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Short note

Fabrication of protein gradients for cell culture using a miniature squeegee

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Abstract

We present a straightforward method to create spatial gradients of substrate bound protein for live cell studies using only mechanical parts. Protein concentration gradients on a micron scale can be fabricated in several minutes for a relatively low cost using a method that is generally applicable to any protein and substrate combination. We describe the details of the device construction, and provide examples of mammalian cells grown on substrates patterned with protein concentration gradients using this technique.

Keywords: Protein gradient; Protein patterning; Axon guidance; Substrate modification

1. Introduction

The aim of reproducing in vitro the spatial distributions of proteins found in vivo during key events in the development of organisms requires simple and reliable ways to fabricate protein concentration gradients. Axonal guidance and cell migration [1–5] are two essential processes in which spatial gradients of chemoattractants and chemorepellents play a fundamental role. Here we report a method to fabricate substrate bound protein gradients in a straightforward way using only mechanical parts to make the patterns with a resolution of several micrometers.

Technologies allowing precise patterning of proteins at a cellular scale have become the focus of a growing field of research [6] and there has been a constant increase in their applications in biology, both as a tool for basic research [7,8] and also for commercial purposes [9]. Several techniques have already proven useful to create such protein patterns, but none of the reported methods has successfully emerged as a standard. Furthermore, the physical characteristics of different proteins of interest are highly variable, and it is likely that one patterning technique will not be ideal for all proteins. Tradeoffs between the length scales of the patterns to be generated, reproducibility, cost of the necessary equipment and consumables, and ease of use of the available techniques has left the door open to the development of new instruments and methods.

Initial methods that aimed to create gradients of membrane proteins in order to determine their influence on axon guidance in vitro were limited in their capacity to reproduce the steepness and small scale required to mimic gradients of interest found in vivo [5,10]. Photonic techniques offer a promising alternative [11], but the use of UV-lasers and specialized chemical cross-linkers that are not commercially available entail that these methods are not easily accessible to typical biomedical research laboratories. The precise deposition of nanodrops of protein solution [12] and microfluidic devices [13] were also applied to address this challenge, but require specialized equipment and have not been widely used in subsequent studies. Recently, gradients of protein spot densities were fabricated by soft lithography of ephrin-6, and these geometric patterns were shown to influence axon extension by retinal neurons [14]. This latter method allowed the generation of well defined graded patterns at a macroscopic scale across distances of tens of micrometers, however a weakness of the technique is that the

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local concentration of protein within each spot remains constant throughout the whole structure while the density of spots is varied.

2. Materials and methods

The device we present consists of a barrier squeegee made of hydrophobic material that is used to spatially confine a protein solution within a substrate binding area set by the location of the squeegee, and which controls the incubation time by mechanical translation of the barrier. A motorized translation stage is used to precisely move this silicone barrier thus exposing the different areas of the substrate to the protein solution in user defined increments in space and time. By varying the incubation time, or by depleting the protein solution, it is possible to control the coverage of the substrate to achieve a graded pattern, within certain limits that are set by the adsorption kinetics that are protein specific. A photograph of the device is shown in Fig. 1D.

After sequential translation of the mechanical barrier, the first area where the drop of protein solution is placed will adsorb the largest number of proteins as it has the longest exposure (reaction) time. As well, the last exposed area will have the lowest surface concentration of the protein. The differences in adsorbed protein concentration in different regions of the substrate result from two contributing factors. As new areas of the substrate surface are exposed to the solution, the concentration of the protein in solution will decrease thus yielding lower numbers of molecules available to bind to the surface. Additionally, incubation time will also contribute to the binding kinetics. The incubation period of the first step of the gradient is equal to the sum of all incubation times ($t_1+t_2+t_3\ldots+t_n$) for all steps in generating the gradient pattern. The second step is incubated for the entire time that it takes to expose all of the remaining areas ($t_2+t_3\ldots+t_n$) in the stepped pattern and so forth.

The horizontal movement of the squeegee that confines the liquid was performed using a motorized translation stage (Thorlabs, Newton, NJ) and custom routines written in LabVIEW (National Instruments, Austin, TX). The DC motor we used has a built-in optical encoder with a large number of counts per revolution that provides submicron positioning accuracy. The chosen translation velocity (300 μm/s) and the step dwell times are inputs of the program.

The pressure that the squeegee exerts on the substrate has to be adjusted depending on the flexibility of the silicone barrier in order to obtain reproducible patterns. The force applied by the barrier is controlled with a second translation stage and a screw. In order to measure this force, the experiments are performed on a simple laboratory balance with the downward force adjusted to approximately 5 g. The squeegees were made of PDMS (poly (dimethylsiloxane), Dow Corning, Midland, MI) due to its versatility and low cost. A mixing ratio of 10:1 of silicone prepolymer to curing agent was used and was placed in an oven

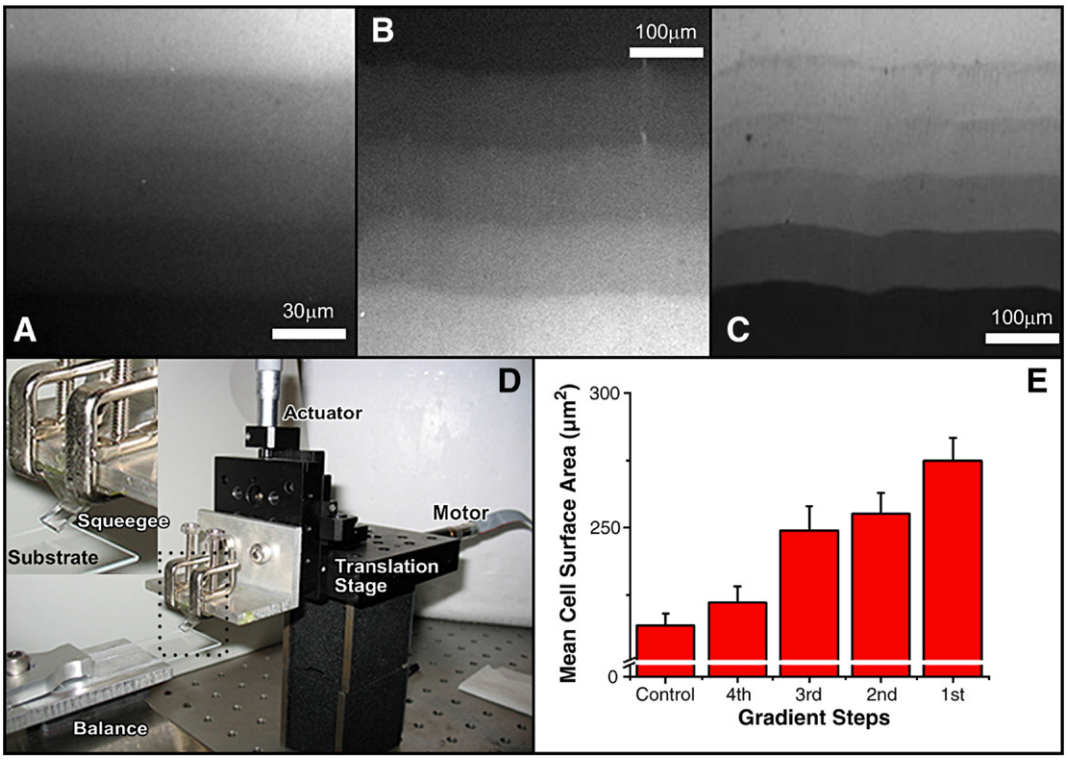

Fig. 1. Three examples of protein gradients at different scales are shown. A and B) are mixtures of fibronectin and Alexa546-human fibrinogen on aldehyde activated slides and C) Alexa488-goat-antimouse antibodies. D) A photograph of the patterning device, including a close up of the PDMS squeegee near the slide surface. E) CHO-K1 cells expressing EGF receptors/GFP were cultured on graded patterns of fibronectin. The cell surface spreading was quantified and shown as cell area function of protein concentration.

at 120 °C for 30 min to fabricate hydrophobic flexible barriers. The thickness and shape of this silicone barrier were chosen so that it slides over the entire substrate surface without leaving gaps through which the protein solution can leak. Typical fabricated squeegees consisted of two parts: a flat slab approximately 2 mm thick and 1 cm long mounted on a base, as shown in Fig. 1D. This barrier was fastened to one end of a glass slide and positioned on top of the active surface, for easy replacement.

3. Results

The capacity of different proteins to adsorb to a substrate can vary widely. Therefore, a method to estimate the concentration of the bound protein at each position along the gradient is necessary. Different examples of this are shown in panels A, B, and C of Fig. 1, where fluorescently tagged antibodies and extracellular matrix proteins were used as concentration indicators. In both cases the substrates used were glass slides with surface activated aldehyde groups (Genetix, Hampshire, UK). These link the protein of interest to the substrate via a covalent bond, and in our experience generate relatively homogeneous patterns. Some proteins may not pattern efficiently using this basic method. We also employed a variation where we used antibodies bound to the surface as scaffolds to link and present the protein of choice.

In the equilibrium state, the surface coverage follows a Langmuir isotherm $K = \Gamma/(C(1-\Gamma))$, where C is a concentration constant and Γ is a the fraction of the surface covered by protein [15]. Hence, the gradient is created by the depletion of proteins from the solution due to binding to the substrate from one substrate area to the next in the step sequence [16]. In practice, a known volume of solvent can be added to the solution at any point during the fabrication process to change the steepness of the gradient.

Given that binding to the glass surface could in principle alter the activity of some proteins, control experiments to test their activity are important. As an example, we tested for the activity of substrate bound extracellular matrix (ECM) proteins. We fabricated concentration gradients of fibronectin (Sigma-Aldrich, St. Louis, MO) and cultured mammalian cells on them. Since cell–substrate adhesion will be influenced by the concentration of fibronectin bound, cell size was used as an indicator of the activity of bound ECM protein.

CHO cells expressing the epidermal growth factor receptor fused with green fluorescent protein (GFP/EGFR) were plated on fibronectin gradients fabricated on aldehyde activated slides (Genetix, Hampshire, UK), and incubated for 60 min at a temperature of 37.0 °C in a 5.0% CO_2 atmosphere. The medium consisted of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine (4 mM). The cells were fixed using a 4% paraformaldehyde solution after plating on the substrate. Fibronectin (45 μg/mL) was mixed at a ratio 1:1 with fluorescently labeled human fibrinogen (Sigma-Aldrich, St. Louis, MO), in order to visualize the gradient steps. The gradient was made by pipetting 5 μL of protein solution behind the barrier and moving it across the substrate in four steps of 100 μm with pause times of 3 min at each step.

Since the cells express the GFP coupled EGFR transmembrane protein, fluorescence images were taken to quantify cell surface area. Using custom Matlab (Mathworks, Natick, MA) scripts, the average size of the cells on the gradients steps was measured from the images and plotted in Fig. 1E as a function of the surface concentration. The increase in cell surface area with ECM concentration indicates that a significant fraction of the substrate bound fibronectin remained active.

4. Conclusions

The new method we describe combines simplicity with easy production of reproducible substrate bound gradients at a scale relevant to individual cells. The construction and operation of the device does not require special skills and all the needed parts are commercially available and relatively inexpensive. The method provides a new alternative to mimic, in vitro, the spatial distribution of proteins found during embryogenesis, with the ability to readily manipulate both the change in concentration across a distance, and the distance across which such changes occur.

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