

Development and Optimization of a Micropatterning Technique Using Soft
Lithographic (Microstamping) Methods

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TABLE OF CONTENTS

	Page Number
1) INTRODUCTION.....	4
2) PROJECT GOALS.....	6
3) METHODS	
3.1) Making the mechanical press.....	7
3.2) Fabricating Stamps.....	8
3.3) Using Fluorescence Microscopy to Gauge Stamp Quality.....	10
3.4) Cleaning the molds.....	10
3.5) Inking the stamps.....	10
3.6) Cleaning the stamps.....	11
3.7) Cleaning glass substrates.....	12
3.8) Printing PDMS stamps.....	13
4) RESULTS	
4.1) Fabricating stamps.....	13
4.2) Using Fluorescence Microscopy to Gauge Stamp Quality.....	14
4.3) Cleaning the molds.....	15
4.4) Inking the stamps.....	15
4.5) Cleaning the stamps.....	16
4.6) Cleaning glass substrates.....	18
4.7) Printing PDMS stamps.....	18
5) DISCUSSION.....	19
6) CONCLUSIONS.....	19
7) REFERENCES.....	20
8) ACKNOWLEDGEMENTS.....	20

LIST OF FIGURES

Figure	Page Number
1a. Process of creating silicon master.....	5
1b. Process of taking a stamp inked with either protein or peptide and stamping a glass substrate.....	6
2. Picture of the mechanical press for stamping.....	8
3. Example of two stamps made from clear PDMS polymer.....	9
4a. Example of a high-quality stamp viewed through white light bright field microscopy.....	14
4b. Medium PDMS grade stamp.....	14
4c. Example of a low quality (unusable) PDMS stamp.....	15
5a. Picture of the fluorescent markings of FITC.....	16
5b. White light view of the 45 minute pattern without clear adsorption.....	16
6a. Stamp that has been soaked in ethanol for cleaning; not yet sonicated.....	17
6b. Picture of stamp (from 6a) being sonicated in ethanol for 15 min.....	17
7. Figure showing the surface of a stamp that was originally adsorbed with FITC and sonicated in ethanol for 20 min.....	18

Abstract:

The ultimate objective of this research project is to optimally develop a micropatterning technique using soft lithography. Optimizing this process depends on perfecting three steps: producing polydimethylsiloxane (PDMS) stamps, "inking" the said stamps with protein, and transferring the protein to a final substrate. Past work has been done using these stamps with biological substrates, but the results have been inconsistent and no reproducible protocol has been developed.

The process begins with Sylgard, a silicon elastomer in liquid form. This elastomer is then poured into silicon molds (not made by our group) with micro-sized features to harden into rubber-like stamps. After curing, the stamps are removed from the molds and analyzed for feature integrity and cleanliness using fluorescence microscopy. Depending on their quality, stamps were either inked with protein or stored for future reference. A few inked stamps were tested for printing onto glass cover slip substrates.

The results from these tests show a continued need to develop a more efficient, reliable microstamping protocol in order to produce successful substrates on which cells with survive and grow.

Introduction:

In the development of a small, biocompatible microchip that can be implanted within the body, the main issue to consider is how to optimize the interface between the implant and the existing biological system. The chip will most likely be interfacing with existing neurons in the body, so the ultimate goal will be inducing the neurons to interact with and extend processes into the chip in a controlled way.

Avoiding an adverse immune response to the implant is another major consideration to consider. The hope is to design a chip that will be "invisible" to the body's immune system by coating the surface with a biological protein such as laminin, or possibly smaller peptide sequences [1]. Before this stage of development can be reached, however, a method of producing reliable, reproducible micropatterns of the desired protein or peptide on a substrate must be found [2].

Still in the early stages of development, our group has chosen to investigate soft lithography as a promising means of achieving these goals. This technique, touted in many other papers, is desirable because it has the advantage of being relatively inexpensive, easy to use, and easily implemented into standard laboratory setting-- as opposed to photolithography or other techniques [3]. Soft lithography is so named because it uses "soft", rubber-like stamps made from PDMS (polydimethylsiloxane) to transfer a micro-sized pattern of proteins or peptides onto a flat substrate [4].

PDMS can be mixed shortly before application, and then poured over thin silicon masters to cure into solid, flexible stamps [4]. See figure (1a) for the process detailing how silicon masters and, subsequently, stamps are made. *It should be noted that the silicon molds used were *not* produced by our group, but had been made previously by the BIOE476 Neural Engineering lab at the at the University of Illinois at Chicago[5].

In addition to the benefits listed above, the mechanical properties of soft

lithography mean that the surface of the substrate does not need to be perfectly flat to obtain an even coating of protein or substance; this is because the surfaces of the stamps are flexible enough to mold to any uneven contours or un-flat areas of the substrate [6]. This makes work with plain glass cover slips, such as the ones used in these experiments, particularly easy and straightforward.

For production, each silicon wafer is coated with photo resist and a masking technique is used, where a laser ablates away the photo resist in certain places and not others. This leaves a pattern of peaks and valleys and results in an intentionally designed pattern with micrometer sized features (anywhere from 15 μm to 30 μm horizontally, with a vertical relief edge between 20 and 50 microns deep). [7] These dimensions are intended to coincide with the sizes of various cells and cell features, helping to enhance directed cellular growth along the inked pattern. Hence, the ideal pattern used will be dependent on the type of cells intended to grow on the substrate, as well how the substrate will be implemented inside the body.

Once the stamps have been made, it is necessary to “ink” the stamp surface with either proteins (such as the extracellular matrix proteins laminin or fibrinogen), or peptides specifically designed for this purpose. This is usually as simple as allowing the protein solution to sit on the surface of the stamp for a given amount of time (typically no less than 30 min for maximum adsorption), and then using a pipetter to suck of the excess solution, rinse the stamp with deionized water and quickly dry with either pressurized Nitrogen or Argon gas. Immediately after drying, the stamp should be brought into contact with the substrate, with a small amount of pressure applied to the stamp [8].

According to almost all literature on the subject, one of the most critical factors in achieving good protein transfer is to keep the time between drying of the stamp and imprinting of the substrate under one minute in length. See figure (1b) for detail of the inking and stamping procedures.

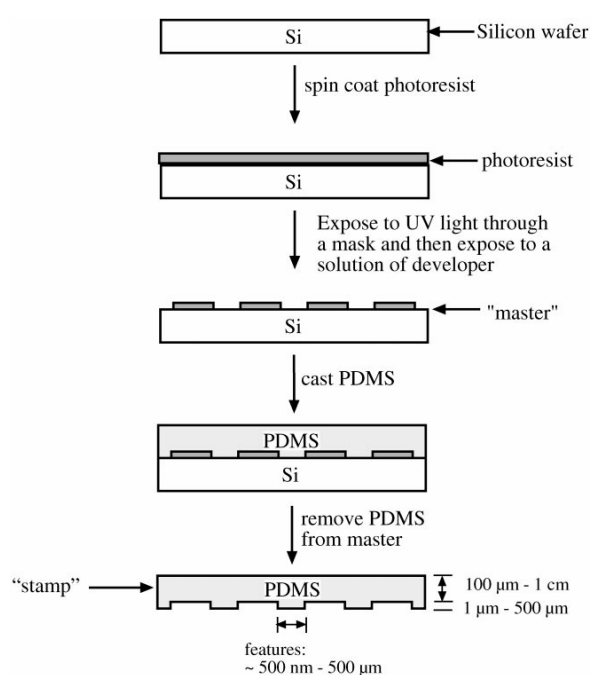
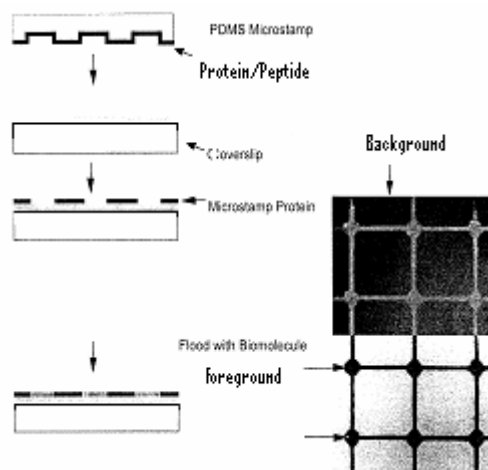


Figure 1a: Process of creating a silicon master up through the creation of a soft PDMS stamp.



[9] Figure 1b: Process of taking a stamp inked with either protein or peptide and stamping a glass substrate with it to form a “foreground”, projected pattern. The “background”, recessed portion of the stamp, can then be flooded with another molecule, differentiating the two areas and materials.

In order to optimally develop this micropatterning technique using soft lithography, the methodology must be split into several “sub-goals”. Each of these sub-goals presents certain technical challenges that have been identified as necessary to overcome before a good protocol can be finalized.

At the time of this paper, certain challenges have been resolved, but a number of problems still remain; unfortunately, due to time constraints, we were not able to fully answer all the questions that arose during the project, especially because along the way the problems become more “process-development” oriented than originally anticipated. The remainder of this paper will address all the problems that arose during this project, those that have been solved and those remaining. It is hoped that another groups will continue our research and make further progress using the foundation we have built.

Project Goals:

In order to clearly define the objective and goals required for this project, there are several steps along the way which must be chronologically addressed. Each step is a goal in its own right and may have several “sub-goals”. These sub-goals may be defined as criteria or standards that are seen as obstacles that need be cleared before the overarching goal is satisfied. The following outlines a chronological listing of goals that our group feels is necessary to address:

Main Goal:

Developing an optimal micropatterning technique using microstamping (soft lithography) with which we can grow and culture retinal cells

Secondary goals:

Fabricating Stamps

- Producing stamps with no air bubbles
- Detergent use?
- Complete Transfer of Features; Methods for Peeling
- Consistently cleaning stamps

- Reliable and robust fabrication protocol

Cleaning the silicon wafer molds

- Molds must be clean within depressed patterned areas to be able to make flawless stamps
- What cleaning solution should be used?
- Must clean without disrupting patterned surface

“Inking” stamps with a protein/ peptide

- Complete protein adsorption to raised features
- Choice of best protein/ peptide
- Possibility of cleaning and re-inking

Transferring Protein to Substrate

- Fluorescent pattern transferred with Uniform Thickness
- Pattern transferred with NO missing features or protein smudging
- Robust, repeatable process

Testing cells on micropatterned substrates

- Choice of Pattern?
- Choice of substrate? (protein/ peptide)
- Choice of cells

The literature will discuss the progress through these secondary goals and necessary steps/procedures to successfully complete the aforementioned objectives.

Methods:

Making the Mechanical Press:

What was logically assumed was that further down path, when patterns need to be stamped onto substrates, a device must be used to eliminate the element of human error. This device is to provide the experiment great mechanical precision. There were several constraints that went into the design of this mechanical press. These included:

- 1) Precision in stamping on the order of millimeters in the up and down (y-directional) plane
- 2) A means to apply various amounts of pressure on the stamp to the surface of application
- 3) Specific way to experimentally measure the amount of force used

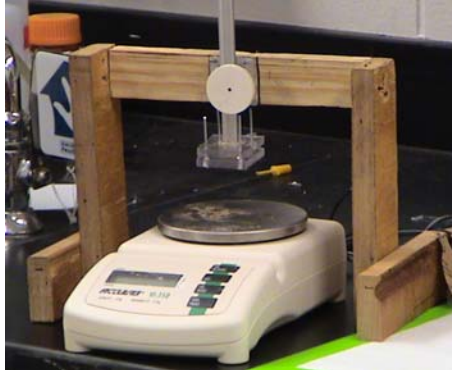


Figure 2: Picture of stamping.

the mechanical press for Although simplistic in design, it

serves the purpose of stabilizing and controlling the stamping process.

4) Mechanical precision and an efficient protocol to reproduce the device through use of the tools and Fabrication laboratory in the Bioengineering Department of UIC, we were able to design and create a simplistic mechanical press for use in the stamping process. Our custom-built device was made almost entirely out of wood pieces (due to availability and easier manufacture than metal pieces), and was designed to have two central legs with supporting “feet” for stability, and a crossbar between them. Attached to this crossbar is a trammel bar, constrained to be capable only of straight vertical up-and-down motion as to limit side to side slippage of the stamp during its contact with the stamping process. The motion is controlled via a large circular knob on the bar, allowing for controlled precision, and very minor adjustments in the height of the bar with greater accuracy. The stamps are attached to a flat plastic plate on the end of the trammel bar; this is achieved by placing a drop of water on top of the stamp and adhering it to the plastic plate using only surface tension forces. There are many benefits of using this method: there is no mechanical compression of the stamps and hence all features on the stamp remain the correct alignment and dimensions; there is no complicated mechanism for trying to hold the stamp in place (i.e. vacuum suction) that could interfere with the stamp-substrate interaction (mechanical sleeve for stamp); the water can easily be removed from the stamp by drying, without leaving any kind of detrimental residue behind, as using something like vacuum grease would.

The entire device is designed to sit over a balance on which the glass substrate is secured. The stamp is attached to the plastic plate relief side down, but fortunately the surface tension is only required to overcome the force of gravity; once the stamp has actually contacted the surface of the glass there is a constant force being applied evenly over the surface of the stamp by the plastic plate. By having the entire apparatus on a weigh balance, the amount of force applied to the stamp and substrate can constantly be monitored and kept constant. Figure (2a) is a picture of our mechanical device, for further visualization of its construction.

Fabricating Stamps

The stamps were made from PDMS (polydimethylsiloxane, Dow Corning) by mixing a hardener and elastomer in a ratio of 1:10. The mixture was stirred for approximately 10 minutes, before being poured over silicone masters with different relief patterns etched into them. Each master had been previously produced in a UIC neuroengineering class by Casey Hathcock, and was loaned to us for the purpose of making the said stamps. Two batches of stamps were made: the first was allowed to cure for 1 day; the second was allowed to cure over the weekend for a course of three

days. It was not initially clear whether or not the amount of time used to cure the stamps would have an effect on their properties, so two different curing periods tested were taken directly from other literature [5] and used previously by Sujata Sundara-Rajan in the lab. In order to contain the liquid PDMS to only the immediately etched area, we used the bottoms of small Petri dishes (using a soldering iron to cut a large hole in the bottom) as “molds”; we then applied a small amount of vacuum grease to the edge of these dishes and, upturned, filled the mold approximately half way with liquid PDMS. This depth was best for producing stamps that were thin enough that all internal bubbles could escape solution while under vacuum, and yet thick enough that they were easy to handle.

Upon pouring, both sets of stamps were placed into a vacuum chamber and held at a constant -25 inches Hg until they were removed. Upon removal from the vacuum, the stamps were carefully peeled off the masters and a razorblade was used to cut the stamps into 5.5 by 5.5 cm squares, as was marked by the edge of the etched portion of the master. The stamps were then stored under deionized water until they were needed for further use. In the past, oxygen plasma treatment had been used on the surface of similar stamps in order to create an oxidized, more hydrophilic surface for the protein solution to adhere to; however, in an attempt to find an easier, less expensive and more reversible alternative to this procedure we decided to store the stamps in water for several days prior to use. Figure (3a) shows a macroscopic view of what the PDMS stamps look like while still wet from cleaning.

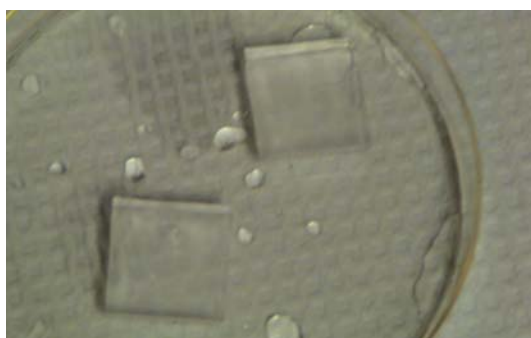


Figure 3: Example of two stamps made from clear PDMS polymer. At this distance, the relief surface is too small to be seen, but it makes up most of the inside of each square.

In an early attempt to improve the number of features reliably transferred from the mold to the stamp, detergent was applied to the silicon surface before pouring the PDMS. It was believed that the presence of a detergent on the mold would allow for easier peeling of stamps, and hence a higher transference of features. Initially, 500 μL of a 10% SDS detergent (v/v, in de-ionized H_2O) solution were pipetted onto the mold's surface, and stamps were allowed to cure on top of this.

After achieving unsatisfactory results with this method, the amount of detergent was reduced to 150 μL , with excess liquid being shaken off before pouring PDMS. Also, the type of detergent was altered to 10% TritonX-100 (v/v, in de-ionized H_2O), to see if this had an effect on the stamp quality. Unfortunately, it did not and detergent was no longer used during the curing process. However, one trial was attempted using Silane as an intermediary material between the stamp and the mold: a cleaned stamp was allowed to sit in a glass Petri dish with several drops of liquid Silane for 1 hour at room temperature. This Silane vaporized and deposited itself on the entire

surface of the mold, presenting a layer that was intended to making peeling of the stamps more successful.

Using Fluorescence Microscopy to Gauge Stamp Quality:

Once stamps had been cured and stored under de-ionized water for several days (since their removal from the masters), the stamps were lightly dried with a Kim Wipe and viewed under white light using a fluorescent microscope. However, an alternate procedure, providing more sanitary stamps, would be to explore not touching the surface of the stamp at all. Drying would then be accomplished under the clean hood, and/or by exposing the stamp to either nitrogen or argon gas. At any rate, the stamps were viewed at a resolution of 10X and 20X, successively. Pictures of the quality of the stamp surface were taken, using a Hamamatsu camera controller (model # 04742-95) interfaced with the microscope and the MetaMorph computer program.

Cleaning the Molds:

In order to ensure the quality of successive stamp fabrication techniques the cleaning of the molds was deemed imperative. Several methods were explored in the cleaning procedure. These included Piranha solution, (concentrated sulfuric acid and hydrogen peroxide in a ratio of 4:1 respectively), treatment with toluene, and treatment with toluene in an oven. Piranha solution was made with 96% sulfuric acid in a 4:1 volume-volume ratio with 30% hydrogen peroxide under the fume hood. The piranha solution was pipetted onto the surface of the mold, covered and let sit for 30 minutes. The mold uncovered, remnants of Piranha were thrown into biohazard wastes, and the mold was washed with de-ionized water and dried with argon gas.

Toluene, already available in the lab, was used in the same manner as Piranha described above, to treat the mold for 24-hours. The mold was cleaned with de-ionized water, dried and observed.

Toluene was also used under the latter conditions, but also placed in the oven at 70 degrees C for 1 day. The mold was cleaned with de-ionized water, dried and observed.

Inking the Stamps:

The methodology of this secondary goal involved a couple different protocols tested to attain the best possible inking results. Prior to initial testing, recently fabricated stamps were gauged for individual quality and robustness. Three of the total 7 initial stamps: six 1-day cured stamps and 1 three day cured stamp were deemed of high enough quality pattern transfer and low enough contamination that they could be used for inking without attempted cleaning first. Each stamp was let sit in the protein “ink” for a certain amount of time.

Three distinct times of inking were tested: 30 min, 45 min, and 60 min. To this point, a specific protocol has not been completely developed to test the main variable in the inking process. This variance is due to the accuracy by which all of the protein completely coats the projected surface of the stamp such that the stamping of the pattern onto glass will yield a perfect duplicate (in protein), of the micropattern.

For initial testing, the peptide used was poly-L-lysine (PLL), fluorescently marked

with FITC (Fluorescein Isothiocyanate), to ink the stamps. Each stamp had a total of 200 μ L of protein solution pipetted onto the relief surface of the stamp, and spread to cover the entire relief area with the tip of the pipette if necessary. Stamps were covered with their respective protein coatings and were left for the appropriate amount of time before being uncovered again. The excess liquid was again sucked off using a pipette, and stamps were rinsed with de-ionized water before being dried with pressurized Argon gas and viewed under a FITC filter using the fluorescence microscope. Pictures of the stamps were again taken for documentation as to the conditions of the stamps, and kept for later reference. Because the protein marker tends to bleach very quickly when left under light, all viewing was done in dark conditions and stamps were stored in a dark drawer when not in use.

A second method, in which recently fabricated stamps were used, was compared to the latter procedure. Four satisfactory stamps that had just been cleaned through sonication with water and ethanol (2:1 H₂O: EtOH), and dried with argon gas, were immediately inked. The same amount of PLL marked with FITC was used to coat the surface of the of the micropatterned PDMS stamp. This time, however, everything was carefully carried out under dim or unlit conditions and the stamps were incubated for one hour. They were immediately taken out and dried with argon gas and immediately taken to be printed onto cleaned glass substrates.

Cleaning the Stamps

Methods for the cleaning of stamps were taken from different literature (Bernard et al. 1998, etc.) and then adjusted accordingly to produce good data. A complete, effective cleaning protocol has yet to be developed, however the methodology of this has developed some good ideas. The four stamps that were deemed to have enough surface contamination to need a cleaning before inking could occur were first placed in a 70% Ethanol solution (v/v) and allowed to soak for 24 hours before being re-examined. Upon the passage of 24 hours in the Ethanol solution, the stamps were rinsed with deionized water and dried with an Argon stream before being viewed under white light with a fluorescence microscope. There did not seem to be a noticeable improvement in the stamp surface after this time period, and so the stamps were placed back in solution and it was decided that sonication in Ethanol would be attempted as a means to clean these stamps. Pictures were taken of all the stamps using the microscope camera and MetaMorph prior to sonication.

Before being sonicated, all stamps were again rinsed with de-ionized water and dried with Argon, and then placed into a sonicator (Cole-Parmer Ultrasonic Cleaner, Model # 08849-00) filled approximately an inch and a half deep with 70% Ethanol solution. These 4 stamps were left in the sonicator for a total of 15 minutes before being removed, rinsed with de-ionized water and dried with Argon for viewing under the microscope.

After sonication, it was clear that three of the four stamps were less contaminated (i.e. looked cleaner). Also, Ethanol seemed to serve no beneficial purpose to the cleanliness of the stamp surface and background. Pictures were taken documenting these phenomena, and then stamps were returned to the sonicator for an additional 10 minutes to see if the additional time would have any added effect.

When viewing was complete, all four stamps were returned to a Petri dish containing 70% Ethanol solution for continued soaking until further testing was needed.

We also used sonication in Ethanol solution as a possible method for removing the proteins that had already been adsorbed onto the stamp surface. The three stamps that had been used for inking were placed in the same 70% Ethanol solution used for the other stamps, and left for a total of 20 minutes before being removed. They were also rinsed with de-ionized water and dried with Argon prior to viewing under the fluorescence microscope. All stamps were viewed under the FITC fluorescence filter, and while they were difficult to see, it was clear that enough fluorescently marked protein remained that the patterns could be determined on two of the stamps, with one having bright spots of FITC but no discernible pattern: we believe that the small features of the stamp led to initial difficulties of protein adsorption only onto the raised features, and that this was not a result of the cleaning process. Also, it must be noted that all stamps that were inked with protein were kept covered in Petri dishes and stored in a drawer with no light exposure when they were not being immediately evaluated. This was necessary in order to prevent bleaching of the fluorescence.

Other tests were run to clean already inked stamps. Three solutions, dilute and concentrated NaOH, and dilute HCl were used to treat the dirty molds. The 0.1M HCl was already available for use in the lab. A separate procedure had to be followed to produce 0.1M NaOH and 10M NaOH. NaOH crystals (FW:40.00) were obtained from under a vacuum chamber. The crystals were weighed out on an exact scale. For the 0.1M NaOH solution, 0.22 grams of crystals were placed in solution with 50 mL of de-ionized water and a stir bar was inserted into the flask. The apparatus was placed on the scale and automatically stirred until the crystals had completely dissolved. For the 10M NaOH, 20.03 grams of crystals were used in the same 50 mL of de-ionized water solvent.

The two base solutions and the acid solution were used to separately treat the previously fabricated and inked (w/PLL) stamps.

Cleaning glass substrates

Prior to testing with biological substrates, non-biological surfaces such as glass cover slips were necessary to test the quality of pattern transfer from the PDMS stamp to the surface. In an effort to keep everything under ideal sanitary conditions, glass cover slips were cleaned through a very simple methodology. Cover slips were soaked in previously made Piranha solution for 20 minutes. Recall that this solution has a 4:1 ratio of Sulfuric acid (H₂SO₄) to Hydrogen Peroxide (H₂O₂). The cover slips were taken out of the Piranha, rinsed with de-ionized water for three times for 5 minutes each. They were sonicated in 2:1 H₂O:EtOH for 5 minutes (Bernard et al. 1998) and then stored in 100% EtOH until they were ready to be used. Just before use they were dried with argon gas.

Printing PDMS stamps:

The cleaned glass substrates were used as surfaces to stamp the recently inked stamps. Immediately after the fabricated stamps were inked, they were placed onto the cleaned glass substrates for 60 seconds, and removed. Note that some stamps had no pressure applied to them, while others had glass cover slips (previously weighed) placed on the surface of the stamp to provide a form of pressure (perpendicular to the glass surface being stamped). The stamp cover slips were rinsed with de-ionized water for 1 minute and dried with argon gas. The surfaces were observed under the microscope. It must be noted that the mechanical press previously mentioned, was not used in the printing of stamps. The preliminary printing tests did not involve mechanical precision; however tests using such a device would be worth exploring in the near future.

Results:

Fabricating Stamps

Once the 1-day stamps had been left under vacuum for approximately 24 hours, they were removed to room conditions and examined visually. While 8 stamps were initially made, only 2 were found to be of useable condition: all of the other 6 had air bubbles present at the relief surface, disrupting the pattern and making further stamping impossible. This is most likely because, as the first attempt to make stamps, the molds were filled with too thick a layer of PDMS, meaning that all bubbles were not able to escape from the liquid before curing occurred. Another possible problem was due to an inadvertent heating of the stamps; accidentally, the stamps were cured at 120°C, instead of at the intended room temperature. By increasing the rate of solidification, this would also lead to an excess of air bubbles being trapped within the stamp.

Our attempt at making the three day stamps was much more successful, with 4 of the 6 total attempted stamps being deemed useable upon initial visual inspection. This seems to be directly related to knowledge gained through the first set of stamps; the 3-day stamps were intentionally made much thinner than the 1-day stamps, and while the vacuum was at a residual 40°C when curing was initiated, it experienced a slow cooling down to room temperature (20°C) over the course of the weekend. So while some stamps did still have an issue with air bubbles trapped at the relief surface and consequently could not be used, the prevalence was significantly less than with the thicker, heated stamps. In general, for both the heated stamps and those cured at room temperature, it seemed that the important curing steps occurred within the first several hours while the PDMS was still in a more “liquid” state. The important curing steps referred to here are those that involve escape of air bubbles and hardening of the mold features. Thus, it was imperative to handle the PDMS filled molds with great care and attention when initially pressurizing them and curing them.

The final conclusions to be made are that re-pressurizing in the vacuum chamber eliminates air bubble accumulation. The quality of the stamps varied to a great extent however this was later determined to be an issue with the molds that were used to fabricate these stamps. Note that the use of any detergent (SDS, TritonX) or the

silanization of a mold prior to curing of PDMS, did not have a significant difference in the detachment of the stamp from the mold, or the quality of the stamps overall.

Using Microscopy to Gauge Inking Quality:

Once the stamps had been fully cured and removed from the masters, they were stored in deionized water until we were able to look at them using the fluorescence microscope. For viewing, the stamps were quickly washed with running deionized water and dried lightly with a Kim Wipe, before being examined under white light at 10x magnification. What we discovered was that although all stamps had a similar appearance macroscopically, they were not at all comparable on the micro level. Indeed, there were three general categories of quality into which the stamps fell: high-quality stamps, having almost no or very few master features fail to transfer onto the stamp surface, and little or no contaminating material present on the stamp's surface; medium-quality stamps, where no more than 20% of the stamp surface had missing features and a small bit of contamination could be see on the stamp surface; and low quality stamps, on which some 50% or more of the relief features were missing (not applicable for further stamping use), and a large amount of contamination was present on the stamp. Pictures were taken of the stamps to document their quality, using the Hamamatsu

Camera Controller described in the methods section. Refer to figures (4a), (4b), and (4c) for examples of high, medium, and low quality stamps, respectively.

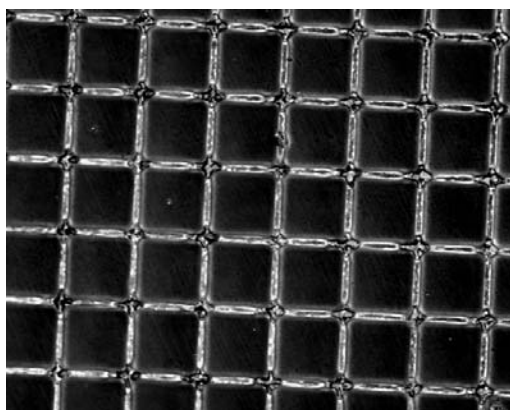


Figure 4a: Example of a high-quality stamp viewed through white light bright field microscopy. All the features have been neatly transferred, and there is nearly no contamination present.

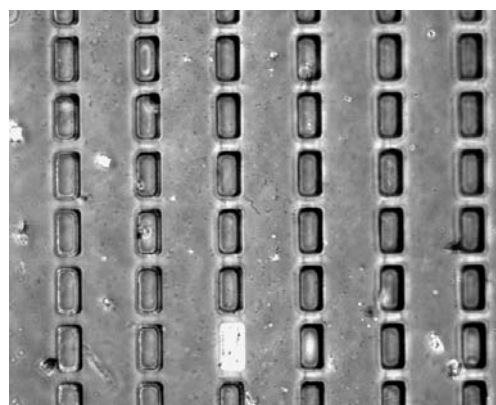


Figure 4b: Medium PDMS grade stamp; nearly all of the features are present (only one raised square missing, see bright rectangle) and there is only minor contamination material present.

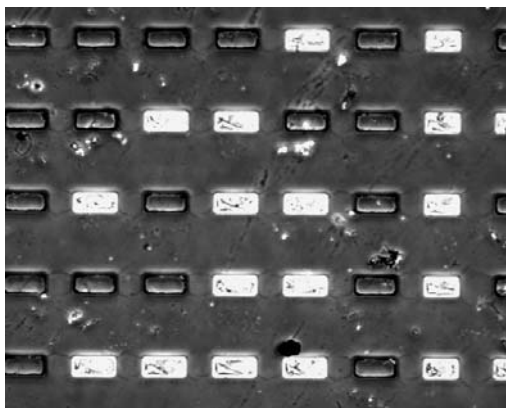


Figure 4c: Example of a low quality (unusable) PDMS stamp, because of the excess of missing features and level of contaminating material present. The dark, bold rectangles represent good presence of raised features. The brighter white rectangles represented recessed, missing features.

It should be noted that, upon viewing the stamps, three were found to be of very high quality and were immediately earmarked for further inking studies. Of the remaining four, two were of medium quality and two stamps were of such poor quality they were considered unusable for further stamping tests; however, all four exhibited an unacceptably large presence of contaminating material on their surfaces, and were kept to be used for further cleaning studies.

Cleaning the molds:

The results obtained from cleaning the molds, is that neither Piranha, nor toluene, nor toluene and heat, are good “cleaners” of the micropattern surface on the silicon wafers. In fact, what was observed was that after cleaning with Piranha solution, the quality of the mold decreased and the mold seemed to have depressions with “cracked” perimeters possibly implying that the Piranha ate away the surface.

Inking the Stamps:

For the three stamps that were adsorbed with proteins for different lengths of time, there appeared to be no significant difference between them that would indicate a longer exposure time results in higher adsorption. This assumption was made by simply analyzing the stamps with the naked eye. However, when viewed under the microscope, it was found that only the 30 minute and 60 minute stamps could be compared, because of a fault with the 45 minute stamp. Both 30 and 60 minute stamps had the same pattern, and thus had very similar results (shown in figure (5a)), and protein adsorbed only onto the pattern could be seen—hence why the pattern is clearly visible under a fluorescent filter. The 45 minute stamp, however, had only random splotches of fluorescence that did not necessarily correspond to the relief pattern; we believe this had to do with a fault inherent in the pattern design, whereby the features are too small and far apart for proper adsorption, and not a fault of the procedure used. See figure (5b) for a white light image of the 45 minute stamp’s pattern, and compare to the more robust pattern of the two stamps that had good absorption patterns.

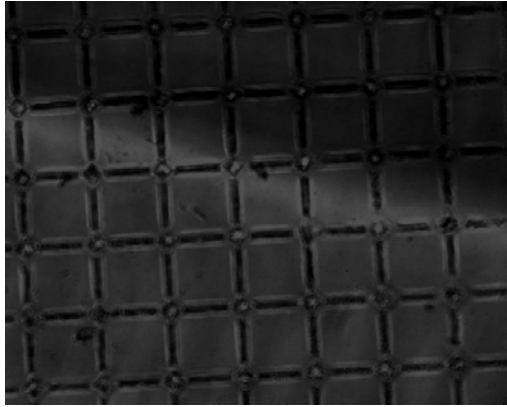


Figure 5a: Picture of the fluorescent markings of FITC –labeled laminin on a stamp (same stamp as figure 4a); protein adsorbed for 60 min. Pattern can clearly be seen through fluorescence microscopy however laminin coating is not clear as most of the surface is dark with the exception of the fluorescent streak through the middle of the pattern.

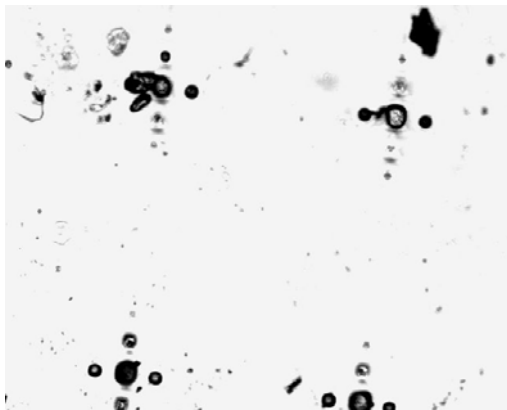


Figure 5b: A white light view of the 45 minute pattern that did not have clear adsorption of protein. Can see this is most likely caused by the small, distant nature of the pattern itself.

The overall results of several inking tests were that the PLL did not adsorb completely on the surface of any of the patterns to the point where uniform, clear inking could be detected under the fluorescent microscope. These results are consistent with those stamps that were inked and incubated for one hour as well. The possibility of other proteins and peptides were not tested to this point, because PLL was the only readily available protein.

Cleaning the Stamps

For the four initially fabricated stamps that were determined to have too much contamination on them for stamping use, we decided to clean the stamps by immersing them in a 70% Ethanol (v/v) solution for 2 days to see if simply soaking them in a material commonly used for cleaning substrates would be sufficient. However, when we removed the stamps from Ethanol, rinsed them with deionized water and dried them with Argon for viewing under the microscope, we found only a very negligible improvement in their condition. Instead, we decided that sonicating the stamps in the same Ethanol solution would be more successful; hence, all 4 stamps were placed in

the same sonicator in approximately 1.5 inch deep Ethanol solution, and left in the bath for 15 minutes. Upon removal, stamps were washed with deionized water and Argon dried before being observed under the microscope with white light. It was immediately obvious that the sonication had greatly improved the quality of the stamps; see figures (6a) and (6b) as examples of the stamps after soaking in Ethanol but before and after sonication, respectively.



Figure 6a: Stamp that has been soaked in ethanol for cleaning but has not yet been sonicated; note the large amount of material present both on and off the relief surface.

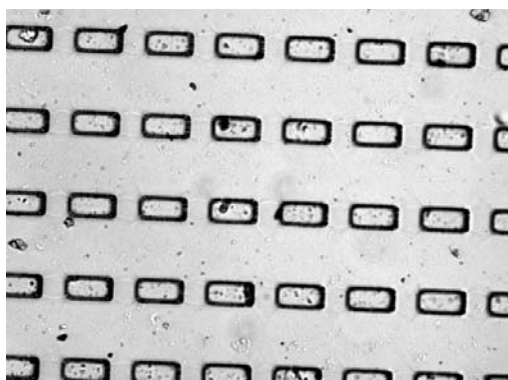


Figure 6b: Picture of the same patten as 6a, (different angle), after being sonicated just 15 minutes in ethanol. Note the drastic improvement in contamination levels and stamp quality.

After this initial success, it was wondered whether the stamps would continue to improve if left in the sonicator for a longer period of time, so the four stamps were returned to the Ethanol solution in the sonicator and left for another 10 minutes. Upon removal, they underwent the same rinsing and drying process as conducted in previous tests, before being observed under the microscope at 10x magnification and white light settings. Contrary to our expectations, the stamps appeared to have improved by only a negligible (not measurable) amount. This still leads to the conclusion that cleaning of non-protein marked stamps can be effectively achieved by sonicating the stamps in Ethanol for 10-15 minutes. The later attempts at sonicating the stamps in a 2:1 ratio of water:ethanol for 5 minutes provided no significant success in cleaning off the surface of the stamp and/or the inked PLL from previous tests.

The initial success with the un-adsorbed stamps led us to conclude that sonication might also be a way to clean the adsorbed protein from stamps between uses. We took the three stamps that had already been treated with protein and placed them in the

same sonicator with the same 70% Ethanol solution that was used for the previous four stamps for a total of 20 minutes. After this time had passed, the stamps were removed from the sonicator and prepared for viewing under the microscope (i.e. washing and drying). These stamps were looked at again under the FITC fluorescence filter, and the amount of protein left on the stamp was visually determined. While it was rather difficult to see the fluorescence, it was definitely clear that the patterns could still be determined and hence too much protein was left on the stamps for this to be an acceptable cleaning method. However, one cannot blame the complete lack of effectiveness of cleaning off protein on the sonication method; instead, we believe that we need to use another, stronger substance besides Ethanol to sonicate the stamps with. See figure (7a) for an image of a protein-treated stamp that has been sonicated in ethanol for attempted cleaning.

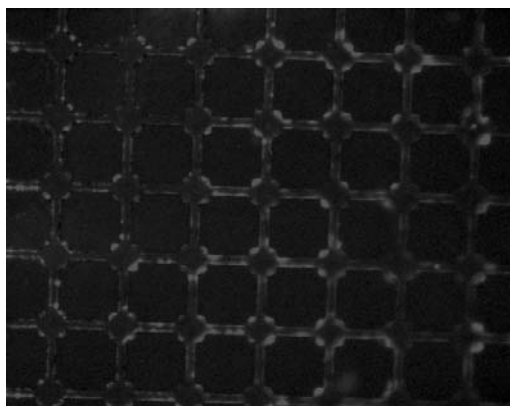


Figure 7: Figure showing the surface of a stamp that was originally adsorbed with FITC-marked laminin and then sonicated in ethanol for 20 minutes. Protein is still clearly present when viewed under FITC filter.

Recall that previously inked stamps were taken and submerged in dilute and concentrated base (NaOH) solutions as well as dilute acid (HCl) solutions. The results from this provided that these three solutions were not able to completely and effectively clean the inked PLL off of the surface of the stamps.

Cleaning the glass substrates:

Tests began on cleaning glass substrates, however only one round of experiments were completed. We have reason to believe that the protocol used to clean the glass was successful; however we have no real results to provide for this hypothesis. Several more trials of cleaning glass substrates are necessary to support this theory.

Printing the stamps:

After observing the glass substrates under the microscope, the results showed that there was not any successful printing of the micropattern from the stamp onto the glass substrate. Therefore, after only one printing trial was run, no significant results can be given relating to the printing of the stamps at this point.

Discussion:

The project objective is to develop an optimal micropatterning technique using soft lithographic methods for the sake of future study in retinal prostheses. The results discussed above have provided sufficient information to lead us down a different, more correct path towards achieving the ultimate goal. In fact, judging the integrity of the results, what we see is that our secondary project goal known as “fabricating stamps” is complete. In this case, we must further our study and understanding on developing a method to clean the molds before fabricating the stamps. The major conclusion is that without clean molds, we can not make clean, flawless stamps. This involves all variables that have been discussed throughout this paper including:

- the integrity of the stamps in relation to the methodology of the initial peeling from the mold
- the cleanliness of the features
- the percentage of features from each mold successfully imprinted in our stamps

We hypothesize that since the patterned surface of the mold is made of poly-methyl methacrylate (PMMA), there is a possibility that Piranha solution, (which is known to attack any organic material), may be corrosive to the patterned surface. Therefore, what should be explored are different solutions/techniques to clean these molds without disrupting the patterned surface.

Once we have figured out the answers to all of our obstacles, we may move on to designing a method and protocol for proper inking of the stamps.

The results retained at this point provide us with a legitimate stepping stone and initial technique by which we can move forward in the project. Analysis of images of self-produced stamps from previously manufactured molds showed that there is inconsistent detail when comparing the features of all of the stamps with one another. Some patterns have approximately 90% or higher detail imprinted on the stamps. Others have quite a bit less efficiency associated with them and must be analyzed to reproduce an exact imprint for future tests.

Conclusions:

The conclusions we may draw from our work at this point is that we understand the logical steps necessary in our approach to optimally designing a micropatterning technique. The variables of each step have been determined and it is a matter of time before we develop a successful protocol for synthesizing near perfect PDMS stamps.

From that point we can move forward in implementing a standard, exact protocol for stamping onto substrates, first glass, then biological substrates. However, as our results conclude, we see that enough real significant data has yet to be determined to move on to the next step which is to culture our retinal cells and stamp onto substrates to test for mechanical force and time of stamping needed for complete imprinting onto a glass surface.

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