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Author manuscript

J Microelectromech Syst. Author manuscript; available in PMC 2015 July 08.

Published in final edited form as:

J Microelectromech Syst. 2014 April ; 23(2): 391–397. doi:10.1109/JMEMS.2013.2278813.

A Macro-to-Micro Interface for the Control of Cellular Organization

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Abstract

The spatial organization of cellular communities plays a fundamental role in determining intercellular communication and emergent behavior. However, few tools exist to modulate tissue organization at the scale of individual cells, particularly in the case of dynamic manipulation. Micromechanical reconfigurable culture achieves dynamic control of tissue organization by culturing adherent cells on microfabricated plates that can be shifted to reorganize the arrangement of the cells. While biological studies utilizing this approach have been previously reported, this paper focuses on the engineering of the device, including the mechanism for translating manual manipulation to precise microscale position control, fault-tolerant design for manufacture, and the synthetic-to-living interface.

Keywords

Biological cells; Micromechanical devices; Tissue engineering

I. Introduction

CELLS in living tissue are engaged in continual communication with one another, sending and receiving a host of signals that combine to determine the function of individual cells as well as the emergent behavior of the larger tissue. The structural arrangements of cells define spatial relationships that strongly influence signal propagation. For example, certain

forms of signaling (cadherins, gap junctions) typically require membrane-to-membrane contact and are thus confined to directly neighboring cells. On the other hand, secreted soluble factors can diffuse much farther, with signal intensity being modulated by transport-related factors such as distance, barriers to transport, and competitive consumption by other cells. Cellular spatial organization therefore plays a fundamental role in modulating intercellular communication for both contact-dependent and contact-independent signaling pathways [1, 2].

From an experimental standpoint, conventional biological tools have traditionally provided little ability to control cell organization at the scale of individual cells, which is on the order of 10 micrometers. This has been changing rapidly in recent years as investigators have adapted methodology from semiconductor microfabrication to control tissue patterning *in vitro*. A number of elegant experiments have leveraged these tools to investigate structure-function relationships in living tissue [1]. These experiments have generally been confined to static patterning, however. Dynamic manipulation is difficult, as little can be done to control the positioning of adherent cells once they attach to the substrate. Methods do exist to select and release specific subpopulations of cells [3-6], but non-destructive pattern reorganization remains very challenging. Probing the dynamics of cell-cell interaction is therefore constrained by the limitations of current tools.

Micromechanical reconfigurable culture is a method to achieve dynamic control of cell patterning through the use of culture substrates that can be broken apart and rearranged much like the pieces of a jigsaw puzzle. This technique has been successfully implemented in studies involving liver hepatocytes and supportive stromal cells [7, 8], however device engineering was previously not fully described. This report will focus on the following: (1) the design of the interface mechanism, which translates macroscale manipulation to microscale positioning control, (2) design for manufacture, emphasizing fault-tolerance and yield, and (3) the synthetic-to-living interface.

II. Design

A. Constant-Adhesion Approach

In tissue culture, the majority of cells are adhesion-dependent, meaning that they must be properly anchored to maintain their phenotype. Once cells are attached to a substrate, however, it becomes a challenge to reposition them in a controlled manner. For example, bulk enzymatic cleavage (e.g. trypsinization) releases all cells from the substrate indiscriminately, thus destroying spatial patterning. While it is possible to detach cells with more specificity [3, 4], there are still consequences that may be undesirable. First, breaking cell-substrate adhesion disrupts cell signaling due to changes in cytoskeletal tension [9] and extracellular matrix signaling. Secondly, long time scales are associated with cell detachment (minutes) and with reattachment and spreading (hours).

In our approach, cells remain fixed on sets of microfabricated plates, and the plates themselves are repositioned to change the spatial configuration of the culture. Hence, the adhesion of individual cells to their local substrate can remain unperturbed while the

organization of the greater tissue structure is shifted. This change in positioning occurs on the order of seconds.

B. Geometric Design

While a variety of biological behavior can be probed through reconfigurable culture, here we focused on controlling cell-cell contact. Specifically, two cell populations must be positioned either close enough together to allow contact-mediated signaling between the two groups, or just far enough apart to prevent contact-mediated signaling while soluble signaling remained unperturbed.

In the case of hepatocytes and 3T3 fibroblasts, it had been previously determined that gaps narrower than 10 μm are readily bridged over, while gaps larger than 40 μm can reliably prevent bridging [10]. For the non-contact configuration, it was hypothesized that it would be important to keep the separation distance minimal in order to preserve short-range paracrine effects. This proved to be true as it was later discovered that the maximum effective range of key soluble factors in this liver coculture model was only about 350 μm [7]. In the final design, the separation distance for the non-contact configuration is 80 μm , and gaps of less than 6 μm have been achieved for the contact configuration (Fig. 1 D-E).

The device is composed of tapered interdigitating fingers, with cells attached to the top surface of the fingers (Fig. 1 A-C). The interdigitated design provides a large interface between the cell populations seeded on opposing sets of fingers, or *combs*. Further, the taper is chosen so as to provide a mechanical transmission ratio of 1:20 when the combs are pushed together. That is, as the combs slide a distance of 1.6 mm, the gap between the fingers changes by only 80 μm . In combination with the integrated positioning mechanism, it thus becomes possible to actuate an 80- μm change in gap width with great accuracy simply by pushing manually with hand-held tweezers. Actuation speed and simplicity are important in order to facilitate the aseptic handling of cell cultures within biosafety cabinets and to minimize time out of the incubator.

Finger width is 250 μm at the tip and 750 μm at the base. These dimensions were chosen based on previous work showing that with circular hepatocyte patterns, a diameter of roughly 500 μm is optimal [11].

C. Positioning Mechanism

The positioning mechanism allows the two complementary parts of the device to be assembled together with high precision. A pair of wedge-shaped latches is mounted to one comb via cantilever springs. These latches snap into matching sets of slots on the complementary comb. There are two sets of slots to choose from, one for the *contact* configuration and another for the non-contact, or *gap*, configuration (Fig. 1B). In the contact mode, the wedge shapes and spring pressure combine to pull the comb fingers into firm contact (Fig. 2A). In the gap mode, the mirror symmetry of the cantilever springs provides self-centering in the y-direction, while the wedge shapes and spring pressure combine to provide self-centering in x and θ (Fig. 2 B-D), such that the gap distance is maintained at $79 \pm 1 \mu\text{m}$. Note that the extruded-2D shape of the parts does not allow for a means to achieve

out-of-plane centering. To achieve alignment in the z-direction, the system relies on having the two parts sit coplanar on a flat surface. In practice, this method of alignment has been found to be sufficient for functional contact-dependent signaling between cells on neighboring comb fingers [7]. In cases when one comb happens to pop out of plane during assembly (<10% of cases), the misalignment can be readily detected by microscope inspection and quickly fixed by disassembly and reassembly. The positioning accuracy of the device has proven adequate for the study of many types of cell-cell interactions [7,8,16]. Contact-dependent signaling is achieved between cell populations on adjoining fingers in the contact configuration, and cells are unable to migrate across the 80 μm separation in the gap configuration.

The spring arms were modeled as simple cantilever beams and designed for a stiffness roughly comparable to a common office paper clip (spring constant of ~ 800 N/m). Beam widths of 300 μm and 400 μm were both fabricated initially; after testing, a width of 350 μm was settled upon as providing the proper balance of clamping force versus ease of motion.

D. Packaging Considerations

Device packaging is not required. The microfabricated parts are robust enough to be directly handled with tweezers. Each comb pair fits into a single well of a standard 12-well culture plate. Once in the plates, standard procedures can be followed for cell seeding, media changes, and biological assays.

In the case of the targeted readout, albumin secretion from hepatocytes, robust detection by ELISA requires about 30,000 cells per ml, for the assay that we employ. Since it takes 750 μl of media per well to cover the surface of the device, this requires that a single comb be designed to fit at least 22,500 hepatocytes on its fingers alone. (Hepatocytes that seed onto the rear of the combs, away from the fingers, are normally scraped off prior to the start of experiments.) In the final design, the measured capacity was 40,000 hepatocytes (counted by hemacytometer).

An elegant aspect of this device is that the entire system, including the positioning mechanism, is integrated into just two extruded-2D shapes that can be batch fabricated in a single-mask process. Manufacturing considerations are discussed in the next section.

III. Manufacture

A. Process Flow

Devices were fabricated by through-wafer deep-reactive-ion-enhanced (DRIE) etching, which has become a well-established process [12]. Briefly, a double-side-polished silicon wafer was thermally oxidized, coated and patterned using thick photoresist, and then etched using a Bosch-process plasma system. To date, manufacturing has been successfully performed in the microfabrication centers at UC Berkeley, MIT, Stanford, and UC Irvine, with minor variations in equipment, process and reagents at each location.

B. Fault-Tolerant Design

In considering system design from a design-for-manufacturing perspective, it became clear that a critical vulnerability arose from the difficulty in controlling DRIE lateral etch. A certain amount of lateral etch is unavoidable in DRIE, such that a 40- μm wide line, as drawn on a mask, can end up as a 46- μm wide trench after etching down 400 μm . The problem is that this lateral etch is difficult to predict and reproduce, so the actual width in a given run could be 52 μm , or perhaps 43 μm . Reproducibility is a particularly difficult issue in research facilities, where the process history of a DRIE system often cannot be controlled from run to run. Fig. 3A illustrates two potential problems arising from variation in lateral etch: (1) the finger edges do not contact precisely, or (2) the latching mechanism does not engage tightly, compromising positioning accuracy.

Instead of attacking this issue from a process control standpoint, design adjustments were implemented to counter the effect of lateral etch variation. First, tapered fingers were used instead of rectangular fingers, and the latch mechanism was designed to push the fingers together until firm contact was established. Second, the rest position of the cantilever latches was designed such tension would be maintained between the latches and notches even if some sidewall erosion took place. These adjustments are illustrated in Fig. 3B. The net effect is that the system is rendered largely insensitive to variations in lateral etch. Indeed, first-pass success was achieved, with a significant number of working devices achieved in the initial processing run, illustrating the strength of pursuing fault-tolerant design.

C. Yield

While lateral etch variation was largely mitigated through design, a different manufacturing issue arose and resulted in poor initial yield. In order to make freestanding parts, the etch must proceed all the way through the bottom of the device wafer. This presents the possibility of etch damage to the wafer chuck, or for unattached parts to scatter and suffer damage. One common solution, which was initially implemented in this work, is to attach the device wafer to an underlying handle wafer using a photoresist bond. Although this was fairly successful, it was also not uncommon for the bond to fail in random regions of the wafer during etching. Specifically, a part would lose solid thermal contact to the handle wafer, and thus the chuck, resulting in rapid sidewall etching and destruction of the part. On average, the yield rate was only about 25% during the initial runs.

The eventual solution was devised in conjunction with A. M. Fitzgerald & Associates (San Carlos, CA) during the outsourcing of manufacturing to this company. Instead of relying on bonding to a handle wafer, the mask was redesigned so that the parts remained attached to the device wafer by small tabs. After the DRIE etch was complete, the parts were released from the frame by using a dicing saw. This solution was very successful and yield was pushed up to almost 80% (88 usable comb pairs out of 112).

D. Polymer Molding

Silicon was chosen as the manufacturing material due to the fact that silicon technology is currently the best developed for precision high-aspect-ratio fabrication, and also because of silicon's excellent mechanical properties, which are important to provide a solid latching

mechanism. Nevertheless, silicon is non-ideal in that it is opaque, making it less amenable for use with typical inverted biological microscopes. Further, through-wafer DRIE etching is an expensive process.

It would thus be highly desirable to produce molded device replicas using a transparent polymer. We employed a method similar to that described by Desai and Voldman [13]. The microfabricated silicon parts were glued to a support wafer by swabbing with a small amount of epoxy (Epo-Tek 301, Epoxy Technology, Bilerica, MA). After silanization to prevent adhesion, a silicone elastomer (Sylgard 184, Dow Corning, Midland, MI) was cast over the silicon parts to form a negative mold (Fig. 4 A-B). The device material was then prepared according to manufacturer instructions and dispensed into the negative mold, using a pipette to fill the cavities to the top (~50 μ l). Finally, the parts were cured and removed from the mold.

Three polymer materials were tested as the device material: Smooth-Cast 310 polyurethane (Smooth-On, Easton, PA), Smooth-On Task 9 polyurethane (Smooth-On) and Epo-Tek 301 epoxy (Epoxy Technology). While Smooth-Cast 310 formed nicely cast parts, the material was opaque and rather soft, and thus unsuitable. The Task 9 was transparent and also stiffer, but the viscosity of the uncured polymer and short curing time made it difficult to avoid forming a large number of bubbles, even with degassing, and so the optical quality of these parts was inadequate for microscopy. Epo-Tek 301 did not share the same limitations: using this material, smooth optically transparent parts were produced that replicated the original device dimensions with good fidelity (Fig. 4 C-F). The spring arms were even stiff enough to hold assembled device pairs loosely in the gap configuration.

However, while many criteria were achieved, the Epo-Tek parts still fell short. For example, greater spring arm stiffness is still required; in the future, a solution might be to design thicker cantilever arms. Also, after some days immersed in cell culture media, the initially transparent parts were found to turn cloudy, ruining the optical quality. It is possible that this can be addressed by increasing the cure time. Finally, as discussed below, cell adhesion on these polymers was found to be inadequate. Therefore, while this work is promising, further development remains before functional devices can be achieved via polymer replica molding.

IV. Synthetic-To-Living Interface

A. Cell-Substrate Adhesion

The premise of this system requires that cells remain firmly attached to the plates that they are sitting on while these plates are repositioned. Adhesion of cells to non-biological materials is typically mediated through proteins that have adsorbed onto the material surface. Since protein adsorption varies for different materials, cell adhesion varies as well. In this case, adhesion of the cell types of interest was found to be unsatisfactory on either silicon or silicon dioxide, hence additional surface engineering was required. Even when the parts were pre-incubated in extracellular matrix proteins such as collagen I, cells would seem to attach and spread normally, only to sheet off of the device later when stressed, such

as during a media change (Fig. 5A). Cell attachment onto the molded polymers from Section III-D was also found to be unsatisfactory (Fig. 5B).

There are multiple reasons why this device design presents a particular challenge for cell adhesion. First, it was observed that sheeting usually would initiate at substrate edges, and the comb shape provides many free edges. Second, when the system configuration is shifted from contact to gap, cell-cell contacts that bridge between adjoining fingers need to be broken. If cell-cell attachment dominates over cell-substrate attachment, large numbers of cells will detach from their intended position as the fingers separate. Considerable effort was thus expended to develop surface treatments that could provide adequate cell-substrate adhesion.

B. Polystyrene Coating

The best solution that we have found for promoting cell-substrate adhesion has been to coat the device surface with polystyrene and follow with a plasma treatment (Fig. 5C). Since standard tissue culture dishes and plates are similarly made out of plasma-treated polystyrene, this coating provides a surface that closely resembles the standard tissue culture environment. Extracellular matrix proteins such as collagen and fibronectin can be readily adsorbed to the polystyrene coating, in a manner similar to standard tissue culture plastic.

Polystyrene was applied by spin coating, which is well established [14]. Briefly, the silicon parts were cleaned in Piranha (2:1 mix of sulphuric acid and hydrogen peroxide) at 120°C for 10 m. Polystyrene (50,000 MW, Polysciences, Warrington, PA) was dissolved in toluene and applied at 2400 rpm for 30 s. The coated parts were baked at 120°C for a minimum of 5 h, then treated in oxygen plasma (200 mT, 200 W) for 60 s. Desai and Voldman have established that following plasma treatment, it is important to wait for 3 h in order to allow the surface to stabilize [15].

Initially, each part was coated individually, since the spin-coater chuck could only vacuum-mount one part at a time. This serial process was slow and tedious, however, and so a more efficient method was devised in which a number of parts were temporarily tacked onto a sheet of elastomer (Sylgard 184) and mounted to a 4" vacuum chuck for batch spin coating (Fig. 5D).

Following the completion of a cell culture experiment, the polystyrene was stripped in toluene, and the parts were cleaned and recoated. The ability to reuse individual devices for many experiments (>20) is an important element to making this system viable in terms of cost and labor.

C. Collagen Conjugation

Another surface treatment approach for promoting cell adhesion was chemical conjugation of collagen to the uncoated silicon surface. The advantage of this approach is that large numbers of parts could be prepared in bulk by simply applying a series of chemical steps. Initial results have been encouraging, showing fairly robust cell adhesion (Fig. 5E-F). However, further characterization is required before it can be established how cell adhesion

and phenotype on this surface compare to polystyrene, which has long been the standard for cell culture.

The treatment procedure was as follows. The starting surface was silicon dioxide, since the thermal oxide layer was not stripped from the surface of the silicon parts following fabrication. Following a Piranha clean, the device parts were thoroughly washed under a continuous stream of ultrapure water for 10 m. The parts were washed 3 times in 100% ethanol and dried on a hotplate at 180°C for 20 m. To aminate, (3-Aminopropyl)trimethoxysilane (APTMS, Sigma-Aldrich), was applied overnight (>12 h) at a concentration of 5.4 M (97%) in 100% ethanol. The parts were again washed 3 times in 100% ethanol, and then heated at 200°C for 3 h. To add a linker, 1 mM Bis(NHS)PEO5 (Thermo Scientific) was applied in 1x sterile phosphate-buffered saline (PBS, Sigma-Aldrich), at room temperature for 2 h with stirring. The parts were washed 3 times in ultrapure water, then incubated at room temperature overnight in collagen solution (0.05 mg/ml) with stirring. Finally, the excess collagen was washed off in 1x PBS. The treated parts were stored in PBS and used for cell culture as soon as possible.

V. Conclusion

To summarize, this report has described the engineering of a new class of MEMS device that can manipulate cellular organization and cell-cell interactions dynamically while preserving cell-substrate adhesion. The paper has detailed the design of a particular device geometry that is optimized for modulating contact between two cell populations, and an integrated positioning mechanism that allows micrometer-scale positioning via manual actuation. Fault-tolerant design-for-manufacturing and process improvements have achieved a high rate of yield, and coating with polystyrene presents a standard tissue culture surface that promotes good cell-substrate adhesion.

This device has proven to be a powerful biological tool and has played a critical role in recent discoveries in our laboratory related to cell-cell signaling in cultured liver tissue [7, 8] and muscle [16]. More broadly, this class of devices should prove to be generally applicable to many kinds of studies involving cell-cell interaction, for example stem cells and supportive feeder layers, or tumors and their surrounding stroma. The specific geometry of the device described here (Fig. 1) may not necessarily be optimal for all situations. Larger combs would be able to fit more cells and reduce the need to pool cell lysate from multiple combs in order to generate sufficient sample volume for protein or nucleic acid quantification [8]. Narrower comb fingers would place a greater percentage of cells near the finger edges where heterotypic cell-cell interactions are maximized. The primary tradeoff is that longer and thinner fingers would be more fragile. In the current device, careful handling is already required in order not to break the cantilever latches.

While this geometric and mechanical design has proven to be highly robust and effective, etching the parts out of silicon presents drawbacks including cost, compatibility with standard biological microscopes, and the additional effort required to modify the surface for cell culture. This paper has reported encouraging progress towards achieving replica-molded parts or batch-modification with conjugated matrix proteins. However, to this point, the

most reliable devices are still the polystyrene-coated silicon parts, and all of our biological work has been accomplished by using such devices. Ultimately, the ideal solution for mass production might be high-precision injection molding of polystyrene parts, which would be cheap, transparent and highly compatible with cell culture.

Acknowledgment

The authors thank Salil Desai for providing assistance in polymer casting, and Ben Chui and Alissa Fitzgerald at A. M. Fitzgerald & Associates for helpful discussions on silicon microfabrication.

This work was supported by the National Science Foundation Faculty Early Career Development Program, National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, and the David and Lucile Packard Foundation. E.E.H. was supported by a Ruth L. Kirschstein National Research Service Award.

Biography



Elliot E. Hui received B.S. degrees in physics and in electrical engineering and computer science from the Massachusetts Institute of Technology, Cambridge, MA in 1994 and the Ph.D. degree in electrical engineering from the University of California, Berkeley, in 2002.

Following postdoctoral work in Sangeeta Bhatia's laboratory at the Massachusetts Institute of Technology, Cambridge, MA as a Ruth L. Kirschstein NRSA fellow, he moved to the University of California, Irvine, in 2008, where he is currently an Assistant Professor of biomedical engineering. His research focuses on the engineering of tools for spatial and temporal control of biological microenvironments.

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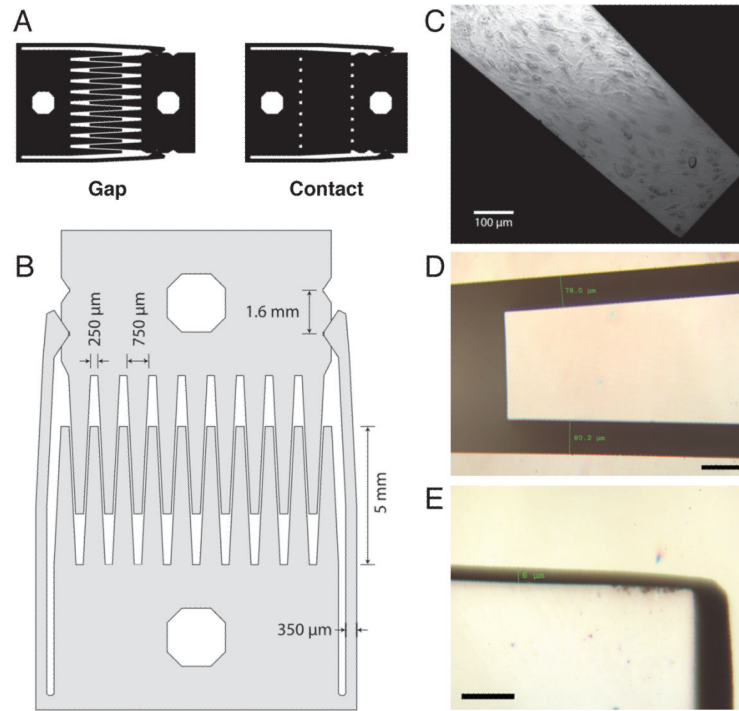


Fig. 1. (A) Schematics of reconfigurable culture device in gap and contact configurations. (B) Device layout and dimensions. (C) 10x brightfield image of 3T3 fibroblasts growing on the device surface. (D) 20x image of device in the gap configuration, showing uniform separation of $\sim 80 \mu\text{m}$ between the fingers. Scale bar is $100 \mu\text{m}$. (E) 100x image of device in the contact configuration, showing minimal separation of $\sim 6 \mu\text{m}$ between the finger edges. Scale bar is $20 \mu\text{m}$.

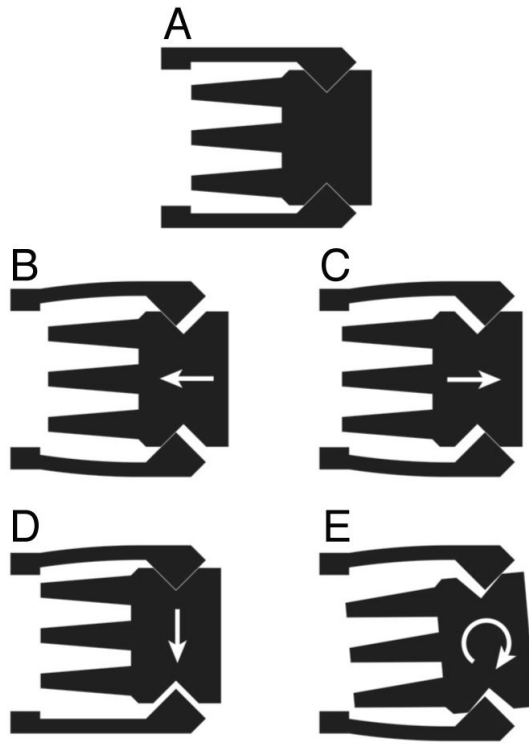


Fig. 2. Precise, self-correcting positioning is provided through the integrated latching mechanism. In this schematic representation, the system is stable when the part is properly centered (A). Upon lateral (B-D) or angular (E) displacement, the spring arms provide restoring forces (arrows) to restore proper positioning.

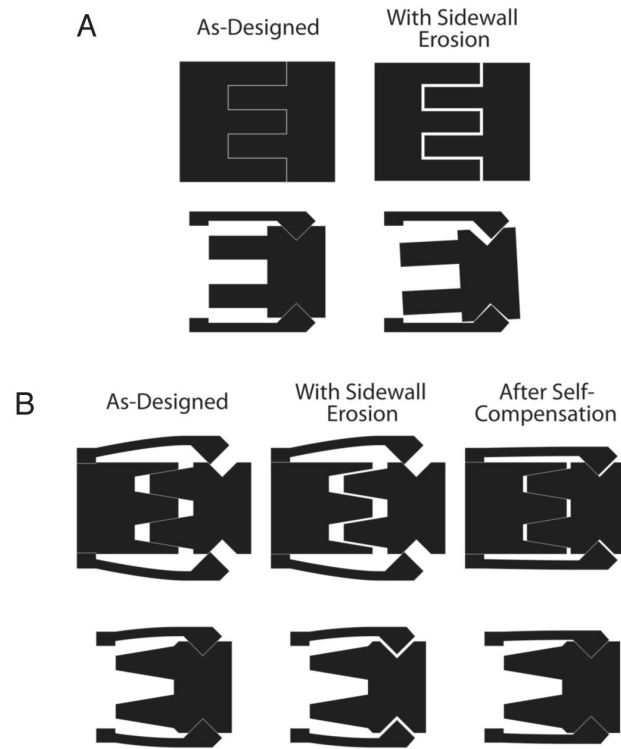


Fig. 3.

Fault-tolerant design compensates for DRIE lateral-etch variation. (A) The device depends on precisely fitting parts, but this can be undermined by sidewall erosion such that the edges of opposing fingers do not properly come into contact (top), or play in the latching mechanism reduces positioning accuracy (bottom). (B) Fingers are tapered and shortened at the tip, and the latch slots are moved back slightly; excessive sidewall erosion is thus compensated for as the combs are simply pushed closer together to achieve tight contact (top). Also, the cantilever springs are pre-loaded so that the latches maintain tension regardless of the amount of sidewall erosion (bottom).

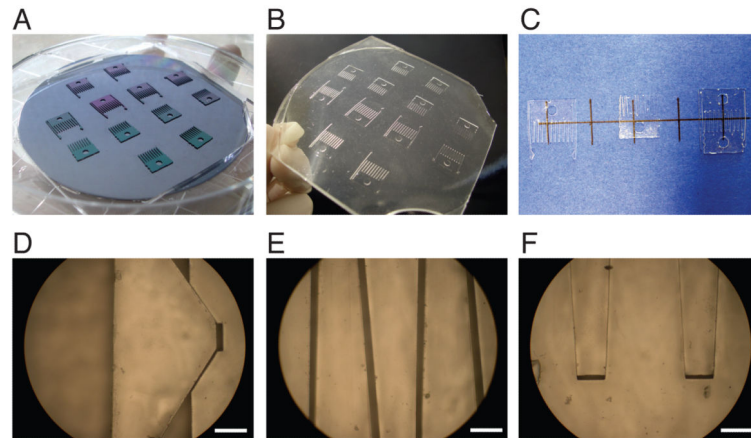


Fig. 4. Replica-molded transparent polymer parts. (A) Microfabricated silicon masters glued to a silicon wafer. (B) Negative PDMS mold cast from silicon masters. (C) Transparent Epo-Tek 301 epoxy parts cast using PDMS mold. High magnification images show excellent precision and fit of the latching mechanism (D), and of the comb fingers in gap mode (E) and in contact mode (F). Scale bars are 250 μm (D-F).

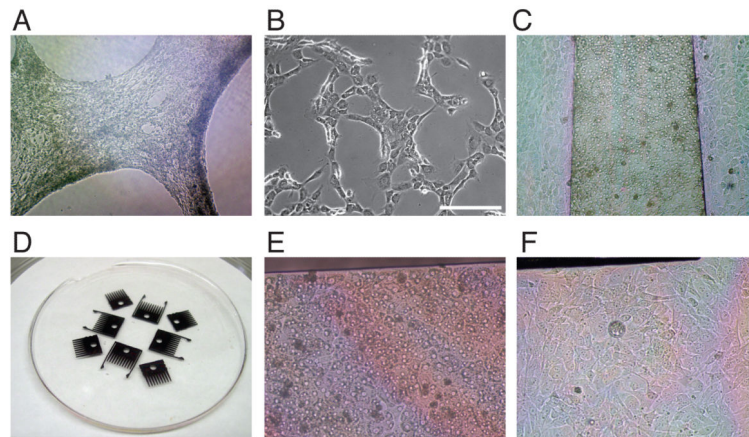


Fig. 5. Surface chemistry mediates cell adhesion. (A) On the as-fabricated silicon dioxide surface, 3T3 fibroblasts attach and spread well initially, however the cells tend to sheet off during perturbations such as media change. (B) 3T3 fibroblasts also adhere poorly on plasma-treated Epo-Tek 301 epoxy. (C) Primary hepatocytes (center) and fibroblasts (left and right) adhere best to the device when coated with plasma-treated polystyrene, followed by collagen adsorption. (D) Polystyrene can be spin-coated by tacking the parts to a PDMS chuck. (E-F) Alternatively, collagen can be chemically conjugated directly to the silicon dioxide surface. Hepatocyte (E) and 3T3 fibroblast (F) adhesion on this surface chemistry is almost as good as on polystyrene.