AN INTEGRATED MECHANOSTIMULATION SYSTEM FOR PROBING ARCHITECTURE BASED CALCIUM SIGNALING IN HUVEC CELLS

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ABSTRACT

Dynamic signal conduction in endothelial networks plays an important role in endothelial function, and characteristics of the network architecture itself are theorized to play a role in this function. We have therefore developed an integrated mechanostimulation system to create spatiotemporal stimuli including geometric cues, fluidic shear, mechanical deformation, and tunable surface stiffness for probing intercellular communication in artificial networks of human umbilical vein endothelial (HUVEC) cells. The system enables detection of architecture dependent (e.g. linear, grid, and branching patterns), spatiotemporal calcium propagation characteristics such as speed, contact length, and repeated stimulation dependence due to mechanostimulation at the single cell level.

INTRODUCTION

Role of Calcium

Calcium ions play numerous roles in cellular function including activation of enzymes, enabling muscle contraction, regulating gene expression, and inducing apoptosis. Due to this importance, the movement of calcium ions is tightly controlled as its concentration can serve as a powerful signal. Many factors of the cellular environment are able to influence the state of calcium ions within a cell which then can affect the functions carried out by calcium ions themselves. Examples of factors which influence calcium ion levels include pH, temperature, signaling chemicals, voltage, type and presence of neighboring cells, shear stress and previous calcium ion levels [1, 2]. Taken together, the complete environment of the cell continuously influences the movement, storage, release and activation of calcium ions.

Endothelial cells, as other cell types do, rely on calcium ions for proper function but additionally are arraigned in a constrained architecture, e.g. vasculature. Being arraigned in such a fashion means that signal conduction may be uniquely affected by the arraignment of the tissue in that messengers must take specific paths which are determined by the architecture itself. Previous mathematical modeling based upon physiological constraints for intercellular exchange of small messenger molecules has highlighted this possibility. This modeling has shown that architecture itself may function as part of the signaling mechanism with branches, loops, or other structures able to influence the movement of messenger molecules [3, 4].

Experimental Investigation

Cells in their native physiological state inhabit microenvironments which are specific per cell type in terms of the inputs that are received. In order to study cell behavior in a rational way, these microenvironments must be replicated inside an experimental apparatus. The current system we have developed, therefore, is based upon using a microscope stage mounted platform providing for continuous observation which can house cells for long time periods (hours to days) and to which several other stimuli are combined. Inside this chamber is placed a microchannel clamped to a Petri dish which contains the cells being studied. The cell surface, additionally, is patterned [5-7] to provide a template for cells that resembles a biological pattern. We have used this approach to study the network dependent signaling behavior of small messenger molecules and in particular calcium ions in networks of HUVEC cells. The calcium wave propagation among HUVEC cells was observed using a combination of a calcium sensitive dye and video fluorescence microscopy. In our experiments, we have observed that networks of HUVEC cells, when mechanically stimulated at the single cell level, show an exchange of calcium that is affected by the architecture of the network. Using this approach, the characteristics of calcium propagation, such as the propagation speed, direction and delay can be measured based on the time of each individual cell to display varying intensity. Furthermore, the effects of repeated mechanical stimulation and the dependence of the contact length between adjacent cells, as well as the dependence of the architecture upon the signaling can be detected in artificial networks of HUVEC cells.

As calcium is involved in diverse processes of cellular function, a means of providing multiple inputs to cells is necessary in order to detect the interplay of the various factors which influence calcium ion movement and storage. Current techniques typically replicate one factor such as surface chemistry or stiffness in order to study and perturb cell behavior. The current method therefore provides a means by which these complex interactions can be replicated and perturbed in a laboratory setting by providing for multiple concurrent inputs. This will allow for uncovering of previously untestable interactions and unobserved phenomenon. Furthermore, the type of network dependent, small messenger, intercellular signaling being studied, which represents the collective, adaptive response of endothelial cells to their physical environment, has not been previously investigated experimentally.

METHODS

Surface Patterning

The surface patterning component of the system is achieved via selective plasma functionalization of surfaces which include biocompatible polymers such as polystyrene and polydimethylsiloxane, (PDMS) and other materials such as glass and silicon [5-7]. Briefly, to achieve patterning, a 3D PDMS mold is placed upon a surface and used to form a series of channels between the surface and the mold itself. The PDMS mold has weight placed on top of it which keeps it in conformal contact when it is next placed inside a plasma chamber. During the plasma treatment, plasma is able to flow only into the channels formed between the mold and the substrate and hence will only react with the surface at these areas and thereby leave a chemical pattern, Figure 1a.

Surface Stiffness

Various substrate stiffnesses were provided to cells via changing mixing ratios of PDMS elastomer base and curing agent as well as culturing on rigid substrates such as glass and polystyrene.

Microchannel Construction

Due to the method by which the surface pattern is created, i.e. via plasma functionalization, typical plasma based sealing of PDMS to glass was not suitable. Additionally, easy channel removal is desirable when probing cells that have been grown in the presence of flowing media, chemical stimulation, or other factors delivered via the microchannels. To achieve formation of a channel that is also removable, the channel was therefore magnetically clamped in place over the desired substrate, Figure 1b.

Single Cell Stimulation

Single cells can be stimulated by different methods including mechanical or chemical inputs. Mechanical stimulation is achieved by either applying shear force via the flow through the microchannel or probing single cells with a needle or capillary tubing attached to a 3-axis micromanipulator. This allows mechanical force to be applied to a cell in degrees ranging from slight stretch to lysis at the single cell level. Chemical stimulation is achieved by delivery of chemicals via the microchannel at the level of single or multiple cells.

Imaging

In order to visualize the small messenger signaling, a calcium ion sensitive dye (Fluro-3 AM, Invitrogen) was loaded into cells with the aid of Pluronic F-127 (Invitrogen). Once loaded into the cells, esterases cleave the dye to a form which cannot leave the cell and which will fluoresce in the presence of calcium ions. Imaging was accomplished with a Nikon TE2000-U inverted phase contrast and fluorescence microscope attached to either a SPOT camera from Diagnostic Instruments (model 2.2.1) for brightfield images or a Cooke SensiCam for live fluorescence images. Cells were rinsed and placed in HBSS buffer containing calcium

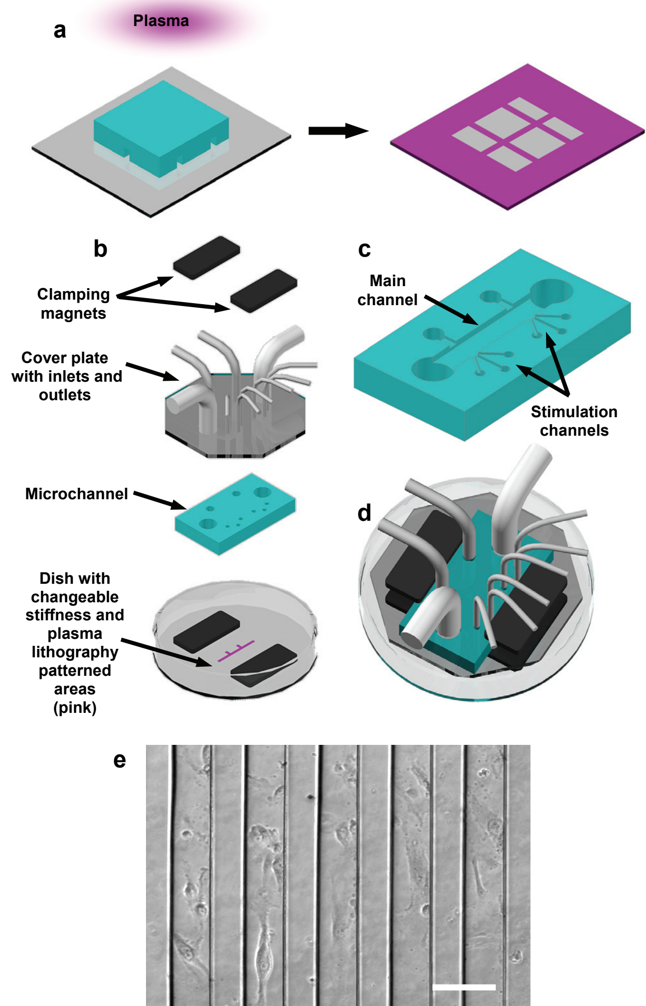

Figure 1: Multiple inputs provided to cells via creation of a microenvironment that provides several distinct stimuli. (a) Shielded plasma exposure via 3D PDMS mold (blue) leaves a chemical pattern that can guide cell attachment. (b) Components of microchannel enclosure system. (c) Detail of microchannel and control features. (d) Assembled channel. (e) HUVEC cells cultured in a series of microchannels, scale bar is 100 μm.

prior to imaging and were maintained on a heated stage during stimulation and image capture. Image sequences were analyzed with ImageJ via a custom macro that recorded whole cell fluorescence levels for export and later analysis.

RESULTS

The cell guidance achieved by the surface patterning is shown in Figure 2, which displays several branching configurations used to test signaling and its dependence upon architecture. The patterning produced is not topographic in any way that cells can sense [6, 8] and is stable for several days to several weeks depending upon cell type. Creation of artificial cell networks as shown in Figure 2 allows for the response of small messengers to be tracked as they move

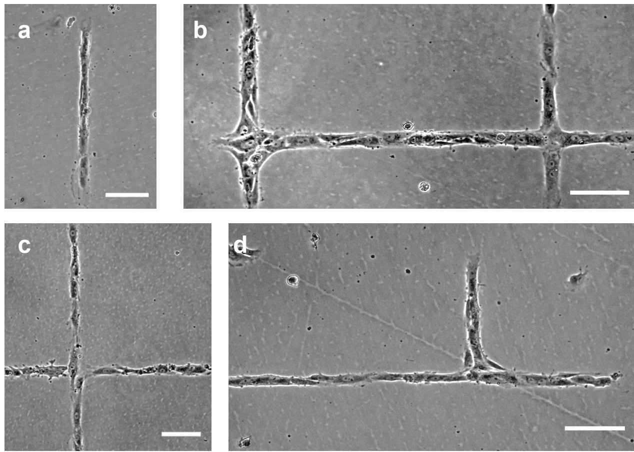

Figure 2: Cell pattering of HUVEC cells on polystyrene substrates. (a) Linear pattern, (b) multiple side branches, (c) 4-way junction, (d) single side branch. Scale bars are 100µm.

throughout these networks in response to stimulation. Typical behavior seen relating to calcium ion levels in cells was observed which included a small delay to pass the signal between adjacent cells, relatively constant times to propagate a signal between cells, a finite number of cells that a signal propagates to before stopping, and the ability of cells to be stimulated multiple times. During multiple stimulation it was also observed that the propagation between cells takes the same amount of time and does not increase or decrease with multiple stimulations. Further, after several stimulations, a stage is typically reached where additional Ca^{2+} stimulation is not possible with repeated probing. An example of signaling behavior can be seen in Figure 3 which shows progression of Ca^{2+} concentration as it moves through a network. In this figure, the propagation of Ca^{2+} levels between cells can be seen as a result of mechanical stimulation at the indicated point. This mechanical stimulation likely causes an increase in Ca^{2+} concentration via opening stretch sensitive calcium channels and activating other cellular means which can increase the free concentration of calcium ions in the cytosol [2, 9]. The probing in general does not drastically harm the cell if the stimulation does not greatly distort the cell. One aspect of the architecture based calcium signaling that can be seen in Figure 3 is that of signal splitting. The initial stimulation of the single cell produces a calcium wave that travels down the branch of the network until it reaches the junction. When it reached this junction, it split and continued to the other 3 arms present in the network downstream from the original stimulation point. Behavior at a junction also highlights a further manifestation of network dependence of signaling that can be seen when signals meet at a junction. What can occur, as seen in Figure 4 is that transmission of a signal can be influenced by the architecture where a propagating signal may or may not pass a junction point. This is likely due to the fact that a junction provides multiple possible paths for the messenger molecules to follow and if the wave is split into multiple parts, conditions needed for the wave to continue cannot be reached, and the wave does not propagate further, Figure 4b. This is illustrated by the fact that if a wave with a high amplitude, or multiple waves reach a junction, then the signaling can continue, Figure 4c. Another observation that has been noted is that signal transmission can depend on the number of signaling waves which are present in a given architecture at one time. When a combination of multiple waves, usually coming from opposite directions, occurs, signals may either cross and continue, or one or both signals will be unable to propagate beyond the meeting point. This varied phenomenon is likely due to the conditions of each wave, and the conditions that their combination produces. Depending upon the magnitude of the waves, the meeting may produce a situation that is either favorable or unfavorable to continued propagation. Additionally, the timing of the wave combination may affect the propagation, as a cell may be unable to be further stimulated if is already in a nonexcitable state when another wave arrives. This is related to another observation where it was seen that signal propagation between cells was dependent upon the type of signal input to the stimulated cell. If, for example a cell was stimulated slightly by the probe and the Ca^{2+} wave was not seen to pass to the neighboring cell, or through a junction, repeated stimulation could often achieve signal propagation. Also, if the cell was allowed to resume its normal, unstimulated Ca^{2+} state after several minutes without being probed, a strong stimulation could produce a signal that

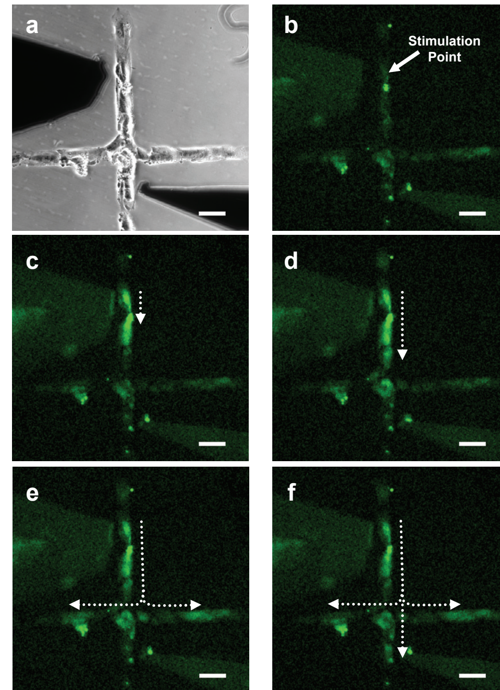

Figure 3: Progression and splitting of a calcium ion wave after mechanical stimulation of a single cell. (a) Brightfield before stimulation. (b) Ca^{2+} Fluorescence before stimulation. (c) Initial stimulation. (d-f) Ca^{2+} wave traveling and splitting at cross junction. Triangular shapes are needles used to stimulate cells. Only top needle is used in this image sequence. Scale bars are 50 µm.

would be carried down through the network.

Additional signaling behavior observed included passing of a calcium ion signal even though a stimulated cell was not in physical contact with a nearby cell (i.e. by diffusion through the buffer) and the occurrence of spontaneous, transient calcium increase without stimulation. Neither of these observations, however, is believed to be the dominant mechanism for changing of calcium ion concentration as they were observed much less frequently than the signaling taking place between cells in direct physical contact.

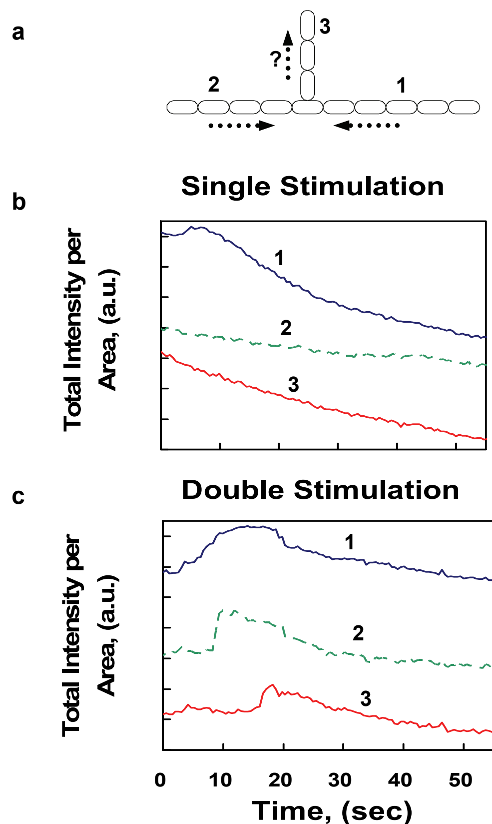

Figure 4: Dependence of signal upon architecture. (a) Schematic showing cell pattern which is similar to Figure 1d. Whole cell Ca^{2+} based fluorescence for stimulation of only cell 1 (b), and for stimulation of both cells 1 and 2, (c). Lines indicating Ca^{2+} concentration have been offset for clarity. Vertical scale and time are equal for both graphs.

CONCLUSIONS

Small messenger signaling plays a key role in many aspects of cell life and operation and its dynamic nature is able to influence these functions. Endothelial cells posses a unique architecture which may affect how concentration information of small messengers is communicated among cells and many aspects of cellular environment including chemical and mechanical inputs influence levels of small messengers. With the current microstimulation system, multiple bio-relevant inputs have been incorporated into a single system to more realistically replicate the myriad types of stimuli that account for physiologic behavior and enable the study of cellular phenomenon which are dependent upon these multiple inputs. We have observed several cases where the signal transmission of the small messenger Ca^{2+} is dependent upon the type of signal being conveyed and on the existing architecture itself. This observation suggests that tissue architecture may play a role in maintaining proper cell function and communication between cells.

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