CHAPTER 16

Flexible Polyacrylamide Substrata for the Analysis of Mechanical Interactions at Cell–Substratum Adhesions

Karen A. Beningo,* Chun-Min Lo,† and Yu-Li Wang*

* Department of Physiology
University of Massachusetts Medical School
Worcester, Massachusetts 01605

† Department of Physics
Cleveland State University
Cleveland, Ohio 44115

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I. Introduction

Cultured cells undergo complex mechanical interactions with the environment through focal adhesions and other adhesive structures. These interactions form the physical basis for cell migration and provide an important means of communication during embryonic development, tissue formation, and wound healing (Martin, 1997; Bray, 2001). However, despite significant advances in understanding the chemical interactions between cells and the environment, only limited progress has been made in the characterization of such mechanical interactions. To gain a thorough understanding of this process, it is important both to measure the forces exerted by a cell, and to characterize cellular responses to external physical forces. Elastic substrata provide an effective means for achieving both purposes.

A number of methods have been developed for the preparation of elastic substrata. Harris and colleagues first introduced the wrinkling elastic substrata (1980), prepared by polymerizing a thin film on the surface of fluid silicone elastomers. Compressive forces introduced by the cell create wrinkles, which provide a limited, qualitative indication of the forces exerted on the substratum. This approach was later improved to allow a more precise control of the elastic property of the substratum (Burton and Taylor, 1997). In addition, quantitative measurements were made with nonwrinkling silicone film embedded with microbeads (Oliver *et al.*, 1998), which move and recoil upon the exertion and relaxation of forces. Molds with etched micropatterns have been used to cast a matrix of dots or a grid on the silicone elastomer films, which allows precise measurements of mechanical forces based on the distortion of the matrix pattern (Balaban *et al.*, 2001). In addition, flexible cantilevers constructed on a microchip have been introduced as an alternative means for force detection (Galbraith and Sheetz, 1997). However, many of these methods are quite involved and costly in their preparation and do not easily allow the modification of their chemical and/or physical properties.

We have developed flexible sheets of polyacrylamide as substrata for studying the mechanical interactions between the cell and the substratum. This approach has several appealing properties. The material is easily prepared with common materials. The polyacrylamide surface is inert and interactions with the cell are mediated by extracellular matrix proteins covalently linked to the surface. The optical properties allow for high-resolution imaging, and the porous nature of the gel provides a more physiological environment than do glass or plastic surfaces. In addition, the material shows nearly ideal elasticity over a range of applied forces, and the flexibility is easily controlled by varying the concentrations of acrylamide/bisacrylamide without changing the chemical properties. By embedding fluorescent beads, the substratum can be used to measure traction forces with a resolution close to 2 microns. When combined with simple micromanipulation procedures, the substratum allows the application of mechanical forces to cultured cells. This chapter provides an updated account of the method, which has been modified and expanded considerably since the previous description (Wang and Pelham, 1998).

II. Preparation of the Polyacrylamide Substratum

A. Preparation of Glass Surfaces

Thin sheets of polyacrylamide are cast by sandwiching a drop of polymerizing acrylamide solution between two coverslips, or a glass slide and a coverslip. Glass slides are used for supporting the gel for upright microscopes, and large No. 1 coverslips (Fisher Scientific) are typically used for inverted microscopes. Thicker coverslips, although easier to handle, should be avoided because of potential problems with focusing and optical aberration with an inverted microscope. The sheet must adhere tightly to the supporting coverslip or glass slide, to prevent the substratum from floating off during experiments. However it should adhere minimally to the other coverslip, to allow easy removal and exposure of the surface for cell attachment. The former is achieved by chemically activating the glass surface for the covalent attachment of polyacrylamide. The latter is facilitated by siliconizing the glass to make the surface hydrophobic. The method of Alpin and Hughes (1981) for chemical activation, based on glutaraldehyde (detailed later), provides consistent bonding that lasts for 3–5 days in a 37°C incubator. The association lasts for at least several weeks when stored at 4°C.

The clean glass surface to be activated is marked (e.g., with a diamond-tipped pen) and passed quickly through the inner flame of a Bunsen burner to make it hydrophilic. The surface is then smeared with a small volume of 0.1 *N* NaOH using the side of a Pasteur pipette and let dry in air. 3-Aminopropyltrimethoxysilane (Sigma) is smeared over the dried NaOH and the glass incubated for 5 min in a hood. The coated glass is then rinsed extensively with distilled water until the treated surface is clear. Next, 0.5% glutaraldehyde in phosphate-buffered saline (PBS) is pooled onto the treated glass surface and incubated at room temperature in the hood for 30 min. The surface is washed thoroughly with distilled water on a shaker and allowed to dry in air. Activated glass can be stored in a desiccator at room temperature for up to a month.

B. Preparation of the Polyacrylamide Gel

If the glass is to be used in a culture chamber, it may be more convenient to mount it into the chamber before casting the gel (Fig. 1). In our simple design, a chamber is formed by sealing the activated side of the coverslip to a piece of acrylic glass with a hole drilled at the center, using high-vacuum grease (Dow Chemical; McKenna and Wang, 1989). We use stock solutions of acrylamide (40% w/v) and *N,N′*-methylene bisacrylamide (BIS; 2% w/v) from Bio-Rad Inc, which are mixed with distilled water and 1 *M* Hepes, pH 8.5 (final concentration 10 m*M*) to obtain a desirable concentration (see later discussion). If the substratum is to be used for measuring traction forces, fluorescent latex beads (0.2-μm Fluospheres, Molecular Probes, Eugene, OR) are sonicated and added to the mixture, typically at a dilution of 1:30. The mixture is then degassed for a consistent period of time, e.g., 20 min for 5 ml, to ensure reliable polymerization. Polymerization is induced by adding 1/200 volume of 10% ammonium persulfate (Bio-Rad) and 1/2000

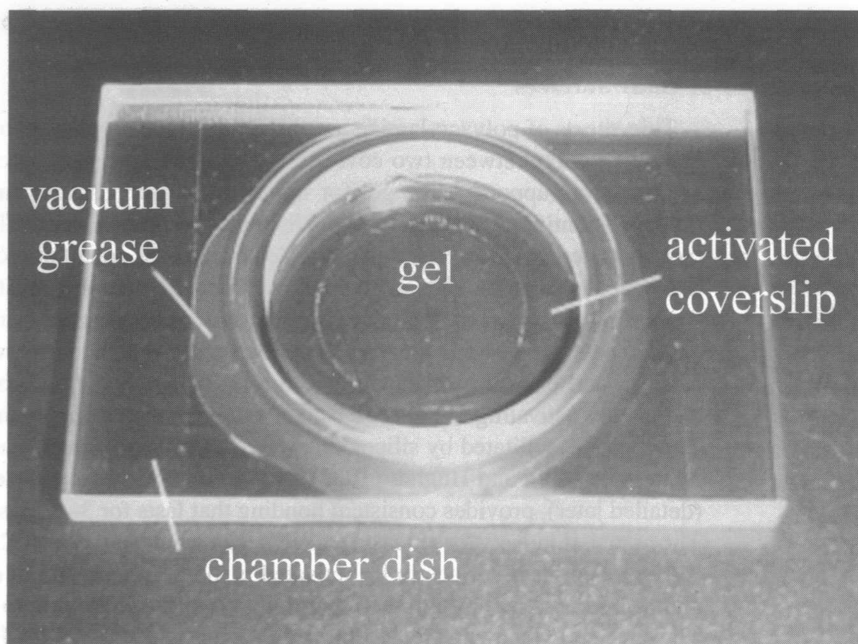

Fig. 1 Cell culture chamber. An image of a 22-μm diameter, 75-μm thick polyacrylamide substratum adhered to a coverglass and mounted with vacuum grease onto a Plexiglas chamber dish. The dish has an overall dimension of $70 \times 48 \times 6$ mm and a hole of 35 mm diameter at the center. An optional groove is machined around the hole for accommodating the lid of a 35 mm petri dish. This simple design allows easy access to the cells and the substratum for micromanipulations.

volume of TEMED (*N,N,N,N'*-tetramethylethylenediamine; Bio-Rad) to the degassed solution. The solution is mixed gently, and a drop of defined volume (discussed later) pipetted immediately onto the activated glass surface. A second coverslip is then placed onto the droplet. The assembly is inverted during polymerization, with the side for cell culture facing down, to encourage settling of the beads to the surface of the substratum. Properly degassed acrylamide should polymerize within 30 min at room temperature. Following polymerization, the chamber assembly is turned right-side-up and the top nonactivated coverslip is removed after flooding the gel with 50 m*M* Hepes, pH 8.5, to reduce the surface tension. We use two pairs of fine-tipped forceps, one for bracing an edge and the second for lifting the opposite edge. The exposed polyacrylamide surface is washed thoroughly with 50 m*M* Hepes, pH 8.5, on a shaker and stored in PBS at 4°C.

C. Helpful Suggestions for Gel Preparation

To obtain clean images, the solutions of Hepes and PBS used for gel preparation should be filtered with 0.22 μm filters to remove particles. The thickness of the gel is determined by the volume used for casting. Typically 15–20 μl is used for a surface

22 mm in diameter and 75–100 μm in thickness. Too small a thickness (<50 μm) would reduce the deformability of the surface, because of the constraint imposed by the bonding between polyacrylamide and the supporting glass surface. Too large a thickness (>100 μm) would cause serious degradation of the image quality.

A wide range of flexibility of the substratum can be obtained by adjusting the concentrations of acrylamide/BIS. The optimal flexibility depends on the cell type and the scientific questions being addressed. For example, to measure the traction forces under an NIH3T3 fibroblast we use 5% (w/v) acrylamide and 0.1% (w/v) BIS. For cells with weaker traction forces, such as neutrophils and neurons, we have used concentrations as low as 5% acrylamide/0.03% BIS. Although it is possible to increase the flexibility by lowering the concentration of either acrylamide or BIS, too low a concentration of BIS would lead to cracking surfaces and eventually failure of polymerization. Thus surface cracking can usually be eliminated by lowering the acrylamide concentration while increasing the BIS concentration.

It is important to avoid excessively flexible substrata, since cell morphology and growth can be adversely affected (Pelham and Wang, 1997; Wang *et al.*, 2000). However, too stiff a substratum would make any displacement difficult to measure. Through systematic trial and error, an optimal composition of acrylamide/BIS (cell dependent) can usually be identified, to yield a displacement of approximately about 10 pixels at the experimental magnification without significantly affecting the cell behavior. If the purpose is to determine the response to different flexibility, then a range of stiffnesses can be obtained by varying the concentration of BIS cross-linker while maintaining a constant total concentration of acrylamide.

D. Conjugation of Matrix Proteins to the Polyacrylamide Substratum

Since tissue cultured cells adhere poorly to naked polyacrylamide, the surface must be coated with extracellular matrix proteins. We have used two types of reagents for covalent conjugation, a photoactivatable heterobifunctional reagent or a classical carbodiimide such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl). The former is quick and effective; however, the latter has worked more consistently for certain proteins such as BSA.

Sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-SANPAH; Pierce Chemical) is a heterobifunctional reagent, one end of which contains a phenylazide group that reacts nonspecifically with polyacrylamide upon photoactivation. The other end contains a sulfosuccinimidyl group, which reacts constitutively with primary amines. A solution of 1 m*M* sulfo-SANPAH in 50 m*M* Hepes, pH 8.5, and 0.5% DMSO is prepared immediately before use (although a more acidic pH may promote the stability of the sulfosuccinimidyl group, it also reduces the solubility of the reagent). The reagent is best solubilized by adding DMSO to the powder followed by the addition of room-temperature Hepes while vortexing. Sufficient solution is added immediately to cover the gel surface, typically 200 μl for a surface of 22 mm diameter. The substratum is then exposed to UV light generated by 302-nm bulbs (VWR #21476-010) at a distance of 2.5 in. for 5–8 min. The photoactivation should cause the sulfo-SANPAH solution to

darken considerably. The solution is then removed and the process repeated with fresh sulfo-SANPAH to ensure activation of the entire surface. The substratum is rinsed with 50 m*M* Hepes, pH 8.5, two to three times to remove any excess reagent. Because of the aqueous instability of the sulfosuccinimidyl group it is important to add the matrix proteins immediately after activation. We typically layer either 0.2 mg/ml type I collagen in PBS or 15 μg/ml fibronectin in Hepes onto the substratum and allow the proteins to react on a shaker at 4°C overnight, although 1–4 h at room temperature should suffice. Other proteins used include fibrinogen, polylysine, and the ECL matrix mixture (Upstate Biotechnology). Laminin is sensitive to chemical modifications and is best coated secondarily on top of a surface of polylysine. Coated substratum is rinsed with several changes of PBS on a shaker and may be stored for up to 6 weeks in PBS at 4°C.

The carbodiimide method relies on the reaction of EDC (Pierce Chemicals) with a free carboxyl group, forming an amine-reactive intermediate for protein conjugation (Grabarek and Gergely, 1990). To supply a free carboxyl group, acrylic acid is included in a solution of 8% acrylamide/0.05–0.2% BIS solution to a final concentration of 0.2%. This concentration of acrylic acid should be adjusted in proportion to the concentration of total acrylamide, to avoid inhibition of polymerization. After polymerization, the substratum is washed thoroughly in 0.1 *M* MES (2-[*N*-morpholino]ethanesulfonic acid; Sigma), pH 4.9. A solution of EDC at 26 mg/ml in 0.1 *M* MES is pooled onto the polymerized substratum and incubated at room temperature for 2 h on a shaker. The substratum is then rinsed three times with the MES buffer and the protein of interest (diluted in MES) is pooled immediately onto the substratum and incubated overnight at 4°C on a shaker. After the reaction, the substratum is rinsed with PBS and stored a 4°C. The amine-reactive intermediate of EDC is highly unstable in aqueous solutions. This may be compensated by using a high concentration of both EDC and protein as in the present procedure, although NHS (*N*-hydroxysuccinimide; Pierce) may be included in the solution for an increased stability (Staros *et al.*, 1986). It should be noted that the buffer used to solubilize proteins can affect the coupling reaction with either method. In particular, Tris buffer should be removed by dialysis and the pH should not deviate substantially from neutrality.

III. Characterization of the Gel

A. Characterization of Protein Coating

The density of coated protein is determined by standard indirect immunofluorescence procedure, using fluorescent secondary antibodies with a different color from that of the fluorescent beads in the substratum. The intensity of fluorescence is measured with a cooled slow-scan CCD camera attached to the microscope. This method provides a simple means for determining the relative density of coating. However, it does not take into account the possibility that some proteins may be able to penetrate into the substratum, and may not be available for interactions with the cell. To determine the density of proteins on the surface, we use secondary antibodies conjugated to the surface of fluorescent particles (1 μm diameter; Polysciences), in a manner identical to the

application of standard secondary antibodies (Lo *et al.,* 2000). Since large beads may create steric hindrance for the binding, the smallest available size should be used for this purpose.

B. Characterization of Mechanical Properties

Young's modulus is a measurement of elasticity. It is required not only for the calculation of traction forces but also for the qualitative comparison of cell behavior as a function of substratum flexibility. We have used three methods to measure the Young's modulus of polyacrylamide substratum. The gel strip method, as previously described by Pelham and Wang (1997), measures the elongation of strips of gels upon the application of a defined weight. Young's modulus is calculated by the equation

$$Y = (F\perp/A)/(\Delta l/l)$$

where l is the original length of the gel strip, Δl is the change in length, A is the cross-sectional area, and $F\perp$ is the applied force (1 g of weight applies 980 dynes of force). The microneedle method measures the horizontal deformation of the gel when lateral forces are applied through a bending microneedle (Lee *et al.,* 1994). The calculation is based on the deformation of the gel and the corresponding deformation of the needle, whose bending behavior is calibrated with known submilligram weights. Although conceptually straightforward, these methods become very difficult as the flexibility of substrata increases and the gels become increasingly susceptible to tear.

An alternative method, suitable for a wide range of stiffness, measures the depth of surface depression caused by the weight of a steel microball (0.64 mm in diameter, 7.2 g/cm^3, Microball Company, Peterborough, NH; Lo *et al.,* 2000). The theoretical basis is similar to that used for reological measurements in atomic force microscopy (Radmacher *et al.,* 1992). A microball is dropped with a pair of fine-tipped forceps onto the surface of the polyacrylamide substratum containing fluorescent beads, placed on the stage of a leveled inverted microscope. The microscope is first focused on the fluorescent beads immediately underneath the center of the microball. The microball is then removed with a magnet, and the upward movement of the substratum surface determined with the focusing mechanism of the microscope. Young's modulus is calculated as

$$Y = 3(1 - \nu^2)f/4d^{3/2}r^{1/2}$$

where f is the force exerted on the gel (calculated based on the volume and density of the microball and corrected for buoyancy), d is the indentation, r is the radius of the steel ball, and ν is the Poisson ratio. This method can be most easily performed with a motorized focusing mechanism.

When measuring Young's modulus with any method, it is critical that the substratum be equilibrated with the medium and at the temperature for the actual experiment, because of the shrinking and swelling of the gel in response to osmolarity and temperature. For the same reason, the thickness of the gel cannot be simply calculated from the volume of the solution for polymerization. The measured thickness at steady state is typically 3–4 times that of the calculated value.

Calculation of traction forces also requires an estimate of the Poisson ratio, which measures the compressibility of the material. Accurate measurement of Poisson ratio is difficult; however, the value does not vary dramatically with the acrylamide concentration (Li *et al.,* 1993). A value of ~0.3 was reported in a published study (Li *et al.,* 1993).

IV. Data Collection for the Analysis of Traction Forces

A. Substratum Preparation

A variety of cell types have been studied on the polyacrylamide substratum including fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, cardiomyocytes, macrophages, neutrophils, and neurons. To prepare the coated substratum for seeding, the substrata are exposed for 10 min to the irradiation from the germicidal (UV) light of a tissue culture hood. This "quasi-sterilization," in combination with a mixture of antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin), keeps contamination under control for at least 2–3 days. The substratum is then incubated for 1 h in growth medium in an incubator, to ensure equilibration of the space within the gel with the medium. For the study of traction forces, it is important that cells be plated at a low density and that neighboring cells be separated by at least one cell's length, to minimize the superimposition of deformation by forces from multiple cells. We typically seed fibroblasts at ~5000 cells per substratum of 22 mm diameter. If the density is too high, a microneedle mounted on a micromanipulator may be used to clear unwanted cells immediately before the experiment.

B. The Microscope Setup

To maintain healthy cells during data acquisition, it is essential that temperature, humidity, and CO_2 be controlled and monitored carefully. Various aspects of cell culture on the microscope stage have been described in detail previously (McKenna and Wang, 1989). We use custom-designed, Plexiglas incubators that completely enclose the stage of an inverted microscope and the space surrounding the objective lens (Carl Zeiss, Inc). To minimize evaporation of the medium, mineral oil (Sigma #400-5) is layered onto the surface of the medium (however, this also prevents the subsequent replacement of the medium or addition of drugs). We prefer a simple chamber with easy access to the cells (Fig. 1). Although a number of microscope incubators/chambers are commercially available, many of them confine cells within a sealed space and limit the accessibility for cell manipulation, which is required for a number of purposes as discussed later.

Besides suboptimal culture conditions, excessive light used for imaging fluorescent beads also causes cell damage. To minimize the light exposure we use a 12V–100W quartz halogen lamp connected to a variable power supply, and set the input voltage at a level just sufficient for imaging. Heat from the lamp is removed with a heat-absorbing filter (BG-38) and/or heat-reflecting mirror. To further reduce the radiation damage, the light is controlled with an automated shutter. Although fluorescent beads are brightly

labeled, it is recommended that a cooled, high-sensitivity CCD camera be used to reduce the level of excitation light.

A microscope equipped with a 40× phase objective is typically used for the measurement of substratum deformation. The strong signal from fluorescent microspheres allows nonimmersion lenses to be used for most purposes. However, if additional fluorescence images such as GFP signals are to be collected, a high-N.A. immersion lens may be necessary. Because of the thickness of the substratum, image quality may be seriously degraded by spherical aberration when using an inverted microscope. The degree of aberration varies with the lens design, and some objectives have proven to be surprisingly tolerant (the Zeiss 40× Plan-Neofluor, phase 2, N.A. 0.75, works well). An ideal, although costly, solution to this problem is to use a long-working-distance, water-immersion lens designed for deep focusing in confocal microscopy.

In addition to fluorescent beads in the substratum, the cell itself may be labeled with fluorescent probes such as microinjected analogs or GFP-tagged proteins, to allow simultaneous observations of dynamic changes in focal adhesions or cytoskeletal components (Beningo *et al.*, 2001; Kaverina *et al.*, 2000). Microinjection of cells on flexible substrata is possible, although considerably more difficult than the injection of cells on coverslips, because of the movement of the substratum upon needle penetration. In addition, the injection can cause profound, reversible effects on traction forces; therefore the cell should be allowed to recover for a period of 30–60 min before observation. On the other hand, GFP-tagged proteins, expressed through transient and stable transfection, can be readily observed in conjunction with red fluorescent beads (Beningo *et al.*, 2001), for determining the relationship between traction forces and focal adhesions.

C. Image Collection

Generally a target cell is selected with a dry 40× objective lens based on several criteria. First, the cell must have a normal healthy morphology. Second, the beads under the cell should be evenly dispersed and as dense as possible while allowing individual beads to be resolved. Third, the distance of the cell from the edge of the substratum (which can be marked before observation with a Magic Marker) and from neighboring cells should be at least 200 μm. For each time point, a phase image of the cell and a fluorescent image of the beads are collected. Some find it helpful to also collect an image with simultaneous phase and fluorescence illumination. These images should be recorded quickly within seconds of each other.

Since the displacement of beads decreases rapidly as a function of the depth into the substratum, it is important to focus the microscope on the layer immediately underneath the cell. The most challenging aspect of data collection is to maintain a consistent level of focusing. Because the beads and the cell do not lie on the same focal plane, refocusing is mandatory when taking each set of phase and fluorescence images. Some practice in manual focusing is required to acquire a set of bead images where the corresponding beads are focused consistently throughout the sequence. Generally it is easier to focus on a unique pattern of beads that lie at some distance from the cell. Alternatively,

automatic, motorized focusing mechanisms on some new microscopes may be used to great advantage for this purpose.

After the desired number of images is collected, a bead image without the cell is collected, which shows the position of beads without forces. This "null-force" image is compared with each previous image to determine the direction and distance of displacements. The simplest approach to remove cells is to treat the dish with Triton or trypsin. Unfortunately this also sacrifices the rest of the dish. Alternatively, cytochalasin B or D may be used to inhibit fibroblast traction forces (Pelham and Wang, 1999), although it requires several hours after extensive washes before the dish can be used again. A more selective method is to simply "pluck" the cell off the substratum with a microneedle and a micromanipulator, or to microinject a substance such as Gc-globulin to relax the forces.

V. Analysis of Traction Forces

The images of beads may be analyzed qualitatively or quantitatively to extract the information on traction forces. Here we will describe the analysis in qualitative terms. A more rigorous discussion of the algorithm involved will be described in a separate article (Marganski, Dembo and Wang, in press).

A. Generation of Deformation Data

Before analysis, it is essential to align all of the bead images to correct any movements due to the drift of the stage or sample. An area far away from the cell, with clearly defined, presumably stationary beads, is selected as the reference region. A computer algorithm based on pattern recognition is then used to search for the corresponding pattern throughout the set of bead images, and the images are translated accordingly to make the reference region stationary. Alternatively, manual inspection can be used to register a pair of images, by displaying the images alternatively on the screen and "panning" the images relative to each other until no wobbling of the reference region is observed.

Once the images are registered, the second step is to map the deformation of the substratum. Although manual comparisons may be performed to determine relative movements of individual beads in a pair of images (Kaverina *et al.,* 2000), this is tedious and time-consuming. We have developed an automated approach based on computer pattern recognition. The program divides the image into many small regions, each with a unique pattern of beads. Positions containing the corresponding patterns are identified in the null-force image and the image with cell-exerted forces, and a map of vectors is generated accordingly.

B. Analysis of Substratum Deformation

Qualitative analysis of traction forces may be performed based on the deformation vectors. Regions with many large vectors generally correspond to regions with strong

tractions, and the direction of these large vectors is generally close to the direction of exerted forces. However, it is important to note that the deformation propagates and superimposes across the substratum surface, and the magnitude of deformation does not simply correspond to the magnitude of traction forces at the corresponding position. Therefore a precise value of traction force cannot be obtained simply by multiplying the deformation by the Young's modulus.

Quantitative analysis of traction forces can only be obtained by a "deconvolution" process, which mathematically identifies a set of forces within the cell boundary that best recreates the observed deformation (Dembo and Wang, 1999). The cell boundary provides an important condition for the calculation and can be defined interactively with the phase image using a drawing program. The calculation generally yields a map of traction stress, i.e., forces per unit area.

C. Visualization of Traction Forces

The distribution of traction stress vectors can be visualized as a map of arrows, as standard practice for studying vectorial fields (Fig. 2A). However, an equally useful approach is to render the magnitude of traction stress vectors as color images, with "hot" color corresponding to strong forces and "cool" color corresponding to weak forces (Fig. 2C). Vector plots have the advantage of displaying both the directional and magnitude information. Color rendering, on the other hand, proves to be superior for visualizing dynamics in time-lapse studies (Munevar *et al.*, 2001).

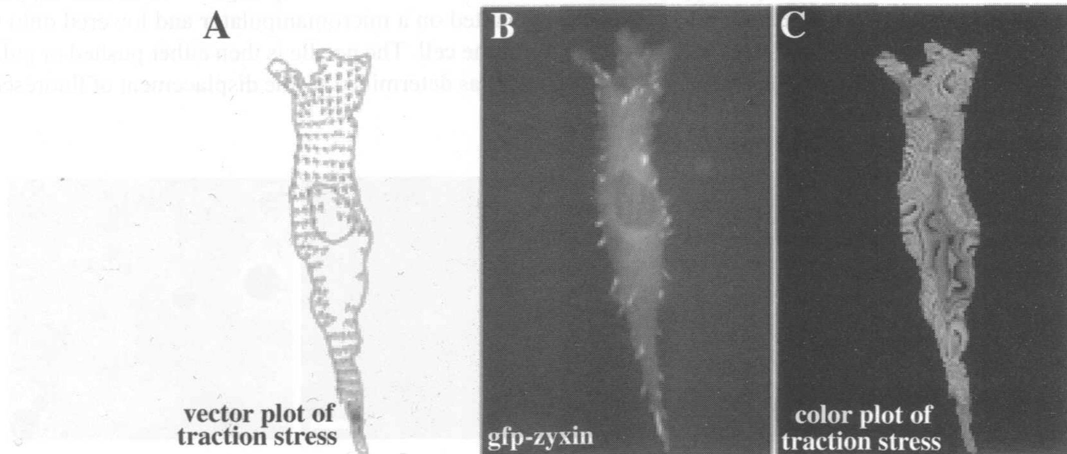

Fig. 2 Visualization of traction stress. (A) A vector map of traction stress shows the direction and magnitude of forces per unit area generated by a fish fin fibroblast. (B) GFP-zyxin identifies the focal adhesions. (C) Color rendering of the magnitude of traction stress, where "hot" colors (red) indicate strong forces and "cool" colors weak forces. (See Color Plate.)

VI. Other Applications using Polyacrylamide Substrata

The chemical characteristics (flexibility, inertness) and optical clarity of polyacrylamide have made it useful for addressing a number of biological questions of a mechanical nature. In addition to the measurement of traction forces, we have used the polyacrylamide substrata for the application of mechanical stimulation, for observing cellular responses to a gradient of substratum flexibility, and for testing the effects of target flexibility on phagocytosis.

A. Application of Mechanical Stimulations

This technique involves pushing and pulling the substratum with a blunted microneedle that grips the substratum near a cell (Fig. 3A). Forces are transmitted across the substratum and through the adhesion sites into the cell, eliciting interesting responses (Lo *et al.*, 2000). Unlike many other methods of mechanical stimulation, this approach allows a highly localized and defined mechanical input to be exerted and the immediate responses of the cell to be observed.

A relatively stiff polyacrylamide substratum of 5–8% acrylamide/0.1% bisacrylamide is typically used, to minimize tearing of the gels. Blunted microneedles are prepared by first pulling glass capillary tubing in a micropipette puller, then shaping the tip with a microforge (Narashige). The latter is done by placing the tip near the heating element of the microforge until the tip melts into a tiny sphere. The shank of the microneedle is then softened by heating, to create a kink several hundred microns behind the tip. This allows the tip to approach the substratum surface at a steep angle to minimize slippage. The blunted microneedle is then mounted on a micromanipulator and lowered onto the substratum within 50–100 microns of the cell. The needle is then either pushed or pulled to create the desired local deformation, as determined by the displacement of fluorescent

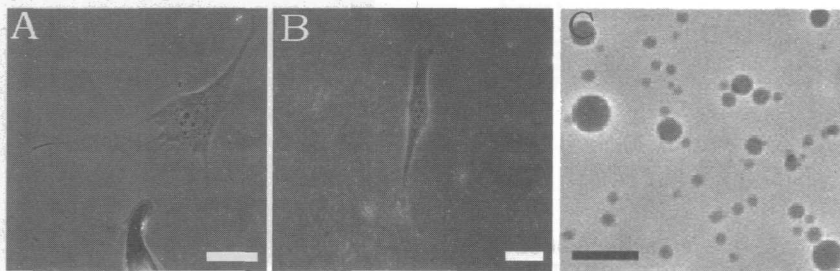

Fig. 3 Other applications using polyacrylamide substrata. (A) Localized mechanical stimulation is exerted on the cell by pushing or pulling on the substratum with a blunted microneedle (Scale bar = 40 μm). (B) Cellular responses to changes in substratum rigidity are tested with a gradient substratum. Since only the soft side contains fluorescent beads, changes in substratum rigidity is visualized as changes in the bead density (Scale bar = 40 μm). (C) Polyacrylamide microbeads of 1–6 μm generated in a microemulsion system (Scale bar = 10 μm).

beads underneath the tip. The magnitude of exerted forces can be estimated based on the displacement of fluorescent beads under the tip of the needle (and the Young's modulus of the material). The actual force exerted on the cell is strongly dependent on the distance from the needle, such that significant forces are generally limited to the region proximal to the microneedle.

B. Preparation of Substrata with a Gradient of Stiffness

Substrata with a gradient of stiffness have been used to test the hypothesis that cells are able to read local mechanical cues for the guidance of their migration (Lo *et al.*, 2000). The substratum is created by placing two-10 μl droplets of activated acrylamide/BIS acrylamide mixtures at different concentrations next to each other on activated glass, followed by careful placement of a coverslip to flatten and merge the two droplets. In this procedure, only the concentration of BIS is varied while the total acrylamide concentration, (acrylamide plus BIS), is maintained at a constant level. The boundary between hard and soft substrata is identified by adding fluorescent beads to one of the mixtures (Fig. 3B). The transition area between high and low rigidity is typically 50–100 μm in width, as judged from the distribution of beads. It is important to perform experiments on such gradient substrata at a low cell density, as both direct cell–cell interactions and forces generated by neighboring cells and transmitted across the substratum can obscure the effect of the change in substratum flexibility.

C. Preparation of Polyacrylamide Beads

Microbeads of polyacrylamide (Fig. 3C) have been used to test the sensitivity of phagocytosis to the flexibility of the target (Beningo and Wang, 2002). The beads are prepared in a microemulsion system, using bis(2-ethylhexyl) sulfosuccinate (AOT) (Fluka Chemicals) in toluene as the emulsifier. This agent is capable of forming inverse micelles (i.e., micelles of aqueous solution in an organic solvent) of relatively uniform size. The size of the beads is determined by several factors, including the AOT concentration and the relative volume of the aqueous solution to the organic solvent (Kunioka and Ando, 1996; Candau and Leong, 1985).

For phagocytosis studies we have generated beads 1–6 μm in diameter. The solution of acrylamide is prepared as described earlier, except that, instead of fluorescent beads, a high molecular weight FITC dextran (464 kDa, Molecular Probes) is added at a concentration of 20 mg/ml. This becomes is trapped within the polyacrylamide beads and serves as a label. AOT is dissolved in toluene in a fume hood at a concentration of 10.2 mg in 1 ml. While stirring under a stream of nitrogen, degassed acrylamide mixture is added to the AOT solution. The beads are allowed to polymerize for 1 h with constant stirring under nitrogen. Beads are recovered by centrifugation at 23*g* for 5 min and washed repeatedly (3–5 times) in methanol to remove the emulsifier. Following multiple washes in PBS, the beads can be stored at 4°C for up to 8 months. Conjugation of proteins to the beads is carried out as for polyacrylamide substrata, although the method with EDC has yielded more consistent results. Because the beads swell and

shrink in response to changes in osmolarity and temperature, it is important to acclimate them to the experimental condition before use. Protein-conjugated beads are used within 2 days.

VII. Summary

We have described a powerful tool for the study of mechanical interactions between cells and their physical environment. Although the approach has already been used in a variety of ways to measure traction forces and to characterize active and passive responses of cultured cells to mechanical stimulation, it can be extended easily and combined with other microscopic approaches, including fluorescent analog imaging (Beningo *et al.*, 2001), photobleaching, calcium imaging, micromanipulation, and electrophysiology. This method will be particularly useful for studying the functions of various components at focal adhesions, and the effects of mechanical forces on focal adhesion-mediated signal transduction. In addition, the method can be extended to a 3D setting, e.g., by sandwiching cultured cells between two layers of polyacrylamide to create an environment mimicking that in the tissue of a multicellular organism.

Whereas chemical interactions between cells and the environment have been investigated extensively, many important questions remain as to the role of physical forces in cellular functions and the interplay between chemical and physical mechanisms of communication. The present approach, as well as other approaches capable of probing physical interactions, should fill in this important gap in the near future.

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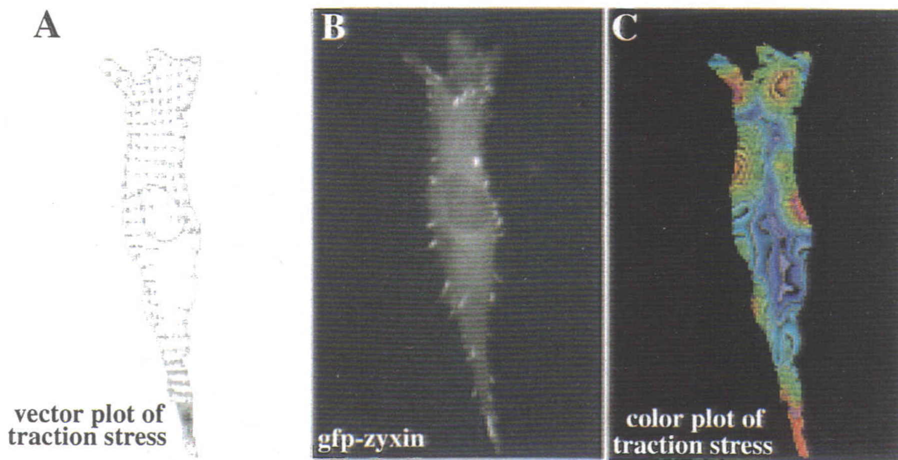

Fig. 16.2 Visualization of traction stress. (A) A vector map of traction stress shows the direction and magnitude of forces per unit area generated by a fish fin fibroblast. (B) GFP-zyxin identifies the focal adhesions. (C) Color rendering of the magnitude of traction stress, where "hot" colors (red) indicate strong forces and "cool" colors weak forces.