CHAPTER TWO

INVESTIGATING INTEGRIN REGULATION AND SIGNALING EVENTS IN THREE-DIMENSIONAL SYSTEMS

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Abstract
There has been much recent interest in working with cells cultured in three-dimensional (3D) matrices to better model the properties of the extracellular matrix environment found in vivo. However, working within 3D matrices adds several difficulties to experiments that have become routine in two-dimensional (2D) culture systems. Biochemical
approaches are made difficult by the presence of milligram quantities of matrix protein, while cell biology approaches are more difficult to assess and image. Moreover, 3D imaging adds complexity to fluorescence studies, including the inherent challenge of a 3D volume as opposed to a 2D image, increased depths of field, and problems of light scatter. The purpose of this chapter is to provide a few overall strategies for working within 3D culture systems, focusing on biochemical and molecular imaging approaches.

1. Introduction

Integrin localization and function has been historically studied on rigid two-dimensional surfaces coated with various extracellular matrix (ECM) proteins. Early identification of integrins as key architects of focal adhesions relied on their immunolocalization within cells cultured in this manner (Burridge et al., 1992). Indeed, the rapid advances in the field of integrin signaling, imaging of molecular events underlying cell migration, and understanding of focal adhesions as signaling scaffolds would not have been possible without these two dimensional culture systems.

Despite the value of classic culture approaches, it is becoming increasingly apparent that the response of cells cultured in three-dimensional (3D) matrices is not completely consistent with responses on coated 2D surfaces (Weaver et al., 2002; Wozniak et al., 2003). Cellular responses in 3D systems are driven in no small part by contractility-based signaling linked to the Rho small GTPase (Wozniak et al., 2003). Given the extreme rigidity of a 2D surface, regulation by contractile events is likely to be quite different in 3D systems versus 2D. Indeed, it appears that the “compliance” of the 3D matrix is a key aspect of cellular response and signaling regulation (Paszek et al., 2005; Wozniak et al., 2003). Moreover, while cells make adhesive structures in 3D matrices, it is clear that these structures are smaller and differ from their 2D counterparts, and thus have been termed “matrix adhesions” rather than “focal adhesions” (Cukierman et al., 2001).

Working within 3D matrices adds several difficulties to experiments that have become routine in 2D culture systems. Biochemical approaches are made difficult by the presence of milligram quantities of matrix protein, which get in the way of cellular proteins during routine analysis. Cell biology is more difficult to assess and image. Moreover, 3D imaging adds complexity to fluorescence studies, including the inherent challenge of a 3D volume as opposed to a 2D image, increased depths of field, and problems of light scatter. The purpose of this chapter is to provide a few overall strategies for working within 3D culture systems, as well as some specific approaches in use in our laboratory. This is not to say that 2D systems are not important complements to the 3D approach, and indeed we find that some of our studies are still best accomplished under 2D culture systems (see section 6). In this chapter, we will address the issues of performing biochemistry, cell biology studies, and imaging within the context of 3D collagen matrices. Included are some basic nonlinear optical approaches for imaging within 3D matrices using multiphoton microscopy.
1.1. Types of 3D matrices

A few different types of 3D extracellular matrix culture systems have been developed to great advantage. Commonly used are 3D gels composed of rBM or matrigel, a purification of the basement membrane components made in abundance by the EHS tumor and containing laminin, collagen IV, proteoglycans, and several other more minor components (Kleinman et al., 1982). Matrigel or rBM is commercially available, or can be produced in one’s own lab, but with particular resources needed to generate and harvest tumors and purify the basement membrane fraction. While rBM has several applications for the study of epithelial structure (e.g., see Barcellos-Hoff et al., 1989; Muthuswamy et al., 2001), gelling properties vary tremendously lot to lot, and the commercial supply is sometimes inconsistent. Moreover, for our purposes, we find that these gels are too compliant for addressing our particular questions related to mechanical signal transduction, and do not seem to adequately mimic certain *in vivo* tissue environments.

Gels composed of Type I collagen, derived from rat tails, are a less expensive and more consistent source of 3D matrix. Collagen gels can be cast in concentrations ranging from ~0.7 mg/ml up to 5 mg/ml (if one has a sufficiently concentrated source of collagen I), which makes them readily customized to the specific structural needs of each individual cell type and the scientific question at hand (see “Collagen Gels of Varying Densities,” below). Thus, collagen gels can be created that cross a range of elastic moduli appropriate to mimic the physical properties of various tissue environments.

The disadvantage of collagen gels is that they do not specifically reproduce the biochemical components of the basement membrane in the way that rBM/matrigel does. However, we have found that after a period of time (≥7 days, usually), “normal” mammary epithelial cells will deposit a thin basement membrane, and thus provide a decent mimic of the 0.2-μm thick basement membrane found *in vivo*. Alternatives are to make mixed gels of matrigel/collagen I, or to “precoat” clumps of cells by incubating them for 10 min in ungelled matrigel, prior to casting them into collagen I gels. It should be noted, however, that mixing matrigel with collagen gels will alter the physical properties of the resultant gel.

An alternative approach to such “exogenous” matrices is to allow cells to deposit their own 3D matrix. The most commonly used are fibroblasts, already specialized for making and assembling 3D matrices. Once a matrix is created, the cells are extracted and the resulting cell-derived matrix used in subsequent experiments. This approach will not be specifically discussed further here, but the reader is encouraged to see Cukierman et al. (2001) for more information.

1.2. Challenges of working in 3D matrices

Several challenges must be overcome to obtain consistent and satisfying results from experiments performed with cells cultured in 3D matrices. Standard biochemical experiments, such as immunoprecipitations, pull-down assays, and molecular signaling events (see sections 4 and 5) can be complicated by the presence of milligram quantities
of ECM protein in the lysate. Following are examples of issues that we have encountered:

- **2D versus 3D.** In comparisons of samples from cells cultured in 2D versus a 3D matrix, the presence of contaminant ECM protein is so abundant in the 3D sample that this sample will run aberrantly on SDS-PAGE compared to the 2D sample. To solve this, we create an equal-volume “dummy” gel/matrix that is added to the 2D sample upon lysis.

- **Releasing cells from 3D gels with collagenase (or other proteases).** It is possible to recover cells cultured in 3D collagen gels by treating the gels with collagenase for about 15 to 30 min, according to the specific activity of the collagenase purchased and the concentration of the gel. This approach is acceptable for a subset of experiments, such as cellular growth curves, in which disrupting the cell–matrix interactions will not alter the outcome of the experiments. However, this approach is not appropriate for signaling studies, gene expression studies, or even protein expression, as 15 to 30 min in a protease is enough time for the cell to completely change the response being investigated. Thus, we lyse cells in residence within the 3D collagen gel.

- **Protein assays are not recommended.** Unfortunately, there is far too much contaminating ECM protein, present in milligram quantities, to ever get an accurate read by any standard protein assay.

- **Differences in cell growth rates.** When comparing different conditions in 3D over several days prior to cell lysis, a complicating factor is that sometimes the growth rates differ (Wozniak et al., 2003), such that one is not comparing equal cell numbers by the time the experiment is performed. Because it is not possible to determine equal protein levels in the samples, alternative approaches must be used. One solution is to culture a parallel set of gels from which cells will be released by collagenase treatment and counted, or that will be stained with DAPI and cell number counted via nuclei. This allows adjustment of sample volumes either at lysis or when loading SDS-PAGE gels.

- **Key to all experiments is inclusion of loading controls, such as parallel blot for GAPDH.** We do not favor actin as a reference protein, as we find changes in actin levels across cells cultured in 2D versus 3D.

- **RNA isolation remarkably easier than protein work in 3D matrices.** We find that using standard RNA reagents, such as Trizol (Invitrogen) and following the manufacturer’s suggestion for isolation of RNA from tissue, rather than from cell culture, works quite well.

Cell biology and imaging approaches are also more difficult in 3D matrices, but there are approaches to work around these difficulties.

- Phase–contrast images of cells in 3D matrices complicated by lack of single focal plane, making satisfactory images difficult. This is particularly true when working with floating gels, which do not stay still long enough to acquire an adequate image. To assist in this, we find that removing the liquid from the culture, and allowing the 3D gel to “sit” on the bottom of the culture dish aids in obtaining better phase–contrast images.
Immunostaining. Satisfying results can be obtained immunostaining within 3D matrices, without the need to section (Wozniak et al., 2003). We have previously developed and published protocols for this (Wozniak and Keely, 2005). The key point is that the gels must be handled delicately, and washed much more extensively, than when working on 2D surfaces.

Fluorescence microscopy. There are inherent issues imaging a cell that has significant 3D volume compared to one that is relatively two-dimensional. Imaging of immunofluorescent staining, or GFP-labeled proteins, by standard epifluorescence is vastly improved by the use of 3D deconvolution software or the use of a confocal microscope. We find identical results whether we use confocal microscopy or standard epifluorescence combined with 3D deconvolution analysis; the latter approach is used, as it is resident within our laboratory.

Multiphoton laser scanning microscopy (MPLSM). In addition to the inherent challenge of a 3D volume as opposed to a 2D image, low signals and light scatter create challenges when imaging 3D samples. The use of MPLSM mitigates much of the difficulties of working within 3D samples, and also has the added benefit of allowing the simultaneous imaging of unstained collagen matrices (see “Imaging Cell–Matrix Interactions in 3D Collagen Gels” below).

2. Collagen Matrices of Different Densities

For the purposes of our particular set of studies, in which we are investigating the effects of collagen density on cell behavior, we often compare cells cultured in collagen gels of varying densities. Gels composed of rat tail collagen can be poured in concentrations from 0.7 mg/ml to several milligrams per milliliter. In practical terms, we have not exceeded 5 mg/ml of collagen, as it is necessary to have a concentrated stock from which to make these gels. It is our experience that the physical properties, or elastic modulus, of these gels range in a predictable way that matches published data (Roeder et al., 2002). The ability to cast gels in a wide range of concentrations, and thus achieve a range of elastic moduli, is one of the advantages of working with gels composed of Type I collagen.

We find that different cells vary in their contractile behavior, and thus the optimum concentration of collagen for a desired cellular behavior varies as well, and may need to be determined empirically. For example, T47D breast carcinoma cells, which are not very contractile, undergo tubulogenesis at an optimum collagen concentration of 1.0 to 1.3 mg/ml. In contrast, this concentration is a bit too compliant for the more contractile normal murine mammary cell line, NMuMG, which undergoes optimum tubulogenesis ~3.0 mg/ml. Fibroblasts are generally more contractile as well, and prefer gels of higher collagen concentrations.

As collagen is acid soluble, sterile solutions are usually commercially available in dilute acid (i.e., BD Biosciences, San Jose, CA provides type I collagen in 0.03 N acetic acid at concentrations of 3 to 5 mg/ml or 8 to 11 mg/ml). Collagen will gel spontaneously upon neutralization at 37°C. To accomplish this, we neutralize collagen by the addition of an equal volume of 2× PBS/100 mM HEPES at pH 7.3.
To make more concentrated gels, one-half volume of 4× PBS/200 mM HEPES can be used. Gels can be cast into tissue culture dishes of various sizes, but typically we use six-well plates and cast a total volume of 1 ml per well. Thus, to cast a 2-μg/ml collagen gel from a 5 mg/ml stock solution, one would use 0.4 ml of collagen, 0.4 ml 2× PBS/100 mM HEPES, and the needed number of cells in 0.2 ml of medium. For tubulogenesis assays with mammary epithelial cells, we generally cast 100,000 to 125,000 cells/ml of gel. Depending on the specific experiment, cell concentrations up to 10 × 10⁶ cells/ml have been used.

When casting collagen gels, it is important to keep the components cold or to work quickly, to mix thoroughly being careful not to introduce air bubbles, and to add the cells last to the mixture. The viscous solution of neutralized collagen plus cells is immediately pipetted into individual wells, and the tissue culture dish placed in a 37°C incubator for 60 min to overnight to allow the gel to polymerize. For floating gels, the polymerized gels are gently released by edging the well with a Pasteur pipette, adding culture medium (2 ml for a 1-ml gel), and gently shaking the gels to release them from the sides and bottom of the dish. For more detail, an excellent protocol for pouring collagen gels has already been published (Wozniak and Keely, 2005).

3. Gel Contraction as a Measure to Quantify Cell Contractility

Because much of the response of epithelial cells to 3D matrices, and the ability of the cells to sense the density of the matrix appears to involve signaling through Rho-mediated contractile events (Wozniak et al., 2003), measurements of collagen gel contraction is a useful approach to assess the response of cells to a particular matrix. Moreover, collagen gel contraction allows the assessment of the contractility of a population of cells relative to another set of cells, or relative to a different set of culture conditions. Collagen gels are cast in a six-well plate as described above for morphogenesis assays (e.g., 100,000 T47D cells/1 ml of collagen gel). Denser gels will be contracted to a lesser extent, and this should be taken into consideration when deciding the purpose of the experiment. Gels are polymerized overnight at 37°C, and released the next day. The day at which the gels are rendered floating is considered Day 0. For example, if the gels are poured in a six-well plate, the diameter of a gel at Day 0 would be 35 mm. The gel diameter should be measured every 24 h for up to 10 days. Generally, the culture medium is changed every 4th day. Cells that are more highly contractile should be followed for shorter intervals. Measurements may be taken using a small ruler or using imaging software on gel images acquired from a dissecting microscope. In general, T47D breast epithelial cells will contract a 1.3-μg/ml collagen gel ~7.5 mm in 8 days, while the more contractile MCF10A cell line will contract a similar gel ~10 mm (Wozniak et al., 2003).
4. Rho Activity Assay from Cells Cultured in 3D Collagen Gels

Recently we have been able to optimize the G-LISA RhoA activation assay (luminescence based) from Cytoskeleton, Inc. (Denver, CO) to analyze RhoA activity using breast epithelial cells cultured within 3D collagen gels. In the past the only way to analyze the activity of RhoGTPase was to perform a very labor-intensive Rho pull-down assay. While a very reproducible Rho pull-down protocol was developed in our lab to assay breast epithelial cells in 3D collagen gels (Wozniak and Keely, 2005), the assay had several limitations. In order to produce a detectable signal, the pull-down assay required the use of approximately 10 to 20 million cells per 1 ml of gel. A concern with this approach was the effects on cellular signaling due to a cell density far greater than that normally used for these 3D collagen-tubulogenesis assays. Due to the high cell number needed for this assay, analysis was restricted to time points that could be completed within a few hours and could not be carried out to 10 days (the time needed for tubulogenesis to occur). Using the G-LISA RhoA assay, not only can significantly lower cell numbers be used for short-term experiments (i.e., 1 hr), but importantly the cells can be cultured in 3D matrices for 10 or more days and Rho activation can be measured at the same time point that tubulogenesis is assessed.

4.1. Cell culture

The example given here uses T47D breast carcinoma cells, but can be adapted for several other types of cells. For the 1-h protocol, cells are harvested in 0.5 mM EDTA in PBS, resuspended in RPMI media supplemented with 5 mg/ml of fatty acid–free BSA, and just 2 million cells per 1 ml of gel (cultured in a six-well plate) are used for each condition to be analyzed. The collagen gels are allowed to polymerize for 1 h at 37°C after which 1 ml of RPMI/BSA is added, and the gels rendered floating by gently displacing their attachment to the dish, or left attached. Following 1 h of additional incubation at 37°C the gels are ready to be assayed.

To measure RhoA activity after 10 days of culture in 3D collagen gels, cells are cast into 3D collagen gels using the same number of cells that we typically use for tubulogenesis endpoints: 100,000 cells per 1 ml of gel. Using full growth media (RPMI with 10% heat-inactivated FBS and 8 mg/ml of insulin), 1-ml gels are poured and allowed to polymerize for 8 to 16 h prior to rendering them floating or leaving them attached in 2 ml of full media. After 10 days in culture, replacing 1 ml of full media every 4 days, the gels are ready to be assayed.

4.2. G-LISA assay

The protocol provided in the G-LISA RhoA activity assay (Cytoskeleton, Inc., Denver, CO) is generally followed with a few modifications. Briefly, the gel is carefully removed from the well with forceps and quickly rinsed in approximately
2 ml of ice cold PBS. Residual PBS is removed by carefully blotting the edge of the gel on a Kim wipe, and then the gel is promptly lysed on ice in a microfuge tube containing 400 µl of the provided lysis buffer, ice cold with protease inhibitors. The gels are quickly sheared through an 18-gauge needle and then the sample is spun at 14,000 rpm for 2 min. Remove 80 µl of supernatant and mix with 80 µl of binding buffer to load 75 µl per well of a G-LISA plate in duplicate (transfer the remainder of the supernatant to a fresh tube). The negative control is also made up so that 75 µl can be loaded per well; however, the positive control is added as recommended in the kit protocol (50 µl per well) due to the fact that increasing the volume can result in values outside the detectable range. The remainder of the assay is preformed as described in the protocol provided with the kit. Briefly, the plate is incubated at 4°C, shaking at a minimum of 200 rpm, for exactly 30 min. After washing the wells, anti-RhoA primary is added and the plate is incubated for 45 min while shaking at room temperature. Again the wells are washed and then the secondary antibody is added for an additional 45-min incubation followed by the addition of HRP detection reagent. The plate is then read on a luminometer.

Although the cell number is counted when loading the gels, we find that the reproducibility of the assay is optimal when relative RhoA levels are quantitated. For quantitation of relative RhoA activity, the total Rho level in each sample also needs to be determined. Total Rho can be measured by loading a constant volume of supernatant (30 µl of supernatant mixed with 10 µl of 3 x Laemmli sample buffer) on a 6% SDS page gel followed by Western blot using anti-Rho antibody (Transduction Laboratories). Densitometry is performed and active RhoA (luminescence values)/total Rho (densitometry) values are calculated. The results can then be expressed as relative RhoA activity.

5. CO-IMMUNOPRECIPITATION OF INTEGRIN-ASSOCIATED PROTEINS FROM CELLS CULTURED IN 3D COLLAGEN GELS

Co-immunoprecipitation has emerged as a useful approach to elucidate signaling complexes, and to understand the molecular interactions among signaling molecules in cells. A few modifications to classic immunoprecipitations are necessary to obtain satisfactory results from 3D matrix cultures. The example given here allows for the analysis of integrin-associated signaling molecules, or other early signaling events associated with changes in the physical properties (density) of the ECM in breast epithelial cells.

For both T47D and NMuMG cells, approximately 10 million cells are required for each co-immunoprecipitation sample, and cast into a 1-ml gel. Cells are mixed with the appropriate concentration of collagen I and then allowed to polymerize for 1 h. After 1 h, gels may either be released from the culture dish (floating) or maintained in the attached state. After 1 h more, cells are lysed using 1 ml of 2 x lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 100 mM NaF, 0.2% BSA, 2% Triton X-100, 1 mM sodium pervanadate, and protease inhibitors). It is important to break up the collagen gel by carefully triturating cells with a pipettor until the gel is a
suspension uniform in consistency. To ensure adequate lysis, the lysate is incubated for 15 min at 4°C on a rotating wheel, followed by centrifugation for 10 min at 4°C to clear the lysate, which is transferred to new tube. For immunoprecipitations, add 3 to 4 μg of antibody plus 30 μl GammaBind G-sepharose (Amersham Biosciences) and incubate lysate for 2 h to overnight at 4°C on a rotating wheel. The length of incubation depends on the antibody used for the immunoprecipitation and the proteins that are being co-immunoprecipitated.

After immunoprecipitation, briefly centrifuge the lysate at 4°C at low speed in a microfuge to pellet the sepharose beads, discard the supernatant, and wash the beads with 1× lysis buffer, resuspending and centrifuging after each wash, for a total of three washes. Remove excess lysis buffer using a syringe attached to a 27-gauge needle, leaving damp beads without any remaining supernatant. Immunoprecipitated proteins are released by adding 30 μl of 1× Laemmli sample buffer to the rinsed beads and heating the samples for 3 min at 95°C. The samples can then be analyzed using standard SDS-PAGE and immunoblotting approaches (for an example, see Fig. 2.1).

6. **β1-Integrin Endocytosis Assays**

Because cell adhesion is affected by the amount of adhesive receptors on the surface of the cell, the mechanism and efficiency of β1-integrin endocytosis will affect adhesion and migration. We hope at some point to adapt these approaches for comparisons to cells cultured in 3D matrices, but for now have worked out approaches in 2D culture.

6.1. **Time-lapse imaging of integrin trafficking in cells cultured on 2D surfaces**

Considering recent interest and abilities in imaging cellular signaling events during cell adhesion and migration, the ability to also visualize integrin endocytosis and trafficking processes in live cells would prove to be highly informative. The use of
commercially available kits that fluorescently label antibodies make this more readily possible. Direct labeling of $\beta_1$-integrin antibody (sc-9970, mouse monoclonal IgG1 [4B7R], Santa Cruz) is carried out using a Zenon Alexa Fluor-555 mouse IgG1 labeling kit from Molecular Probes (Eugene, OR), according to the manufacturer’s instructions.

Cos-7 cells, obtained as a generous gift from Richard Anderson (Madison, WI) and maintained in DMEM (containing high glucose, l-glutamine, sodium pyruvate and pyridoxine hydrochloride) plus 10% fetal bovine serum at 5% CO2, are plated into poly-L-lysine coated glass bottomed dishes (MatTek Ashland, MA) for 24 h at low confluency to minimize cell–cell interactions. Labeled antibodies are applied to cells for 30 min at 25°C. Excess antibody is then washed out with PBS, followed immediately by live cell imaging.

6.1.1. Microscopy

Images were acquired using a CoolSnap FX (Roper) CCD camera mounted to an inverted microscope working in the epifluorescence mode. The emission intensity of a 100-W mercury arc bulb was attenuated by the appropriate excitation filter (545/30 for red fluorescence), directed to the cells with a dichroic mirror (565 DCLP), and fluorescence emission filtered (620/60) before reaching the camera. Exposure times ranged between 100 and 500 msec and the oil immersion objective used was 40× N.A. = 1.3. We find it useful to perform no-neighbors deconvolution to remove out-of-focus fluorescence from the image. Digitized images can be exported to Image J for further analysis.

Figure 2.2A is a deconvolved epifluorescent image of a cell incubated with AlexaFluor555–tagged $\beta_1$-integrin antibody (4B7R, Santa Cruz). The staining was dimly fluorescent around the cell periphery where, in addition, higher-intensity microdomains of staining were also observed. Interior regions of the cell contained punctate and vesicular staining, which likely were trafficking vesicles and endosomal compartments. Figure 2.2A was the first image acquired in a series, and therefore some internalization of surface bound antibody has already occurred. This was necessary, as this technique required a 30-min incubation period with the fluorescent antibody followed by washout; otherwise cells would be unobservable during the incubation period due to high fluorescence, but at the same time require a proper amount of labeling. Compare the image in panel A with that of a cell stained using conventional immunocytochemistry techniques (panel B). The fixed cell did retain the phenotype of plasma membrane microdomain staining of $\beta_1$-integrin (arrow), and because the cell was permeabilized, there was accumulation of the antibody in the perinuclear region and labeling of intracellular, trafficked $\beta_1$-integrin. The advantage of potentially increased sensitivity obtained using immunocytochemistry, as witnessed by a greater number of small puncta of labeled $\beta_1$-integrin, is offset by the doubt raised concerning the specificity of staining. However the main advantage of avoiding the use of a fixed cell lies in the ability to perform time-lapse imaging experiments to look at $\beta_1$-integrin dynamics. Figure 2.2C shows a series of images of the boxed region in A acquired at approximately 2-min intervals. In this manner, one could observe how endocytosis of the microdomain of $\beta_1$-integrin occurred not through invagination of the membrane into a large vesicle, but rather...
the entire ribbon of staining internalized slightly and then subsequently dissipated into several individual small vesicles that were visible at the 6-min mark (Fig. 2.2, arrows). The high spatiotemporal resolution of this technique can also be used to visualize the formation of new microdomains of $\beta_1$-integrin at the plasma membrane as well as trafficking of internalized $\beta_1$-integrin to endosomal compartments.

### 6.1.2. Immunocytochemistry
Cos-7 cells were rinsed in PBS to remove DMEM, fixed in 4% paraformaldehyde for 15 min at room temperature (RT), followed by quenching of excess PFA in 0.15-M glycine for 10 min. Cells were blocked in 1% donkey serum and 1% fatty acid–free BSA for 30 min after which primary antibody (1:100) was applied overnight at 4°C. The antibody solution consisted of blocking solution supplemented with 0.01% Triton–X–100. Following washout of the primary antibody solution with PBS, the secondary antibody (Alexa Fluor555 goat anti-mouse; 1:200) was applied for 1 h at RT followed by several rinses with PBS. Cells were imaged the same day as secondary antibody application, using the epifluorescence microscope described above.

### 6.2. Endosome protection biotinylation assay
As a quantitative adjunct to the visualization of $\beta_1$-integrin endocytosis, we have developed an endosome protection biotinylation assay. The basic principle, which is applicable to any plasma membrane protein with an extracellular epitope, is to discern the partitioning of protein remaining on the cell surface from the fraction
that has become internalized. Intact cells are biotinylated to label the pool of proteins remaining exposed to the surface during the test period, allowing the subsequent removal of cell-surface proteins on strepavidin beads. Thus the pool of protein that is internalized is protected from biotinylation and is quantified.

The experiment can be performed as follows: cells are grown in 100-mm Petri dishes for 24 h, and then incubated with anti-β1-integrin antibody (4B7R, Santa Cruz) for 30 min at 37°C to allow antibody to be internalized with β1-integrin, and washed. Whole cells are subsequently biotinylated using Pierce EZ-Link Sulfo-NHS-LC-Biotin at >20-fold molar excess for 30 min on ice at 4°C. Two control conditions should be performed: (1) one dish should not be biotinylated as a control for total β1-integrin, and (2) one dish should be exposed to antibody and then immediately biotinylated as a negative control. Following washout of excess biotin, cells are lysed in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1mM EDTA, 1 µM NaF, and protease inhibitor cocktail) and biotinylated antibody was cleared using a 40-min incubation with strep-avidin beads and centrifugation in a microfuge at low speed for 20 sec. A total cell lysate sample should be saved, to later determine that equal amounts of initial protein were used. To recover the protected anti-β1 integrin antibody, the cleared supernatant is incubated in gamma-bind sepharose beads (GE Healthcare BioSciences) for 40 min followed by three rounds of washing in 1× lysis buffer. Proteins are released from the beads using 1.5× Laemmli buffer containing β1-mercaptoethanol at 95°C for 3 min. Samples are run on an 8% SDS-PAGE gel, transferred to a PVDF membrane, probed for β1-integrin antibody, which appears as the 50-kDa heavy chain on the western blot. Variations on this assay can be performed to determine the effect of various signaling intermediates or trafficking pathways, as cells can be transfected or exposed to pharmacological inhibitors prior to initiating the assay.

7. Imaging Cell–Matrix Interactions in 3D Collagen Gels

The use of MPLSM, which uses non-linear excitation to image a narrowly defined plane deep within tissue noninvasively, has the advantages of reduced phototoxicity (Squirrell et al., 1999) and the ability to image deeper into turbid samples (Centonze and White, 1998) than other live cell imaging techniques. Moreover, its nonlinear excitation method is compatible with another nonlinear optical method—second harmonic generation (Campagnola et al., 1999). Since collagen fibers are strong harmonophores, they can be readily imaged without additional contrast or staining approaches. This makes MPLSM an ideal approach to image cells and simultaneously image the 3D collagen matrix.

7.1. Cell culture

Mouse embryonic fibroblasts (MEFs) and the highly invasive MDA-MB-231 breast cancer cell line are maintained in T75 tissue culture flasks (Corning, Lowell, MA) containing DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco,
Carlsbad, CA) at 37°C and 10% CO₂. Cells are passaged by trypsinization (0.5%; Cellgro, Herndon, CA) followed by 1:5 to 1:10 dilution into fresh media every 3 to 4 days.

7.2. Stable transfection
Due to the fact that the transfection efficiency in breast epithelial cells is relatively low, generation of stably expressing cells may by desirable. To generate stable MBD-MB-231 cells lines expressing GFP-vinculin, or GFP as a control, cells at ~85% confluency are transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Prior to transfection, 20 μg of DNA (in 1.9 ml of serum-free DMEM) is combined with 75 μl of Lipofectamine 2000 (in 1.9 ml of serum-free DMEM) and DNA-Lipofectamine complexes are allowed to form for 20 minutes. The DNA-Lipofectamine complexes are then added to the culture plate with 12 ml of DMEM plus 10% FBS for 18 h. After 18 h, the cells are passed 1:20 into multiple T75 flasks containing culture medium supplemented with 1 mg/ml G418, where the cells grow and then die off over a 10- to 14-day period. Stable transfectants are then pooled and sorted under flow cytometry to obtain a population of cells with desired level of expression.

7.3. Transient transfection
Since it is not always necessary or unproblematic to create a stably expressing cell line, transient transfection of the desired construct may be utilized. However, due to the low transfection efficiency for breast epithelial cells (typically 20 to 30%) a fluorescent marker, such as GFP, is necessary for cell biology experiments. For transient transfection, seed ~5 × 10⁵ MEFs or MDA-MB-231 cells into 12-well culture plates 2 days prior to imaging. After 24 h, or after the cells are more than 80% confluent, cells are transfected with complexes resulting from the mixture of 1 μg of DNA (in 100 μl of serum-free media) and 4 μl of Lipofectamine 2000 (in 100 μl of serum-free media). At 18 h post-transfection, pools of transfected cells are harvested by trypsinization and seeded into collagen gels.

7.4. Three-dimensional collagen gels
Following transfection, transient transfectants or stably expressing GFP-vinculin cells are detached and cells collected by centrifugation. Cells are resuspended in standard culture media and 1 to 5 × 10⁵ cells seeded into a 3.0 mg/ml type I collagen gel. For 2D control experiments cells are plated onto collagen coated (30 μg/ml) glass bottom culture dishes. Cell-seeded gels are allowed to polymerize at 37°C and maintained under standard culture conditions for 24 h. After 24 h, the gels are moved to glass bottom dishes (MatTek Corp., Ashland, MA) and imaged with multiphoton laser-scanning microscopy.
Figure 2.3 3D matrix adhesions in MEFs transiently expressing GFP-vinculin. Use of combined multiphoton excitation (MPE) and second harmonic generation (SHG) microscopy facilitates imaging of both the cell–matrix interaction and reorganization of the extracellular matrix (ECM). (A) Bright-field transmission image of a MEF on a collagen-coated coverslip for 24 h. (B) MPE image of GFP-vinculin–positive, 2D focal adhesions in the cell shown in (A) following two-photon excitation at 890 nm and filtering of the emission signal to isolate fluorescence from GFP (480-to-550-nm band-pass filter). (C) Bright-field transmission image of a MEF in a 3.0 mg/ml collagen gel after 24 h. (D) Concurrently acquired (nonfiltered) MPE/SHG image of the cell shown in (C) following two-photon excitation at 890 nm to simultaneously excite GFP and generate second harmonic signals from collagen. (E) MPE image of GFP-vinculin–positive, 3D matrix adhesions in the cell shown in (C) following two-photon excitation at 890 nm and filtering of the emission signal to isolate fluorescence from GFP (480-to-550-nm band-pass filter). The arrow in (E) indicates the region that is enlarged in the subpanel displayed in (E) showing punctuate 3D matrix adhesions at the leading edge of the cell where collagen has been pulled in toward the cells resulting in reorganization of the extracellular matrix. (F) SHG image of fibrillar collagen, following excitation at 890 nm and filtering of the emission signal to isolate SHG (445-nm, narrow band-pass filter), that has been reorganized by a contractile cell transmitting force through 3D matrix adhesions to the ECM. Note: See color pallet for pseudocolored image with merged MPE and SHG signals. Scale bars = 25 μm.
Figure 2.4 3D matrix adhesions in highly invasive and migratory MDA-MB-231 breast carcinoma cells stably expressing GFP-vinculin. Multiphoton excitation (MPE) and second harmonic generation (SHG) microscopy facilitate imaging of 3D matrix adhesions and extracellular matrix reorganization. (A) MPE image of GFP-vinculin–positive, 2D focal adhesions in MDA-MB-231 cells plated on collagen-coated glass coverslips for 24 h. GFP is excited at 890 nm and the emission signal filtered to isolate fluorescence from GFP (480–550 nm band-pass filter). (B) Bright-field transmission image of a MDA-MB-231 cells in a 3.0-mg/ml collagen gel after 24 h. (C) MPE
7.5. Nonlinear optical imaging of collagen–3D matrix adhesion interaction

Multiphoton laser-scanning microscopy (MPLSM) is used to generate multiphoton excitation (MPE) and second harmonic generation (SHG) (Cox et al., 2003; Provenzano et al., 2006; Zipfel et al., 2003; Zoumi et al., 2002). In combination, these methodologies allow high-resolution live cell imaging as well as imaging of collagen structure. In MPE two or more low-energy (usually near-infrared) photons simultaneously excite a fluorophore, with the emission dependent on the square of the intensity, the probability of which is steeply dependent on the plane of focus. Thus, MPE produces optical sectioning that improves the axial resolution over standard confocal imaging (Centonze and White, 1998; Denk et al., 1990). In contrast, SHG signals depend on nonlinear interactions of illumination with a noncentrosymmetric environment, such as the highly crystalline structure of fibrillar collagen, resulting in a coherent signal at exactly half the wavelength of the excitation (Mohler et al., 2003).

MEF or MDA-MB-231 cell-seeded collagen gels are imaged with a MPLSM workstation assembled around a Nikon Eclipse TE300 (Wokosin et al., 2003). Excitation is produced with a 5W mode-locked Ti:sapphire laser (Spectra-Physics-Millennium/Tsunami, Mountain View, CA) tuned to 890 nm. The beam is focused onto the collagen gels with a Nikon 60\(\times\) Plan Apo water-immersion lens (N.A. = 1.2). To discriminate MPE and back-scattered SHG signals, a 445-nm, narrow band-pass filter is used to isolate SHG emission, while GFP signals are isolated with a 480-to-550–nm (band-pass) filter (all filters: TFI Technologies, Greenfield, MA). Without the use of these filters, a single image of GFP and collagen can be obtained, separation of the signals is not possible. The power of this approach is shown in Figs. 2.3 and 2.4, in which MPLSM imaging of GFP-vinculin expressing cells and their interaction with the collagenous matrix shows 3D matrix adhesions at the cell–collagen interface, as well as cell-mediated reorganization of the collagen matrix.

![image of GFP-vinculin-positive, 3D matrix adhesions](image.png)

The image shows examples of adhesions illustrated with arrows in the cell shown in (B) following two-photon excitation at 890 nm and filtering of the emission signal to isolate fluorescence from GFP (480-to-550–nm band-pass filter). (D) SHG image of fibrillar collagen, following excitation at 890 nm and filtering of the emission signal to isolate SHG (445-nm, narrow band-pass filter), demonstrating cell-mediated collagen reorganization that correlates with 3D matrix adhesion location. (E to L) Progressive images taken at 1-\(\mu\)m intervals from an 8-\(\mu\)m z-stack showing GFP-vinculin-positive, 3D matrix adhesions (arrows indicate the region that is enlarged in the inset). On 2D substrates, matrix-engaged focal adhesions are located at the cell substrate interface (A), and are therefore primarily located within a narrow imaging window “below” the cell. In contrast, cells in 3D microenvironments (B to L) possess 3D matrix adhesions around the volume of the cell, and therefore are in multiple focal planes. Note: See color pallet for pseudocolored image with merged MPE and SHG signals. All images are the same magnification; scale bar = 25 \(\mu\)m. (Color pallet: Merged MPE and SHG images from Fig. 3(A) and Fig. 4 (B). MPE of GFP-vinculin isolated with a 480-to-550–nm band-pass filter pseudocolored in green, SHG image of collagen isolated with a 445-nm, narrow band-pass filter pseudocolored in red.)
REFERENCES


