

ENDOTHELIAL CELL ADHESION AND MIGRATION

Cynthia A. Reinhart-King

Contents

1. Introduction	46
2. Cell Preparation	48
3. Preparation of Well-Defined Surfaces	49
4. Preparation of Polyacrylamide Substrates for Cell Adhesion Studies	50
5. Quantifying Cell Adhesion	54
5.1. Observation of endothelial cell spreading dynamics	54
5.2. Centrifugation assay	55
6. Quantifying Endothelial Cell Migration	56
6.1. Collective cell migration: The wound-healing assay	57
6.2. Individual cell motions: Calculation of cell speed	59
References	61

Abstract

Endothelial cell adhesion and migration is fundamental to a number of physiologic processes, including vascular development and angiogenesis. It has been investigated in a variety of contexts, including tumorigenesis, wound healing, tissue engineering, and biomaterial design. The chemical and mechanical extracellular environments are critical regulators of these processes, affecting integrin-matrix binding, cell adhesion strength, and cell migration. Understanding the synergy between matrix chemistry and mechanics will ultimately lead to precise control over adhesion and migration. Moreover, a better understanding of endothelial cell adhesion is critical for development of therapeutics and biomaterials for the treatment of endothelial cell dysfunction and the progression of vascular disease. This chapter will focus on the specific interactions between endothelial cells and the extracellular matrix that mediate adhesion and migration. Several engineering methods used to probe and quantify endothelial cell adhesion and migration will be discussed.

Department of Biomedical Engineering, Cornell University, Ithaca, New York

Methods in Enzymology, Volume 443
ISSN 0076-6879, DOI: 10.1016/S0076-6879(08)02003-X

© 2008 Elsevier Inc.
All rights reserved.

1. INTRODUCTION

The endothelium is the single cell layer lining blood vessels, establishing a semipermeable barrier between blood and surrounding tissue. The formation of blood vessels, from larger arteries to micron-sized capillaries, depends on endothelial cell adhesion to the extracellular matrix and well-organized cellular movements. During the closure of a wound, for example, angiogenesis is essential to revascularization within the newly healed tissue. Endothelial cells migrate from preexisting blood vessels, proliferate, and reorganize with vascular smooth muscle cells and pericytes to form a new capillary network (Davis and Senger, 2005; Lamalice *et al.*, 2007). Similarly, angiogenesis occurs in response to tumor-secreted angiogenic signals that trigger the formation of a vascular network to provide a blood supply to growing tumors (Eliceiri and Cheresh, 2001). Endothelial cell adhesion and migration is also critical in the formation of larger vessels and has been the subject of intense investigation for the optimization of vascular stents. It is now well established that without proper endothelialization, stents tend to occlude because of thrombosis and intimal hyperplasia (Sayers *et al.*, 1998). Therefore, a greater understanding of cell adhesion and migration could be exploited for the design of stents that support rapid endothelialization.

Endothelial cell adhesion and migration is primarily mediated through integrin binding to the extracellular matrix. Integrins are $\alpha\beta$ heterodimeric cell surface receptors that recognize specific extracellular matrix ligands. Integrins not only serve to anchor the cells to their matrix, but they also function as transducers of chemical and mechanical signals between the extracellular and intracellular environments (Miranti, 2002). Because of their critical role in mediating cell adhesion, integrins are important regulators of several endothelial cell-related processes, including vasculogenesis and angiogenesis. Endothelial cells express several integrins associated with several different ECM ligands (Table 3.1). Evidence indicates that specific integrins have specific functions in various vascular processes. For example, α_v is believed to be the subunit primarily responsible for angiogenesis (Friedlander *et al.*, 1995). Additional studies with α_v knockout mice indicate that other integrin subunits may also have a role in angiogenesis and can compensate without the expression of α_v (Bader *et al.*, 1998). Consistent with this finding, α_5 knockout mice also displayed impaired vasculogenesis during development (Yang *et al.*, 1993). The importance of integrin-ECM connections has been confirmed through the use of ECM fragments as anti-angiogenic agents (Eliceiri and Cheresh, 2001). Although there is still much to be learned about the individual contributions of individual integrin subtypes, it is clear that integrin-matrix interactions are requisite for endothelial cell adhesion and migration associated with formation of the vasculature.

Table 3.1 Endothelial cell integrins and ligands

Integrin Subunits	ECM Ligands
$\alpha 1\beta 1$	collagen/laminin
$\alpha 2\beta 1$	collagen/laminin
$\alpha 3\beta 1$	laminin
$\alpha 5\beta 1$	fibronectin
$\alpha 6\beta 1$	laminin
$\alpha v\beta 3$	vitronectin/fibronectin
$\alpha v\beta 5$	vitronectin

In vitro assays of endothelial cell adhesion and migration have led to critical insights into the mechanisms of angiogenesis and vasculogenesis. By investigating adhesion and migration *in vitro*, specific extracellular matrix signals to the cells can be precisely controlled. Most studies involving adhesion-mediated signals have paid special attention to the chemical nature of adhesion-related signals. That is to say, many studies have focused on the specific interaction between certain endothelial integrins with particular extracellular matrix proteins, including fibronectin, laminin, and collagen. Of interest is how the matrix type or density affects adhesion-related cell response. These studies have been useful in dissecting the relative roles of various integrins on endothelial cell adhesion, migration, and tube formation.

More recently, the role of matrix mechanics has emerged as an area of intense interest. It is becomingly increasingly evident that matrix stiffness (or modulus) can alter cell adhesion and subsequent cell signaling responses in a variety of cell types, including vascular smooth muscle cells (Peyton and Putnam, 2005; Wong, 2003), fibroblasts (Lo *et al.*, 2000), mammary epithelial cells (Paszek *et al.*, 2005), neurons (Georges *et al.*, 2006), and endothelial cells (Reinhart-King *et al.*, 2003). Substrate stiffness has been shown to alter cell-substrate adhesive strength, cell contractility, focal adhesion formation, migration speed, cell-cell interactions, and cell assembly. Because *in vivo* compliance varies within tissues and changes in pathologic conditions (Guo *et al.*, 2006; Paszek *et al.*, 2005), the role of matrix mechanics in cell regulation is gaining increasing attention.

Our laboratory, in particular, is interested in how both the chemical (i.e., matrix type, density, and presentation) and the mechanical (i.e., substrate stiffness and external applied forces) environments affect endothelial cell adhesion, migration, and subsequent adhesion-related signaling. In this chapter, methods to prepare cells for the study of adhesion and adhesion-related signals and protocols to control the chemical and

mechanical cellular microenvironment will be described, as well as methods to quantify cell adhesion and migration.

2. CELL PREPARATION

In cell adhesion and migration experiments, it is important that the cells are synchronized and adhesion-related background signals are at a minimum, particularly before assaying for adhesion-related signal transduction. To synchronize the cells and minimize adhesion-related signals, the following protocol can be followed:

1. Culture endothelial cells until confluence.

NOTE: The cells should not be overgrown, because they can begin to peel up as cell sheets rather than remaining adhered to the dish.

2. Serum-starve the cells for 16 h. Serum starvation is most important for signaling assays to minimize all background signaling; however, for measurements of adhesion strength and migration, it is often sufficient to synchronize the cells using only step 1.

Media used for serum starvation varies with the type of endothelial cells and culture conditions before starvation. For example, we have found bovine aortic endothelial cells can be serum starved for 16 h by completely removing the serum; however, human umbilical vein endothelial cells can require 2% serum to remain viable over the 16 h window.

3. Wash the cells twice with ample PBS to remove all residual media and then trypsinize. Because the length of time the cells are exposed to trypsin can affect their ability to re-adhere (Brown *et al.*, 2007), it is important to keep this time constant throughout all adhesion experiments. In addition, to help maintain consistency, freshly thawed aliquots of trypsin should be used for each experiment. To reduce trypsin activity after the cells have released from the dish, trypsin inhibitor should be added to the trypsinized cells at an equal or greater volume to the amount of trypsin used to detach the cells.
4. The cells are then centrifuged to a pellet for 5 min at 100g and resuspended in serum-free media.
5. To further minimize adhesion-related signaling events, cells can be placed in suspension in serum-free media for 30 min in conical tubes coated with 1% heat-inactivated BSA. The BSA coating minimizes cell adhesion to the walls of the tube.

The cells can now be plated on the substrate of choice at the desired density.

3. PREPARATION OF WELL-DEFINED SURFACES

To study endothelial cell adhesion and migration quantitatively, a well-defined adhesive substrate must be prepared. This chapter will focus on primarily on 2D adhesion and migration. Advances in the design and use of 3D scaffolds for cell adhesion and migration significantly lags behind the widespread use of 2D supports. Matrigel is perhaps the most prevalently used 3D matrix for the study of endothelial cell adhesion and migration; however, its chemical composition is not well defined. A number of investigators are creating tailored 3D matrices for tissue engineering scaffolds and for use as platform for basic science studies of endothelial cell adhesion and migration (Jun and West, 2005; Raeber *et al.*, 2005), but these systems are not at the “off-the-shelf” stage and are, therefore, not yet widely used. As the 3D materials platform continues to mature, we expect that methods to measure cell adhesion and migration will also evolve. A number of differences exist between 2D and 3D adhesion. Most importantly, in 2D versus 3D, integrins bind the ECM on all sides, thereby altering cell adhesion, morphology, migration, and signaling (Berrier and Yamada, 2007). Quantitative methods to study adhesion strength and migration behavior in 3D are requiring a significant shift in approach to address these fundamental differences.

Preparing a 2D adhesive substrate typically involves immobilizing specific extracellular matrix proteins onto glass or plastic. In general, multiwell plates are used, because the format allows for simultaneous replication of adhesive conditions and minimal use of protein and cells. The regions not occupied by specific cell adhesion proteins are typically backfilled with a blocking agent, most often 10 mg/ml heat-denatured bovine serum albumin (BSA). This is prepared by dissolving the appropriate amount of BSA in calcium and magnesium-free Dulbecco's PBS, sterile-filtered, and heated to 85 °C for 10 min in small volumes to ensure equal heating. Proper heat-inactivated BSA should appear homogenous and slightly hazy. Aggregates indicate that the BSA has been overheated, whereas a clear solution indicates that the BSA is not denatured. To uniformly coat a glass or plastic dish with a specific protein, the following short protocol can be followed.

1. Dissolve the ECM protein of interest in the appropriate buffer. This is typically PBS, but may vary depending on the protein. Salt can be added to maintain the conditions that are oftentimes specified by the supplier of the specific protein. In the case of full-length fibronectin, for example, dissolving the lyophilized protein requires the addition of water and a 30-min incubation time before being mixed or aliquoted for use or storage. The 30-min incubation period helps minimize aggregation.
2. Add the dissolved protein to the well to be coated at the desired concentration, typically ranging from 1 $\mu\text{g}/\text{ml}$ to 50 $\mu\text{g}/\text{ml}$.

3. Incubate overnight at 4 °C or 37 °C overnight on a level surface.
4. Aspirate off the remaining solution and wash twice with PBS. Add 1% BSA solution (described previously) and incubate for 1 h at 37 °C.

NOTE: Because time, temperature, and concentration can alter the amount of protein bound to the culture dish, it is best to choose one time and temperature for all experiments.

One of the drawbacks of this particular method for creating an adhesive surface is that cells have the ability to remodel the protein on the surface of the glass or plastic by secreting additional matrix or degrading the protein already on the surface. Therefore, coating glass or plastic is only typically used if the study to be performed is short term, over just a few hours. To perform longer term adhesion studies, we have adopted the use of polyacrylamide sheets (Wang and Pelham, 1998).

4. PREPARATION OF POLYACRYLAMIDE SUBSTRATES FOR CELL ADHESION STUDIES

The use of polyacrylamide as a cell attachment substrate was first proposed by Pelham and Wang, in 1998 (Wang and Pelham, 1998). Their goal in this study was to create a deformable substrate to assay cell response to changes in substrate stiffness. However, because polyacrylamide is typically inert to cell adhesion and protein adsorption, it also provides an easy-to-characterize, stable platform for controlling protein presentation to cells. That is to say, it is relatively easy to chemically conjugate specific proteins to the polyacrylamide gel, and because polyacrylamide is not easily remodeled by cells, the desired protein presentation changes relatively little over the duration of the experiment compared with protein on glass or plastic (Nelson, 2003). The compliance of the polyacrylamide is varied by controlling the relative ratio of acrylamide to cross linker.

Since the original publication describing polyacrylamide as a cell substrate, the method has been implemented and published by a number of groups (Johnson *et al.*, 2007; Kandow *et al.*, 2007; Klein *et al.*, 2007). In our laboratory, we have adapted the use of polyacrylamide gels to study endothelial response to covalently conjugated ECM proteins at varying densities and changes in mechanical stiffness (Reinhart-King *et al.*, 2003; 2005). Although the specific interactions between endothelial integrins and ECM ligands have received much attention over the past several decades, the effect of ECM mechanics on endothelial cell adhesion and migration has received relatively little attention. By use of polyacrylamide matrices of varying compliance and matrix chemistry, we have shown that both matrix chemistry and mechanics can alter cell spreading, cell migration, and the

formation of stable cell–cell contacts (Reinhart-King *et al.*, 2003; 2005). Polyacrylamide substrates have another advantage in addition to their mechanical and chemical tunability; they can be used as substrates for use in traction force microscopy—a method by which the contractile forces exerted by cells on its substrate are measured (Fig. 3.1) (Dembo and Wang, 1999). As cells exert force on the polyacrylamide substrate, the polyacrylamide deforms. These deformations are detected on the basis of fluorescent markers embedded within the substrate. The bead movements are translated into a strain map, and these strains are used to calculate the forces exerted by the cell. The method to perform traction force microscopy will not be presented here, because it typically requires a computationally intensive, custom algorithm to translate substrate strains to traction stresses and is described in detail elsewhere (Dembo and Wang, 1999). In addition,

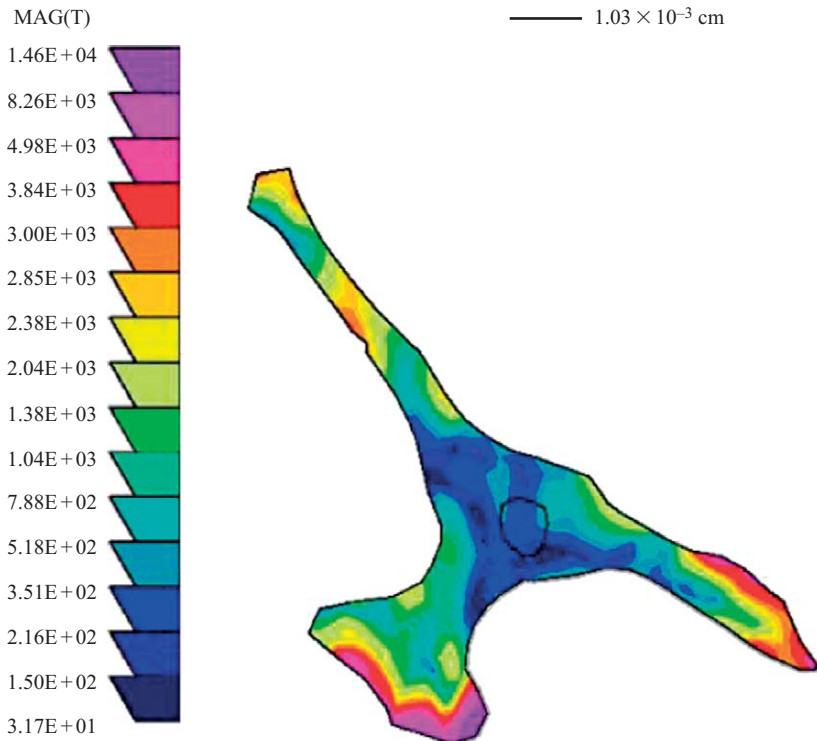


Figure 3.1 Color contour map depicting the traction forces exerted by a bovine aortic endothelial cell on a deformable polyacrylamide substrate derivatized with an RGD-containing peptide. The image was obtained by use of the LITRC traction algorithm written by Micah Dembo at Boston University; he is also the inventor of the basic theory that underlies traction force microscopy.

there are other methods available to calculate cellular traction forces that are based on the movement of microfabricated cantilevers (Galbraith and Sheetz, 1997) or posts (Tan *et al.*, 2003) by cellular forces.

It is becoming increasingly obvious that the role of mechanics cannot be ignored in the study of endothelial cell adhesion and migration. To control chemistry and mechanics, we use the following protocol to synthesize polyacrylamide substrates of well-defined compliance, presenting well-defined ECM protein chemistries, which has been adapted from Wang and Pelham (Wang and Pelham, 1998).

1. The first part of the protocol involves activating glass coverslips used as a support for the gels. The coverslip size is chosen on the basis of the experiment. For migration/adhesion assays, 22-mm square, No 1 coverslips fit into 6-well plates, allowing for easy manipulation.
2. Briefly pass the glass coverslip through a flame. Heating the glass helps the NaOH in step 3 to spread easily; however, if the glass gets too hot, it will break.
3. With a clean cotton swab, immediately apply 0.1 N NaOH to the flamed side.
4. Repeat steps 1 and 2 until the required number of slides have been coated. Allow the coverslips to dry, producing a thin film of NaOH over the surface.
5. In fume hood: Add $\sim 30 \mu\text{l}$ of 3-aminopropyl-trimethoxysilane (APTMS) to each coverslip and spread quickly by use of a glass Pasteur pipette. This amount varies on the basis of the size of the coverslip—the amount should be just enough that it covers the surface of the coverslip.
6. Allow coverslips to dry for approximately 10 min inside the chemical fume hood.
7. Rinse the coverslips thoroughly with DI water.
8. Incubate the coverslips in a solution of glutaraldehyde in PBS (1:140, v/v) for 30 min.
9. Wash each coverslip three times with distilled water. Incubate for 5 min between each rinse.
10. Allow the coverslips to dry.
11. Coat Corning No. 1½, 18-mm circular glass coverslip with Rainex. (The size of this coverslip is based on size of glass used in step 1). The two coverslips act as a sandwich used to cast the gel. This coverslip should be smaller than the one used above. Apply Rainex with a clean cotton swab and allow to dry for at least 5 min. Buff off the excess with a Kimwipe.
12. Combine the following components in a 50-ml centrifuge tube to prepare a gel with stiffness of 2500 Pa: 2.5 ml 40% (w/v) acrylamide (BioRad, Hercules, CA); 1.0 ml 2%(w/v) n,n' methylene-Bis-acrylamide (BioRad,

Hercules, CA); 2.6 ml 0.25 M HEPES, pH 6.0; 12.39 ml ddH₂O; and 10 μ l TEMED (BioRad, Hercules, CA).

Compliance is based on the amount of acrylamide and bisacrylamide.

To vary the compliance, the amounts of acrylamide and bisacrylamide should be adjusted (Yeung *et al.*, 2005).

13. pH solution to 6.0 by adding ~4 to 5 drops of 2 M HCl.
14. Remove 925 ml of acrylamide mixture and degas solution for 30 min under vacuum.
15. Weigh out 5.6 mg of *N*-succinimidyl ester of acrylamidohexanoic acid (N6). The linker allows for the covalent conjugation of proteins through linkage through primary amines. This particular linker is synthesized by use of the protocol from Pless *et al.* (Pless *et al.*, 1983); however, a number of other linkers are commercially available (Kandow *et al.*, 2007).
16. Add 70 μ l of 200 proof ethyl alcohol (molecular biology grade) to the *N*-succinimidyl. Pipette the solution up and down until well mixed and add it to the 925 μ l of acrylamide mixture.
17. To initiate polymerization, add 5 μ l freshly prepared 10% ammonium persulfate (APS). Mix gently by pipetting up and down, being careful not to introduce bubbles.
18. Add 20 μ l of gel solution to the activated coverslips from step 1.
19. Gently press the drop of gel solution with Rainex-coated coverslip by carefully touching the coverslip to the edge of the drop and then lowering it slowly with forceps.
20. Allow polymerization to occur for 30 min—*not longer*. The edges of the gel should recede beneath the top coverslip.
21. A few minutes before the gel is done polymerizing, dilute the ECM protein of choice in 50 mM HEPES buffer (pH 8.0) to the desired concentration (typically 1 μ g/ml to 1 mg/ml).
22. Peel off coverslip from each gel with a clean razor blade.
23. Add 200 μ l of the dissolved protein to the gels; 200 μ l is chosen, because it seems to be the minimum amount needed to cover the gel. Spread the protein across the gel by gently pipetting the liquid over the gel's surface, completely covering the gel.
24. Incubate at 4 °C for 2 h.
25. Mix a 1:1000 volume of ethanolamine with 50 mM HEPES, pH 8.0. Each gel requires 200 μ l of solution.
26. Dispense 200 μ l of the ethanolamine/HEPES solution directly onto the gels. There is no need to rinse off the protein first.
27. Incubate gels at room temperature for 30 min.
28. Place gels in cold ddH₂O water and store at 4 °C for up to 2 weeks. Cells can be plated on the substrates just as they would be plated onto glass or plastic.

5. QUANTIFYING CELL ADHESION

Cell adhesion is a complex process, involving the initial contact of a cell to a surface, coordinated receptor–ligand binding and actin polymerization, and finally, establishment of a well-spread state. Several methods have been tailored to understand and quantify each step in this process (Dobereiner *et al.*, 2004; Dubin–Thaler *et al.*, 2004; Giannone *et al.*, 2004; Reinhart-King *et al.*, 2005). Protocols to observe and measure cell adhesion during initial cell–substrate contact, spreading, and complete adhesion will be presented here.

5.1. Observation of endothelial cell spreading dynamics

In recent years, there has been significant interest in the observation of cells during initial adhesion and spreading to understand how intracellular and extracellular forces drive spreading (Cuvelier *et al.*, 2007; Dubin–Thaler *et al.*, 2004; Reinhart-King *et al.*, 2005). We have found that the extracellular matrix composition can alter endothelial cell–spreading dynamics, namely the rate of spreading and the shape changes cells undergo during spreading (Fig. 3.2) (Reinhart-King *et al.*, 2005). We have used measurements of area and perimeter to show that cells on high densities of ECM protein tend to spread isotropically, whereas cells on less ligand tend to spread anisotropically, through extension of thin membrane protrusions. This work has been accomplished by extensive time-lapse studies. Unlike conventional time-lapse studies of cell migration, we have found that endothelial cells are particularly sensitive to light before they have become well attached. To observe cells from their rounded state through to a fully

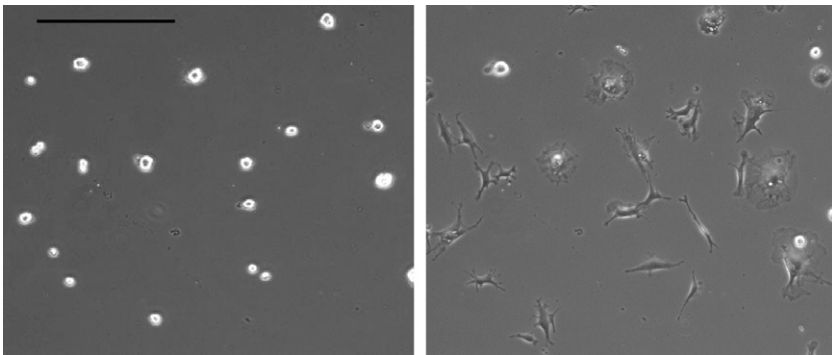


Figure 3.2 Images of bovine aortic endothelial cells plated on an RGD-coated polyacrylamide gel of 2500 Pa stiffness, taken at the time of plating and 4 h later. Scale bar is 200 μm .

spread state, the following considerations increase cell viability on the microscope stage.

1. Bright-field light should be minimized through the use of a shutter. In addition, the bulb intensity should be decreased and the exposure time on the camera should be increased accordingly. In our setup, the light is decreased such that it is virtually undetectable through the eyepieces and requires a 12-sec exposure by use of a Spot RT CCD camera.
2. A green filter is placed into the light path (Olympus, IF550).
3. A temperature and CO₂-controlled chamber is used. One hour before the experiment, the controls are turned on to allow the chamber to equilibrate. In previous experiments, we used a homemade custom-designed chamber that enclosed the microscope, from beneath the objectives to the area above the condenser. We have recently installed an enclosure from Precision Plastics (Beltsville, MD) that is sealed around the microscope with a gasket and is equipped with temperature, CO₂, and humidity control. This also works well for this purpose.
4. The experiments are performed in a dark room to minimize light. In addition, there is minimal traffic in the room to minimize air disturbances and temperature fluctuations.
5. If fluorescence microscopy is used, neutral density filters should be installed in the light path to minimize light on the sample.

If these guidelines are followed, cell spreading can be tracked through the entire process: from touchdown of the cell to a fully extended state.

5.2. Centrifugation assay

Because ECM chemistry and mechanics can alter the strength of endothelial cell adhesion and rate of spreading, it is of interest to measure the affinity between the cell and substrate. The centrifugation assay was developed as a method to quantify receptor-ligand affinity during early adhesion events (McClay *et al.*, 1981). It can be used to measure changes in cell adhesion strength as a function of changes in the ECM mechanics or chemistry. Cells are plated in 96-well plates coated with ECM proteins. After several minutes of adhesion, the plate is inverted and centrifuged to detach cells from the substrate. The data can be reported in terms of either the amount of force needed to detach a certain fraction of cells or as the fraction of cells at each condition detached by a given force (Asthagiri *et al.*, 1999; Guo *et al.*, 2006). In either case, the amount of force applied should be experimentally determined for a given set of conditions.

1. Typically, multiwell plates are used for this assay and are selected so that the wells are as small as possible for a given experiment. This helps in the process of removing air from the wells as explained in step 3. Most often,

- a 96-well flat bottom plate is used and coated with the desired ECM proteins and blocked with BSA as described previously. A positive control well should be plated with poly-L-lysine. It is preferable to use the inner wells of the plate for the assay to prevent media from leaking out of the plate when it is inverted for centrifugation. If the assay is to be performed on cells plated on polyacrylamide gels, then 6-well plates can be used, where the polyacrylamide glass support is fixed to the bottom of the wells.
2. Cells are plated in the wells in serum-free media. For a 96-well plate, we use 1×10^5 cells/ml in 100 μ l of media. The plates are then incubated at 37 °C, 5% CO₂ for 15 min.
 3. At this time, the wells should be filled to the top with serum-free media and sealed. To seal the wells before centrifugation, packing tape should be pressed down over the wells. Air and excess media should be pushed out as the tape is pulled across the wells. It is critical that no air is trapped in the well before being inverted in the next step.
 4. The plate is then inverted onto a plate carrier and centrifuged for 10 min at room temperature. Typical speeds might range from 1000 to 2000g. The speed of centrifugation should be determined for the conditions being tested and will vary with the substrate, time of incubation before centrifugation, and time of centrifugation.
 5. Plates should be removed from the centrifuge. At this point, cells remaining attached to the plate should be counted. The most straightforward way to do this is to count the cells with an inverted microscope and compare this number to the number of the cells still adherent in the positive control. If there are many conditions being tested, it can be easier to take images of each well and count them later with automated imaging software like ImageJ. Alternately, the nonadherent cell can be aspirated off and the wells can be fixed with 3.7% paraformaldehyde for 10 min, washed with PBS, and then counted.

6. QUANTIFYING ENDOTHELIAL CELL MIGRATION

Endothelial cell migration is critical to processes like wound healing and angiogenesis. A number of methods have been developed to measure cell migration. In this chapter, we will focus on approaches to investigate cell migration in uniform conditions; although there are a number of methods impose chemical gradients for the study of chemotaxis and haptotaxis. Most notably is the Boyden Chamber assay, in which cell migration is measured on the basis of the number of cells that migrate from a chamber containing no chemical factor through a filter into a chamber containing a chemotactic cue (Boyden, 1962). More recently, several methods have been developed to measure cell migration in chemical gradients created with microfluidic

platforms (Irimia *et al.*, 2007; Saadi *et al.*, 2006; Schaff *et al.*, 2007; Wu *et al.*, 2006). Microfluidics allows for precise control over the imposed gradient and most often permit the simultaneous observation of cell migration relative to the chemical gradient with a standard inverted microscope.

Here, two approaches are presented: one to measure collective motion and the second to measure individual cell motion. The first is the traditional wound-healing assay, in which the ability of endothelial cells to migrate into an imposed wound is measured. The second is a method to quantitatively measure migration speed and persistence of individual cells.

6.1. Collective cell migration: The wound-healing assay

The wound-healing assay is used to study the ability of cells to initiate migration once a denuded area is created in a confluent culture. The method has been in use for more than 40 years (Todaro *et al.*, 1965) and has been useful in characterizing a number of factors involved in cell migration, including the role of ECM proteins, the role of cell-cell connections, and the role of various intracellular proteins in mediating cell directionality. In this assay, a confluent monolayer of cells is “scratched” away, and cell migration is measured on the basis of the amount of time it takes the bordering cells to repopulate the denuded area. The wound-healing assay is particularly relevant to the healing of the endothelium that occurs *in vivo*. When the endothelium is injured because of a wound or denuded because of balloon angioplasty, for instance, endothelial cells migrate as a sheet into the injured area to re-endothelialize the area. The wound-healing assay is a relatively easy, straightforward method to study endothelial cell migration that can be accomplished with tools readily available in most cell biology laboratories.

1. Culture dishes are coated with the ECM protein of choice and blocked with BSA as described previously.
2. Endothelial cells are plated on the dishes and grown to confluence. Because the wound healing is due to both cell migration and cell proliferation, actinomycin C can be added to the medium after the cells reach confluence at a concentration of 1 ng/ml to inhibit proliferation.

NOTE: The assay can be performed on native or transfected cells. If transfected cells are to be used, the cells should be transfected with the plasmid of interest and a reporter plasmid such as GFP and grown to confluence before scratching the monolayer.

3. Scratch a “wound” in the monolayer by dragging a (p200 or p1000) pipette tip in a straight line across the monolayer (Fig. 3.3).
4. Mark the location of the wound with a marker on the underside of the dish. This will make it easier to find the wound in the following steps.

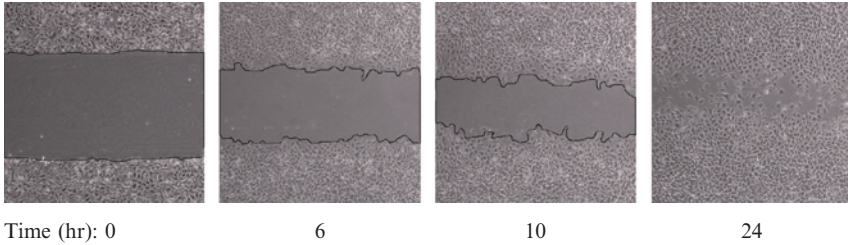


Figure 3.3 Example of images acquired during a wound healing experiment. Bovine aortic endothelial cells were scraped from a dish with a pipet tip and images were taken at 0, 6, 12, and 24 h by use of phase-contrast microscopy as the cells repopulate the wound. To measure the rate of healing, the area between the wound edges is measured and compared relative to the area of the original wound at $t = 0$. Images provided by Joseph Califano.

5. Aspirate the media from the dish and replace with fresh, warmed media.
6. Use the markings created in step 5 as reference points to find an area of the wound that will be imaged throughout the experiment and acquire an image of the wound under phase-contrast microscopy.
7. Return the dish to the incubator for 6 h.
8. Locate the same area imaged in step 6 with the reference markings and take an image with phase-contrast microscopy.
9. Repeat steps 7 and 8 until the wound has completely filled in. This time will be based on the conditions and cell type, but typically takes approximately 24 h.

To quantify cell migration, the area of the initial wound is compared with the area of the healing wound at various time points after the scratch is imposed (See Fig. 3.3), where

$$\% \text{Healed} = \left[\frac{\text{Area of original wound} - \text{Area of wound during healing}}{\text{Area of original wound}} \right] \times 100.$$

This can be plotted against time to determine the rate of healing, a measure of cell migration. To calculate the area, automated programs have been written to calculate the denuded area in a given field of view (Bindschadler and McGrath, 2007; Sottile *et al.*, 2007). Alternately, this can be done manually by use of program such as ImageJ. Although some have quantified wound healing on the basis of the distances between the two wounded edges, we have found that measurements of area result in much less error in the sampling.

Although this method is relatively easy, it does injure cells at the border of the wound. To minimize this injury, techniques to create denuded areas without scratching the surface have been developed (Kumar *et al.*, 2005). In these methods, typically a barrier is placed in culture while the cells grow to

confluence. It is then removed to allow cells to migrate into the area previously occupied by the barrier. These methods attempt to eliminate injuring the cells at the wound edge that can introduce debris that could affect migration.

6.2. Individual cell motions: Calculation of cell speed

An important aspect of cell migration is not only whether cells are capable of migrating, but also how fast they migrate. However, the process of calculating cell speed by use of time-lapse microscopy is not as straightforward as observing a cell at $t = 0$ and then at $t = 1$ h and on the basis of the distance traveled, calculating the migration speed. During normal chemokinesis, cells migrate in a random walk (Lauffenburger and Linderman, 1993). As depicted in Fig. 3.4, if cell movement were measured as the distance between the starting point A and the finishing point B, all of the information about the cell's path would be lost, and the total distance traveled would be incorrectly reported. Therefore, it is important to choose an interval that is appropriate for the cell and conditions. For endothelial cells, we have found that 5- to 10-min intervals are optimal. Given the ability to observe cells at 37 °C and 5% CO₂ as described previously, cell migration speed and persistence time can be calculated as follows:

1. Treat culture plates with ECM proteins and block with BSA as described previously.
2. Plate the cells sparsely and return the dish to an incubator for 6 h, allowing the cells to adhere and spread.
3. During the last hour of incubation, turn on the microscope incubator to allow the temperature and CO₂ to equilibrate.

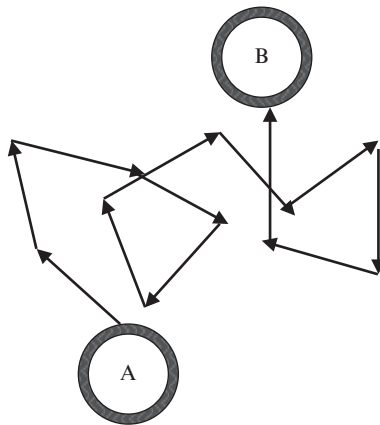


Figure 3.4 Cartoon of a sample trajectory of a cell migrating on a 2D substrate, starting at point A and stopping at B during the course of observation.

4. Place the cells on the microscope stage and image the cells by use of a $10\times$ objective under phase-contrast microscopy. The cells should be sparse such that cell-cell collisions during migration are minimal.
5. Take one image every 5 to 10 min for the duration of the experiment, which typically ranges from 6 to 24 h, while shuttering the light to prevent exposing the cells unnecessarily.
6. Track the motion of individual cells from frame to frame by either:
 - a. Tracking the position of the nucleus (i.e., the centroid of the cell)
 - b. Tracing the cell outline and use a program such as ImageJ to calculate the center of the cell on the basis of the outline.
7. Use these pixel coordinates, converted to micron displacements, to calculate the mean-squared displacement ($\langle d^2 \rangle$) for the range of time intervals.
8. The speed, S , and direction persistence time, P , can be determined by fitting the mean-squared displacement ($\langle d^2 \rangle$) and the time interval, t , to the persistent random walk equation: $\langle d^2 \rangle = 2S^2P(t - P(1 - e^{-(t/P)}))$ with nonlinear least squares regression analysis (Lauffenburger and Linderman, 1993).

The Matlab code to perform this analysis is included below. This type of analysis has been used extensively to characterize the motion of cells (Peyton and Putnam, 2005; Stokes *et al.*, 1991) and particles (King, 2006) in 2D. A more detailed description of the derivation of the equation can be found in Lauffenburger and Linderman (1993). The code requires input of x,y coordinates, entered as micron measurements, which are loaded into the code from sheet 1, column 1, and column 2, respectively, of an Excel file. The following code is commented in areas designated by “%.”

```
%File name is cellmig.m
%uses a second file "SSEcellmig.m" included below
global ts MSDs
x=Sheet1(:,1); y=Sheet1(:,2);
%x and y are the location of the cell in microns,
  %converted from pixels
dt=10; %10 minutes between images
n=length(x);
Nfit=50;
MSD=zeros(n-2,1);
% calculate the mean squared displacement for range of
  %time intervals
for i=1:n-2
MSD(i)=0;
for j=1:n-i-1
MSD(i)=MSD(i)+(x(j+i)-x(j))^2+(y(j+i)-y(j))^2;
end
MSD(i)=MSD(i)/(n-i);
```

```

end
t=[1:length(MSD)]*dt;
ts=t(1:Nfit);
MSDs=MSD(1:Nfit);
% to determine S and P, perform nonlinear least squares
% regression of migration model to MSD data.
% Uses separate %m-file "SSEcellmig.m" included below.
c=fminsearch(@SSEcellmig,[.005;400]);
migration_speed=c(1) %in units of microns/min
persistence_time=c(2) %in units of min
% plot of cell path in x-y space
Fig3.1
plot(x,y,'b')
xlabel('x (\mum)')
ylabel('y (\mum)')
% plot of MSD as a function of dt, with best-fit model
Fig.3.2
plot(t,MSD,'bo',t,2*c(1)^2*(c(2)*t-c(2)^2*(1-exp
(-t/c(2)))),'b-')
xlabel('t (min)')
ylabel('MSD (\mum^2)')
% File name is SSEcellmig.m
% SSEcellmig.m is a function to determine the Sum of
% the Squared Errors, for the nonlinear regression of
% migration model.
function temp=SSEcellmig(c)
% time and MSD are passed between this function and main
% program cellmig as global variables
global ts MSDs
S=c(1); P=c(2);
temp=0;
% sum the squared difference between the model and the data
% for i=1:length(ts)
temp=temp+(2*S^2*(P*ts(i)-P^2*(1-exp(-ts(i)/P)))-
MSDs(i))^2;
end

```

REFERENCES

- Asthagiri, A. R., Nelson, C. M., Horwitz, A. F., and Lauffenburger, D. A. (1999). Quantitative relationship among integrin-ligand binding, adhesion, and signaling via focal adhesion kinase and extracellular signal-regulated kinase 2. *J. Biol. Chem.* **274**, 27119–27127.

- Bader, B. L., Rayburn, H., Crowley, D., and Hynes, R. O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* **95**, 507–519.
- Berrier, A. L., and Yamada, K. M. (2007). Cell-matrix adhesion. *J. Cell. Physiol.* **213**, 565–573.
- Bindschadler, M., and McGrath, J. L. (2007). Sheet migration by wounded monolayers as an emergent property of single-cell dynamics. *J. Cell. Sci.* **120**, 876–884.
- Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.* **115**, 453–466.
- Brown, M. A., Wallace, C. S., Anamelechi, C. C., Clermont, E., Reichert, W. M., and Truskey, G. A. (2007). The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials* **28**, 3928–3935.
- Cuvelier, D., Thery, M., Chu, Y. S., Dufour, S., Thiery, J. P., Bornens, M., Nassoy, P., and Mahadevan, L. (2007). The universal dynamics of cell spreading. *Curr. Biol.* **17**, 694–699.
- Davis, G. E., and Senger, D. R. (2005). Endothelial extracellular matrix: Biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ. Res.* **97**, 1093–1107.
- Dembo, M., and Wang, Y. L. (1999). Stresses at the cell-to-substrate interface during locomotion of fibroblasts. *Biophys. J.* **76**, 2307–2316.
- Dobereiner, H. G., Dubin-Thaler, B., Giannone, G., Xenias, H. S., and Sheetz, M. P. (2004). Dynamic phase transitions in cell spreading. *Phys. Rev. Lett.* **93**, 1–4.
- Dubin-Thaler, B. J., Giannone, G., Dobereiner, H. G., and Sheetz, M. P. (2004). Nanometer analysis of cell spreading on matrix-coated surfaces reveals two distinct cell states and STEPs. *Biophys. J.* **86**, 1794–1806.
- Eliceiri, B. P., and Cheresh, D. A. (2001). Adhesion events in angiogenesis. *Curr. Opin. Cell. Biol.* **13**, 563–568.
- Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A. (1995). Definition of two angiogenic pathways by distinct alpha v integrins. *Science* **270**, 1500–1502.
- Galbraith, C. G., and Sheetz, M. P. (1997). A micromachined device provides a new bend on fibroblast traction forces. *Proc. Natl. Acad. Sci. USA* **94**, 9114–9118.
- Georges, P. C., Miller, W. J., Meaney, D. F., Sawyer, E. S., and Janney, P. A. (2006). Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys. J.* **90**, 3012–3018.
- Giannone, G., Dubin-Thaler, B. J., Dobereiner, H. G., Kieffer, N., Bresnick, A. R., and Sheetz, M. P. (2004). Periodic lamellipodial contractions correlate with rearward actin waves. *Cell* **116**, 431–443.
- Guo, W. H., Frey, M. T., Burnham, N. A., and Wang, Y. L. (2006). Substrate rigidity regulates the formation and maintenance of tissues. *Biophys. J.* **90**, 2213–2220.
- Irimia, D., Charras, G., Agrawal, N., Mitchison, T., and Toner, M. (2007). Polar stimulation and constrained cell migration in microfluidic channels. *Lab. Chip.* **7**, 1783–1790.
- Johnson, K. R., Leight, J. L., and Weaver, V. M. (2007). Demystifying the effects of a three-dimensional microenvironment in tissue morphogenesis. *Methods Cell. Biol.* **83**, 547–583.
- Jun, H. W., and West, J. L. (2005). Endothelialization of microporous YIGSR/PEG-modified polyurethaneurea. *Tissue Eng.* **11**, 1133–1140.
- Kandow, C. E., Georges, P. C., Janney, P. A., and Beningo, K. A. (2007). Polyacrylamide hydrogels for cell mechanics: Steps toward optimization and alternative uses. *Methods Cell. Biol.* **83**, 29–46.
- King, M. R. (2006). Anisotropic Brownian diffusion near a nanostructured surface. *J. Colloid Interface Sci.* **296**, 374–376.

- Klein, E. A., Yung, Y., Castagnino, P., Kothapalli, D., and Assoian, R. K. (2007). Cell adhesion, cellular tension, and cell cycle control. *Methods Enzymol.* **426**, 155–175.
- Kumar, G., Meng, J. J., Ip, W., Co, C. C., and Ho, C. C. (2005). Cell motility assays on tissue culture dishes via non-invasive confinement and release of cells. *Langmuir* **21**, 9267–9273.
- Lamallice, L., Le Boeuf, F., and Huot, J. (2007). Endothelial cell migration during angiogenesis. *Circ. Res.* **100**, 782–794.
- Lauffenburger, D. A., and Linderman, J. J. (1993). “Receptors: Models for Binding, Tracking and Signalling.” Oxford University Press, New York.
- Lo, C. M., Wang, H. B., Dembo, M., and Wang, Y. L. (2000). Cell movement is guided by the rigidity of the substrate. *Biophys. J.* **79**, 144–152.
- McClay, D. R., Wessel, G. M., and Marchase, R. B. (1981). Intercellular recognition: quantitation of initial binding events. *Proc. Natl. Acad. Sci. USA* **78**, 4975–4979.
- Miranti, C. K. (2002). Application of cell adhesion to study signaling networks. *Methods Cell Biol.* **69**, 359–383.
- Nelson, C. M., Raghavan, S., Tan, J. L., and Chen, C. S. (2003). Degradation of micro-patterned surfaces by cell-dependent and -independent processes. *Langmuir* **19**, 1493–1499.
- Paszek, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., Reinhart-King, C. A., Margulies, S. S., Dembo, M., Boettiger, D., Hammer, D. A., and Weaver, V. M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell* **8**, 241–254.
- Peyton, S. R., and Putnam, A. J. (2005). Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell. Physiol.* **204**, 198–209.
- Pless, D. D., Lee, Y. C., Roseman, S., and Schnaar, R. L. (1983). Specific cell adhesion to immobilized glycoproteins demonstrated using new reagents for protein and glycoprotein immobilization. *J. Biol. Chem.* **258**, 2340–2349.
- Raeber, G. P., Lutolf, M. P., and Hubbell, J. A. (2005). Molecularly engineered PEG hydrogels: a novel model system for proteolytically mediated cell migration. *Biophys. J.* **89**, 1374–1388.
- Reinhart-King, C. A., Dembo, M., and Hammer, D. A. (2003). Endothelial cell traction forces on RGD-derivatized polyacrylamide substrata. *Langmuir* **19**, 1573–1579.
- Reinhart-King, C. A., Dembo, M., and Hammer, D. A. (2005). The dynamics and mechanics of endothelial cell spreading. *Biophys. J.* **89**, 676–689.
- Saadi, W., Wang, S. J., Lin, F., and Jeon, N. L. (2006). A parallel-gradient microfluidic chamber for quantitative analysis of breast cancer cell chemotaxis. *Biomed. Microdevices* **8**, 109–118.
- Sayers, R. D., Raptis, S., Berce, M., and Miller, J. H. (1998). Long-term results of femorotibial bypass with vein or polytetrafluoroethylene. *Br. J. Surg.* **85**, 934–938.
- Schaff, U. Y., Xing, M. M., Lin, K. K., Pan, N., Jeon, N. L., and Simon, S. I. (2007). Vascular mimetics based on microfluidics for imaging the leukocyte endothelial inflammatory response. *Lab. Chip.* **7**, 448–456.
- Sottile, J., Shi, F., Rublyevska, I., Chiang, H. Y., Lust, J., and Chandler, J. (2007). Fibronectin-dependent collagen I deposition modulates the cell response to fibronectin. *Am. J. Physiol. Cell Physiol.* **293**, C1934–C1946.
- Stokes, C. L., Lauffenburger, D. A., and Williams, S. K. (1991). Migration of individual microvessel endothelial cells: Stochastic model and parameter measurement. *J. Cell Sci.* **99**(Pt 2), 419–430.
- Tan, J. L., Tien, J., Pirone, D. M., Gray, D. S., Bhadriraju, K., and Chen, C. S. (2003). Cells lying on a bed of microneedles: an approach to isolate mechanical force. *Proc. Natl. Acad. Sci. USA* **100**, 1484–1489.
- Todaro, G. J., Lazar, G. K., and Green, H. (1965). The initiation of cell division in a contact-inhibited mammalian cell line. *J. Cell. Physiol.* **66**, 325–333.

- Wang, Y. L., and Pelham, R. J., Jr. (1998). Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells. *Methods Enzymol.* **298**, 489–496.
- Wong, J., Velasco, A., Rajagopalan, P., and Pham, Q. (2003). Directed movement of vascular smooth muscle cells on gradient-compliant hydrogels. *Langmuir* **19**, 1908–1913.
- Wu, M., Roberts, J. W., Kim, S., Koch, D. L., and DeLisa, M. P. (2006). Collective bacterial dynamics revealed using a three-dimensional population-scale defocused particle tracking technique. *Appl. Environ. Microbiol.* **72**, 4987–4994.
- Yang, J. T., Rayburn, H., and Hynes, R. O. (1993). Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **119**, 1093–1105.
- Yeung, T., Georges, P. C., Flanagan, L. A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P. A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell. Motil. Cytoskeleton* **60**, 24–34.