Direct Patterning of Proteins with the Alvéole PRIMO

A standard operating procedure for the Alvéole PRIMO system, submitted to the Stanford Nanofabrication Facilities in partial fulfillment of course requirements for ENGR 241

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Executive Summary

This document was prepared to introduce users to the Alvéole PRIMO tool in the ExFab at the Stanford Nanofabrication Facilities. The tool as officially added to the ExFab in December of 2017, and aside from the brief user guide on the SNF website, there are very few published resources for working with the device. For that reason, the primary aim of our E241 project has been to develop a manual that will guide a novice in using the tool. This document cannot replace in person training and mentorship, but rather should be used as a starting point in process development.

We recommend that potential tool users start by looking at the "Quick-start guide" in Section 1--meant to give the big picture overview of creating a first protein pattern. Then, it is beneficial to review the process flow as laid out in Section 4.1. This diagram shows a cross section of a sample substrate throughout processing to highlight the surface level changes accompanying each step. Some users may then find it helpful to review the mechanics of the system by reading Section 3.1. In this system overview we describe the mechanism by which PRIMO is able to achieve micron scale resolution.

For those readers that aren't sure if protein patterning is necessary, we recommend reading Section 2. This section gives a brief overview of the importance of protein patterning and some examples of its application in modern mechanobiology literature. Stanford students and post-doctoral scholars wishing to learn more can inquire about the ME/BioE/BIOPHYS 342A class: "Mechanobiology and Biofabrication Methods."

To summarize the results of our 10-week long project: protein patterning is a task that requires fine tuning. There are many parameters to be adjusted, some more important than others. In our experience, we found these 'tips and tricks' to be most helpful, and we expand upon these ideas throughout the report:

- "Masks" can be made with ImageJ (open source software, popular in biology
- We recommend a minimum feature spacing of 15 pixels (~4.2 um)
- Glass must be very clean and particle free
- Plasma treating the glass improves the quality of patterning
- Maintaining PEG hydration throughout handling is critical
- Consideration must be given to protein solution:
    - Protein concentration
    - pH
    - salt concentration
    - Incubation time and temperature
- 100% power, 60 second duration is a good starting point, but individual users may want to optimize these parameters to improve
    - Uniformity

    - Resolution
- PRIMO is easier to learn and has fewer barrier to entries that lift off or microcontact printing
- For large scale operations, Heidelberg lift-off (not direct writing) may be quicker. Refer to Section 7 for considerations needed before this possible.
- Future users may want to try diluting the PLPP and increasing exposure time as an approach to improving minimum feature size

Many scholars have invested significant energy into developing the set of knowledge presented here. Erica and Joy are both co-advised by Beth Pruitt, Professor of Bioengineering--whose group has made great efforts in developing devices with protein patterns for mechanobiology assays. We are grateful for all of the mentorship we have received from our labmates, including but certainly not limited to: Gaspard Pardon, Leeya Engel, Sasha Denisin, Robin Wilson, Alison Schroer, Alexandre Ribiero, Jens Moeller, and alumni of the Stanford Microsystems for Mechanobiology group, PI Beth Pruitt.

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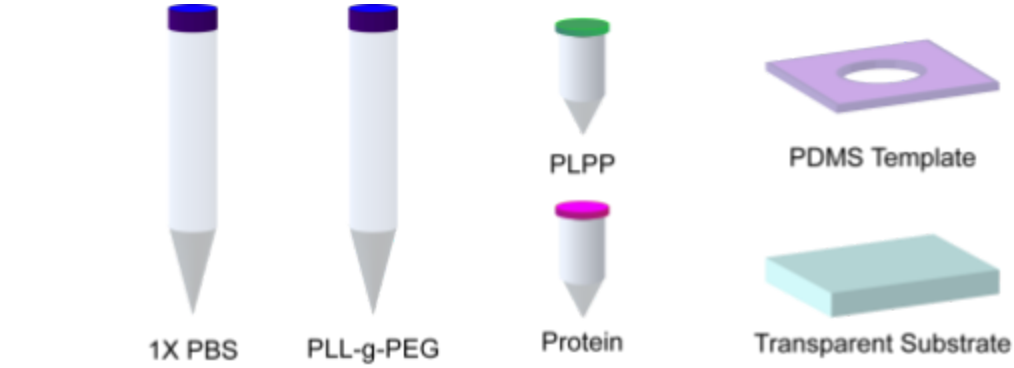
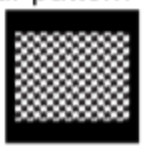
1. Quick-start Guide

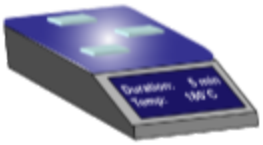


1. Make your pattern using ImageJ

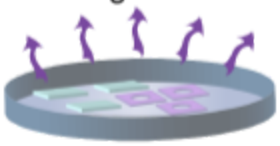


2. Clean coverslips

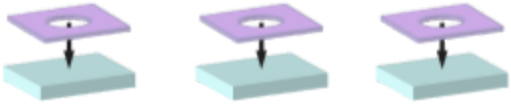
3. Dry coverslips for 5 min at 180˚C



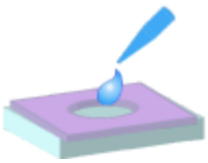
4. Plasma treat glass and PDMS



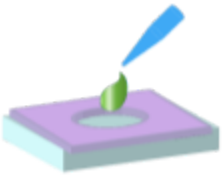
5. Assemble PDMS and glass



6. Incubate PLL-g-PEG for 1 hr

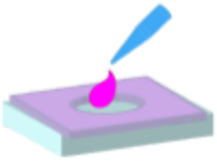


7. Thoroughly rinse surface with PBS and keep it hydrated until PLPP application

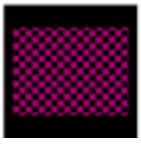


8. Perform PRIMO UV exposure

9. Thoroughly rinse surface with PBS and keep it hydrated until protein application



10. If using a flourescently labeled protein, image the results



2. Background: Why pattern protein?

Growing cells or tissues *in vitro*, away from an organism, is a common approach to studying biological systems. The fact that the cells are growing outside of the native environment is specifically why this approach is so appealing: it allows us to directly probe the role of the cell's microenvironment. Regardless of the scientific questions being asked, typically the cell's must be attached to a substrate such as a cell culture dish or hydrogel. Generally, a cell will only attach to this substrate if the substrate has been functionalized with a protein. Figure 2, below, displays a highly simplified model of how this type of adhesion works. A generalized, eukaryotic cell attaches to its extracellular environment through integrin binding sites. These integrins are a special type of protein made by the cell that extend from the cell's membrane into the microenvironment. The integrins are often complex proteins that have binding sites for specific protein types. In culturing cells, an important step is to determine the protein to which a specific cell type will bind. Examples of proteins include fibronectin, Matrigel, and gelatin.

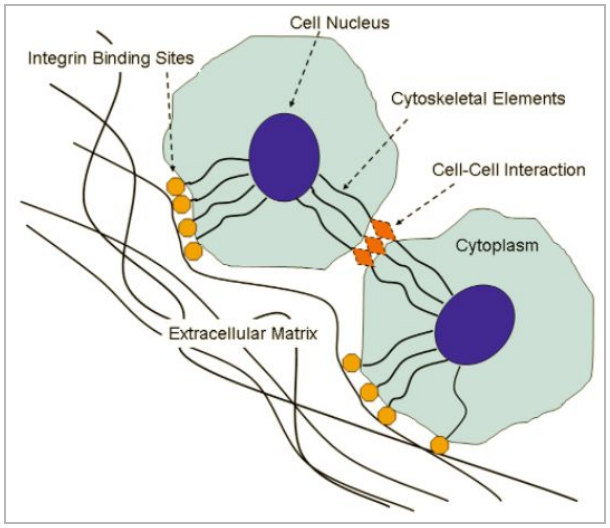


Figure 2. Cells rely on extracellular proteins for anchoring.[1]

Although scientists have been culturing cells and tissues on protein for decades, more recent work has placed an emphasis on how the micro-mechanical environment affects biology. This field is called "mechanobiology." Being able to control the location of protein in the 2D or 3D space around the cell has allowed scientists to explore cell morphology, development, and mechanical output. Figure 3, below, presents three different applications of protein patterns. Figure 3A shows cells, labeled with green fluorescence, attaching to a variety of patterns, shown in red fluorescence. Figure 3B shows cardiomyocytes, heart muscle cells, grown on protein patterns with varying aspect ratios. Using traction force microscopy, Ribiero *et al*. see that pattern aspect ratio influences force production. Figure 3C, from Grevesse *et al*., shows an example where patterns were used to encourage a standard cell morphology. Had the cells not been cultured on well defined protein patterns, the cell shape would difficult to tune. This approach allowed the researchers to obtain more repeatable and tunable mechanics measurements.

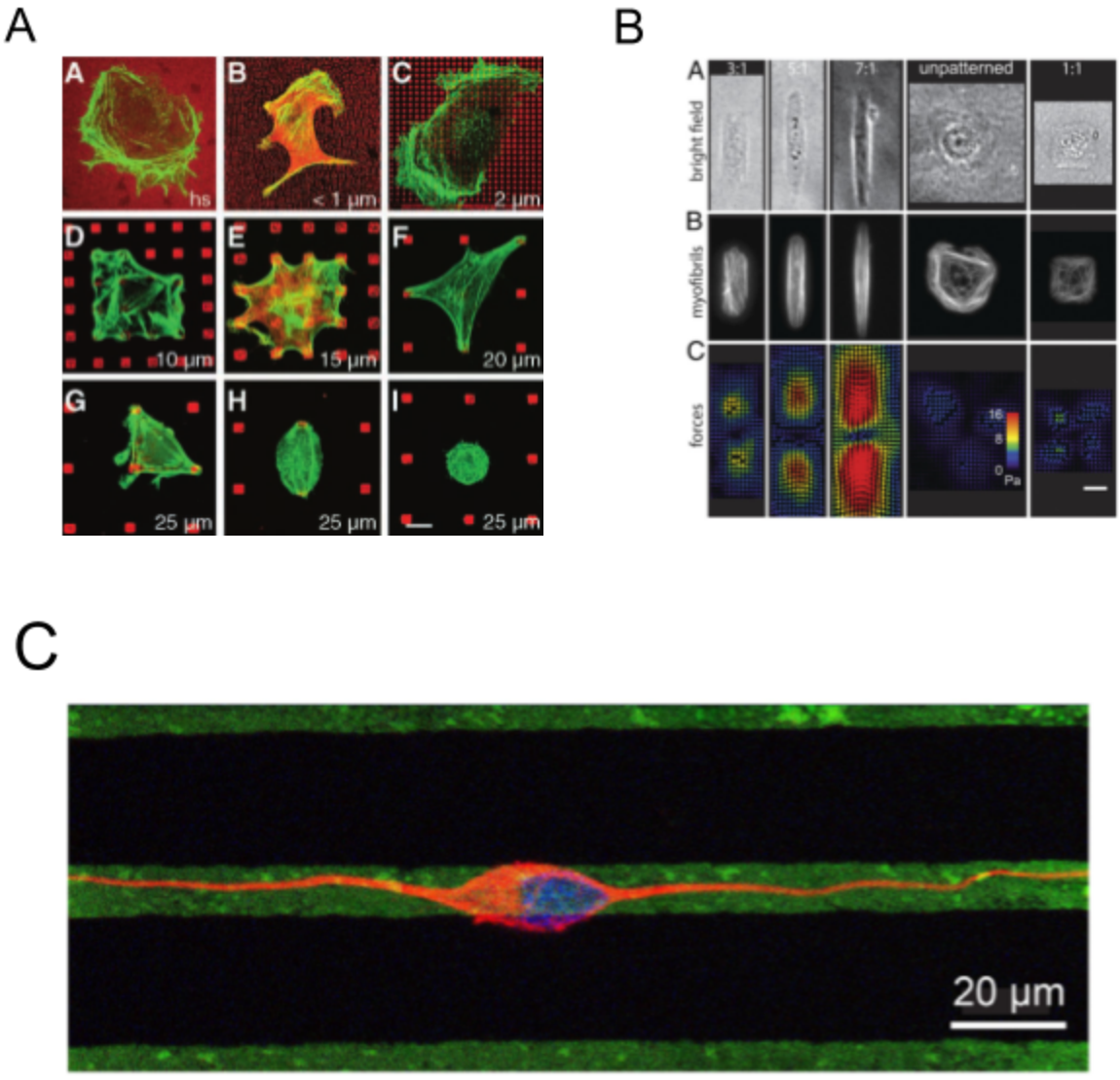


Figure 3. Examples of protein patterning in mechanobiology literature. A) Cell morphology controlled by protein patterns[2]. B) Cardiomyocyte output varies with protein pattern aspect ratio [3]. C) Patterning neurons improves the repeatability of mechanics assays [4].

3. An Introduction to the PRIMO Tool

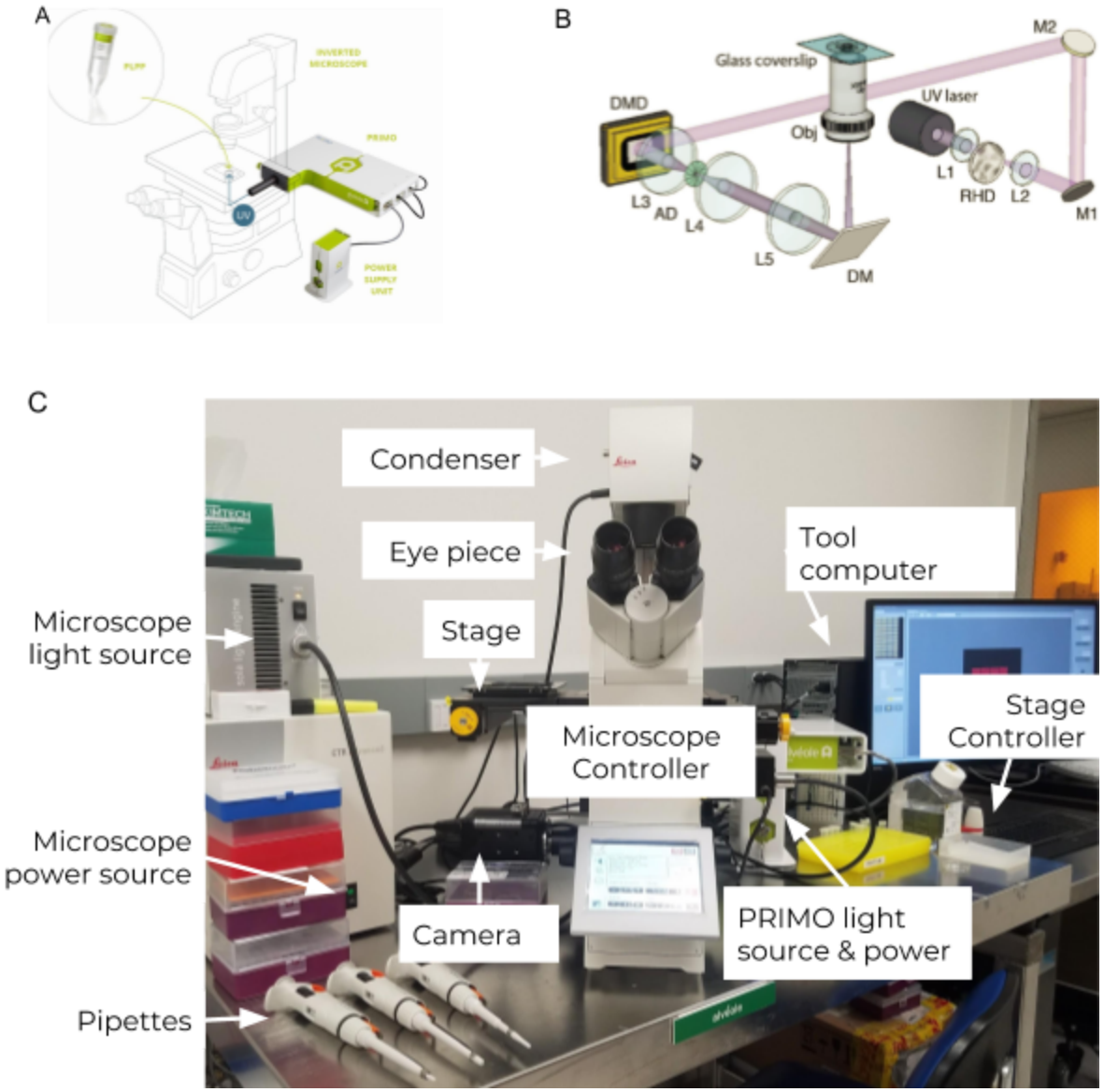


Figure 4. The Alvéole PRIMO. A) The PRIMO tool shown with a generic inverted light microscope[5] B) Light path from the UV laser source, to the PRIMO (DMD) to the glass coverslip[6]. C) The Alvéole PRIMO workspace in the Ocean room of the ExFab at the SNF.

3.1 System Overview

The Alvéole PRIMO system is comprised of the tool itself, Figure 4A above, the proprietary photoinitiator PLPP, and the control software Leonardo that operates as

a plugin for the open source software Micro-Manager. For a visual demonstration of how the system works, we refer the reader to Section 1 of this document. A generic process flow for working with the PRIMO is also available for reference in Section 4.1. Figure 4B, adapted from Strale *et al*., shows the path traveled by the UV light as it moves from source to the final location of the glass coverslip. When the light enters the DMD, the array of mirrors selectively reflects the UV light based on the user's specified pattern. The Leonardo software controls the positioning of the DMD mirrors. The software also allows the user to specify the number of times to expose the pattern on the substrate. If the user specifies more than one, the Leonardo software controls the motorized stage (Figure 4C) so that the substrate automatically moves to the next pattern location.

3.2 Health & safety warnings

- Laser - do not look into the laser's light path.
- PLPP - must wear gloves as PLPP can cause irritation if it comes in contact with skin, eyes, or mucous membranes. Acute toxicity by ingestion.
- Never pipette by mouth.
- Keep fingers away from the motorized stage immediately following power up--the microscope automatically moves the stage during this period.

3.2 Cautions

- Be careful when raising the objective so as to not ram the objective into the sample. After each use, we recommend setting the objective to lowest z height.
- Use caution when tilting the condenser between positions (i.e., gently tilt it back rather than letting it fall back on its own)
- Never put oil or water onto the objectives
- When possible, position the stage using the controller rather than the yellow knobs

3.3 Interferences

- The focal plane of the objective must be on the surface of the substrate where the PLPP-UV interaction is desired. It is recommended that to orient or sandwhich sample such that light path goes through the minimum number of layers.
- Conducting a proper calibration of the device before using a new substrate is critical to achieving high quality results.
- At times, Leonardo software or MicroManger stop working. Recommended to exit micromanger and restart. Saving the calibration file is a good practice and will save time in case of software stops during a session.

3.4 Personnel qualifications & responsibilities

- Users should be comfortable operating an inverted microscope in brightfield

3.5 Equipment and supplies

- Glass coverslips (22x22mm)
- Straight Tapered Flat Point Tweezers
- Acetone
- Isopropanol Alcohol
- DI or Milli Q Water
- Silicone spacer (wells made with a biopsy punch)
- Plasma Asher
- Petri dishes/ 6-well plates
- 0.1 mg/ml (poly(l-lysine)-graftpoly(ethylene glycol) (PLL-g[3.5]-PEG (2kDa), SuSoS AG
- PBS (1X, pH 7.4)
- Individual pipettors (10uL, 20uL)
- Pipette tips
- PLPP (1X, Alveole)
- CAD drawing of pattern of interest
- Protein (ex. Peanut Lectin, 500ug/mL ; ELP-RGD 100 ug/mL)

4. Procedure for using the Alvéole PRIMO

4.1 Process flow

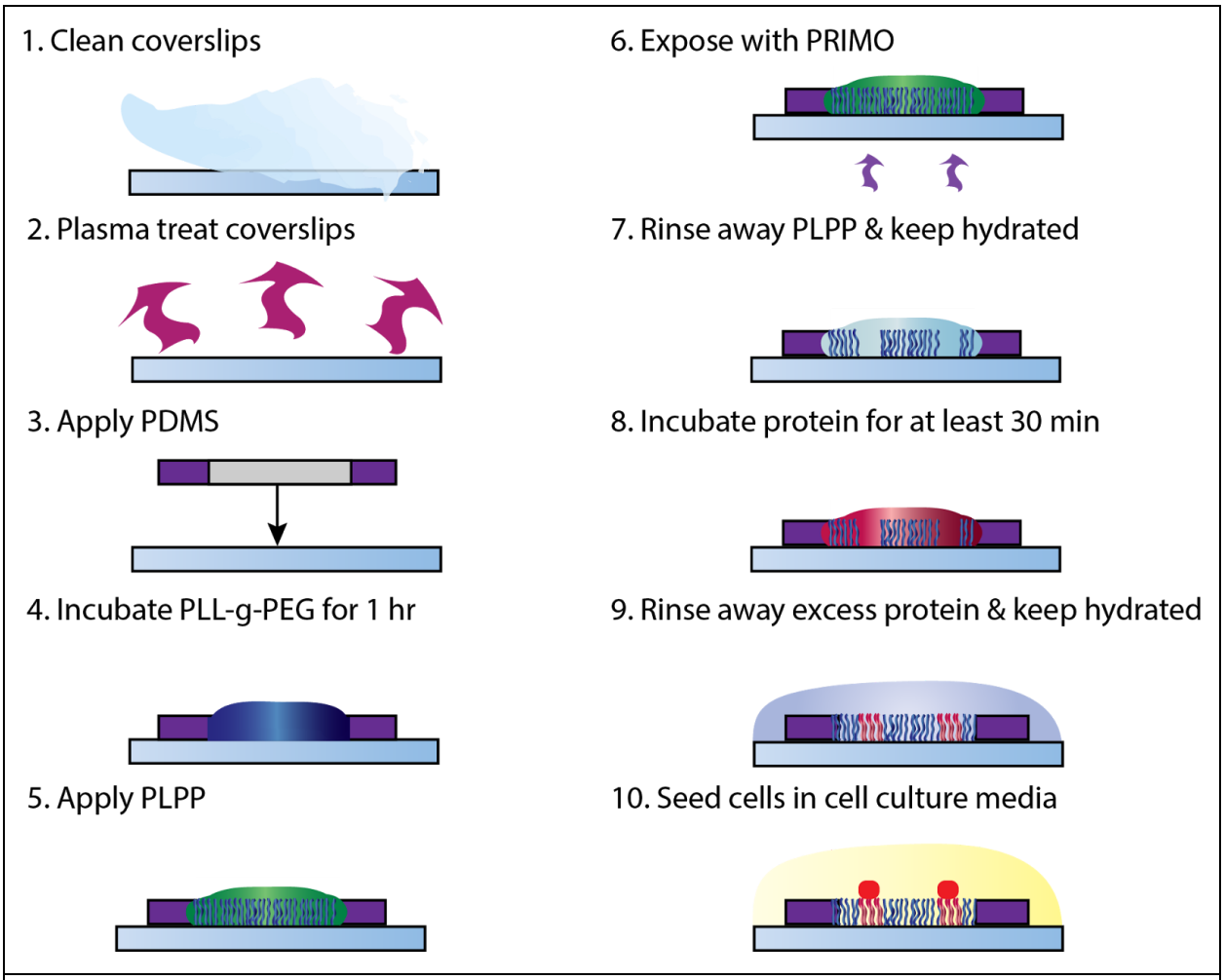


Figure 5. Basic process flow for patterning protein with the Alvéole PRIMO tool.

Figure 5, above, is meant to demonstrate the basic process flow required to selectively pattern protein on glass. We encourage potential users to go through each of the steps as detailed throughout Section 4. In brief, we suggest the following when considering this overview:

- Clean coverslips - Rinse each glass slide starting with Acetone, Isopropanol Alcohol, and thoroughly rinse in DI water. Use Nitrogen to air dry, followed by 5min on 180C hot plate.
- Plasma treatment - 20 seconds at 80W
- Pll-g-PEG - 100ug/mL in 1X PBS (pH 7.4), 1hr at room temperature (RT)
- It is important to rinse thoroughly between steps 4 and 5, 7 and 8, and in step 9. This will ensure (1) properly washing away any excess (2) properly wash away

any component not adsorbed. 5 times rinse in PBS (1X, pH 7,4) is recommended.
- Although omitted from the graphic above, we have had success in sterilizing the device for cell culture by placing it under a UV lamp between steps 7 and 8 for at least one hour. However, we anticipate those results will vary greatly between UV lamps, substrates, and cell types. Some cell types are notoriously more sensitive to contaminants. Be sure to check that the UV lamp being used is the proper wavelength for lysing DNA and that it is within its acceptable lifetime.

4.2 Making a pattern

There are many ways to make a suitable pattern to use with the PRIMO tool. Whichever method is used, the image must be:

- 8 bit
- .tif format
- 1824 x 1140

In our hands, the easiest approach to drawing a pattern was to use the NIH open-source software ImageJ or FIJI (FIJI is just ImageJ). To do this:

1. Open ImageJ
2. File -> New -> Image
    a. Type: 8-bit
    b. Fill with black
        i. Black = 0; no UV exposure
        ii. White = 255; full power UV exposure
        iii. Gray = 1 - 254; Leonardo will accept an image with any pixel having a value between 0 and 255.
    c. Width: 1824 pixels
    d. Height: 1140 pixels
    e. Slices: 1
3. Use any of the region of interest tools to specify a shape.
    a. Use the left and right arrows to move the selection
    b. The floating toolbar will display X and Y positions, as well as height and width of the selection
    c. When running on Mac OSX, holding the option key while using the left and right arrows will allow you to change the size of a rectangle one pixel at a time.
4. Edit -> Invert: After this step the region of interest should have become white*.

An alternate approach, one which we did not try but might work in theory, is to use MatLab to create two-dimensional, 8-bit array of size 1824 and 1140. You could then assign a value between 0 and 255 to each element in the array. Elements with a value of 0 will see no UV light. Elements with values between 1 and 254 will be on the gray scale, and those with a value of 255 will read as pure white. This variable would

then need to be exported into a .tif format. It is possible that you could achieve a similar outcome using Python.

4.3 Making PDMS templates

The PDMS template plays a very important role throughout the patterning process. During PLL-g-PEG incubation it keeps the solution within the region of interest, and similarly so for the PLPP and protein steps. As such, it is very important that the PDMS is securely attached to the glass. For that reason we suggest the following:

- Work with the PDMS in a very clean environment--any dirt or oils will prevent the PDMS from sticking to glass
- Use Scotch tape to clean the PDMS
- Try plasma treating the PDMS before applying it to the glass
- Apply gentle, even, firm pressure to the PDMS to promote full PDMS-glass bonding

There are multiple ways to make the PDMS template, as it is also possible to purchase templates directly from Alvéole. Users may find it easiest to cut their PDMS membranes to the desired shape using a laser cut. For our E241 experiments we used a sheet of very thin PDMS that ships with protective plastic on both sides, shown in Figure 6, below. We then did the following:

1. Thoroughly clean the working surface with 70% ethanol
2. Mark the cut lines on the outer plastic with a pen
3. Use scissors to roughly cut the desired area
4. Peel away the protective plastic
5. Use an X-acto knife or whole punches to cutter the inner template area
6. Apply the sticky side of Scotch tape to the PDMS to remove debris and residue
7. Plasma treat the PDMS prior to bringing it into contact with the glass

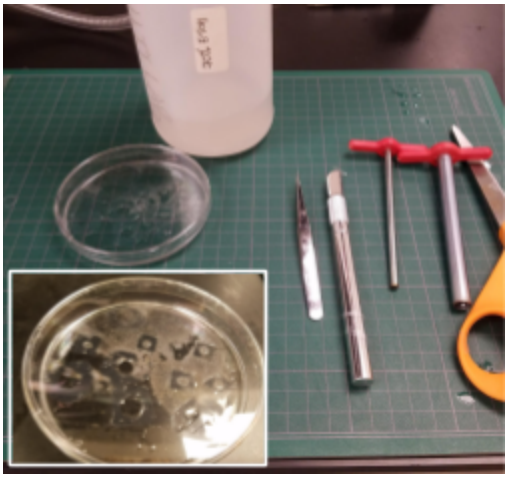


Figure 6. Example of PDMS template making supplies.

4.4 Preparing the substrate

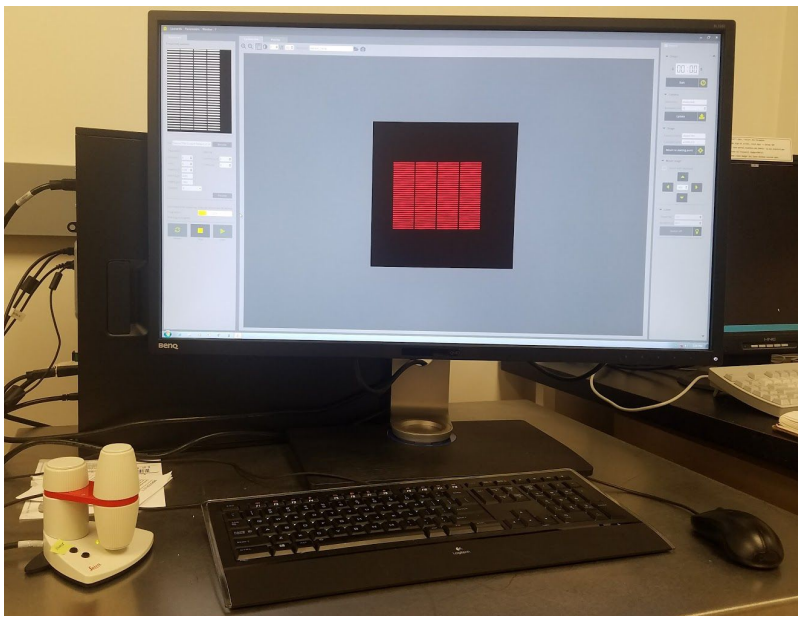
A well prepared substrate is the key to a quality protein pattern. This cannot be emphasized enough. Any particulate or residue on the surface will interfere with the protein adsorption and will thus reduce the uniformity and potentially the resolution of the resulting pattern. Precise steps for preparing the substrate will vary between users and applications. It will be important that all users devote time to developing a technique that is best suited for their application. For our E241 experiments where we used glass coverslips as our substrate, we followed these steps.

1. Clean the coverslips with double distilled water (ddH20), followed by acetone, methanol, isopropyl alcohol, and then ddH20. After removing the coverslips from the final ddH20 wash the water should freely roll away from the surface. If the water clings to the surface then the surface is still dirty and this process should be repeated.
2. Thoroughly dry the coverslips by placing them on a hot plate set to 180 $^{\circ}$C for 5 minutes. This should be done in a protected environment so as to prevent particles in the air from falling onto the coverslips.
3. Plasma treat the coverslips for 20s and 80W (durations and power settings will vary between plasma ashers).

Here we have found it useful to use the same approach to handling the substrates as when working with silicon wafers: never speak to an open wafer. Whenever transporting the substrate, it is best to cover the substrate and to protect it from the surrounding environment.

4.5 Powering up the tool

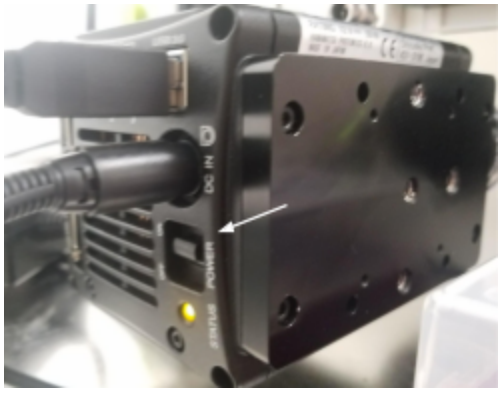
1. Turn on the computer



2. Turn on the Sola Light Engine rear switch

3. Turn on the Sola Light Engine front switch and the Leica CTR advanced microscope power switch

4. Turn on the Hamamatsu camera



5. Turn on the Alvéole PRIMO rear power switch

6. Turn the Alvéole PRIMO laser key to the ‘on’ position

4.6 Calibration

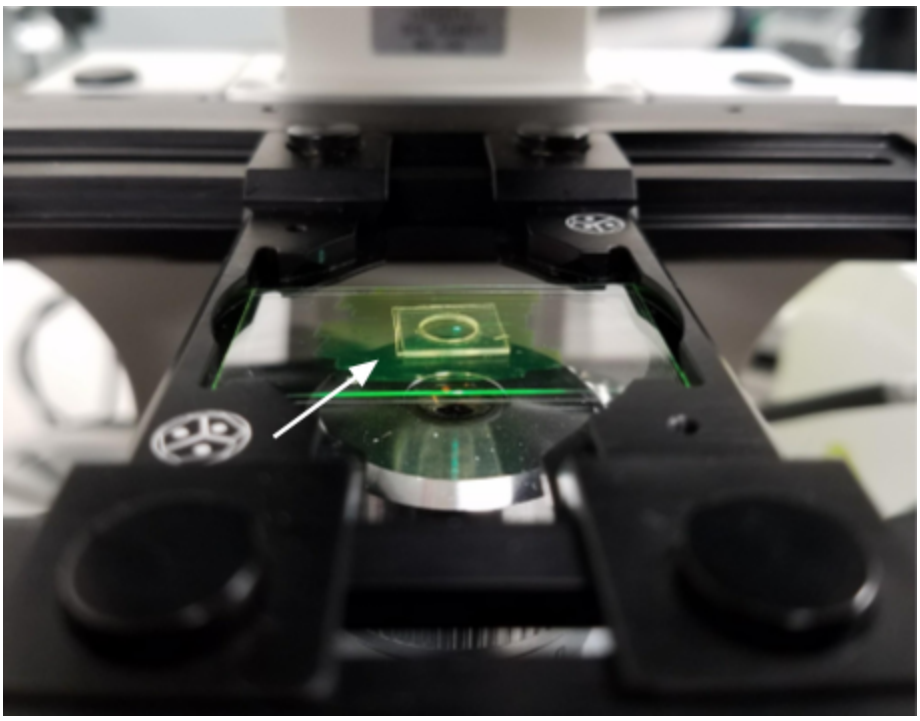


Figure 7. Calibration setup for the PRIMO tool. The white arrow is pointing to a transparent substrate of the same thickness as the desired sample, coated with yellow highlighter ink. At the center of the larger circle is a light green dot. This is the UV light shining from the PRIMO tool onto the surface.

As described in Section 3.3, proper device calibration is essential to achieving quality results. In this step, you are guiding the Leonardo software so that it can calculate the needed values to focus the UV light on the surface of your substrate. To calibrate the PRIMO tool:

1. Calibration requires having a substrate of the same transparency and thickness as the one you will use for your exposures. An example is shown in Figure 7 above:
    a. Use the yellow highlighter near the PRIMO tool to mark the surface thoroughly
    b. Mount the substrate on the stage as shown in Figure 7
    c. Raise the 20x object to approximately the height shown in Figure 7
2. After powering on all of the equipment, open Micro-Manager
3. Menu -> Tools -> Devices
    a. Change transpose X value from 0 to 1
    b. Change transpose Y value from 0 to 1
4. Menu -> Plugins -> Leonardo: the Leonardo software should open and will guide you through the calibration process as shown in Figure 8 below.
    a. When prompted, use a magnification value of 19.8
    b. In the black square shown in Figure 8, the white PRIMO logo may not be readily visible. Often this is because the 20x objective needs to be

raised significantly. Please be extremely cautious when doing so in order to avoid crashing the objective into the glass coverslip.

c. Use the fine control on the remote microscope controller to bring the PRIMO logo into focus
d. Adjust the laser power until no pixels are saturated (i.e., white). In our hands this was typically around 30%.
e. Keep the exposure time set to 25 ms
f. When ready, click 'ok.'
g. Be sure to save the calibration by going to the Leonardo software menu -> Parameters -> Calibration tab -> 'Save'

Figure 8. A screenshot from the Leonardo software during the calibration process.

4.7 Applying the PLPP

Once calibration is completed, it is time to remove the PBS from your substrate and to replace it with PLPP. During this step it is very important to keep the surface hydrated, but to simultaneously not dilute the PLPP--all while not disrupting the PDMS template. This is a challenging task that will require practice. We suggest practicing with PBS a few times before using PLPP. Always use a pipette to apply the PLPP. Approximately 10 µl of PLPP is sufficient for an area of diameter .75 cm. Once the PLPP has been applied to the substrate, mount your substrate onto the stage in a way similar to that shown in Figure 7. Be sure that the surface is level and secure.

4.8 Exposure

In order to achieve the best quality you will need to adjust the focal plane so that you are focused on the desired substrate surface. Figure 9, below, shows the Leonardo software during this step.

1. Using the microscope control panel, set the brightfield shutter to open and increase the white light intensity to ~ 50.
2. In the Leonardo software, load your pattern using the 'Browse' button on the left hand side of the window. It will appear in white in the uppermost left corner of the screen. Click the icon that is a set of squares next to the magnification buttons to make the pattern appear in red above the field of view.
3. In the Leonardo software, locate your desired position. We used our PDMS template as a reference point in determining the location of our focal plane.
4. Indicate the number of patterns you want to complete (1 pattern = 1 column and 1 row). Use the preview button to see how large of an area this pattern will span.
5. When you've positioned your substrate, using the microscope controller:
    a. Keep the brightfield shutter open
    b. Turn down the light intensity to zero
    c. Set the Z position
    d. Set Z-AFC to 'Hold'
6. When you are ready to expose, in the Leonardo software:
    a. Adjust the laser power
    b. Adjust the duration
    c. Press the 'Play' button
7. Verify that the laser light has turned on by holding a piece of white paper above the condenser lens. You should see a blue spotlight on this piece of paper.

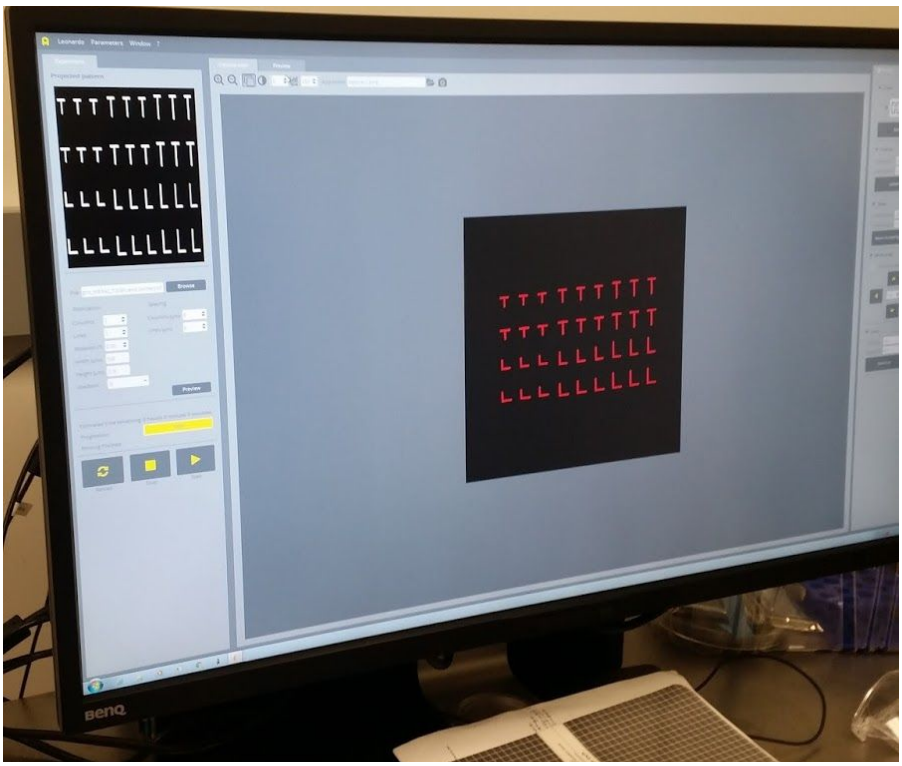


Figure 9. Leonardo software during pattern positioning.

4.9 Removing the PLPP

Following exposure, use the pipettes to replace rinse away the PLPP with PBS, but be careful to maintain surface hydration. We recommend rinsing 5x with 1X PBS, though this is a point upon which other users may vary.

4.10 Tool shutdown

When you have finished using the tool, shut down the equipment in this order

1. Exit Leonardo
2. Exit Micro-Manager
3. Shut down the computer
4. Turn the Alvéole Laser key to the 'off' position
5. Turn off the back laser switch
6. Turn off the Hamatsu camera
7. Turn off the Leica CTR Advanced power source
8. Turn off the front switch of the Sola Light Engine
9. Turn off the back switch of the Sola Light Engine

4.11 Sample storage

Between removing the PLPP and applying the protein it is very important that the PLL-g-PEG layer be hydrated. The PEG's bio-passivation properties rely on it

being hydrated. Some members of our research group (Microsystems Lab) have been able to store the passivated devices in PBS at 4˚C for one week without trouble. We recommend users experiment with this parameter to find their optimal solution.

4.12 Sterilization

Cells and tissues need a sterile environment for culture. Unfortunately, it isn't possible to preserve the sterility of the substrate and the PDMS throughout processing. In our experience, one method to sterilizing the substrate after processing is to place it under a UV lamp for at least one hour. Of course, the UV lamp needs to emit the correct wavelength in order to breakdown the DNA of whatever potential contaminant you are dealing with. The duration of UV exposure will also vary depending on the age of the UV bulb, intensity, and distance from the substrate. Individual users will need to experiment with this parameter in order to find the ideal solution for their situation. In our experiments we found that placing the substrate in a biosafety cabinet with the UV light on for 1 hr was sufficient, though others in our group have required doing the same thing overnight to achieve acceptable results.

4.13 Protein application

When the sterilization step is complete and you are ready to apply your protein, you will need to exercise care when removing the PBS so as to not let the surface dry out. If the surface dries out, and then you apply protein, the protein will adsorb to undesired regions of the surface. That is, the PLL-g-PEG loses its biopassivation properties when it is dehydrated. As with the PLPP application, this is a tricky process and will require practice. It may be beneficial, for this reason, to start with a protein concentration higher than normal so that it will not be overly diluted when applied to the surface. Protein incubation times and temperatures will vary between substrates and protein types. In our experiments with peanut lectin we were able to achieve good results by allowing the protein to sit on the substrate for at least 30 min at room temperature. After this time you will need to thoroughly rinse the surface to clear any excess protein. Excess protein can tend to make the cells clump together, which is typically not desired.

Protein chemistry is outside the scope of this report, but there is extensive literature on working with protein. As general tips to potential users, here are some things we have found to be very important:

- pH
- Salinity
- Storage conditions
    - Make your dilution
    - Aliquot to 100 µl
    - Freeze at -20˚C
    - Thaw until just before use
    - Vortex/pipette up and down just prior to use

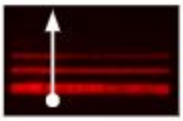
- Caution, some proteins are very temperature sensitive and prone to clumping and therefore one would need to:
    - Work with the protein on ice
    - Never pipette vigorously or vortex
    - If pipetting only use a 1000 µl pipette tip

Due to the dramatic variation in results achieved between users and protein types, we highly recommend that all users plan to first work with a fluorescently labeled version of their protein to work out the details of their own protocol.

5. Using Fluorescently Labeled Protein to Assess Pattern Quality

It is recommended to visualize protein patterns using fluorescently labeled protein. This can be achieved by protein having a fluorophore attached or by spiking in some other fluorescently labeled protein to your protein of intrest. Image protein pattern under a fluorescent microscope and save image. Depending on protein adsorbed and fluorophore, adjust the exposure time (~ 10ms - 500ms) and dynamic range.

For the purposes of this project, we were intrested in quantifying the following parameters: area uniformity, resolution, and line width consistency. The table below describes the analysis output and the ideal trend desired for each parameter. The images within the table depict the area/line drawn in imageJ for analysis. Imaging scripts written in all in Matlab.

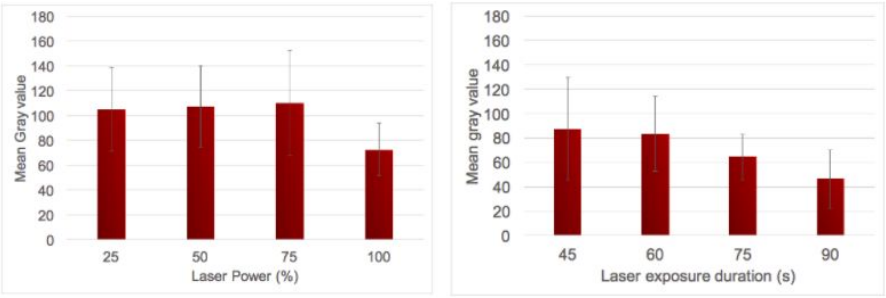
Parameter	Analysis Output	Ideal Trend Desired
Area Uniformity 	Standard deviation	**Minimize STDEV** Less spread
Resolution 	SNR = Imax/Inoise 	**Maximize SNR** obtain smallest resolution
Line Width Consistency 	Standard deviation	**Minimize STDEV** Less spread

6. Project Overview: E241 - Autumn 2017

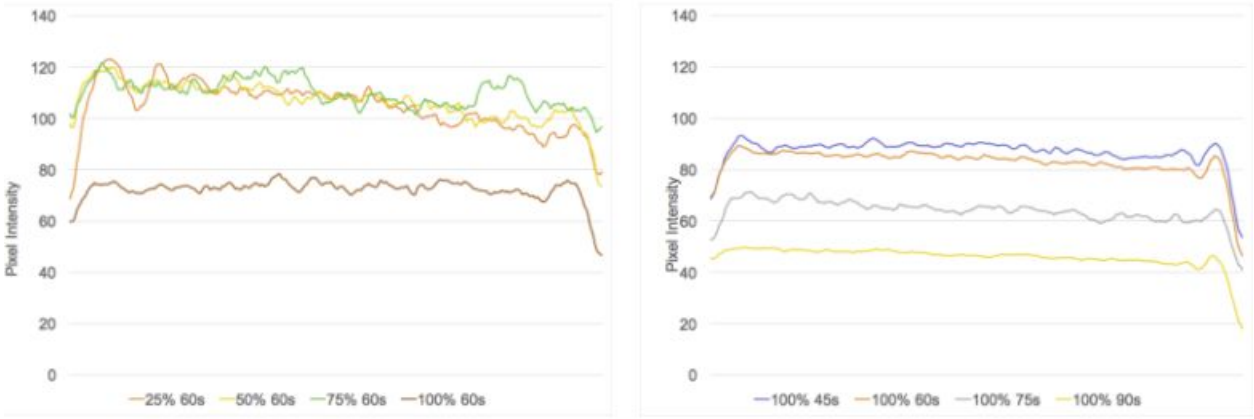
Below is table describing our project motivation and objectives.

Protein | Substrate Matrix	Glass	TEM Carbon Grids
Peanut Lectin (Fluo)	❑ Motivation: study neurons ❑ Integration of new protein ❑ Resolution Study	❑ Motivation: study protein structure ❑ Integration of new substrate ❑ Feasibility
ELP-RGD (Fluo)	❑ Motivation: engineered protein ❑ Integration of new protein ❑ Feasibility	X

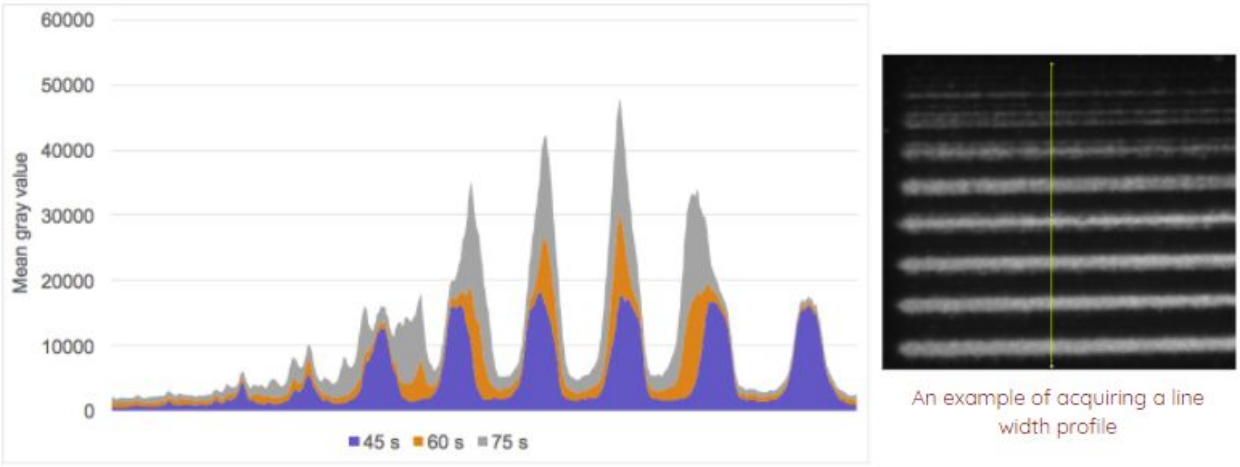
6.1 Peanut Lectin on Glass



Laser power and duration do not affect average protein signal from patterns.

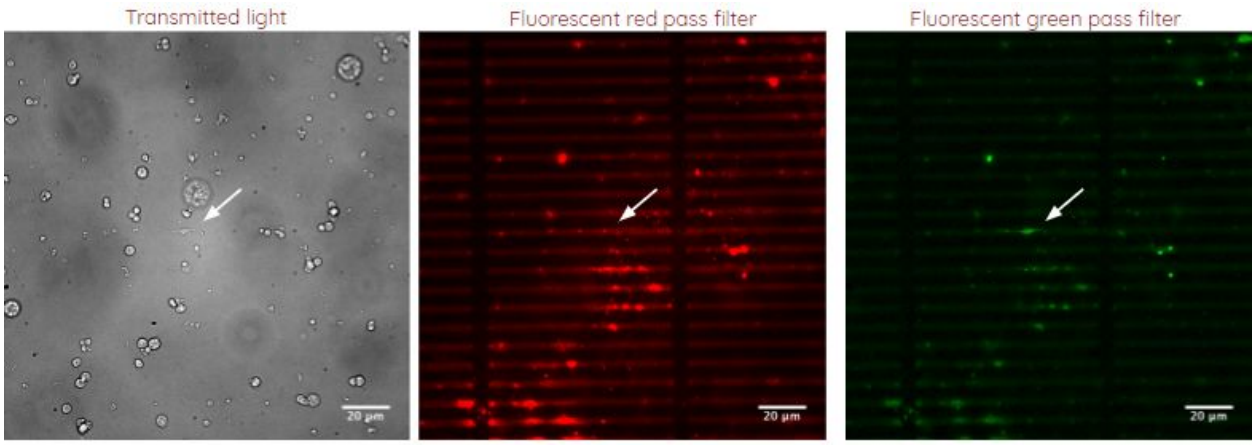


High laser power and longer duration may improve signal unformity.



An example of acquiring a line width profile

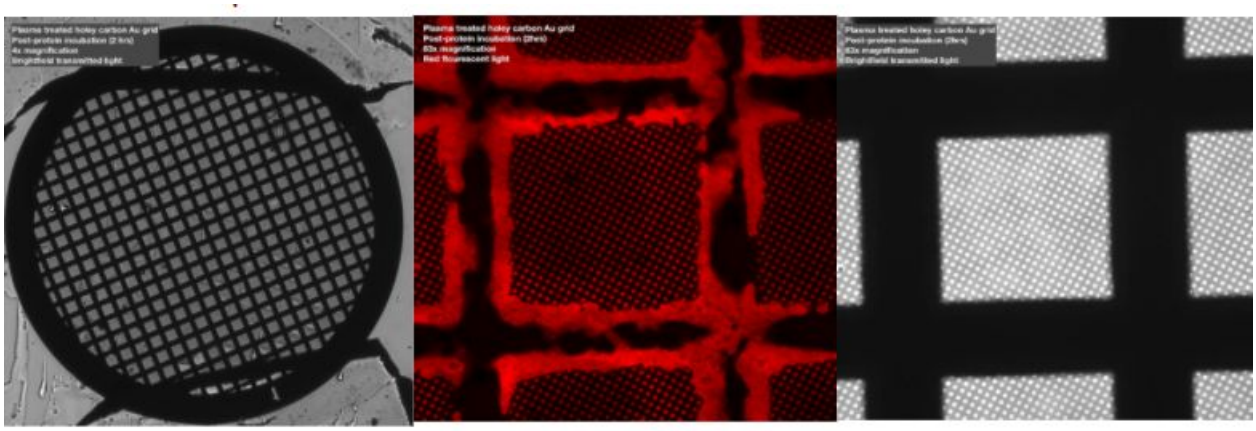
Shorter expsure duration results in narrower lines.



Proof of concept: cells can grow on PRIMO patterns

6.2 Peanut Lectin on TEM Grids

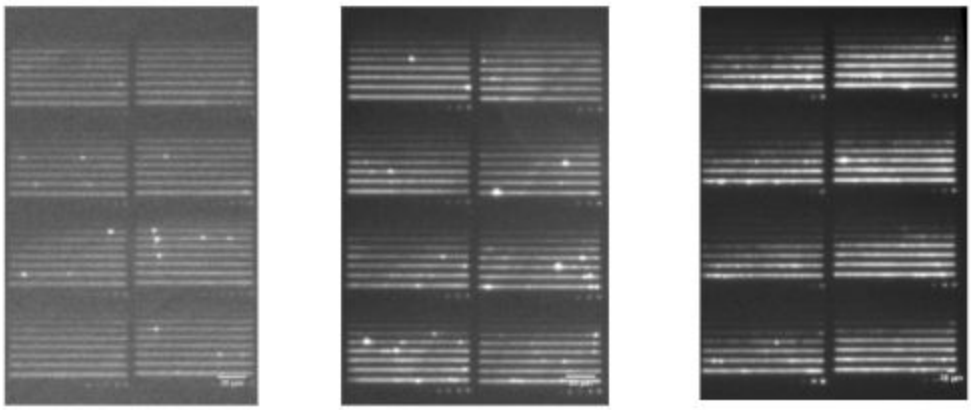
- With some handling practice, the grids (12 nm carbon on Au grid) can withstand *all* treatment steps
- Imaging the grids presents the greatest challenge
- For future users:
    - Try first on glass coated with carbon. Removes handling complexity.
    - Adjust protein incubation time
    - Consider protein pH & salinity
    - Use critical point dryer after protein incubation if imaging dry.



TEM Grids imaged after all steps in protocol detailed above.

6.3 ELP-RGD on Glass

- Confirmed feasibility. Used standard parameters (100% Power, 60sec exposure), n=3 coverslips. Imaged below, scale bar 20um.



7. Project ideas

Below are potential project ideas using PRIMO ALVEOLE.

- Characterization of alignment between multiple proteins
- Characterization of protein gradients
- Expansion of substrates used
- Expand into covalent chemistry (currently only adsorption based)
- Expand into different biopassivation components (degraded by PLPP + UV light)
- Alignment issues for large patterns. There appears to be offsets between each 300x500um exposure region. This could be a software issue, but there may be way to work around this manually.

Note: PRIMO Alveole version 1 was used for work descirbed here. At the time of writting report, the tool purchase was finalized. The purchase will be for PRIMO Alveole version 2. Thus some of the ideas suggested above may be improved in version 2.

Below is a table detailing considerations for other ExFab tools if objective is patterning using the same principles of PRIMO Alveole. The objective of this table is determine potential concerns, sample size limitations and resolution. The table below also includes two other methods for 2D protein patterning for a reference comparison. For a more detailed comparison between microcontact printing and lift off technique we refer the reader to following article (Moelle, J., Denisin, A. K., et al, plosONE Dec 2017). https://www.biorxiv.org/content/early/2017/02/24/111195

Method/Tool	Sample Size Limitations	Resolution	Comments
Microcontact printing	--	~um Limited by PDMS stamp features	Requires protein to be dried and then transfered onto glass. Can have a high degree of variability from pattern to pattern.
Lift off patterning (LOP)	--	~um Limited by s1818 structures	Protocol briefly consists of S1818 patterns on glass, biopassive pll-g-peg, NMP (N-Methyl-2-pyrrolidone) to lift off S1818 structures and backfill of protein. Critical steps require a hydrate substrate;,proteins never dried

			Repetable patterns of high quality (protein transfer efficiency, pattern accuracy).
Primo Alveole	Limited to microscope stage holder size 22x22mm 3inx1in	1.2um	Down-top tool. Light comes from the bottom. Critical steps require a hydrate substrate; proteins never dried. Repetable patterns of high quality (edge sharpness, uniformity). Multiple proteins *no other available commercial tool as of December 2017
Heidelberg Maskless Aligner (SNF setup)	Up to 6in wafer 100um-6mm thick substrate	1um	Top-down tool. Light comes from the top. Concerns: Major concern: Focusing mechansim relies on back pressure. This could be potential issue since plpp and pll-g-peg layer need to hydrated. Tool at SNF uses 405nm wavelength. 375nm available for purchase. This could be potential issue since PRIMO laser wavelength is 395nm. Need to test is PLPP is cleaveable at 405nm. Liquid falling into the chuck or camera (both located in the bottom). This could be potential issue since plpp and pll-g-peg layer need to hydrated. Overall, doesn't lend itself to patterning using pll-g-peg and plpp as in PRIMO. If desired, could be possible with lots of modifications.
Nanoscribe (SNF setup)	Up to 4in wafer	~500nm	Depending on the operating mode, substrate has working distance limitation. This would limit the substrate thickness Standard Mode: light → substrate with photo-active resist on top. Working distnace of 300um. Dip-in laser Mode: objective is submerged in resist. Requires a 0.05 diffraction index change to focus on substrate.

			Concerns: Tool at SNF uses 780nm 2-photon exposure system (390nm). This could be potential issue since PRIMO laser wavelength is 395nm. Major concerns: Working distance and 0.05 diffraction index change required
Optomec (SNF setup)	Up to 6in wafer	>15um	Aerosol jet, dispensing 3-5um droplets towards substrate

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Appendix

Matlab scripts used in analysis. For further image automation refer to (Moeller, Denisin, et al refence).

%% Uniformity

```
% 11.14.2017
% Erica Castillo

%Import Intensity vs Position data
% Home>Import data> Excel File remove first row
% datapos  vs dataint

%calculate average
average=mean(dataint);

%calculate standard deviation
stdev=std(dataint);

%plot
Vavg=dataint.*0 + average;
Vpstdev=dataint.*0 + average + stdev;
Vmstdev=dataint.*0 + average - stdev;

plot( datapos,dataint,datapos,Vavg,'-.r', datapos,Vpstdev,'--r',datapos,Vmstdev,'--r')
legend('line profile','average','+1 stdev','-1 stdev')
 xlabel('horizontal position (pixels)')
 ylabel('intensity')
```

%% Resolution

```
% 11.14.2017
% Erica Castillo

%Import Intensity vs Position data
% Home>Import data> Excel File remove first row
% datapos  vs dataint
```

```
plot(datapos,dataint)
legend('line profile across pattern')
 xlabel('position (pixels)')
 ylabel('intensity')

% dataintSmooth=smooth(dataint);
% plot(datapos,dataintSmooth)

%MANUALLY: Find average noise by taking start/or end of vector
noise=mean(dataint(1:30,1))

findpeaks(dataint,datapos,'MinPeakDistance',0.0001)

%MANUALLY input peak values
%disregard some peaks visually, can play with the last entry to findpeaks
%function to get add/delete peaks
Peakint=[1870,1454,1049,348]; %taken visually using data cursor,last entry is where
not distinguishable
SNR=Peakint./noise

%output SAVE : SNR
```

%% Line Width Uniformity

```
% 11.14.2017
% Erica Castillo

%Import Intensity vs Position data
% Home>Import data> Excel File remove first row
% datapos  vs data

% adapated from
https://www.mathworks.com/matlabcentral/answers/310113-how-to-find-out-full-wid
th-at-half-maximum-from-this-graph

%% FWHM
% Find the half max value.
halfMax = (min(data) + max(data)) / 2;
```

```
% Find where the data first drops below half the max.
index1 = find(data >= halfMax, 1, 'first');
% Find where the data last rises above half the max.
index2 = find(data >= halfMax, 1, 'last');
fwhm = index2-index1 ; % FWHM in indexes.
% OR, if you have an x vector
fwhmx = datapos(index2) - datapos(index1);


%Visualize Plot
plot(datapos, data,
datapos(index1,1),data(index1,1),'*r',datapos(index2,1),data(index2,1),'*r')
legend('line profile across width','FWHM p1','FWHM p2')
 xlabel('position (pixels)')
 ylabel('intensity')
```