Protein Photopatterning on PDMS in 3D with the Alveole PRIMO

ENGR 241: Autumn 2018-19

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1. Executive Summary

This purpose of this document is to provide a progress update for our project using the Alveole PRIMO photopatterning tool, as well as an SOP for SNF users to use PRIMO for patterning of proteins on PDMS. The main objective of the project over the two quarters (Fall 2018 and Spring 2019) is to develop the capabilities of the PRIMO at SNF as a tool for patterning on complex PDMS substrates beyond simple glass coverslips. Ultimately, one goal of the project is to generate protein micropatterns inside a PDMS microfluidic channel as a biophysical tool for studying the effects of fluid flow and spatial patterning on cell behavior. The primary goal for the fall quarter was to perform characterization experiments using slanted PDMS substrates to explore the limitations of PRIMO photopatterning on non-planar surfaces and develop a general SOP for PDMS photopatterning. Using PDMS adds a layer of complexity to the patterning process from an optical and surface chemistry perspective. This report provides a list of some important considerations that users must be aware of when preparing the PDMS samples, calibrating the PRIMO tool, designing a pattern, selecting patterning parameters, and imaging the final results.

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3. Project Objective

3.1 Brief Background

Standard cell culture techniques fail to recapitulate the native tissue organization, which provides key biophysical cues directing cell behavior and function. Better control of the 3D cellular microenvironment (e.g. by micropatterning) is crucial for basic biological research and has applications in quantitative mechanobiology, tissue engineering, disease modeling, drug discovery, and biosensor development.

3.2 Project Goals

The overall goal of this project is to expand the capabilities of the Alveole PRIMO tool at SNF for protein photopatterning on complex 2.5D and 3D substrates. This will benefit the SNF community by developing a toolbox for researchers to create custom, well-defined 3D cellular microenvironments by rapid and flexible photopatterning. This includes the ability to pattern within microfluidic channels for applications such as organs-on-chips.

4. Project Methods

Given the objective of the project, the goal for the autumn quarter was to develop an understanding of the key considerations for generating protein patterns within microfluidic channels and on complex 2.5D/3D PDMS substrates. Generation of such patterns requires an understanding of several factors:

1. What depth of field can we achieve with the PRIMO laser while maintaining acceptable pattern resolution?
2. How will pattern distortion through PDMS on slanted surfaces affect pattern quality?
3. Through what thickness of PDMS can we pattern through while maintaining acceptable resolution?
4. How does PRIMO dosage change for new substrates?

These questions will influence pattern design and stitching when creating features which span multiple focal planes, set limitations on feature sizes and microfluidic channel geometries which can be patterned, and determine parameters for PRIMO exposure. In order to understand these factors, we used a simple test system for characterizing the performance of PRIMO photopatterning by patterning on slanted PDMS surfaces.

4.1 Preparing PDMS Wedges

PDMS wedges were prepared by mixing the prepolymer and curing agent in 10:1 ratio by weight. The PDMS was then poured onto glass slides set at defined angles inside a petri dish and then left overnight for curing. Extra devices of each thickness were prepared for later use in calibration. (Note: PDMS must be cast on a smooth surface such as glass slide to achieve optical transparency.)

After PDMS curing, sample and glass side were plasma air etched for 60 seconds and then stuck together. (Note: Plasma treatment is optional and was not done for every experiment.)

Once the PDMS was cured and removed from the mold, a score mark was placed at the z height matching the thickness of the control sample which would be used for calibration (see image below). After marking the PDMS sample, a stencil was placed with the score mark passing through the middle of the stencil well.

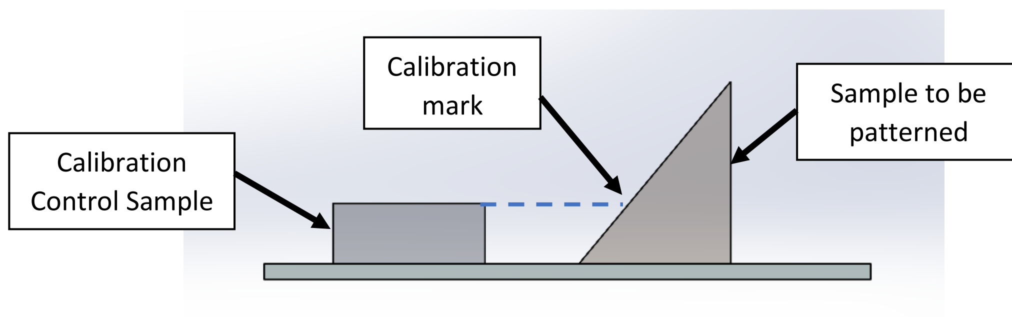


Figure 1: Calibration at the desired z plane with planar PDMS slab

The prepared sample was passivated with PLL + PEG-SVA per the PRIMO PROTOCOL: PHOTOPATTERNING ON PDMS WITH PRIMO Rev 2 (see section 8.2.1 for detailed steps).

4.2 PRIMO Patterning

Before patterning on a new substrate, you must perform calibration. This is extremely important, as the resulting protein pattern can only be as good as your calibration. Tube lens calibration is especially important before patterning on PDMS to correct for stigmatism and ensure that the DMD laser projection plane is conjugated with the objective focal plane. For detailed calibration steps see Section 9.3

A droplet of UV highlighter was pipetted onto the PDMS control surface and covered with a coverslip as shown in the figure below. PDMS is hydrophobic and the coverslip was used to prevent the highlighter droplet from shrinking. (Note: Placing a glass coverslip on top of the highlighter caused some visible reflections in the calibration image, so generally we removed the coverslip immediately prior to calibration. It is unclear what effect, if any, these reflections might have on the software's calibration calculations.)

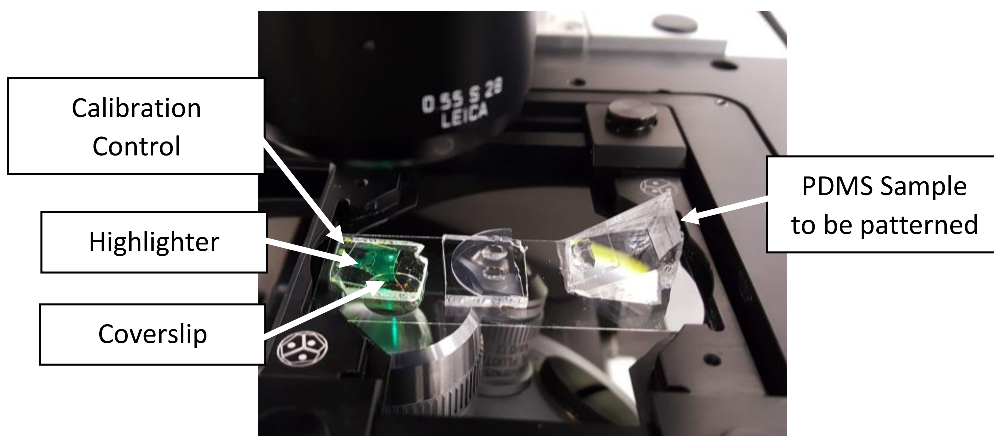


Figure 2: Calibration and patterning setup

After calibration, the Leonardo software was used to pattern PDMS substrates as described in section 9. The test image shown below was the primary pattern used for characterization. This pattern consisted of lines and spaces 1, 2, 4, 8, 16, 32, and 64 pixels wide. This was used to assess pattern resolution for different conditions.

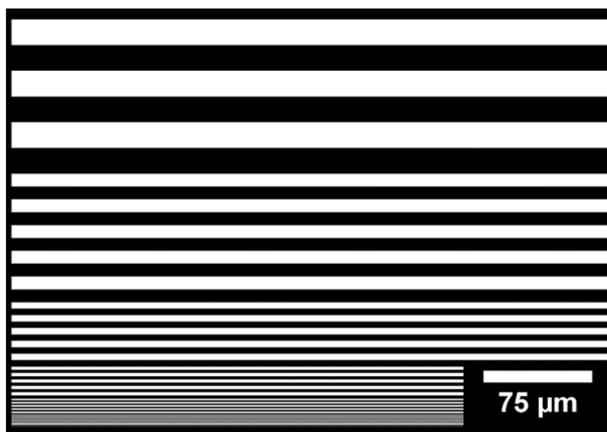


Figure 3: Pattern used for PDMS flat and wedge experiments

For PDMS wedges, this test pattern was oriented with respect to the slanted surface such that the length of the columns extended across multiple focal planes (i.e. the left side of the pattern is at a higher focal plane than the right side). The pattern was centered on the score mark corresponding to the calibration plane, such that the pattern would be in good focus at the centerline and drift out of focus equally on both sides.

A range of UV exposures were tested to determine the optimal dosage (i.e. avoid overexposure while providing sufficient degradation of the passivation layer). After exposure the wells were incubated overnight with fluorescent protein as described in section 9.8

4.3 Pattern Analysis

The patterns were imaged using the Leica microscope. To assess pattern quality, line profiles of the fluorescent intensity along the patterns were generated in ImageJ.

5. Project Results and Future Plans

There are several key considerations to be aware of when patterning in 2.5D/3D:

- Limitation of z-depth across pattern (depth of field)
- Pattern distortion/stretching
- Resolution degradation for different thickness (related to working distance)
- Exposure/dosage optimization for PDMS with this setup

5.1 Evaluating Depth of Field

As expected, pattern resolution is lost as the substrate goes out of focus. The highest resolution is achieved in the middle of the pattern (section 3) because the calibration was performed with the control sample whose thickness matched the z height at the middle of the pattern. Section 1 which is furthest away from the center of the pattern shows greatest blurring of features.

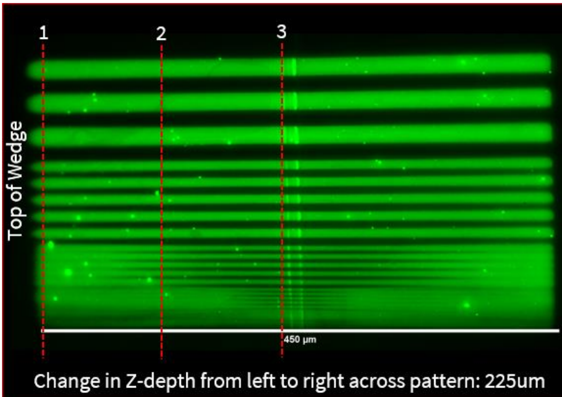


Figure 6: Pattern

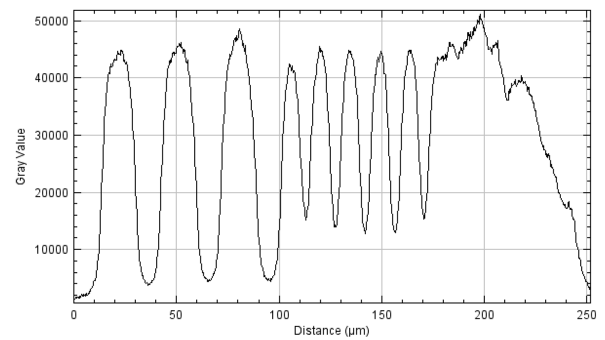


Figure7: Intensity at Section 1

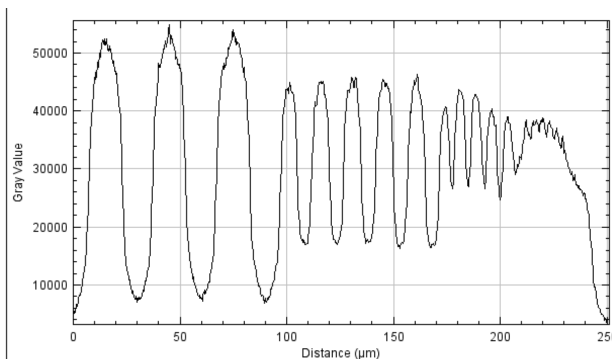


Figure 8: Intensity at Section 2

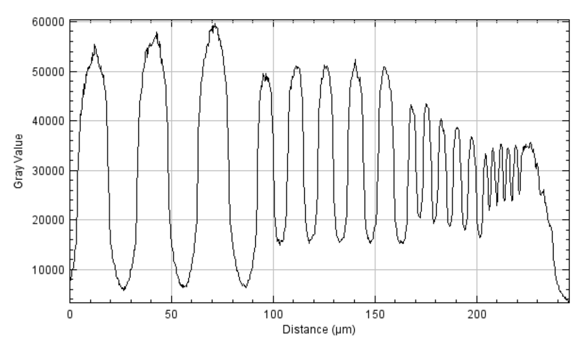
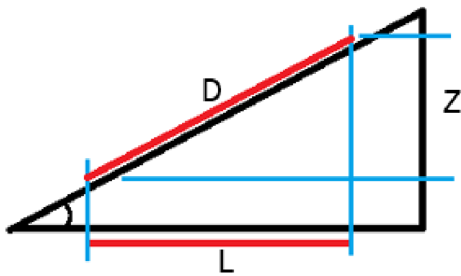


Figure 9: Intensity at Section 3

In order to apply this information towards future guidelines for patterning on 3D surfaces, an acceptable resolution threshold may be specified in order to determine the maximum z-range which can be spanned by a single laser projection. When creating large patterns, the loss in resolution should be considered to determine the size of each pattern to stitch together. This may depend on the feature size (line width and spacing), as the relative distortion of the pattern is greater for thinner lines.

5.2 Pattern Distortion/Stretching

As the PDMS surface is angled with respect to the DMD projection, the pattern gets stretched. Therefore, when designing the pattern this stretching should be considered and pattern should be drawn smaller than the true design dimensions.



D = L / cos(theta)
Z = L*tan(theta)
D = Elongation length
L = Original pattern length
Z = Height of pattern

5.3 Pattern Resolution Degrades with Increasing Thickness

As the thickness of the PDMS increased the pattern resolution degrades as shown in the calibration images figure below. Alveole reports that patterning through PDMS should be possible for layers up to 2mm thick using the 20x Nikon S plan flour objective (default on SNF setup), with resolution remaining at 1μm for layers up to 250μm. Again, the acceptable thickness of PDMS through which you can pattern will depend on the acceptable pattern resolution as dictated by your specific application. However, based on these preliminary results we would suggest limiting PDMS substrate thickness to under 1mm.

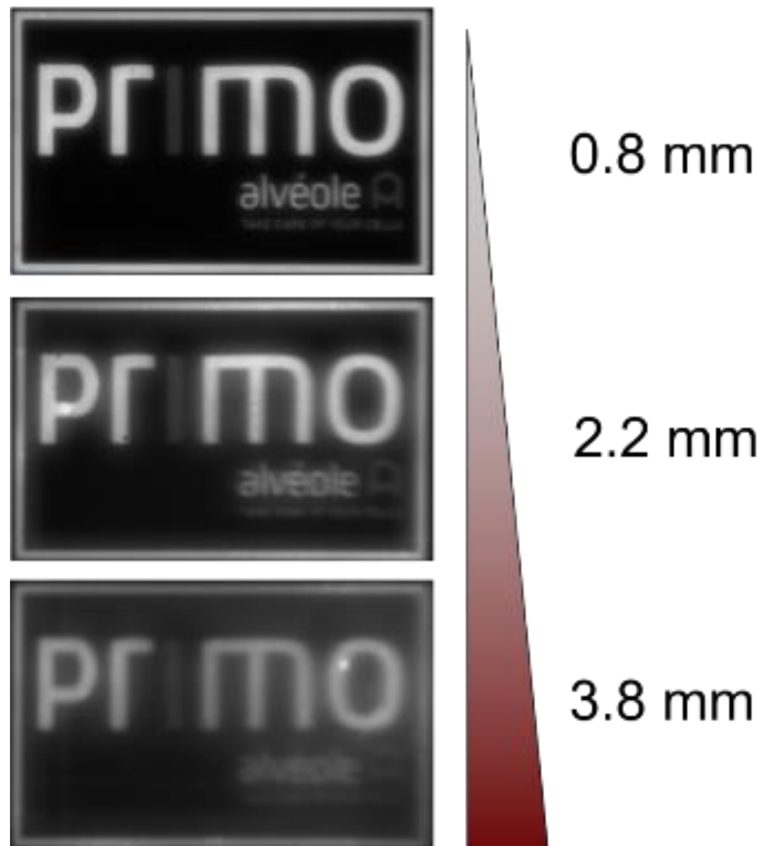


Figure 10: Pattern degradation with increasing thickness

5.4 Exposure/Dosage Optimization for PDMS

UV exposure dosage affects the resolution of the patterns as shown in figures below. At higher doses, rounding of the corners of the pattern clearly indicates overexposure. The optimal dosage for thin PDMS layers of roughly 0.8mm thickness with this setup was determined to be between 750 mJ/mm^2 and 1000 mJ/mm^2.

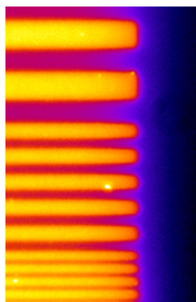


Figure 11: Pattern when exposed to 1000 mJ/mm^2

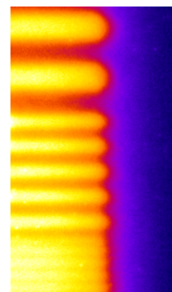


Figure 12: Pattern when exposed to 1500 mJ/mm^2

5.5 Future Plan

In the upcoming quarter, the plan is to expand to patterning inside a microfluidic channel. The major steps of the process will include channel fabrication, calibration, patterning, and imaging. Two channel configurations are currently being explored: a rectangular and a round cross-sectional channel. The rectangular cross section will be patterned first to troubleshoot issues we expect to encounter when moving from flat surfaces to enclosed geometries (e.g. reagent handling and transfer, optical shadowing and reflection effects inside the channel. Development of repeatable calibration strategies and imaging techniques will also be required for enclosed geometries. From an engineering perspective, one motivation for this project is to develop the ability of the tool to generate flexible and rapid micropatterns within complex, user-defined geometries. From a biological perspective, this technology would provide steps towards in-vitro modeling of human organ systems (e.g. blood vessel on a chip), as well as be applied as a biophysical tool for studying the mechanobiology of endothelial cell responses to combinatorial mechanical cues.

5.6 Team Member Contribution

Task	Contributing team member
PDMS Preparation	Frank and Chinmay
Angled Wedge Fabrication	Chinmay
Reagent Preparation	Frank
Sample Preparation (etching, incubation, etc.)	Frank and Chinmay
PRIMO Patterning	Frank and Chinmay
PRIMO Imaging	Frank and Chinmay
Class Presentation	Frank and Chinmay
Poster Presentation	Frank and Chinmay
Project Report	Frank and Chinmay

6. Budget

Item	Amount
Budget Allocated	$5190
Materials/Supplies Cost	$1115
Equipment Usage Cost	$200
Training Cost	$480
Total Expenses	$1795

7. SOP: PRIMO Patterning on PDMS Surfaces

The objective of this SOP is to provide guidelines for SNF users who wish to generate protein patterns with PRIMO on PDMS substrates. This document includes step-by-step instructions for substrate preparation, calibration, and photopatterning, as well as some tips and considerations for patterning PDMS that we have accumulated during the first half of the project.

7.1 System Overview

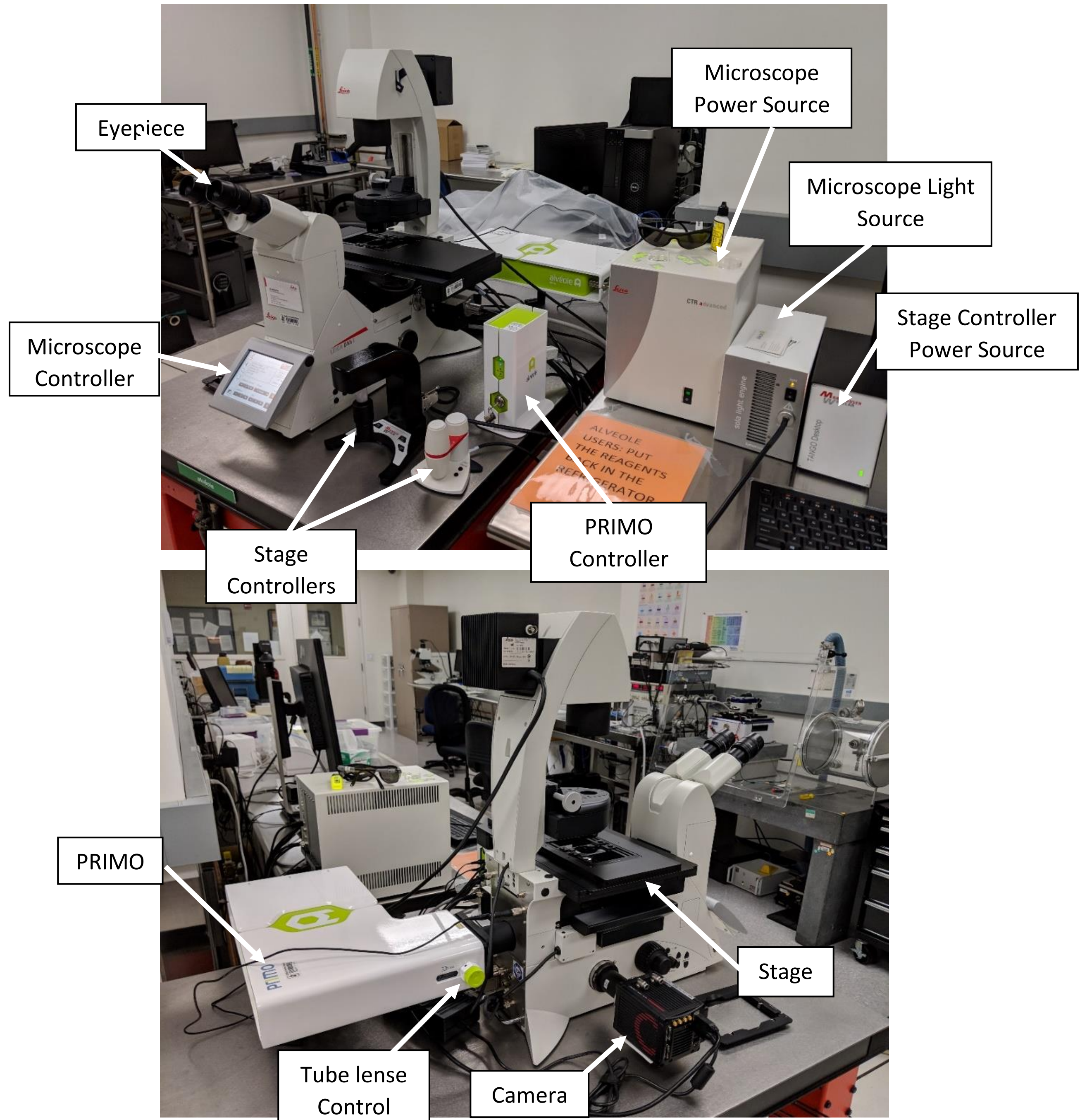


Figure 13: PRIMO system overview showing the main components

7.2 Workflow Schematic

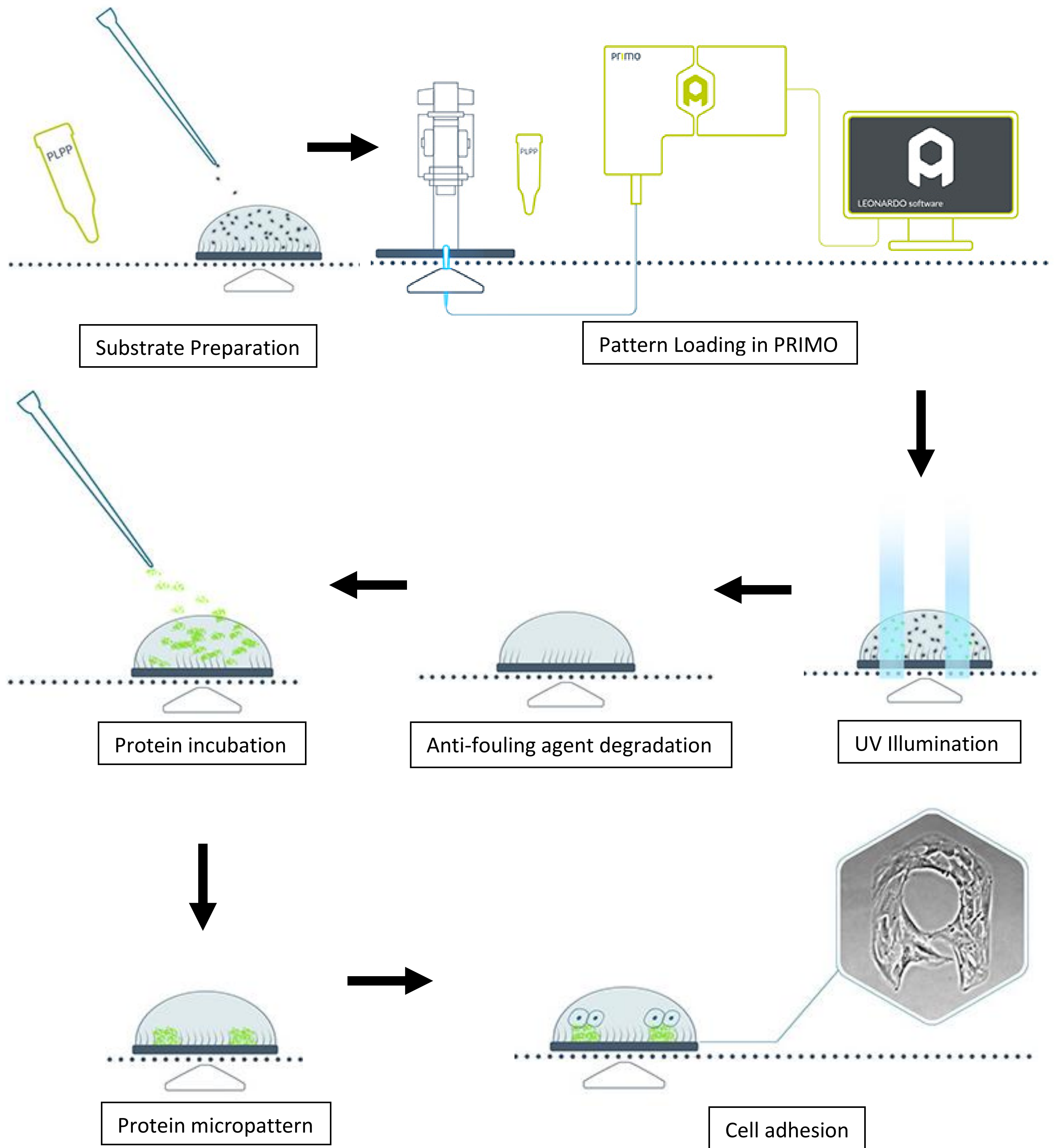


Figure 14: PRIMO workflow schematic showing the major steps

7.3 Materials List

- Glass coverslips or slides
- Tweezers
- X-acto knife
- PDMS stencils
- Plasma Etcher
- Petri dishes or 6 well plates
- PBS (1X, pH 7.4)
- Individual pipettors (10uL, 20uL, 200uL)
- Pipette tips
- PLPP (Alveole)
- Protein of interest (e.g. Gelatin, Oregon Green 488 Conjugate, 100ug.ml^{-1})
- PDMS (prepolymer and curing agent, e.g. Sylgard 184)
- Buffer, 8<pH<8.5 (e.g. HEPES 100mM. DO NOT USE primary amine buffers such as tris-buffer)
- Poly-L-Lysine (Sigma-Aldrich)
- mPEG-SVA (Laysan Bio, ref: M-SVA--5K)
- Misc. consumables: scotch tape, kimwipes, etc.

8. Substrate Preparation

8.1 Applying PDMS Stencils

The PDMS stencils (shown below) contain the reagents (PLL, mPEG-SVA, PLPP, etc.) within the region of interest and allow smaller volumes to be used. These stencils can be purchased from Alveole or cut in-house from thin sheets of PDMS. We recommend working with the stencils in a clean environment, using scotch tape to clean the PDMS, and covering the sample during transport to prevent contamination. Plasma treating the stencils before bonding to the PDMS substrate to be patterned may help in the case of adhesion difficulties.

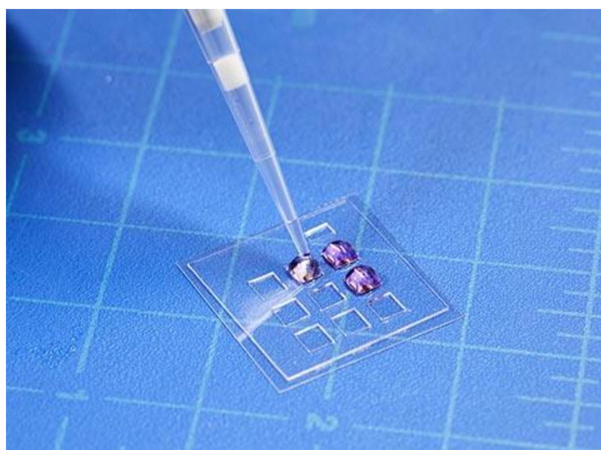


Figure 15: PDMS stencil

Image source: https://www.alveolelab.com/our-products/pdms-stencil-multiwell-plate/

8.2 PDMS Passivation

While there are many ways to passivate PDMS, we have had success using a protocol provided by Alveole (PRIMO PROTOCOL: PHOTOPATTERNING ON PDMS WITH PRIMO Rev 2).

8.2.1 Passivation steps:

1. (Optional) Plasma air treat for 30 to 60 seconds
2. Place stencil on PDMS if needed
3. Incubate with PLL at 500ug.ml^{-1} for 30min
4. Prepare the HEPES buffer at 8<pH<8.5 if not already done
5. Rinse 3 times with HEPES
6. Prepare a solution of PEG-SVA in HEPES at 50mg.ml^{-1}. **This must be prepared just before use, as the half-life of the SVA ester is 10min at pH 8.5**
    - It may be helpful to weigh out aliquots of the powdered mPEG-SVA into Eppendorf tubes in advance for convenience.
7. Incubate with PEG-SVA solution for 1hr. **Be sure that the substrate remains hydrated.**
8. Wash 5 times with PBS

8.2.2 Considerations and Tips

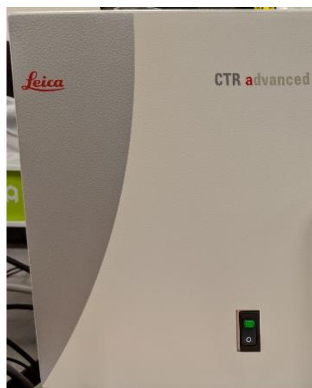
- **IMPORTANT:** Make sure to maintain hydration of the PEG layer during rinsing and handling, otherwise the PEG brush will collapse and gelification may occur (see Substrate Preparation Tutorial Video for rinsing technique)
- Consider patterning a flat PDMS slab along with each run when trying a new substrate or a more complicated geometry. This can serve as a control to help diagnose reagent and handling issues
- PDMS can absorb some reagents (e.g. PLPP). Rinsing is therefore very important, as it can allow these reagents to come back out of the substrate
- Sharpie marks or score marks made with an X-acto knife are often quite useful as reference points for setting focal planes during patterning.
- Pay attention to the alignment of the PDMS in relation to coverslip, if that is important for your application (e.g. lining up direction of greatest slope of wedge perpendicular to coverslip edge)

9. Patterning with PRIMO

9.1 Powering up the Tool

Step 1: Turn on the Marzhauser TANGO Desktop stage controller (switch is in the back)

Step 2: Turn on Sola Light Engine: First turn on the power switch (located in the back) then turn on the light switch



Step 3: Turn on the Leica microscope controller

Step 4: Turn on the PRIMO laser controller: First turn on the power switch (located in the back) then turn the key clockwise to on position

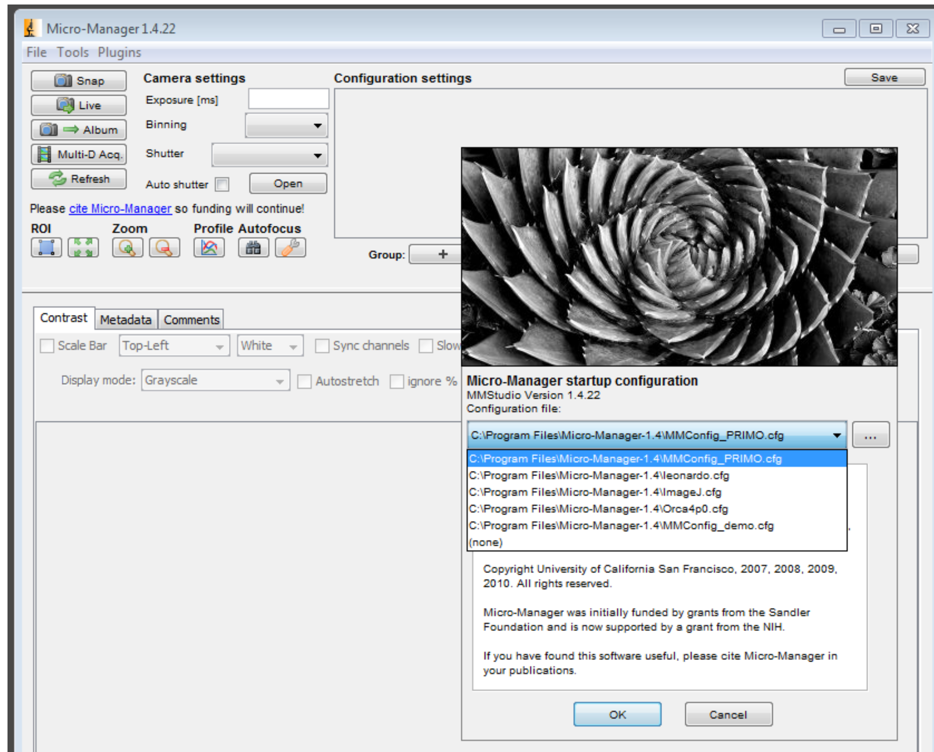
Step 5: Turn on the camera

Figure 16: Startup procedure for the PRIMO tool

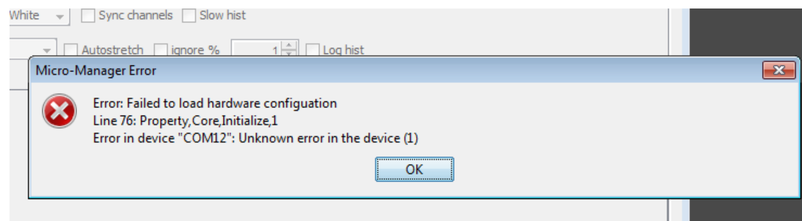
9.2 Launching Leonardo

Step 1: Open Micro-Manager

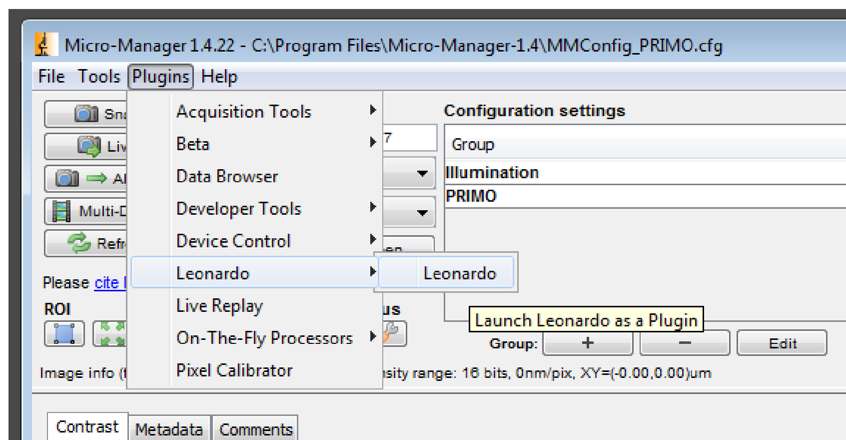
Step 2: Select *C:\Program Files\Micro-Manager-1.4\MMConfig_PRIMO.cfg* configuration file, click OK.



Note: If the following error message appears then make sure all the devices are properly powered on. Close Micro-Manager, restart all the devices and then open Micro-Manager again.



Step 3: Launch Leonardo from the Plugins tab as shown



9.3 Calibration

Upon launching Leonardo, the following Leonardo main screen should open with the following three options: Calibrate, Load a calibration, or Calibrate tube lens

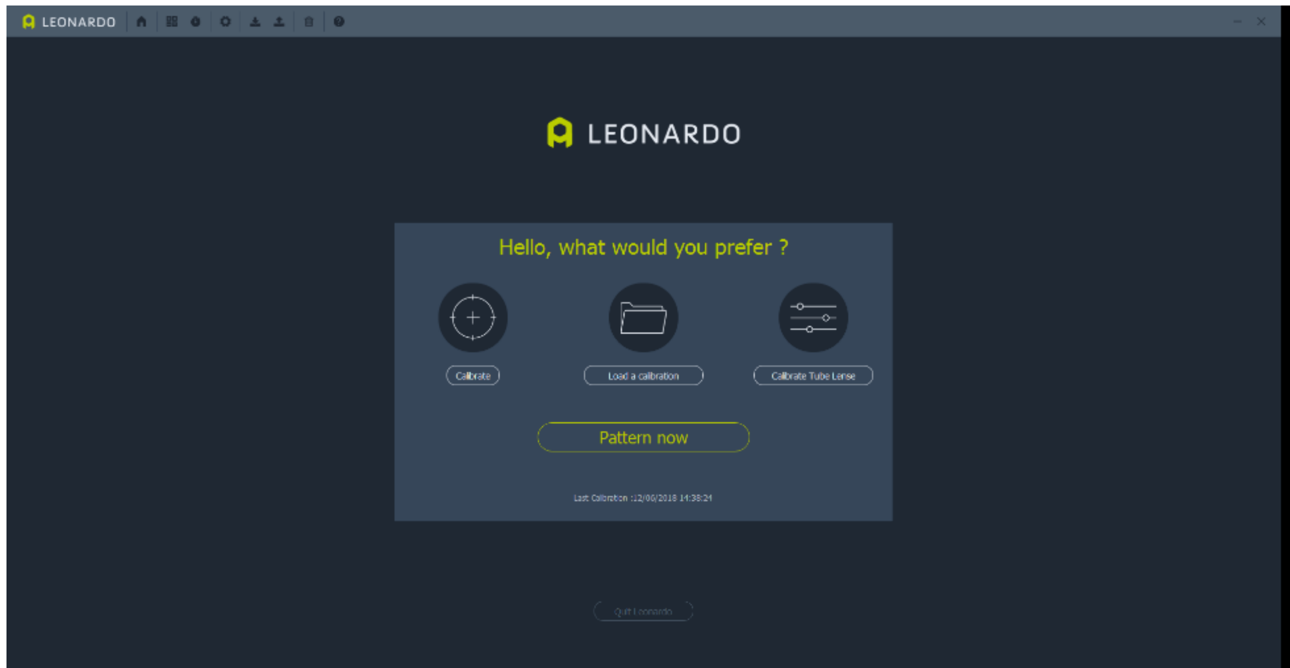


Figure 17: Leonardo main screen

Calibration of the device before patterning with a new substrate is crucial for obtaining high quality results. Calibration of the tube lens should be performed any time the microscope objective is changed or when a different type of substrate is used. This step is especially important for patterning on PDMS substrates where sample thickness may vary between experiments.

It is recommended to save calibration files as these can be reloaded to save time in the event of a software crash, and as a record of the calibration parameters (e.g. um/px ratio) used in an experiment.

Step 1: Prepare PDMS substrate for calibration

Select "Calibrate tube lens" and follow the onscreen instructions. Place a PDMS layer of the same thickness as the sample to be used in the experiment on a glass slide and cover with highlighter.

Figure 18: Calibration setup

Leave the default optical parameter settings: magnification value of 19.91 and default laser power of 5.20 mW.

Step 2: Correction collar adjustment

With the laser off, first use the correction collar on the microscope objective and the microscope stage controller (Z-adjustment) to obtain good focus on the PDMS surface in brightfield. Use a sharpie mark or score mark as a reference point to be sure you are focusing on the correct plane.

Figure 19: Correction collar: Default value for glass coverslip is indicated with a black mark

NOTE: If the sample is not visible make sure the brightfield transmitted light shutter is open and the intensity is not all the way down to 0.

Figure 20: Image of the Leica microscope control panel with transmitted light (TL) shutter open and brightfield light intensity set to 41%.

Step 3: Image Projection

Select an image to be projected onto the highlighter for calibration. Alternatively, your desired pattern can be projected.

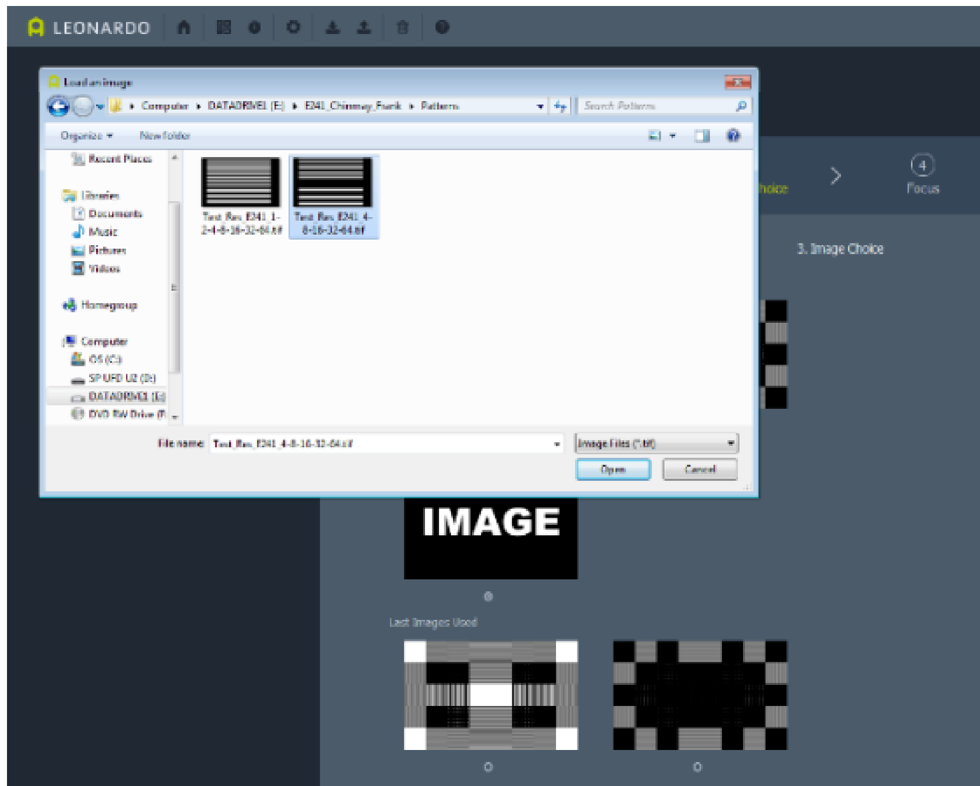


Figure 21: Selection of a custom pattern for use in calibration

Step 4: Adjust tube lens

Reduce the laser power (%) to eliminate any saturated pixels in the image histogram.

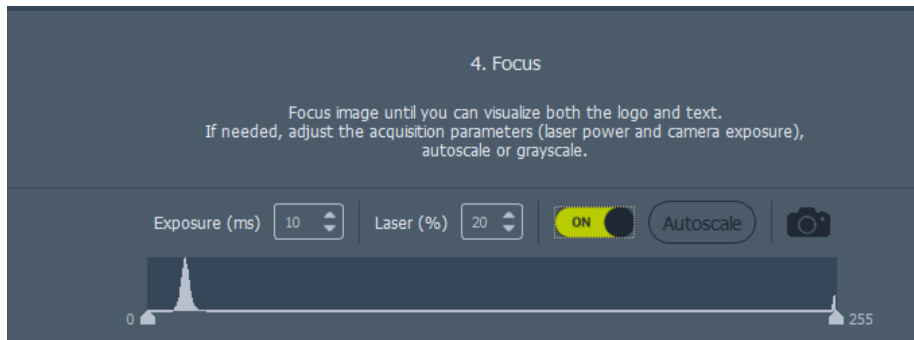


Figure 22: Leonardo screenshot during calibration. Note that the laser power is at 20%, but there are still saturated pixels (indicated by a spike in the image histogram of pixels with a value of 255).

Figure 23: Calibration image as laser power is reduced. Note the change in the saturation level of the pattern.

Use the green tube lens adjustment knob to obtain a clear and focused imaged of the pattern. This will conjugate the DMD with the lens focal plane, and is very important for small patterns (<10μm). Make note of position of the tube lens before making any adjustments, and leave a note for the next user or return the knob to its original position once done patterning (the default setting is currently +5).

Figure 24: Tube Lens Adjustment Knob (located on the side of the PRIMO optical case)

Step 5: Complete calibration

Once a clear and focused image has been obtained, click ok to complete the calibration. The software will calculate the horizontal and vertical shifts and rotation angle needed to align the DMD projection plane with the objective focal plane. Be sure to save the calibration file.

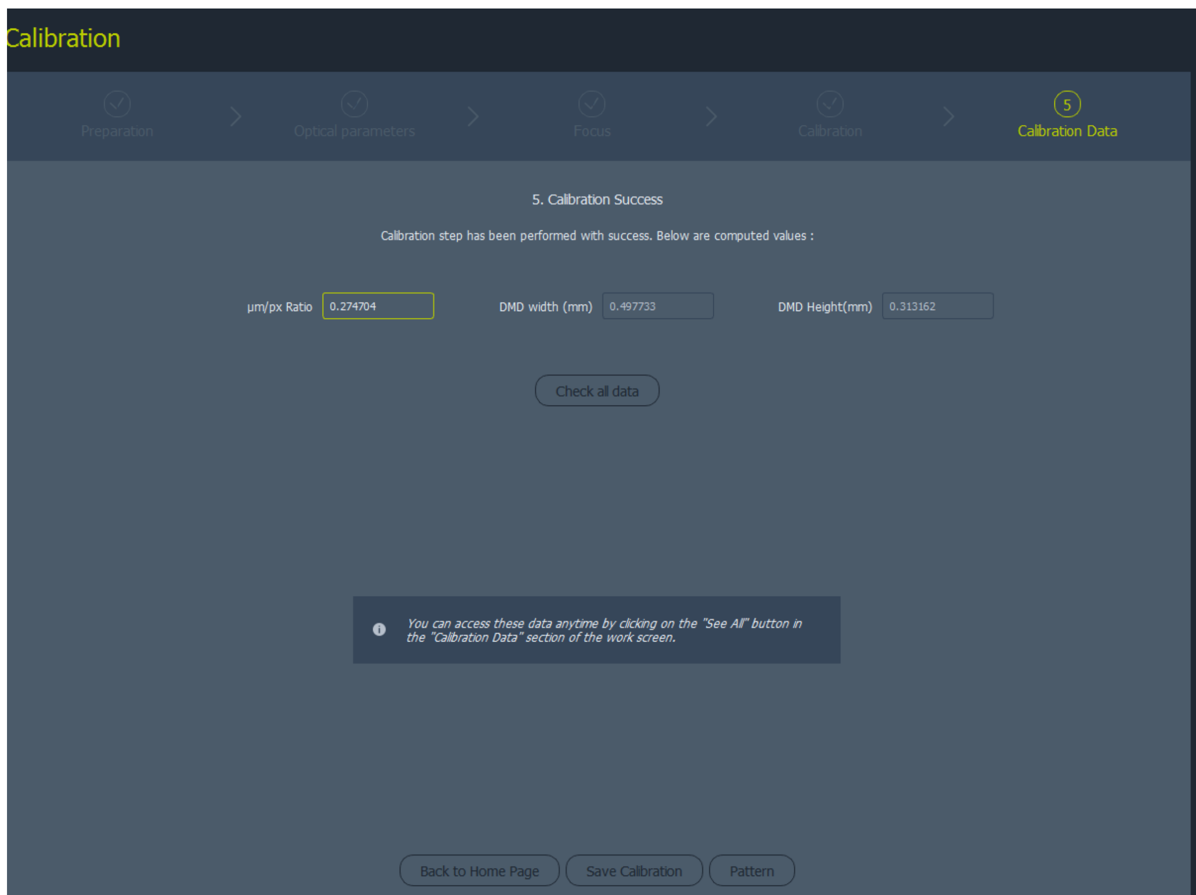


Figure 25: Post-calibration screen, displaying parameters and option to save the calibration file.

9.4 Setting up the Pattern in Leonardo

Set up your regions of interest and locations for pattern exposure. For more information about defining regions of interest and setting up pattern templates in Leonardo, please refer to “ALVEOLE – PRIMO User Manual Leonardo”.

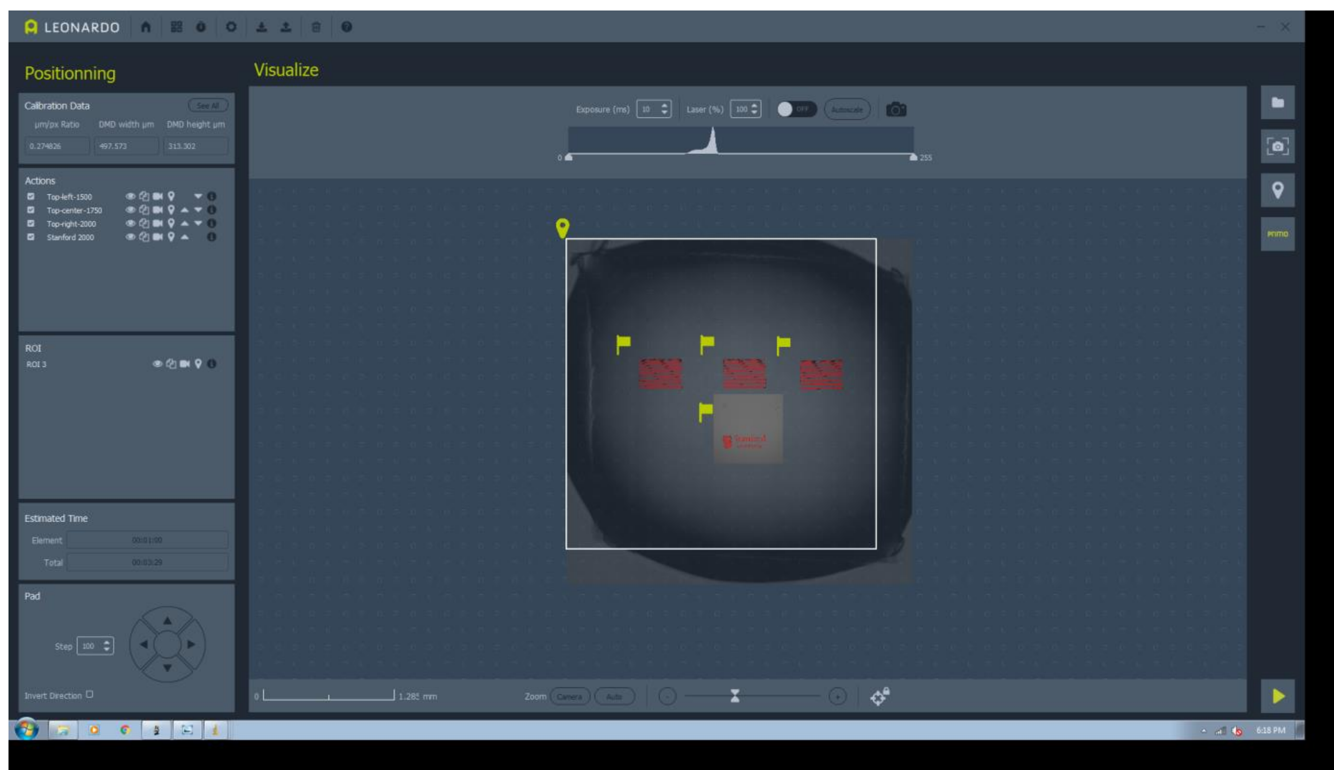


Figure 26: Four different patterns (red) to be generated within a square ROI (square PDMS stencil).

9.5 Applying PLPP

After calibration and pattern setup, remove the PBS from the substrate and add 10µL PLPP, **while making sure to maintain surface hydration.**

9.6 Exposure

Set the UV exposure for each pattern and press play to begin exposure. We have found that a range of 750 to 1000 mJ.mm^{-2} is effective for thin PDMS layers (under 1mm), though this may vary depending on the setup conditions and particular reagents.

9.7 Special Considerations for Photopatterning on PDMS

- For thick layers of PDMS, you may wish to use an inverted patterning configuration as described in the Alveole PRIMO PDMS protocol
- Given the pattern resolution degradation for thicker PDMS layers, if you require total passivation of the unpatterned regions for your application it might be best to lean towards underexposure rather than overexposure to avoid bleedthrough

- The Z-AFC system included as part of the Leica microscope uses a laser bouncing off the surface of the sample to theoretically keep the substrate in focus. The top surface of the PDMS it often out of the Z-AFC operating range but it would likely not function properly on PDMS anyway. Having Z-AFC activated (even if Z-AFC Hold is not turned on) will lead to loss of some of the laser signal since it has its own filter cube in the light path. For this reason, it may be preferable to keep Z-AFC off when not in use.
- Be careful when raising the objective to avoid crashing into the sample, especially for thick PDMS samples. After each use, it is recommended to set the objective to lowest z height.

9.8 PLPP Removal, Sample Storage, and Protein Incubation

Following exposure, rinse away the PLPP with several changes of PBS, being sure to maintain surface hydration. The biopassivated PEG layer must remain hydrated between removal of the PLPP and protein application. We have found that storing devices at 4°C in PBS in a petri dish wrapped with parafilm to prevent evaporation generally maintains surface hydration for extended periods.

To complete the patterning process, incubate with your protein solution. For robust pattern visualization, we used fluorescent gelatin 488 at 100ug.ml^{-1} and incubated for 1hr at room temperature or overnight at 4°C. Following incubation, rinse 5X with PBS. This step is important for removing unabsorbed protein and reducing the background fluorescence when imaging your patterns.

If there are issues with protein clumping (as identified by small bright dots in the fluorescence image), start from a fresh protein stock solution. Micro-centrifuging the solution and taking the supernatant may also help to avoid clumps.

9.9 Tool Shutdown

Be sure to shut down the equipment and log out of Badger before leaving.

10. Pattern Imaging

Following protein incubation and rinsing, use a fluorescent microscope to image your patterns.

10.1 Imaging Considerations

- If patterning on a flat but non-horizontal surface (e.g. PDMS wedge), flipping the device over onto a clean glass coverslip can greatly simplify imaging, as long as hydration is maintained, and excess protein does not get onto the patterned region
- Try to avoid photobleaching:
    - One strategy for avoiding photobleaching is to find the focus in fluorescent channel away from the pattern, close the shutter, move a defined distance over to where you know the pattern is, then open the shutter/image
    - Binning should be ok if you are oversampling enough to resolve the lines (if pixel size is still below the resolution of the PRIMO anyway, then you won't lose info
    - Can change the lookup table (LUT) for the MicroManager display to 12bit while imaging to see better at lower exposure

- Users will likely need confocal imaging to get good results on thicker PDMS devices or within microfluidic channels and resolve imaging artifacts from the patterning result itself

11. Troubleshooting Strategies

One method which can be quite useful is using the calibration image as a direct observation of what you would expect your pattern to look like on/within more complex geometries (i.e. load your pattern as the calibration image). As another intermediate step, you could use the Norland UV-curing glue to visualize the patterning while avoiding the complications of the surface chemistry, etc.

12. Additional Resources

- For updated PRIMO protocols: contact Alveole After-Sales Support
- For a review of the many additional ways to passivate PDMS: Zhang et al., J. Med. Biol. Eng., 2015
- For tutorial videos from Alveole: https://www.alveolelab.com/resources-support-center/tutorial-videos/

13. References

[1] Multiprotein Printing by Light-Induced Molecular Adsorption, Strale et. al. Advanced Materials, 2015

[2] Alveole PRIMO PROTOCOL: PHOTOPATTERNING ON PDMS WITH PRIMO Rev 2

[3] ALVEOLE – PRIMO User Manual Leonardo