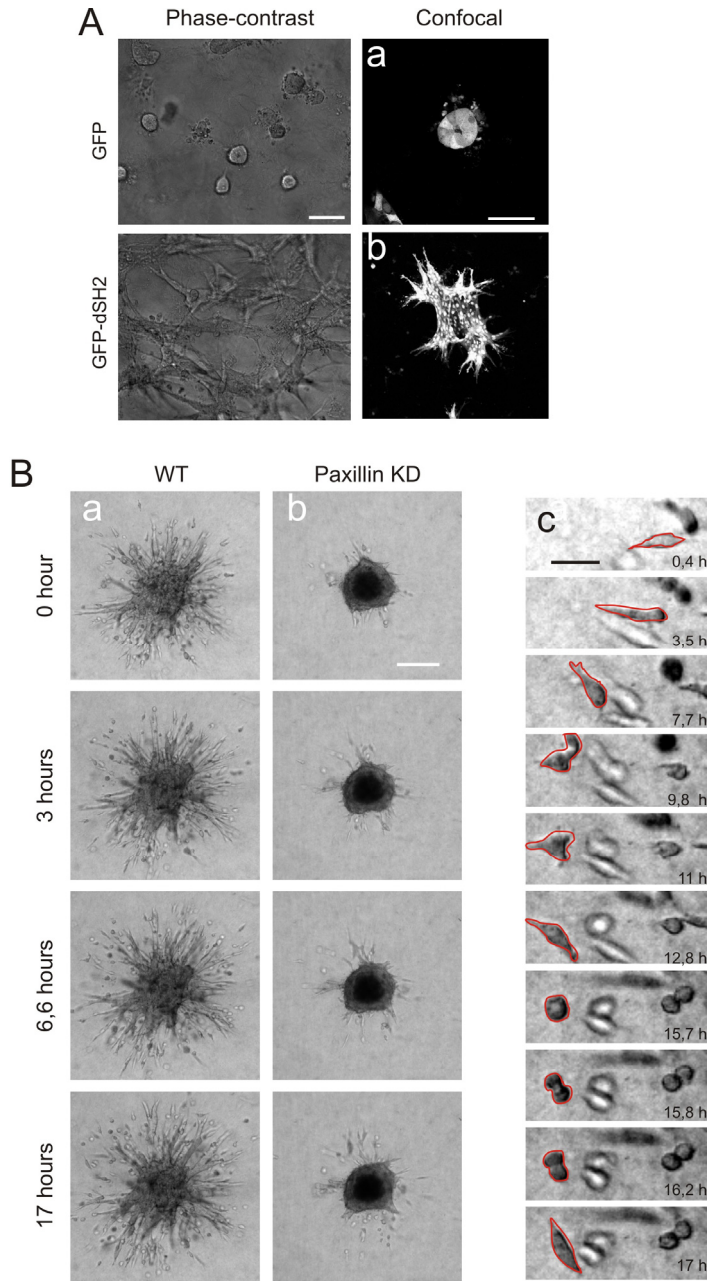


Figure 5: Imaging adhesion and cell migration in 3D culture system *in vitro*. (A) Phase-contrast (scale bar is 100 μm) and confocal pictures (scale bar is 50 μm) of tubulogenesis assays conducted with LLC-PK1 cells overexpressing either GFP alone (a) or GFP-dSH2 (b) in Matrigel-collagen gels. (B) Time lapse series (of 17 hours) of 4T1 mouse mammary carcinoma cells control (a) and paxillin knock down (b) invading 3D collagen gels (made with the help of H. Truong). Scale bar is 200 μm . (c) Detailed time lapse serie of one migrating 4T1 control cell. Scale bar is 50 μm .

4.1 *Imaging adhesions and tumor invasion in 3D culture systems*

Although studying the role of matrix adhesion in migrating cells in a 2D environment has provided a wealth of data, it is not surprising that there is an ongoing effort on setting up 3D invasion models adapted for dynamic live cell imaging that allows a more robust understanding of tumor cell invasion (see recent review⁹¹). A number of experimental methods incorporating different types of ECM substrates (Type I collagen, fibronectin, Matrigel, matrix polymers and tumor associated 3D matrices) have been developed to study 3D tumor invasion *in vitro*⁹²⁻⁹⁷. Despite these recent advances in 3D substrates, it remains a technical challenge to visualize first the entire cell body of moving cells and second the matrix adhesions self. Several handicaps include background due to the matrix itself, signal to noise and the depth of the sample which limit the choice of objectives. Phase-contrast imaging is a convenient imaging approach if signal to noise is not an issue for further data processing (Fig. 5A). It provides cellular and temporal resolution which is already enough to characterize the type of 3D invasion which can be depicted as either single (mesenchymal or amoeboid) or collective migration^{1,91,92,98}. Confocal microscopy is the most suitable imaging technique to collect fixed endpoints or time-lapse sequences of three-dimensional data of migrating cells and matrix adhesion activities⁹⁹⁻¹⁰¹. Advanced bioinformatics is needed to further process the data and apply multiparametric image analysis to describe with several parameters cell behaviours^{102,103}. Next to the dynamic imaging of cell migration, imaging of matrix adhesion turnover in a 3D environment is technically extremely challenging. In Fig. 5A, we show how confocal imaging allow high resolution imaging of the GFP-dSH2 reporter overexpressed in epithelial cells that have the ability to form tubulogenesis. Thanks to this reporter combined with fast confocal microscopy, we will be able to monitor tyrosine phosphorylation at adhesion sites when cells are migrating collectively through the collagen gels (Le Dévédec et al., unpublished). Furthermore, by using our established 3D invasion assay for 4T1 mammary tumor cells together with GFP-tagged adhesion protein overexpression we are able to image adhesion dynamics in a 3D environment during tumor cell migration (Fig. 5B).

4.2 *Imaging migration and adhesions in vivo.*

Studying adhesion dynamics in migrating cells in an *in vitro* 3D matrix is already technically challenging. Obviously, it is even more challenging to do so within intact organisms. Suitable *in vivo* models for both cancer progression and high resolution imaging are necessary.

4.2.1 Zebrafish model for metastasis analysis

Recently, the zebrafish and its transparent embryos became a new model system to investigate tumor development, cancer cell invasion and metastasis formation¹⁰⁴⁻¹⁰⁶. In the transparent zebrafish embryos invasion, circulation of tumor cells in blood vessels, migration and micrometastasis formation can be followed in real-time. Moreover, a number of unique features make this animal model very attractive: zebrafish are inexpensive to maintain, breed in large numbers, develop rapidly *ex vivo*, and can be maintained in small volumes of water. Several independent studies have now shown that human melanoma cells and other cancer cell lines are able to induce neovascularization when xenografted in the zebrafish¹⁰⁶⁻¹⁰⁸. The role of the small GTPase RhoC in tumor formation, angiogenesis and cell invasion was investigated in real-time in 1-month-old immunosuppressed zebrafish xenografted with the human breast cancer cell line MDA-435¹⁰⁶. This study achieved high-resolution imaging of the dynamic cell-vascular interface in transparent juvenile zebrafish. All these innovative studies established the use of the zebrafish xenotransplantation model for the analysis of cancer cell lines as we also did in our lab (Fig. 6). We screened a number of cancer cell-lines and established that very aggressive phenotype after injection in the yolk (Fig. 6A-a), were able to outgrow and disseminate throughout the animal body (Fig. 6A-b) (Ghotra et al., manuscript in preparation). We also demonstrated that certain cell-types could trigger the angiogenesis process (Fig. 6A-c and 6A-d) (Ghotra et al., manuscript in preparation). In a very recent study, it was shown that zebrafish embryos can even be used to directly transplant human tumor tissue and primary human tumor cells¹⁰⁹. Zebrafish embryos thus provide a simple, fast and cost-effective method to test the metastatic behaviour of human cell-lines and primary tumors in an *in vivo* vertebrate animal model that also permits high throughput drug screening (see later section).

4.2.2 Mouse metastasis model for imaging of tumor cell migration

The availability of multi-photon intravital microscopy has allowed researchers to visualize the dynamic behavior of cancer cells *in vivo*^{100,101,110,111}. Multiphoton microscopy uses longer wavelengths (up to 1200 nm) that are able to penetrate deeper into tissues and allows us to visualize more than 100 μm deep into the primary tumor. Multi-photon excitation causes also less photo-damage, permits good optical sectioning and 3D resolution¹¹² and non-invasive visualization of the extra-cellular matrix thanks to the second harmonic generation phenomenon^{113,114}. In the past years, intravital imaging has been mainly used to follow up individual or group of cells fluorescently labelled within the primary tumor and study the interaction of moving cells with their microenvironment

such as collagen matrix (Fig 6B)^{111,115}. Recently, a new technical development, the mammary imaging window (MIW) has been shown to be advantageous for studying cell movement and adhesion with high resolution. The use of photoconvertible fluorophores such as Kaede or Dendra2 allows a precise monitoring of cellular movement *in vivo* not anymore over hours but over days^{116,117}. Despite all this technological advances, it was still not possible to visualize adhesions in migrating cells *in vivo*. Just recently, for the first time, a study on E-cadherin dynamics in living animals has been reported¹¹⁸. Photobleaching and photoactivation was used to compare the mobility of cell adhesion and plasma membrane probes *in vitro* and in tumors grown in mice and consequently demonstrate critical differences in molecular dynamics *in vitro* and *in vivo*.

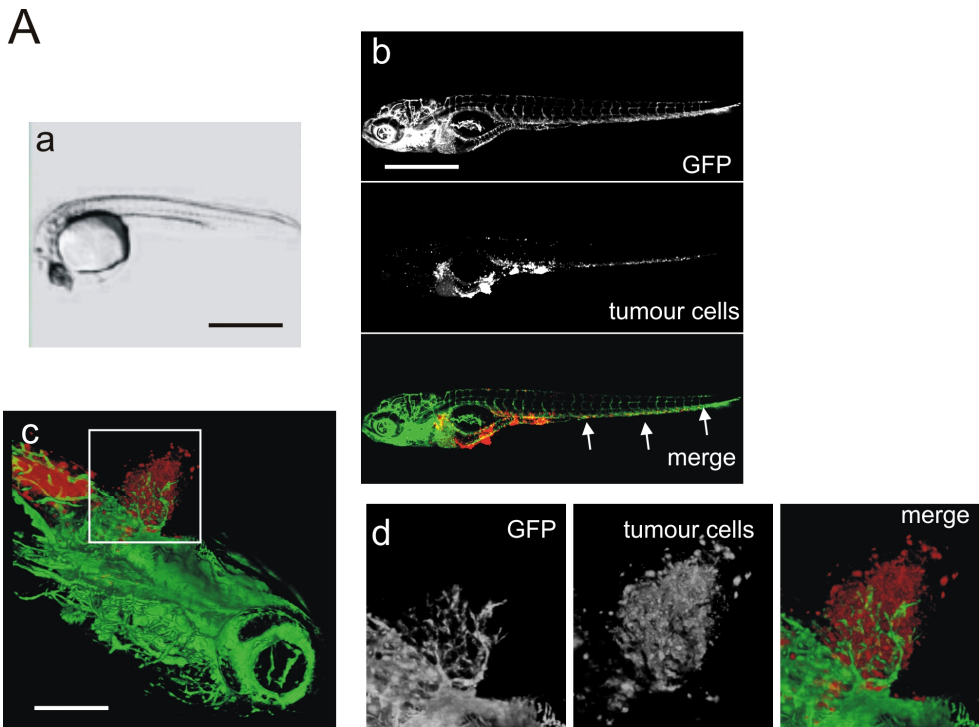


Figure 6A: Imaging tumor cell migration *in vivo*. Migration and cell mass formation of human tumor cells injected into the yolk sac of zebrafish embryos (Pictures obtained from V. Gothra, S. He, BE Snaar-Jagalska, and EHJ Danen). (a) phase-contrast overview picture of the yolk sac of zebrafish embryos.. (b) an example of spreading of human tumor cells (red) in transgenic zebrafish embryos expressing GFP under an endothelial promotor. Cells invaded, migrated and formed distant micrometastases, which are indicated with arrows.. (c) An example of angiogenesis formed through the tumor cell mass formed. (d) Higher magnification of the delineated region; green is GFP endothelial cells, red are tumor cells.

B

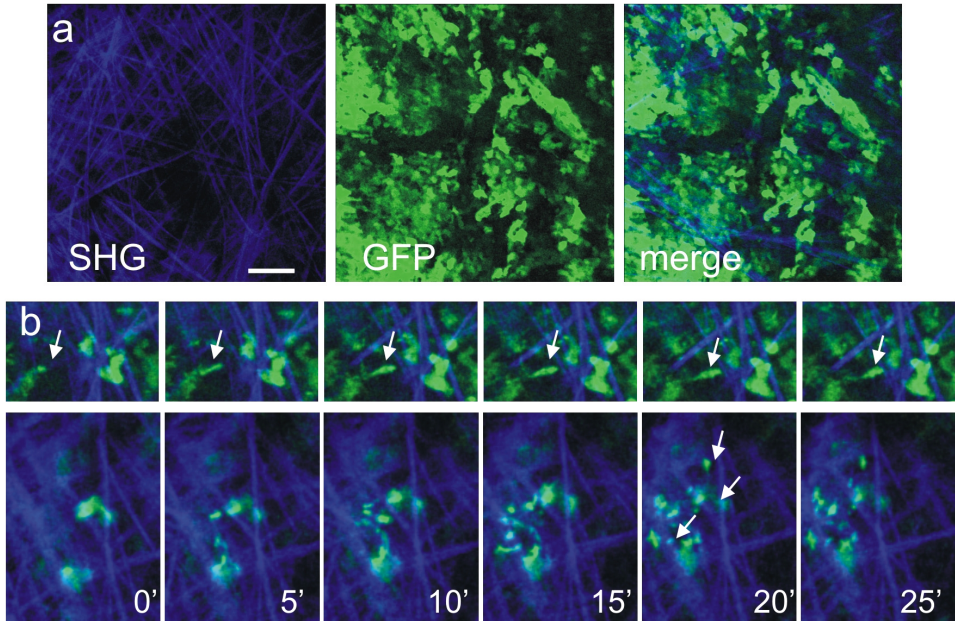


Figure 6B: Imaging tumor cell migration *in vivo*. Rat mammary carcinoma MTLn3 cells in orthotopic mammary tumors move show high motility *in vivo* with an amoeboid. (a) Multiphoton microscopy shows tumor mass (green) and extra cellular matrix visualized by second harmonic generation (blue). Scale bar is 100 μm . (b) Time-lapse images of MTLn3 carcinoma cells as they extend protrusions along ECM fibres (arrowheads). Images shown are at 5 min intervals.

5. Future directions and concluding remarks

5.1 FRAP/FRET, FRAP/TIRF and FRET/TIRF

An interesting alternative to the FRAP method is the combination of both FRAP and FRET that has already been applied in the nucleus^{78,119}. In the acceptor photobleaching FRET methodology, the bleaching of the acceptor results in an increase of the donor intensity. By applying FRAP on cells expressing adhesion proteins tagged with both FRET partners e.g. CFP and YFP, and simultaneously recording in the bleached region the acceptor recovery and the redistribution of the increased donor signal, it is possible to compare the mobility of the interacting proteins (donor redistribution) relative to the mobility of the total pool of proteins (the YFP redistribution as in conventional FRAP experiment). Of course it would be even more attractive to apply our previously described FLIP-FRAP methodology together with FRET to understand the complexity of both protein dynamics and interactions. Another very attractive combination of imaging technique is TIRF together with FRAP and/or FRET, technique that is currently experiencing rapid growth in application¹²⁰⁻¹²². This combination of imaging technologies will ensure an improved insight into adhesion protein dynamics and complexation in migrating cells since TIRF microscopy enhances enormously spatial resolution of fast moving matrix adhesions.

5.2 High through put techniques (2D and 3D) for target identification

To explore the mechanisms underlying the regulation of cell migration by matrix adhesion dynamics, we described various qualitative and quantitative approaches that study adhesion dynamics and cell migration in a 2D and 3D environment. Such dynamic studies are of particular relevance to understand cancer cell motility. To provide a systematic analysis of genes that regulate cell migration or to study effect of potential drugs on tumor cell migration, high throughput screening (HTS) is the most recent advance in imaging technology. The Geiger lab published two different screens which provide helpful methodologies and data on cell adhesion and migration: the first used high resolution microscopy to profile the effect of a library of natural extract on cell adhesion¹²³, the second used a modified phagokinetics tracks with MCF7 and identified novel pro-migratory, cancer associated genes¹²⁴. Very recently a third screen using high-throughput, high-resolution, microscopy based assay together with human kinases, phosphatases and adhesome libraries was performed as well and provide a model for the molecular hierarchy of FA formation¹²⁵. Another very elegant study used the traditional wound healing assay with MCF-10A breast epithelial and screened siRNAs targeting 1,081 human genes encoding phosphatases, kinases and proteins predicted to influence cell

migration and adhesion¹²⁶. Extensive validation of all the hits yielded 66 high confidence genes that, when downregulated, either accelerated or impaired migration; 42 of these high confidence genes were not previously associated with motility or adhesion¹²⁶. Although, the results of those screens are very promising and provide new data on cell migration the analysis was performed with fixed samples. Indeed, despite the recent described advances, a number of technical and analytical challenges remain to be solved to allow functional genomics together with dynamic imaging in a 2D and 3D environment. The major experimental challenge is the need to perform fast image acquisition and the need to obtain “high content” information about the migratory behaviour of many cells, including dynamic features such as migration velocity. Only fast microscope with high sensitive CCD camera and adapted bioinformatics can fulfill those requirements. In our lab, we are setting up a screen based on dynamic imaging of fast moving cells to obtain cell behavior measurements. Post-image acquisition we also fix the plate and stain for focal adhesion markers so that we can correlate changes in cell motility with altered focal adhesion morphometry (Fig. 7). Current speed of image acquisition is fast enough for scanning a 96 wells plate.

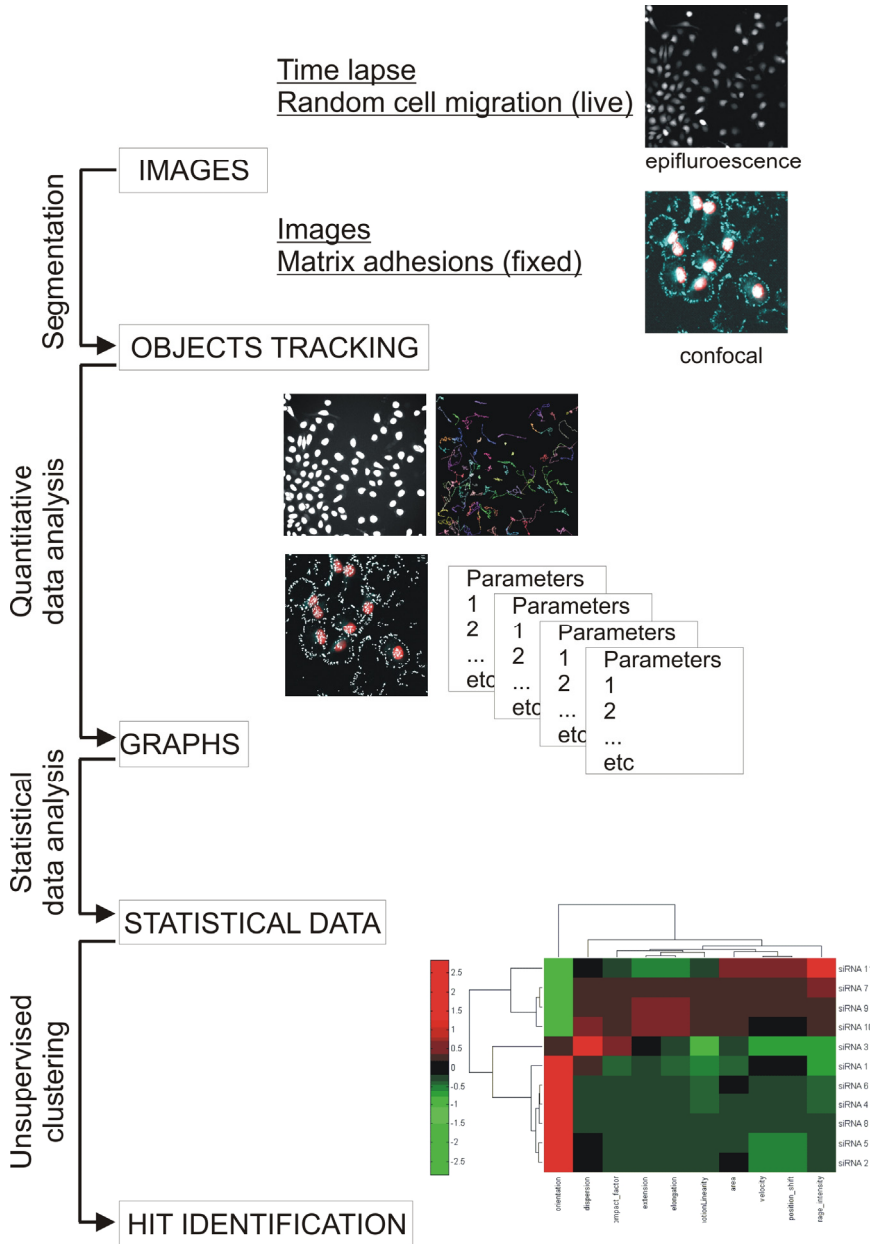


Figure 7: Image analysis of combined HTS screen (live and fixed) to understand the role of matrix adhesion in migrating cells. Image segmentation defines objects (individual cells or matrix adhesions) and allows cell tracking for the live cell migration. Object-by-object morphological and fluorescence intensity parameters are saved for every image. Statistical tests compare each parameter for all control wells with those in each treated well. Every parameters for each treated well are analysed with unsupervised clustering which help identify new hits (adapted from¹²³).

In this review, we presented a non-exhaustive overview of the different imaging techniques developed over the past years to study in a dynamic way cell adhesion and migration. In Table 1, we summarized the different techniques to study protein dynamics and interactions in matrix adhesions. The application of dynamic live cell imaging technology to both 2D migration, 3D invasion model of tumor cells and *in vivo* will enhance our understanding of tumor cell migration and consequently metastasis formation¹⁰³. In Table 2, an overview of the techniques used to model the various modes of tumor migration is given. Optimization of current techniques and systems that combine diverse techniques will improve both our spatial and temporal resolution of the role of matrix adhesions in migrating cells. Not only imaging techniques will improve but also more and more bioinformatics tools will be generated for fast and detailed image analysis and data processing. The additional knowledge obtained will hopefully provide insight into the molecular mechanisms behind tumor cell migration and help developing new anticancer therapies.

Acknowledgments

The authors would like to thank Leo Price and Hoa Truong for technical support for the 3D invasion assays, Veerander Ghotra for providing pictures of zebrafish embryos. This work was financially supported by grants from the Dutch Cancer Society (UL 2007-3860), the EU FP7 Health Program Metafight (Grant agreement no.201862) and ZF Cancer (Grant agreement no.201862), the Netherlands Organization for Scientific Research (902-21-229 and 911-02-022) and TI Pharma (T3-107).

TABLES

Technology	Biology	What's next	References
Photoactivation	Measure high resolution diffusion, trafficking and stability of protein <i>Actin dynamics, protein movement, integrin receptor trafficking</i>	For <i>in vivo</i> imaging, to track in the long term photoconverted cells and study protein dynamics.	48-50
FRAP or FLIP	Measure $t_{1/2}$, k_{on} , k_{off} , diffusion coefficient but individual measurements <i>Integrin, FAK, paxillin, zyxin, vinculin, and actin</i>	Combined FRAP and/or FLIP with TIRF and /or FRET	53-64
FRAP-FLIP	Measure $t_{1/2}$, k_{on} , k_{off} , diffusion coefficient and protein mobility parameters. Include all bleached and unbleached matrix adhesions. <i>FAK, paxillin, vinculin, zyxin and actin</i>	Combined FRAP-FLIP with TIRF and /or FRET	(Le Dévédec <i>et al.</i> ; submitted)
FCS, ICS, RICS	Determine rates of diffusion, degree of aggregation, number of fluorescent entities and flow velocities (mainly used in solution). <i>$\alpha 5$-integrin, α-actinin, FAK, paxillin and actin</i>	In living cells to study protein distribution, dynamics and interactions at high time and spatial resolution.	70-74
FSM	Movement of structure, assembly dynamics, and subunit turnover <i>Actin, integrin, FAK, talin and α-actinin</i>		65-69
FRET	Protein-protein interaction and protein activity <i>Src, FAK, Rho GTPases, and matrix adhesion proteins</i>	Combined with FRAP and/or TIRF and <i>in vivo</i>	76,80-85,88

Table 1: Imaging techniques to study protein dynamics and interactions in adhesion

Environment	Models	Microscopy	Obtained information	References
<i>In vitro/2D</i>	Matrix coating Patterned	Widefield, confocal	Insights into the organization of molecular machineries underlying cell adhesion and migration	19,38,101,110,127-131
<i>In vitro/3D</i>	Matrigel On/in collagen gel	Widefield, confocal, confocal reflection microscopy, SHG	Distinguish aspects of cell movement / invasion (collective/individual; mesenchymal/amoeboid)- Visualize interactions cell- ECM (in particular collagen fibers I).	20,91,92,94-96,98,99,102,103,113,127,132
<i>In vivo</i>	Zebrafish Mouse Rat	Confocal, confocal reflection, Multiphoton (SHG and FLIM)	Aspect of cell movement in the primary environment-visualize interaction between tumor cells and tumor environment (ECM, host cells and blood vessels)-visualize intravasation event when blood vessels are counterstained.	1,34,101,104,106-116,133-139

Table 2: Modeling distinct modes of tumor migration

REFERENCE LIST

1. Friedl, P.; Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol.* 10: 445-457, 2009.
2. Ridley, A. J.; Schwartz, M. A.; Burridge, K. et al. Cell migration: integrating signals from front to back. *Science.* 302: 1704-1709, 2003.
3. Linder, S. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol.* 17: 107-117, 2007.
4. Destaing, O.; Saltel, F.; Geminard, J. C.; Jurdic, P.; Bard, F. Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. *Mol Biol Cell.* 14: 407-416, 2003.
5. Yamaguchi, H.; Lorenz, M.; Kempf, S. et al. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *J Cell Biol.* 168: 441-452, 2005.
6. Yamaguchi, H.; Pixley, F.; Condeelis, J. Invadopodia and podosomes in tumor invasion. *Eur.J Cell Biol.* 85: 213-218, 2006.
7. Baldassarre, M.; Ayala, I.; Beznoussenko, G. et al. Actin dynamics at sites of extracellular matrix degradation. *Eur.J Cell Biol.* 85: 1217-1231, 2006.
8. Chan, K. T.; Cortesio, C. L.; Huttenlocher, A. FAK alters invadopodia and focal adhesion composition and dynamics to regulate breast cancer invasion. *J Cell Biol.* 185: 357-370, 2009.
9. Heath, J. P.; Dunn, G. A. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron-microscope study. *J Cell Sci.* 29: 197-212, 1978.
10. Izzard, C. S.; Lochner, L. R. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J Cell Sci.* 21: 129-159, 1976.
11. Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell.* 110: 673-687, 2002.
12. Geiger, B.; Bershadsky, A.; Pankov, R.; Yamada, K. M. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat Rev Mol Cell Biol.* 2: 793-805, 2001.
13. Zamir, E.; Geiger, B. Molecular complexity and dynamics of cell-matrix adhesions. *J.Cell Sci.* 114: 3583-3590, 2001.
14. Zaidel-Bar, R.; Itzkovitz, S.; Ma'ayan, A.; Iyengar, R.; Geiger, B. Functional atlas of the integrin adhesome. *Nat.Cell Biol.* 9: 858-867, 2007.
15. Huveneers, S.; Danen, E. H. Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci.* 122: 1059-1069, 2009.
16. Zamir, E.; Katz, B. Z.; Aota, S. et al. Molecular diversity of cell-matrix adhesions. *J.Cell Sci.* 112 (Pt 11): 1655-1669, 1999.
17. Zaidel-Bar, R.; Cohen, M.; Addadi, L.; Geiger, B. Hierarchical assembly of cell-matrix adhesion complexes. *Biochem.Soc.Trans.* 32: 416-420, 2004.
18. Zamir, E.; Geiger, B.; Kam, Z. Quantitative multicolor compositional imaging resolves molecular domains in cell-matrix adhesions. *PLoS.ONE.* 3: e1901-2008.
19. Geiger, B.; Spatz, J. P.; Bershadsky, A. D. Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol.* 10: 21-33, 2009.
20. Cukierman, E.; Pankov, R.; Stevens, D. R.; Yamada, K. M. Taking cell-matrix adhesions to the third dimension. *Science.* 294: 1708-1712, 2001.
21. Kirchner, J.; Kam, Z.; Tzur, G.; Bershadsky, A. D.; Geiger, B. Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption. *J.Cell Sci.* 116: 975-986, 2003.
22. Webb, D. J.; Parsons, J. T.; Horwitz, A. F. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat.Cell Biol.* 4: E97-100, 2002.
23. Small, J. V.; Kaverina, I. Microtubules meet substrate adhesions to arrange cell polarity. *Curr Opin Cell Biol.* 15: 40-47, 2003.
24. Larsen, M.; Tremblay, M. L.; Yamada, K. M. Phosphatases in cell-matrix adhesion and migration. *Nat Rev Mol Cell Biol.* 4: 700-711, 2003.

25. Schlaepfer, D. D.; Mitra, S. K.; Ilic, D. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim.Biophys Acta*. 1692: 77-102, 2004.
26. Tomar, A.; Schlaepfer, D. D. Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr Opin Cell Biol*. 2009.
27. Turner, C. E.; West, K. A.; Brown, M. C. Paxillin-ARF GAP signaling and the cytoskeleton. *Curr Opin Cell Biol*. 13: 593-599, 2001.
28. Golubovskaya, V. M.; Kweh, F. A.; Cance, W. G. Focal adhesion kinase and cancer. *Histol.Histopathol*. 24: 503-510, 2009.
29. Owens, L. V.; Xu, L.; Craven, R. J. et al. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Res*. 55: 2752-2755, 1995.
30. Zhao, J.; Guan, J. L. Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev*. 28: 35-49, 2009.
31. van Nimwegen, M. J.; van de, W. B. Focal adhesion kinase: a potential target in cancer therapy. *Biochem.Pharmacol*. 73: 597-609, 2007.
32. Yafeng M.; van de Water B.; Le Devedec, S. E. Focal Adhesion Kinase inhibition as a potential strategy for anticancer therapies. *Drugs of the Future*. 34: 477-484, 2009.
33. Vadlamudi, R.; Adam, L.; Tseng, B.; Costa, L.; Kumar, R. Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells. *Cancer Res*. 59: 2843-2846, 1999.
34. Chudakov, D. M.; Lukyanov, S.; Lukyanov, K. A. Fluorescent proteins as a toolkit for in vivo imaging. *Trends Biotechnol*. 23: 605-613, 2005.
35. Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. A guide to choosing fluorescent proteins. *Nat.Methods*. 2: 905-909, 2005.
36. Shaner, N. C.; Patterson, G. H.; Davidson, M. W. Advances in fluorescent protein technology. *J.Cell Sci*. 120: 4247-4260, 2007.
37. Wang, Y.; Shyy, J. Y.; Chien, S. Fluorescence proteins, live-cell imaging, and mechanobiology: seeing is believing. *Annu.Rev Biomed Eng*. 10: 1-38, 2008.
38. Dormann, D.; Weijer, C. J. Imaging of cell migration. *EMBO J*. 25: 3480-3493, 2006.
39. de Rooij, J.; Kerstens, A.; Danuser, G.; Schwartz, M. A.; Waterman-Storer, C. M. Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J.Cell Biol*. 171: 153-164, 2005.
40. Entschladen, F.; Drell, T. L.; Lang, K. et al. Analysis methods of human cell migration. *Exp.Cell Res*. 307: 418-426, 2005.
41. Webb, D. J.; Donais, K.; Whitmore, L. A. et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat.Cell Biol*. 6: 154-161, 2004.
42. Hoebe, R. A.; Van Oven, C. H.; Gadella, T. W., Jr. et al. Controlled light-exposure microscopy reduces photobleaching and phototoxicity in fluorescence live-cell imaging. *Nat Biotechnol*. 25: 249-253, 2007.
43. Axelrod, D.; Thompson, N. L.; Burghardt, T. P. Total internal inflection fluorescent microscopy. *J Microsc*. 129: 19-28, 1983.
44. Patterson, G. H.; Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science*. 297: 1873-1877, 2002.
45. Ando, R.; Hama, H.; Yamamoto-Hino, M.; Mizuno, H.; Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc Natl Acad Sci U S A*. 99: 12651-12656, 2002.
46. Gurskaya, N. G.; Verkhusha, V. V.; Shcheglov, A. S. et al. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat Biotechnol*. 24: 461-465, 2006.
47. Ando, R.; Mizuno, H.; Miyawaki, A. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science*. 306: 1370-1373, 2004.
48. Osborn, E. A.; Rabodzey, A.; Dewey, C. F., Jr.; Hartwig, J. H. Endothelial actin cytoskeleton remodeling during mechanostimulation with fluid shear stress. *Am J Physiol Cell Physiol*. 290: C444-C452, 2006.

49. Betzig, E.; Patterson, G. H.; Sougrat, R. et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. 313: 1642-1645, 2006.
50. Caswell, P. T.; Spence, H. J.; Parsons, M. et al. Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. *Dev Cell*. 13: 496-510, 2007.
51. Houtsmuller, A. B.; Vermeulen, W. Macromolecular dynamics in living cell nuclei revealed by fluorescence redistribution after photobleaching. *Histochem.Cell Biol*. 115: 13-21, 2001.
52. van Royen, M. E.; Farla, P.; Mattern, K. A. et al. Fluorescence Recovery After Photobleaching (FRAP) to Study Nuclear Protein Dynamics in Living Cells. *Methods Mol Biol*. 464: 363-385, 2009.
53. Ballestrem, C.; Hinz, B.; Imhof, B. A.; Wehrle-Haller, B. Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J.Cell Biol*. 155: 1319-1332, 2001.
54. Hamadi, A.; Bouali, M.; Dontenwill, M. et al. Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *J.Cell Sci*. 118: 4415-4425, 2005.
55. Cluzel, C.; Saltel, F.; Lussi, J. et al. The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. *J.Cell Biol*. 171: 383-392, 2005.
56. Geuijen, C. A.; Sonnenberg, A. Dynamics of the alpha6beta4 integrin in keratinocytes. *Mol.Biol.Cell*. 13: 3845-3858, 2002.
57. Lele, T. P.; Pendse, J.; Kumar, S. et al. Mechanical forces alter zyxin unbinding kinetics within focal adhesions of living cells. *J.Cell Physiol*. 207: 187-194, 2006.
58. Edlund, M.; Lotano, M. A.; Otey, C. A. Dynamics of alpha-actinin in focal adhesions and stress fibers visualized with alpha-actinin-green fluorescent protein. *Cell Motil.Cytoskeleton*. 48: 190-200, 2001.
59. von Wichert, G.; Haimovich, B.; Feng, G. S.; Sheetz, M. P. Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *EMBO J*. 22: 5023-5035, 2003.
60. Lele, T. P.; Thodeti, C. K.; Pendse, J.; Ingber, D. E. Investigating complexity of protein-protein interactions in focal adhesions. *Biochem.Biophys.Res.Commun*. 369: 929-934, 2008.
61. Wolfenson, H.; Lubelski, A.; Regev, T. et al. A role for the juxtamembrane cytoplasm in the molecular dynamics of focal adhesions. *PLoS.ONE*. 4: e4304-2009.
62. Fraley, T. S.; Pereira, C. B.; Tran, T. C.; Singleton, C.; Greenwood, J. A. Phosphoinositide binding regulates alpha-actinin dynamics: mechanism for modulating cytoskeletal remodeling. *J Biol Chem*. 280: 15479-15482, 2005.
63. Chandrasekar, I.; Stradal, T. E.; Holt, M. R. et al. Vinculin acts as a sensor in lipid regulation of adhesion-site turnover. *J Cell Sci*. 118: 1461-1472, 2005.
64. Goetz, J. G.; Joshi, B.; Lajoie, P. et al. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. *J Cell Biol*. 180: 1261-1275, 2008.
65. Waterman-Storer, C. M.; Desai, A.; Bulinski, J. C.; Salmon, E. D. Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells. *Curr Biol*. 8: 1227-1230, 1998.
66. Ponti, A.; Machacek, M.; Gupton, S. L.; Waterman-Storer, C. M.; Danuser, G. Two distinct actin networks drive the protrusion of migrating cells. *Science*. 305: 1782-1786, 2004.
67. Ponti, A.; Matov, A.; Adams, M. et al. Periodic patterns of actin turnover in lamellipodia and lamellae of migrating epithelial cells analyzed by quantitative Fluorescent Speckle Microscopy. *Biophys.J*. 89: 3456-3469, 2005.
68. Gupton, S. L.; Waterman-Storer, C. M. Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell*. 125: 1361-1374, 2006.
69. Hu, K.; Ji, L.; Applegate, K. T.; Danuser, G.; Waterman-Storer, C. M. Differential transmission of actin motion within focal adhesions. *Science*. 315: 111-115, 2007.
70. Wiseman, P. W.; Brown, C. M.; Webb, D. J. et al. Spatial mapping of integrin interactions and dynamics during cell migration by image correlation microscopy. *J.Cell Sci*. 117: 5521-5534, 2004.

71. Brown, C. M.; Dalal, R. B.; Hebert, B. et al. Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope. *J Microsc.* 229: 78-91, 2008.
72. Digman, M. A.; Brown, C. M.; Sengupta, P. et al. Measuring fast dynamics in solutions and cells with a laser scanning microscope. *Biophys.J.* 89: 1317-1327, 2005.
73. Digman, M. A.; Gratton, E. Analysis of diffusion and binding in cells using the RICS approach. *Microsc.Res.Tech.* 2008.
74. Brown, C. M.; Hebert, B.; Kolin, D. L. et al. Probing the integrin-actin linkage using high-resolution protein velocity mapping. *J Cell Sci.* 119: 5204-5214, 2006.
75. Digman, M. A.; Brown, C. M.; Horwitz, A. R.; Mantulin, W. W.; Gratton, E. Paxillin dynamics measured during adhesion assembly and disassembly by correlation spectroscopy. *Biophys J.* 94: 2819-2831, 2008.
76. Ballestrem, C.; Erez, N.; Kirchner, J. et al. Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. *J Cell Sci.* 119: 866-875, 2006.
77. Parsons, M.; Vojnovic, B.; Ameer-Beg, S. Imaging protein-protein interactions in cell motility using fluorescence resonance energy transfer (FRET). *Biochem Soc.Trans.* 32: 431-433, 2004.
78. van Royen, M. E.; Dinant, C.; Farla, P.; Trapman, J.; Houtsmuller, A. B. FRAP and FRET methods to study nuclear receptors in living cells. *Methods Mol Biol.* 505: 69-96, 2009.
79. van Rheenen, J.; Langeslag, M.; Jalink, K. Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. *Biophys J.* 86: 2517-2529, 2004.
80. Cai, X.; Lietha, D.; Ceccarelli, D. F. et al. Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Mol Cell Biol.* 28: 201-214, 2008.
81. Wang, Y.; Chien, S. Analysis of integrin signaling by fluorescence resonance energy transfer. *Methods Enzymol.* 426: 177-201, 2007.
82. Itoh, R. E.; Kurokawa, K.; Ohba, Y. et al. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol.* 22: 6582-6591, 2002.
83. Nakamura, T.; Kurokawa, K.; Kiyokawa, E.; Matsuda, M. Analysis of the spatiotemporal activation of rho GTPases using Raichu probes. *Methods Enzymol.* 406: 315-332, 2006.
84. Pertz, O.; Hahn, K. M. Designing biosensors for Rho family proteins--deciphering the dynamics of Rho family GTPase activation in living cells. *J Cell Sci.* 117: 1313-1318, 2004.
85. Pertz, O.; Hodgson, L.; Klemke, R. L.; Hahn, K. M. Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature.* 440: 1069-1072, 2006.
86. Parsons, M.; Messent, A. J.; Humphries, J. D.; Deakin, N. O.; Humphries, M. J. Quantification of integrin receptor agonism by fluorescence lifetime imaging. *J Cell Sci.* 121: 265-271, 2008.
87. Bastiaens, P. I.; Squire, A. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. *Trends Cell Biol.* 9: 48-52, 1999.
88. Fan, Y.; Gong, Y.; Ghosh, P. K.; Graham, L. M.; Fox, P. L. Spatial coordination of actin polymerization and ILK-Akt2 activity during endothelial cell migration. *Dev Cell.* 16: 661-674, 2009.
89. Romer, L. H.; Birukov, K. G.; Garcia, J. G. Focal adhesions: paradigm for a signaling nexus. *Circ.Res.* 98: 606-616, 2006.
90. Alderliesten, M.; de Graauw, M.; Oldenampsen, J. et al. Extracellular signal-regulated kinase activation during renal ischemia/reperfusion mediates focal adhesion dissolution and renal injury. *Am J Pathol.* 171: 452-462, 2007.
91. Wolf, K.; Alexander, S.; Schacht, V. et al. Collagen-based cell migration models in vitro and in vivo. *Semin.Cell Dev Biol.* 2009.
92. Sahai, E.; Marshall, C. J. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol.* 5: 711-719, 2003.

93. Yates, C.; Shepard, C. R.; Papworth, G. et al. Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. *Adv.Cancer Res.* 97: 225-246, 2007.
94. Kenny, H. A.; Krausz, T.; Yamada, S. D.; Lengyel, E. Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extra-cellular matrices on adhesion and invasion of ovarian cancer cells to the omentum. *Int J Cancer.* 121: 1463-1472, 2007.
95. Abhyankar, V. V.; Toepke, M. W.; Cortesio, C. L. et al. A platform for assessing chemotactic migration within a spatiotemporally defined 3D microenvironment. *Lab Chip.* 8: 1507-1515, 2008.
96. Benton, G.; George, J.; Kleinman, H. K.; Arnaoutova, I. P. Advancing science and technology via 3D culture on basement membrane matrix. *J Cell Physiol.* 2009.
97. Zaman, M. H.; Trapani, L. M.; Siemeski, A. et al. Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *Proc.Natl.Acad.Sci.U.S.A.* 103: 10889-10894, 2006.
98. Hooper, S.; Marshall, J. F.; Sahai, E. Tumor cell migration in three dimensions. *Methods Enzymol.* 406: 625-643, 2006.
99. Gobel, W.; Kampa, B. M.; Helmchen, F. Imaging cellular network dynamics in three dimensions using fast 3D laser scanning. *Nat Methods.* 4: 73-79, 2007.
100. Wolf, K.; Friedl, P. Functional imaging of pericellular proteolysis in cancer cell invasion. *Biochimie.* 87: 315-320, 2005.
101. Friedl, P. Dynamic imaging of cellular interactions with extracellular matrix. *Histochem.Cell Biol.* 122: 183-190, 2004.
102. Nystrom, M. L.; Thomas, G. J.; Stone, M. et al. Development of a quantitative method to analyse tumour cell invasion in organotypic culture. *J Pathol.* 205: 468-475, 2005.
103. Carragher, N. O. Profiling distinct mechanisms of tumour invasion for drug discovery: imaging adhesion, signalling and matrix turnover. *Clin Exp.Metastasis.* 26: 381-397, 2009.
104. Goessling, W.; North, T. E.; Zon, L. I. New waves of discovery: modeling cancer in zebrafish. *J Clin Oncol.* 25: 2473-2479, 2007.
105. Langenau, D. M.; Traver, D.; Ferrando, A. A. et al. Myc-induced T cell leukemia in transgenic zebrafish. *Science.* 299: 887-890, 2003.
106. Stoletov, K.; Montel, V.; Lester, R. D.; Gonias, S. L.; Klemke, R. High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. *Proc Natl Acad Sci U S A.* 104: 17406-17411, 2007.
107. Haldi, M.; Ton, C.; Seng, W. L.; McGrath, P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis.* 9: 139-151, 2006.
108. Nicoli, S.; Presta, M. The zebrafish/tumor xenograft angiogenesis assay. *Nat Protoc.* 2: 2918-2923, 2007.
109. Marques, I. J.; Weiss, F. U.; Vlecken, D. H. et al. Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC.Cancer.* 9: 128-2009.
110. Sahai, E. Illuminating the metastatic process. *Nat.Rev.Cancer.* 7: 737-749, 2007.
111. Condeelis, J.; Segall, J. E. Intravital imaging of cell movement in tumours. *Nat.Rev.Cancer.* 3: 921-930, 2003.
112. Zipfel, W. R.; Williams, R. M.; Webb, W. W. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol.* 21: 1369-1377, 2003.
113. Campagnola, P. J.; Loew, L. M. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. *Nat.Biotechnol.* 21: 1356-1360, 2003.
114. Kedrin, D.; Wyckoff, J.; Sahai, E.; Condeelis, J.; Segall, J. E. Imaging tumor cell movement in vivo. *Curr.Protoc.Cell Biol.* Chapter 19: Unit-2007.
115. Kedrin, D.; van Rheenen, J.; Hernandez, L.; Condeelis, J.; Segall, J. E. Cell Motility and Cytoskeletal Regulation in Invasion and Metastasis. *J.Mammary.Gland.Biol.Neoplasia.* 2007.
116. Kedrin, D.; Gligorijevic, B.; Wyckoff, J. et al. Intravital imaging of metastatic behavior through a mammary imaging window. *Nat.Methods.* 2008.

117. Gligorijevic, B.; Kedrin, D.; Segall, J. E.; Condeelis, J.; van Rheenen, J. Dendra2 photoswitching through the Mammary Imaging Window. *J Vis.Exp.* 2009.
118. Serrels, A.; Timpson, P.; Canel, M. et al. Real-time study of E-cadherin and membrane dynamics in living animals: implications for disease modeling and drug development. *Cancer Res.* 69: 2714-2719, 2009.
119. van Royen, M. E.; Cunha, S. M.; Brink, M. C. et al. Compartmentalization of androgen receptor protein-protein interactions in living cells. *J Cell Biol.* 177: 63-72, 2007.
120. Bal, M.; Zaika, O.; Martin, P.; Shapiro, M. S. Calmodulin binding to M-type K⁺ channels assayed by TIRF/FRET in living cells. *J Physiol.* 586: 2307-2320, 2008.
121. Wang, L.; Bittner, M. A.; Axelrod, D.; Holz, R. W. The structural and functional implications of linked SNARE motifs in SNAP25. *Mol Biol Cell.* 19: 3944-3955, 2008.
122. Raveh, A.; Riven, I.; Reuveny, E. The use of FRET microscopy to elucidate steady state channel conformational rearrangements and G protein interaction with the GIRK channels. *Methods Mol Biol.* 491: 199-212, 2008.
123. Paran, Y.; Ilan, M.; Kashman, Y. et al. High-throughput screening of cellular features using high-resolution light-microscopy; application for profiling drug effects on cell adhesion. *J Struct.Biol.* 158: 233-243, 2007.
124. Naffar-Abu-Amara, S.; Shay, T.; Galun, M. et al. Identification of novel pro-migratory, cancer-associated genes using quantitative, microscopy-based screening. *PLoS.ONE.* 3: e1457-2008.
125. Winograd-Katz, S. E.; Itzkovitz, S.; Kam, Z.; Geiger, B. Multiparametric analysis of focal adhesion formation by RNAi-mediated gene knockdown. *J Cell Biol.* 186: 423-436, 2009.
126. Simpson, K. J.; Selfors, L. M.; Bui, J. et al. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat Cell Biol.* 10: 1027-1038, 2008.
127. Bershadsky, A. D.; Ballestrem, C.; Carramusa, L. et al. Assembly and mechanosensory function of focal adhesions: experiments and models. *Eur.J.Cell Biol.* 2005.
128. Lauffenburger, D. A.; Horwitz, A. F. Cell migration: a physically integrated molecular process. *Cell.* 84: 359-369, 1996.
129. Roy, P.; Rajfur, Z.; Pomorski, P.; Jacobson, K. Microscope-based techniques to study cell adhesion and migration. *Nat.Cell Biol.* 4: E91-E96, 2002.
130. Webb, D. J.; Brown, C. M.; Horwitz, A. F. Illuminating adhesion complexes in migrating cells: moving toward a bright future. *Curr.Opin.Cell Biol.* 15: 614-620, 2003.
131. Wouters, F. S.; Verveer, P. J.; Bastiaens, P. I. Imaging biochemistry inside cells. *Trends Cell Biol.* 11: 203-211, 2001.
132. Sahai, E. Mechanisms of cancer cell invasion. *Curr.Opin.Genet.Dev.* 15: 87-96, 2005.
133. Peter, M.; Ameer-Beg, S. M. Imaging molecular interactions by multiphoton FLIM. *Biol.Cell.* 96: 231-236, 2004.
134. Condeelis, J. S.; Wyckoff, J.; Segall, J. E. Imaging of cancer invasion and metastasis using green fluorescent protein. *Eur.J.Cancer.* 36: 1671-1680, 2000.
135. Friedl, P.; Wolf, K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat.Rev.Cancer.* 3: 362-374, 2003.
136. Hoffman, R. M.; Yang, M. Subcellular imaging in the live mouse. *Nat.Protoc.* 1: 775-782, 2006.
137. Pinner, S.; Sahai, E. Imaging amoeboid cancer cell motility in vivo. *J Microsc.* 231: 441-445, 2008.
138. Hoffman, R. M. Imaging cancer dynamics in vivo at the tumor and cellular level with fluorescent proteins. *Clin Exp.Metastasis.* 26: 345-355, 2009.
139. Provenzano, P. P.; Eliceiri, K. W.; Keely, P. J. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin Exp.Metastasis.* 26: 357-370, 2009.