Chapter Twelve

Micropost Methods for Cell Biomechanics of the Cardiovascular System

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The cardiovascular system is more than a biological pump and a system of pipes. Unlike a typical plumbing system, the human body actively changes the size of its pump and pipes through a process of mechanotransduction. Understanding this process at a cellular level enables a greater understanding of the entire system at a fundamental level. This chapter focuses on studies using flexible polymer microposts, which can measure the contractile forces of cardiovascular cells with nanoscale precision. Specifically, microposts have been used to understand how cardiomyocytes, endothelial cells, and platelets change their function in response to react to changes in their microenvironment and how these changes relate to diseases and disorders of the cardiac, vascular, and coagulation systems.

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12.1 BACKGROUND ON CARDIOVASCULAR SYSTEM

The cardiovascular system pumps blood throughout the body to regulate the supply and removal of cells, proteins, or molecules that regulate metabolism, growth, healing, and the immune response. The function of the cardiovascular system is classically understood on a macroscale level, but there are opportunities to learn more about its physiology at a microscale level. For example, the function of the heart is to pump blood, but its individual cells must contract with ample power in order to achieve this effect.

Three major cell types in the cardiovascular system are highlighted in this chapter: cardiomyocytes, endothelial cells, and platelets. These cells serve a unique set of functions in the cardiovascular system: pumping, containment, and wound healing. Cardiomyocytes contract together, allowing the heart to beat and force blood through the vascular system. Endothelial cells line the inside of vessel walls, serving as a barrier between blood and the underlying vascular tissue. Platelets act as first-responders in wound healing by attaching to a damaged vessel wall and preventing further blood loss.

Both muscle and nonmuscle cells are able to produce contractile forces via their actin and myosin proteins. In particular, cellular forces are important for adhesion, proliferation, migration, contraction, wound healing, and mechanotransduction. For cardiomyocytes, endothelial cells, and platelets, their cellular forces are important for pumping blood, maintaining their barrier function, or forming stable clots. The study of cell forces is a relatively new field and the techniques to fabricate devices has improved over the years, producing refined tools on the micro- and nano-scale. There is a growing understanding on the relationship between cellular forces and cellular function, which has enabled new technologies and treatments for cardiovascular disease.

12.2 BACKGROUND ON MICROPOSTS

The study of cellular traction forces was pioneered by Harris *et al.* in 1980 using thin films of silicone rubber [6]. Cells seeded on the thin films pulled on the surface of the silicone and produced wrinkles that were visible under an optical microscope. The number and size of these wrinkles indicated the magnitude of the tract force generated by the

cells. Although it was a novel and exciting development, the initial tool was qualitative measurement of cellular traction forces and not a quantitative one.

In response to the shortcomings with wrinkling silicone membranes, traction force microscopy was developed [7]. In order to quantitatively determine cellular traction forces, beads were placed onto a sheet of silicone rubber and when cells exerted forces, the beads moved with the deformation of the silicone sheet. The motion of the beads coupled with the elasticity of the silicone enabled a quantitative measurement of the cellular traction forces. This technique was improved upon with the use of polyacrylamide gels instead of silicone rubber, which was softer and enabled larger deformations that were easier to track [8].

Measuring cellular traction forces took another step forward when cantilevers were used for cell migration studies [9]. In the first incarnation, a cantilever was oriented horizontally and situated underneath a flat surface in which there was a small opening where the cell could attach to the tip of the cantilever. As a cell crawled across the surface, its traction force on the tip of the cantilever caused entire cantilever to bend. The cantilever acted like a spring where the movement of its tip was proportional to the applied force. This tools was novel for it gave insight into the dynamics of cellular traction forces during migration, but it suffered from being able to only report force at one location, rather than at each point of adhesion that a cell has with a substrate. Recently, vertical cantilevers made from silicone rubber were developed to determine cellular traction forces [10]. The mechanics and techniques behind using vertical cantilevers will be discussed in detail in this chapter.

12.2.1 *Overview of Micropost Technology*

Micropost technology consists of an array of vertical posts that bend under traction forces of cells attached onto the tips of the posts. The posts are made out of polydimethylsiloxane (PDMS) and are functionalized with extracellular matrix protein to enhance cell adhesion (Figure 12.1). Once a cell has attached to the tips of the posts, they are able to contract, thereby deflecting the tip of each post while the bottom of each post remains stationary. Consequently, the total deflection of each post can be determined by comparing the location of the tip of each post with the

location of the bottom of each post. Typically, microposts are stained with fluorescent dye and bending of the posts is measured using custom image analysis software. Each post deforms independently of other posts in the array and the force at the tip acts as a single point load such that each post can be treated as a fixed beam cantilever with a point force load on the end. Microposts are commonly made with a circular cross section, which gives the relation between the force and the deflection of the top of the beam in Eq. 12.1, where F is the force, E is the Young's modulus of the material, d is the diameter of the post, L is the height of the post, and δ is the deflection of the top of the post relative to the bottom (Figure 12.1 C).

$$F = \frac{64E\pi d^4}{3L^3}\delta \tag{12.1}$$

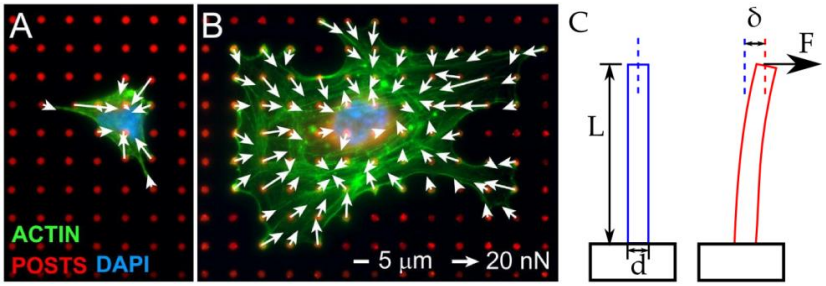

Figure 12.1. A) and B) Cells seeded onto micropost arrays with force vectors. C) Diagram of the deflection of a post with relevant variables.

12.2.2 *Manufacturing Methods of Microposts*

Microposts are created using a soft lithography process. First, a master structure can be fabricated using SU-8 photoresist in a clean room (Figure 12.2). First, SU-8 is spun onto a clean silicon wafer to the desired height of the posts (typically 5-10 µm). Next, the SU-8 is exposed to light in a pattern of dots and then developed to produce a master array of posts. In order to prevent PDMS from adhering to the master when fabricating negative molds, a vapor fluorosilane treatment is performed

on the posts. The fluorosilane creates a self-assembled monolayer of silane on the SU-8 master, preventing adhesion of PDMS to the master. After silane treatment, a PDMS negative mold is fabricated from the master and treated subsequently with silane. The negative mold is then used with a ratio of 10:1 base to crosslinking agent of PDMS to create the final version of posts, typically casting against a glass slide activated with plasma and baking in an oven at 110 °C to fully cross-link the PDMS microposts. After the array has been peeled from the negative mold, they are ready for functionalization with protein to enable adhesion of the cells to the surface.

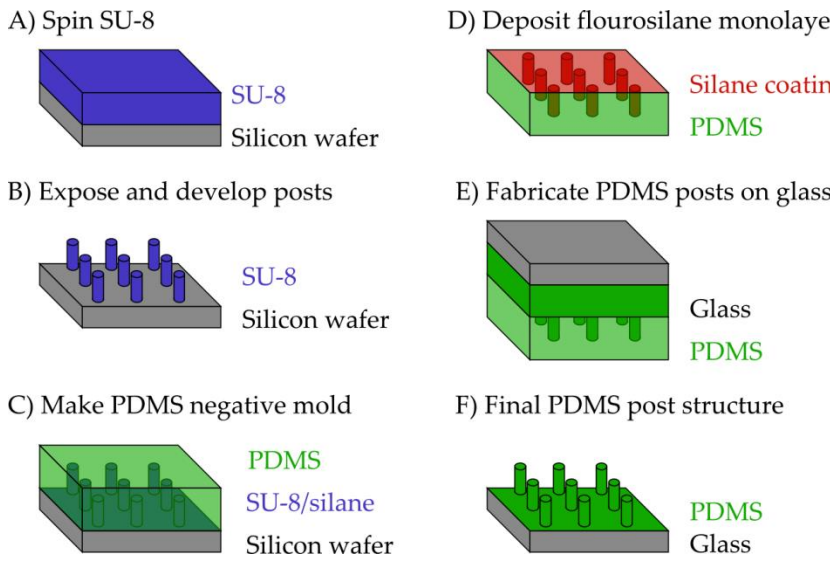

Figure 12.2. Fabrication of PDMS microposts starting with an SU-8 master.

Functionalizing the PDMS microposts for cell adhesion involves cleaning the posts, adhering protein to the tips, and blocking the remaining areas of the posts. First, the posts are cleaned with UV-Ozone to ready them for protein adhesion. Rather than directly seeding protein on the tips of the posts, protein is absorbed onto a stamp that is also made out of PDMS by using a 30:1 base to curing agent ratio to make it more flexible and offset any small difference in height of the post. After the protein has absorbed onto the stamp in a monolayer, the stamp is

dried and then placed in direct contact with the tops of the posts. After removing the stamp, the posts are dipped in ethanol before they are placed in water in order to make them more hydrophilic, preventing collapse. Finally, the posts are placed in media, and then cells are seeded on top of them in a ratio appropriate to the specific experiment being conducted.

Although fabricating PDMS posts from an SU-8 master is fairly straightforward, there has been some research regarding the repeatability of each set of posts in terms of dimensions and properties. A negative mold made out of PDMS is highly desired as it enables the user to create a thin layer of PDMS under the posts and to more easily peel the mold from the glass without collapsing the posts. Unfortunately, thermal fracture of the necessary silane layer on the PDMS has been discovered to create problems when fabricating more than one set of posts from a single negative mold [11]. Because of the thermal fracture, molds can only be used for a maximum of two times and then a new set of molds needs to be made which involves another treatment of silane. Additionally, the silane treatment that is required on the negative mold can affect the actual size of the post if it is not consistent [12], which is particularly important when fabricating posts in the nanoscale range. Furthermore, the properties of PDMS have been questioned as factors such as mixing ratio, mixing time and curing time have been found to affect the Young's modulus [13-15]. Ensuring the same properties of PDMS on each array of posts is vital to ensuring accurate experiments and comparisons.

Aside from a simply passive environment, nanowires have been embedded in the PDMS microposts to apply forces to a discrete region of focal adhesions in a cell and determine how the cell responds to the motion of the post [16]. By embedding cobalt wires into the microposts and applying a uniform magnetic field across the substrate, a single post under a cell was moved up to 0.8 μm. The novel approach of actuating single focal adhesions showed a connection between the cell and the other regions of the cell. The traction forces were either reduced or increased throughout the cell depending on the proximity to the magnetic posts and whether the point of measurement was on the periphery of the cell or the interior.

12.3 CARDIOMYOCYTES CONTRACT ON MICROPOSTS

Cardiomyocytes are vital to the cardiovascular system for they work to pump blood throughout the body. Cardiomyocytes beat constantly and do not typically regenerate once the body has reached maturity. Significant research is focused around how to develop cardiomyocytes to be strong enough in order to graft them into damaged regions of a heart. Additionally, the stiffness of the heart changes significantly from the time of birth until maturity as well as after infarctions [17, 18]. The change in stiffness is thought to have an effect on many properties, and microposts were used to study the effects of stiffness on the force and power generation of cardiomyocytes.

Several researchers have looked at the forces during beating of cardiomyocytes, and have successfully seeded them onto PDMS microposts and had them beat. Zhao and Zhang put cardiomyocytes onto posts and measured the displacement of cardiomyocytes during a beating cycle [19]. After converting the displacement of the posts to forces per Eq. 12.1, they measured the maximum contraction forces relative to the static force of the cardiomyocytes. Kim *et al.* measured the forces throughout the spontaneous beating cycle [20] of rat neonatal cardiomyocytes cultured on PDMS posts and showed the time-varying nature of the forces. These studies showed the ability to measure time-varying forces on microposts and looked at the forces while the cell was beating in an attempt to measure the peak contractile force.

12.3.1 *Power of Neonatal Cardiomyocytes Measured with Microposts*

Rodriguez, *et al.* observed rat neonatal cardiomyocytes on microposts of varying stiffnesses to study the effects of stiffness on intracellular calcium, force, and power of cardiomyocytes [4]. The microposts the researchers used had different heights and diameters to change the stiffness of the posts as given by Eq. 12.1. They discovered that increasing the stiffness of the posts increased the force generation of the cardiomyocytes (Figure 12.3D). Furthermore, the researchers developed a line-scanning program to determine the velocity of each post while the cell was contracting. By analyzing the velocity, the researchers determined the power of the cardiomyocyte and discovered that power

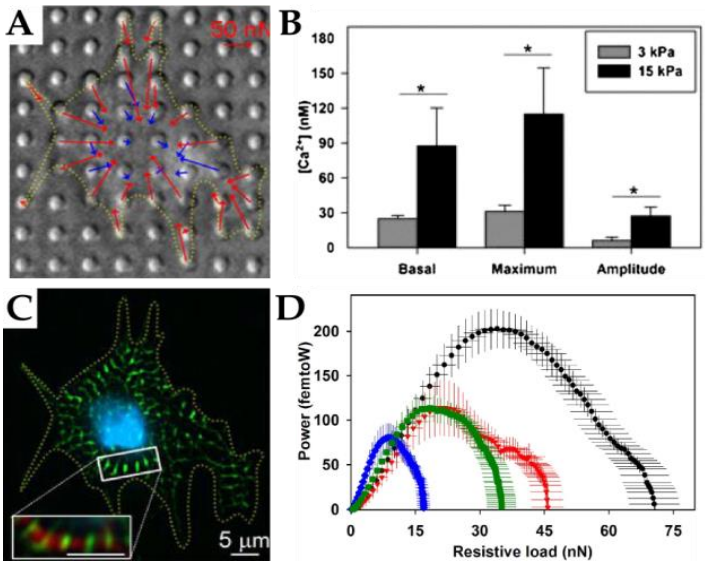

Figure 12.3. Stiffness affects neonatal cardiomyocytes. A) The force is determined by micropost deflection with stronger forces on the periphery (red) than the interior (blue). B) The basal, maximum, and amplitude of the intracellular calcium concentration are higher on stiffer substrates. C) The z-bands are characterized by staining for α-actinin (green) and actin filaments (red). D) Microposts were used to determine the power generation, which also increased for cells on increasingly stiff substrates of 3 kPA (blue), 8kPa (green), 10kPa (red), and 15 kPA (black). Adapted from Rodriguez, *et al.* [4].

also increases with stiffness (Figure 12.3D). Finally, the researchers looked at the intracellular calcium levels and found that calcium was also increased as stiffness was increased (Figure 12.3B). The novel findings of this study show a correlation between the stiffness of the substrate and three main factors of the health of neonatal cardiomyocytes.

12.4 ENDOTHELIAL CELLS PRODUCE FORCE ON MICROPOSTS

Endothelial cells in the cardiovascular system are vital to withstanding the pressure of the blood flow and regulating the transport of cells and molecules to the underlying tissue. The endothelium is a monolayer of cells and is characterized by tight junctions between neighboring cells.

Without the barrier function of endothelial cells, there can be uncontrolled regulation of transport into the walls of the arteries, which is typically thought to be the onset to atherosclerosis [21, 22]. In addition to complications with atherosclerosis, the endothelium plays a role in the growth and spread of cancer [23, 24]. Because of the unique nature of the role of cell force, particularly intercellular forces, microposts have been used with endothelial cells in novel patterns and experiments to study how the endothelial forces are affected through external factors.

12.4.1 *Endothelial Cell Patterning on Microposts*

Previous studies have shown that both cell area and cell stiffness affect the ability of cells to produce force [25, 26]. Recently, a hypothesis has suggested that the spread area of a cell and the traction force the cell generates are closely related. The spread area affects the average focal adhesion size which in turn affects the maximum force generation of the cell (**Figure 12.4**). Additionally, the substrate stiffness also affects the spread area and the force generation [1]. In order to determine the relation between force generation and spread area, the cells needed to be patterned to a specific size rather than allowing the cell to freely spread on a surface. During the stamping step, patterns of squares were stamped off of a 30:1 base to curing agent PDMS stamp and were then stamped onto the tops of the micropost arrays. By stamping the posts in a specific pattern, more information about the cells was obtained. Microcontact printing is a common method to pattern cells, and it has also been successfully applied to microposts to conduct studies on specific patterns and sizes of cells and groups of cells.

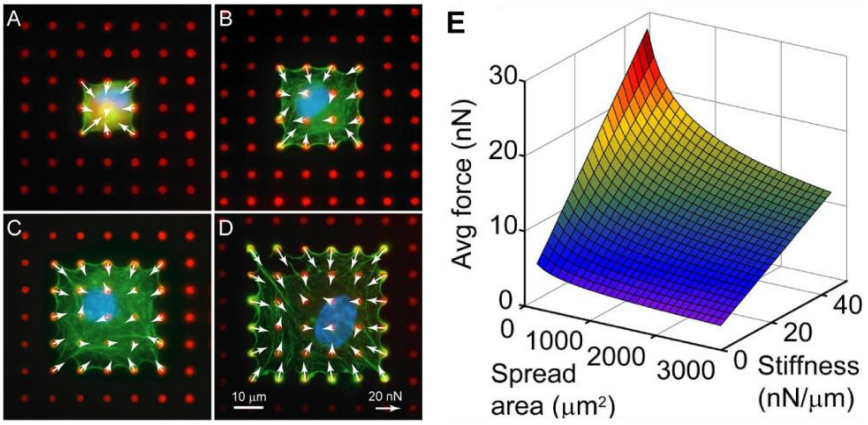

Figure 12.4. Endothelial cells patterned on microposts. A)-D) Cell spread area on microposts can be controlled by microcontact printing. E) Traction forces change in response cell spread area and micropost stiffness. Adapted from Han, *et al.* [1].

12.4.2 *Endothelial Cells in Pairs Studied Using a Bowtie Pattern*

If one were to measure force generation in a gel with pairs of cells, it would be challenging to determine which forces were generated at the intercellular connections, and which forces were generated at the cell-substrate interface. All of the forces are coupled and the motion of the underlying layer can be affected at a distance from where the force was generated, leaving the force generation in each region inconclusive. After utilizing the point-force measurement of microposts, one can determine the total force associated with each cell and the imbalance is the force generated by the intercellular adhesions. Because the sum of forces of each cell must equal zero due to the actin-myosin nature of force generation, the intercellular force is equal to the imbalance in each cell's force vector. All of the remaining forces are transmitted through the cytoskeleton to the micropost substrate, so the remaining forces must be to the other cell.

Liu *et al.* described how to calculate and determine the intercellular forces of a pair of cells, then patterned endothelial cells as bowties to study how they were affected when in pairs or alone (**Figure 12.5**) [3]. Each cell took up one half of the bowtie and then joined to the other cell at the point of the bowtie in a location that was not directly above a micropost adhesion site. The study showed that the average force of each cell remained the same regardless of whether a cell was in contact with another cell or by itself. In fact, the average traction force of a cell was not regulated by the size of the intercellular connection, but the intercellular connection did have a linear relationship with the intercellular force. As the junction size increased, so did the tugging force between cells.

12.4.3 *Transmigration of Leukocytes*

In another study by Liu *et al.*, endothelial cells were patterned on microposts to form a small region of a monolayer [27]. After treating the endothelial cells to promote adhesion, leukocytes were distributed in the media and allowed to adhere and transmigrate through the monolayer. Because the microposts were used to measure the traction forces, there was a region for the leukocytes to migrate below the layer, and the posts were able to report the underlying forces of the layer. The transmigration process is important for immune system response, and the author's

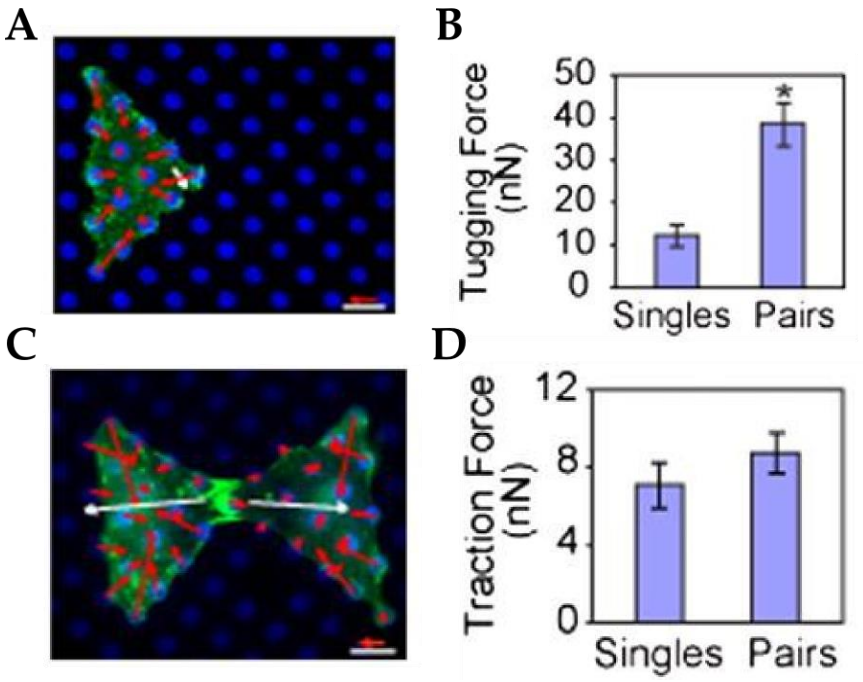

Figure 12.5. Endothelial cells produce more tugging force and more overall traction force in pairs than as single cells. A) Single cells were compared with B) pairs of cells and assessed for adherens junctions through ß-catenin staining (green), while the posts are stained blue. B) The total force of pairs of cells was much greater than single cells, while D) the average force per post remained consistent for either single cells or pairs of cells. Distance scale bar is 10 μm, and force reference arrow is 10 nN. Adapted from Liu *et al.* [3].

found that endothelial monolayers increased in total force when leukocytes were adhered and transmigrating through the layer compared to when the endothelial cells were simply in a monolayer.

12.4.4 *Endothelial Cells Are Affected by the Type of Flow*

An endothelial cell *in vivo* generally has blood flowing across its apical surface. Not surprisingly, the speed and type of flow across the endothelium has been shown to have an effect on the ability of the cells to create tight junctions and remain attached. Several studies have looked at the role of flow across a monolayer of endothelial cells, but have only been able to determine protein expression, not cell tugging

forces [28, 29]. Although this method is useful to show the effect of laminar or disturbed flow, it does not give more than a suggestion about how cell and inter-cellular forces are affected. Ting, *et al.* has shown the effects of monolayer forces and intercellular forces by seeding a monolayer on microposts and applying two types of flow above the cells [2]. In a single chamber, they used a step to apply either a disturbed or laminar flow across a patterned monolayer seeded on microposts (Figure 12.6). The disturbed flow was defined by a recirculation region to mimic a region of disturbed flow in the body, typically at bifurcations or downstream of stents. They found that in the region of disturbed flow, the cells had lower total force, and lower directionality in the force as compared with monolayers exposed to laminar flow.

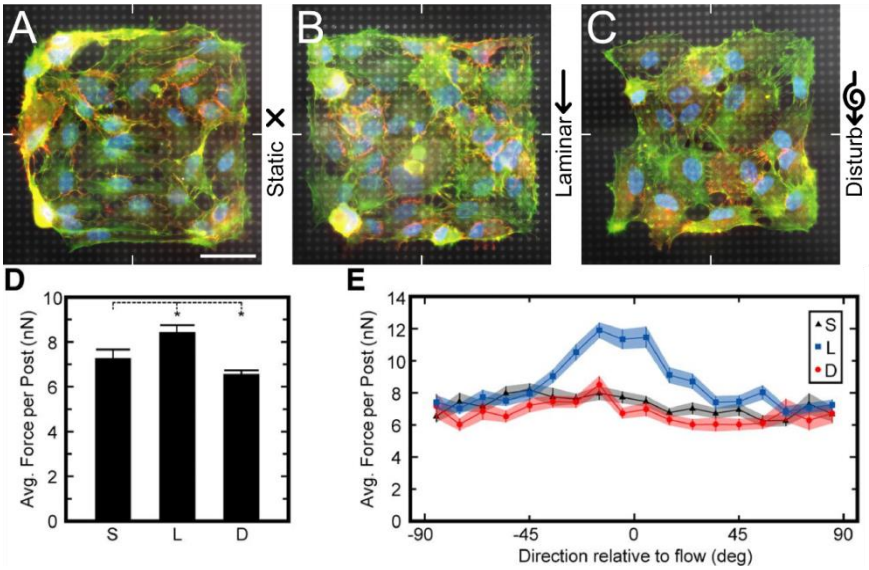

Figure 12.6. Endothelial cell monolayers were seeded on microposts and exposed to A) static, B) laminar, and C) disturbed flow. Cells are stained for actin (green), ß-catinen (red), nucleus (blue), and posts are white. D) Average force per post, and E) direction of force per post of cell monolayers exposed to laminar (L) flow was greater than for either static (S) or disturbed (D) flow. Scale bar is 40 µm. Adapted from Ting, *et al.* [2].

In order to determine the direction of intercellular force, Ting, *et al.* showed that by analyzing the resultant force of a single cell, the vector sum of its tugging forces can be determined (Figure 12.7) [2]. Individual

interactions between any two cells are mathematically indeterminate, but the total imbalance of each cell can be used to determine its force on its neighboring cells. In this manner, information about the intercellular forces can be determined even if each specific interaction cannot. Information about the tugging force vector is a valuable tool to determine force alignment and intercellular tugging based on different types of flows.

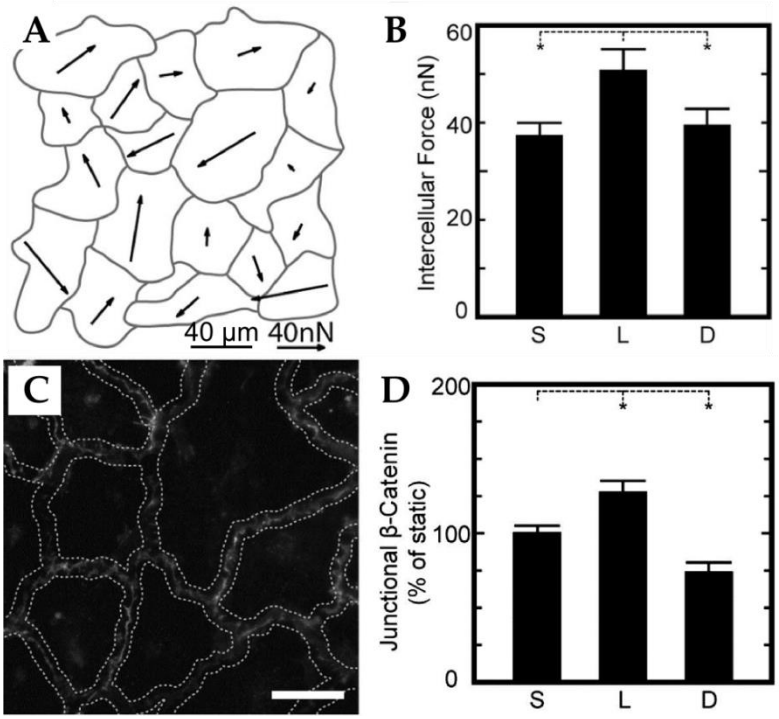

Figure 12.7. A) Each cell tugging force can be mapped as the resultant force for each cell outlined in a monolayer. After cell monolayers are exposed to either laminar (L), static (S), or disturbed (D) flow, the intercellular cell tugging force is greater for cell monolayers exposed to laminar flow, scale bar is 40 µm, force reference arrow is 40 nN. C) Junctional protein β-catenin was measured as the intensity per unit length and divided by the number of cells in each monolayer, with D) the finding of an increase in monolayers exposed to laminar flow, scale bar in C) is 20 µm. Values in D) are intensity normalized by static condition intensity. Adapted from Ting, et al. [2].

When exposed to laminar flow, the actin in an endothelial cell monolayer is aligned in the direction of flow when compared with actin in monolayers under either static conditions or disturbed flow. Furthermore, the traction forces of the monolayer on the substrate are also aligned in the direction of flow under laminar flow (Figure 12.6). Intercellular forces and the junction protein ß-Catenin are also affected by the type of flow and are stronger when exposed to a laminar flow than when exposed to disturbed flow or static conditions (**Figure 12.7**). Because of the novel use of measuring the vector sum of intercellular forces, Ting, *et al.* were able to determine that the intercellular forces were indeed affected by flow conditions as to be expected from previous studies showing an increase in junction protein levels [2]. The micropost method enhanced the previous results to show the actual forces rather than solely the protein expression or the degree to which cells were connected together.

12.5 PLATELET CONTRACTION WITH MICROPOSTS

Platelets serve a vital role in the cardiovascular system, and much of the function is mechanical. Platelets are found throughout the body and play a critical role in wound healing by adhering to damaged endothelial cells and signaling other platelets to adhere to cover the damage to the endothelium [30, 31]. Although an agglomeration of platelets is the first and necessary step to heal a damaged area, the thrombus needs to contract in order to continue the process of tissue repair. A few methods to measure clot strength have been used including thromboelastography (TEG), clot retraction assay, and platelet clot strips, but none of them provide a direct measurement of platelet strength in clots [32]. Micropost arrays are able to use a platelet rich mixture and measure force without further straining the micro-clot that has formed on the tips.

12.5.1 *Formation of Micro-Clots on Microposts*

In order for platelets to adhere to the tops of the microposts, a specific matrix coating for the platelets was required. In the study performed by Liang, *et al.*, both human fibrinogen and human fibronectin were used as a matrix coating on the surface of the microposts as described earlier in this chapter with no significant difference in thrombus force between

coatings [33]. After centrifuging human blood to separate platelets, the platelets were suspended in Tyrode buffer and seeded onto microposts. After 25 minutes had passed, the platelets were stimulated with thrombin to form the micro-clots and allow them to generate force. The micro-clots were fixed and stained for actin and imaged.

12.5.2 *Platelet Force Increases with Time and Thrombin Concentration*

Platelet forces can be affected by a variety of factors, and the amount of thrombin can affect the contractility of a micro-clot on microposts. Thrombin enhances platelet contractility and signals them to start contracting. The study by Liang, *et al.* showed that an increase in thrombin concentration will enhance platelet force generation in a non-linear fashion (**Figure 12.8**) [33]. As higher concentrations of thrombin are used, both the total force of the clots increased and the average force based on clot size increased.

Unlike many aspects of cell signaling and dynamics, platelet force generation takes place over a period of time. A series of micro-clots were analyzed after specific time points up to an hour after seeding adding thrombin to the platelet solution and seeding on microposts. The researchers found that micro-clots continued to have increased forces all the way up to sixty minutes after adding thrombin.

The role of thrombin and time in how platelet micro-clots generate force is important to wound healing and treatment. Platelet force generation is also significant in hemostasis and thrombosis. A clot without significant force may break away from the damaged region and catch onto another point in the body.

12.6 FUTURE DIRECTIONS IN MICROPOST METHODS FOR CARDIOVASCULAR CELL STUDIES

Microposts have been used for a variety of studies of cells, in particular cardiovascular cells and will further the understanding of mechanical forces in the cardiovascular system. New techniques are being developed to both measure and manipulate forces on cardiovascular cells. Future developments in this field may see a trend towards nanoscale dimensions to understand single platelets as well as a higher density of

force measurements. Furthermore, tools may be developed out of the current methods to be utilized in the hospital to diagnose disorders that primarily rely on mechanical force deficiencies. Ultimately, the cardiovascular system will continue to be studied as a mechanical system due to the analogous system of a pump and piping system. Micropost methods enable a look at the individual components of the system in the same way that studying the grain structure of a pipe identifies causes of a crack that led to a leak and future cardiovascular diagnoses will depend on understanding all of the factors that went into instigating a particular disorder.

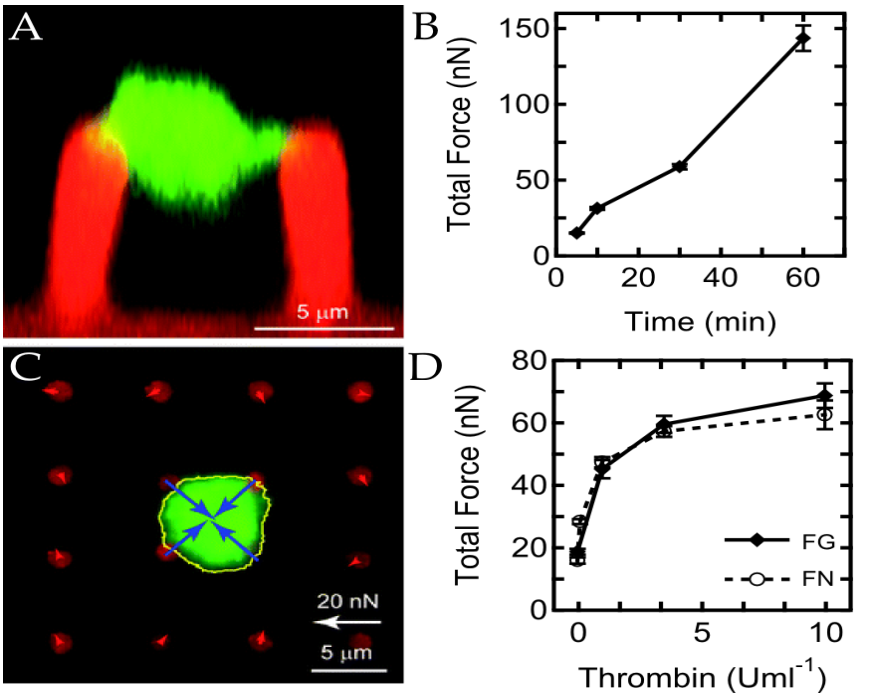

Figure 12.8. Platelets form microclots on microposts. A) Confocal image of a microclot stained for actin (green) suspended between and bending two microposts (red). B) Thrombin is added to platelets on microposts at t=0, and platelet forces increase with time. D) Thrombin concentration affects platelet forces, measured 30 minutes after thrombin addition. Adapted from Liang, *et al.* [5].

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