

Standard Operating Procedure for Protein Photopatterning on PDMS Surfaces with the Alveole PRIMO

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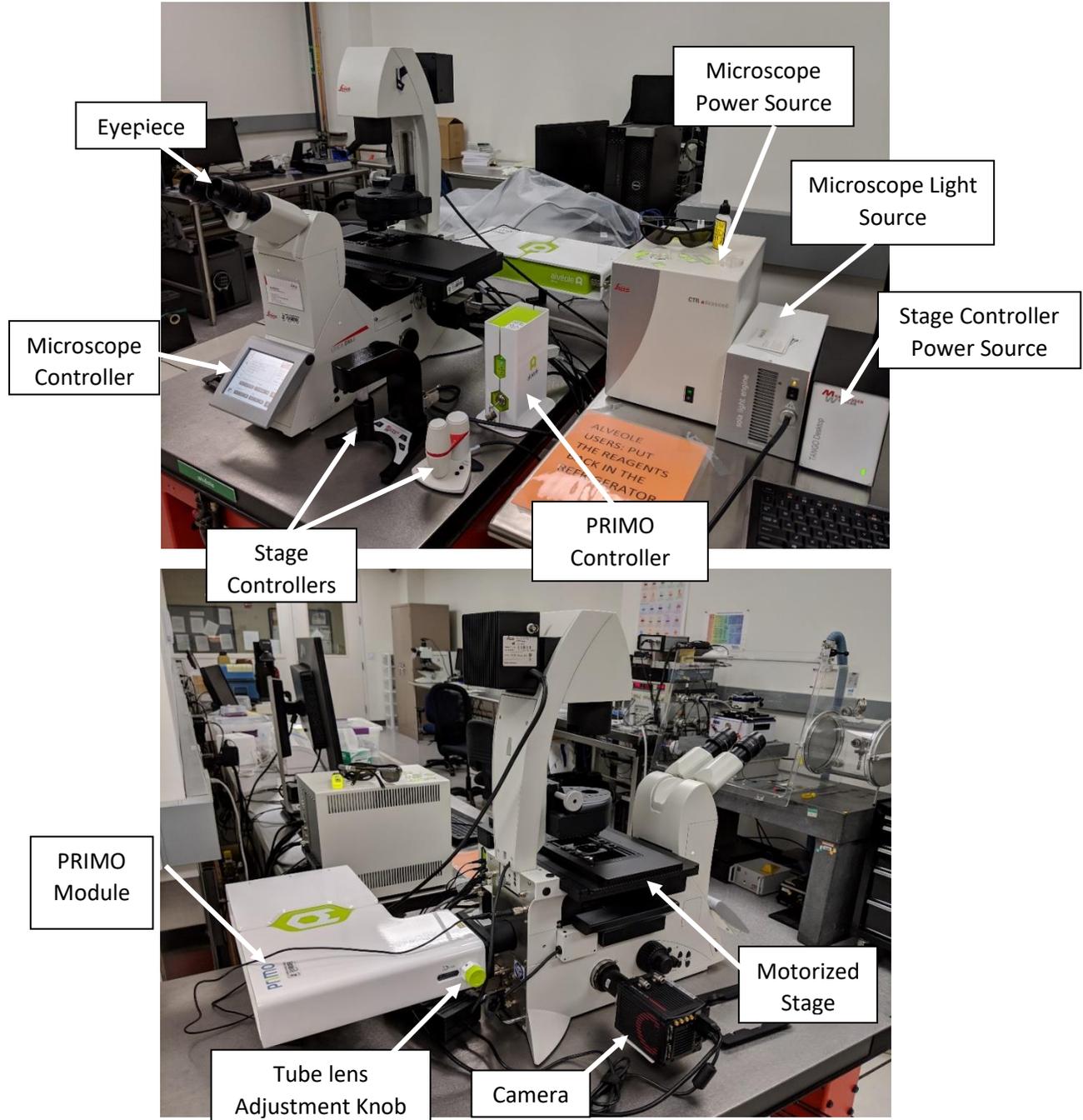
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2. SOP: PRIMO Patterning on PDMS Surfaces

The objective of this SOP is to provide guidelines to 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 that might be useful for certain PDMS patterning applications.

2.1 PRIMO System Overview



2.2 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.

2.3 Substrate Preparation

2.3.1 Applying PDMS Stencils

The PDMS stencils (shown below) are used to physically 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 1: PDMS Stencil

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

2.3.2 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).

1. (Optional) Plasma air treat for 30 to 60 seconds
2. Place stencil on PDMS if needed
3. Incubate with PLL at $500\mu\text{g}\cdot\text{ml}^{-1}$ for 30min
4. Prepare the HEPES buffer at $8 < \text{pH} < 8.5$ if not already done
5. Rinse 3 times with HEPES
6. Prepare a solution of PEG-SVA in HEPES at $50\text{mg}\cdot\text{ml}^{-1}$. **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

2.3.3 Considerations and Tips

- **IMPORTANT:** Make sure to maintain hydration of the PEG layer during rinsing and handling, otherwise the PEG brush may collapse and lose its passivation properties. (see <https://www.alveolelab.com/tutorials/preparing-your-substrates/> for a demonstration of proper rinsing techniques)
- 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 leak 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. Misalignment of the PDMS sample with respect to the coverslip (as shown in figure below) can result in undesired pattern distortion and uneven shifts in focal plane for a given field of view.

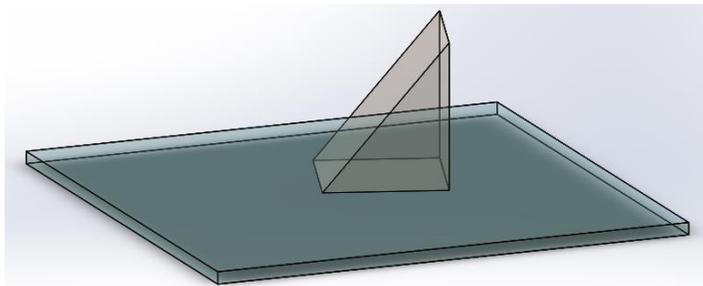


Figure 2: PDMS wedge (sample) rotates externally with reference to the bottom coverslip.

2.4 Patterning with PRIMO

2.4.1 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



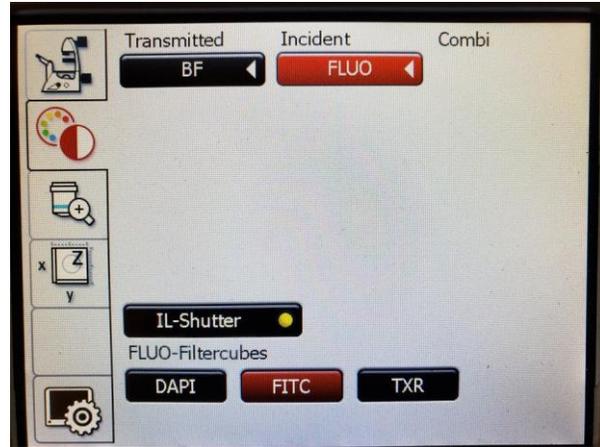
Step 5: Turn on the camera

Checking the Incoming Light Source

For the PRIMO laser to be projected through the microscope objective onto the sample, the incoming light source (indicated by the “Infinity Port” setting) must be correctly set to the PRIMO module, which connects at the rear of the microscope. While in brightfield mode, the Leica settings panel will not display this option. To verify that optical path is correctly set up for patterning, follow the steps below after powering up the tool:



Step 1: Default microscope settings for patterning with the PRIMO laser diode (using the dedicated PRIMO EMP_BF filtercube)



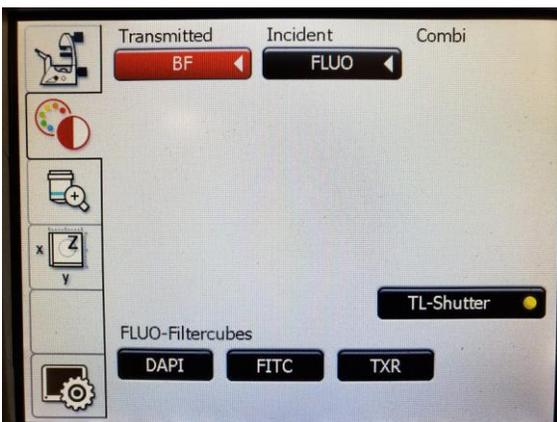
Step 2: To verify that the incoming light source is correct, switch to fluorescence mode (FLUO)



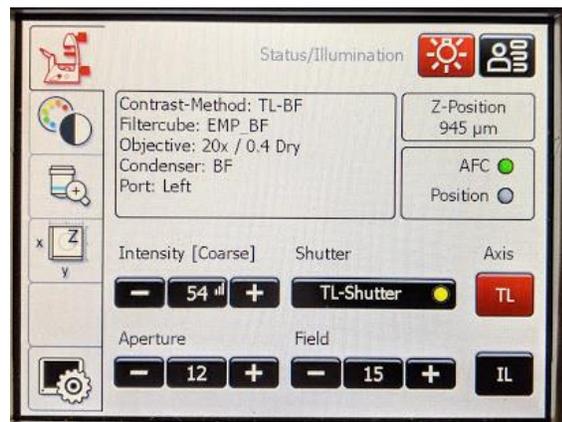
Step 3: **Verify that Infinity Port is set to Rear.** This corresponds to the PRIMO Module



Step 4: If Infinity Port is set to something other than Rear (i.e. Side), toggle using the CHG RL button on the Leica controller



Step 5: For all calibration and patterning steps, return to brightfield (BF) mode and ensure that the transmitted light (TL-Shutter) is open



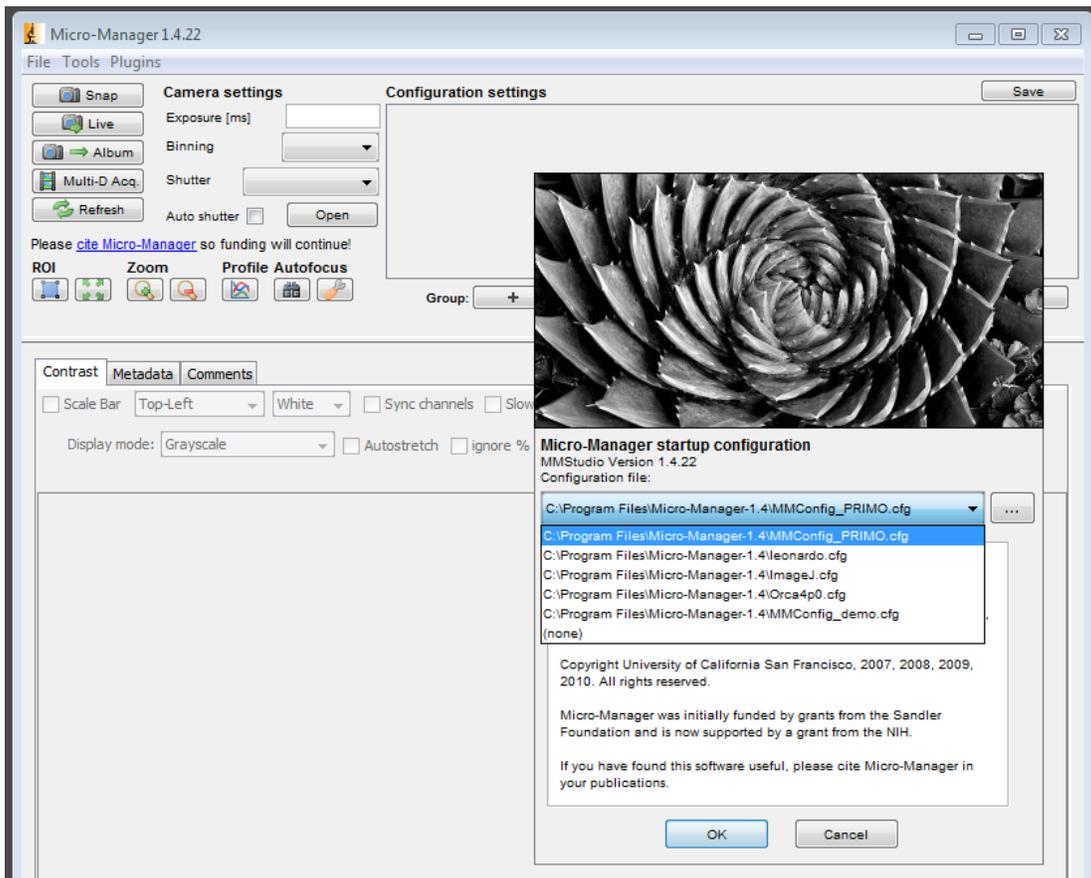
Verify that Port is still set to Left, so that the output is directed to the camera. If set to Right or Eyepieces, use the COMBI button on the Leica controller to toggle to the correct option

2.4.2 Launching Leonardo

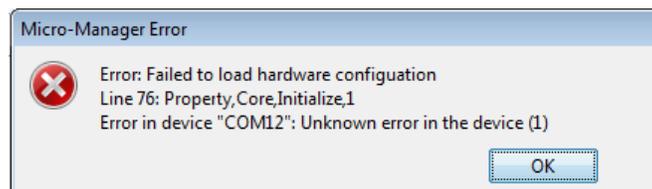
Step 1: Open Micro-Manager



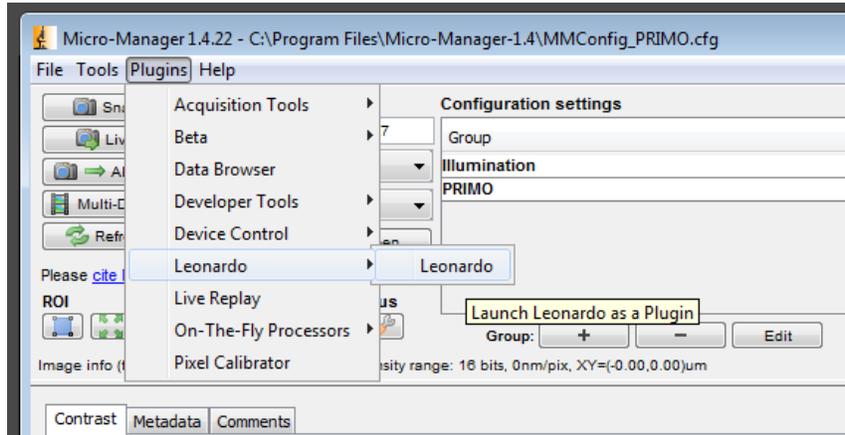
Step 2: Select `C:\Program Files\Micro-Manager-1.4\MMConfig_PRIMO.cfg` configuration file, click OK.



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



Step 3: Launch Leonardo from the Plugins tab as shown



2.4.3 Calibration

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

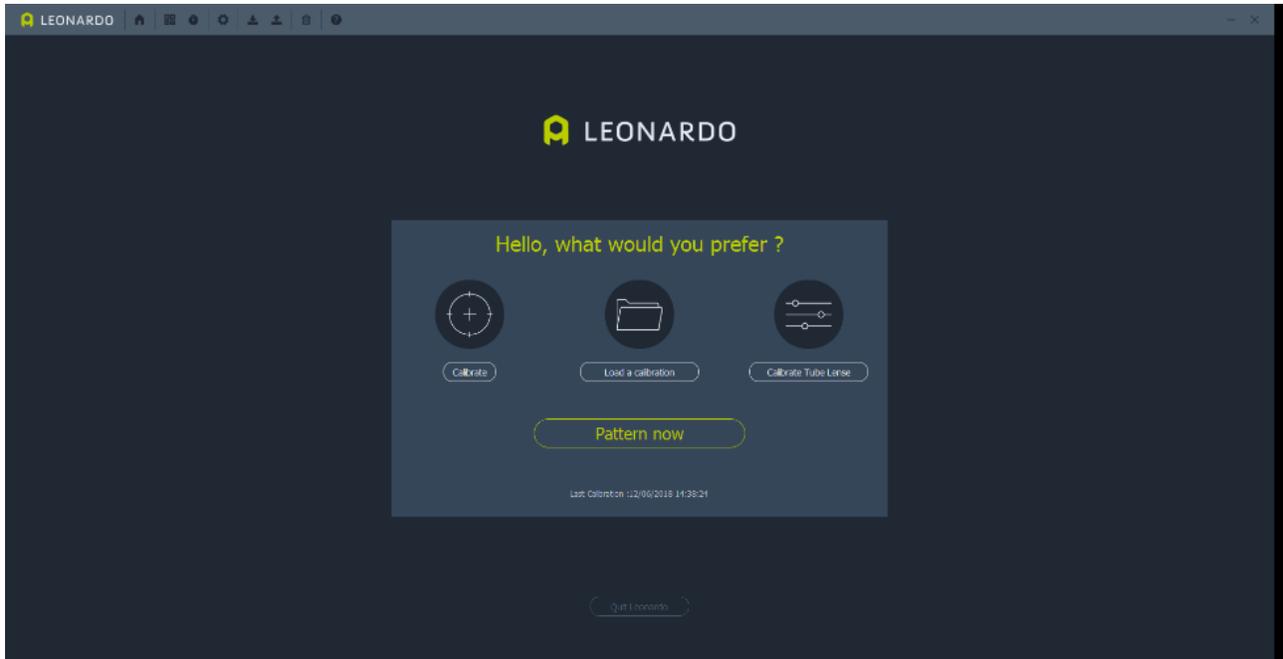


Figure 3: Leonardo main screen

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. $\mu\text{m}/\text{px}$ 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.

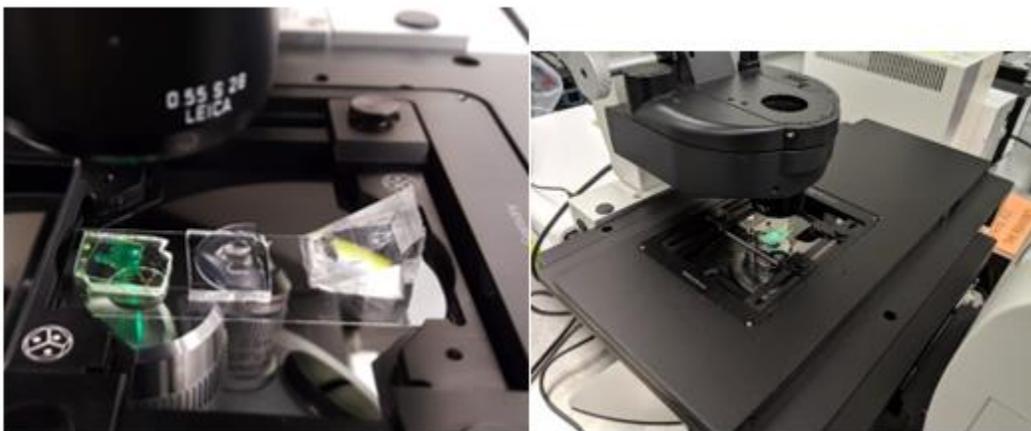


Figure 4: Calibration setup for PDMS wedges

Leave the default optical parameter settings: magnification value of 19.91 and default laser power of 5.20 mW. (These values may change slightly after calibrations of the lens alignment, so check with Swaroop for the latest settings.)

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 5: 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.

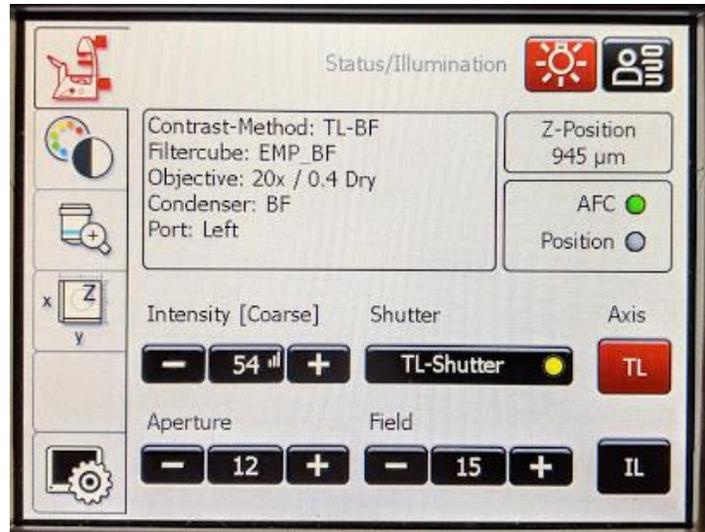


Figure 6: Image of the Leica microscope control panel with transmitted light (TL) shutter open and brightfield light intensity set to 54%.

Step 3: Image Projection

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

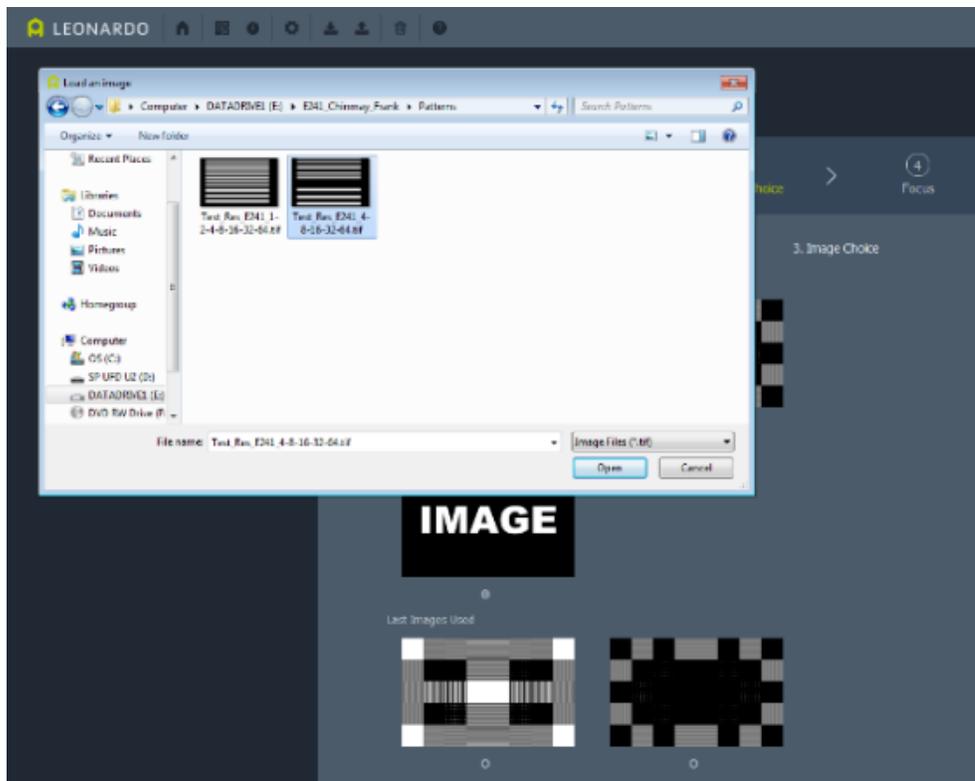


Figure 7: Selection of a custom pattern for use in calibration

Step 4: Adjust tube lens

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

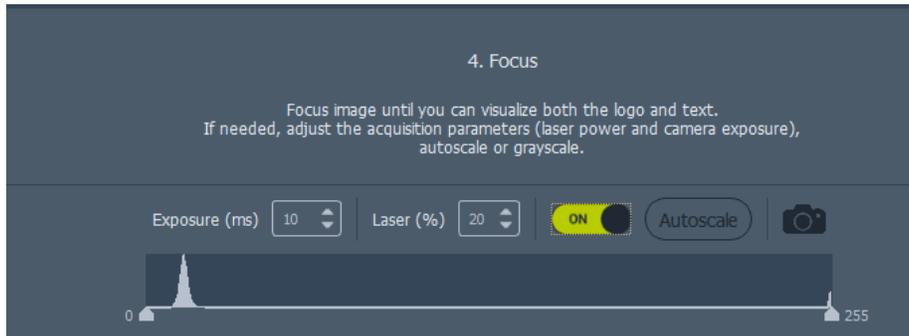


Figure 8: Leonardo screenshot during calibration. Note that the laser power is at 20%, but there are still saturated pixels (indicated by a spike in the image histogram of pixels with a value of 255).

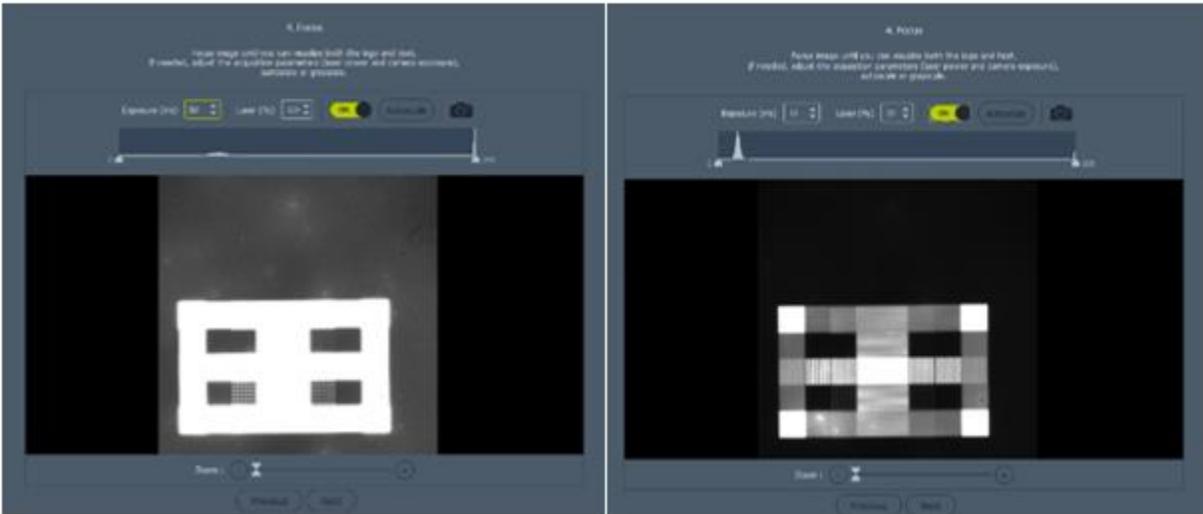


Figure 9: Calibration pattern. Note the change in the saturation level of the pattern.

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 (<10 μm). 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 patterning is completed (the default setting is currently +5).



Figure 10: 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.

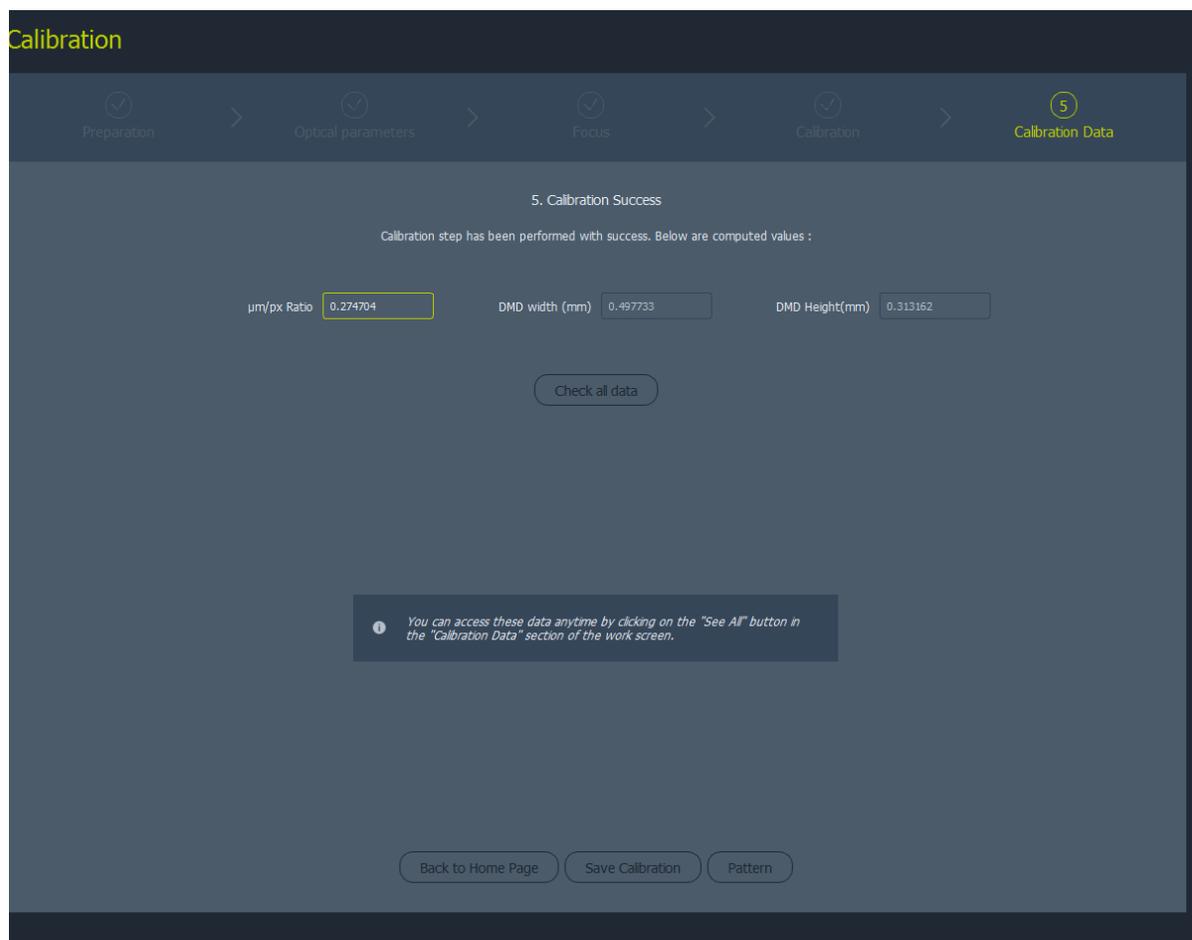


Figure 11: Calibration data

2.4.4 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”.

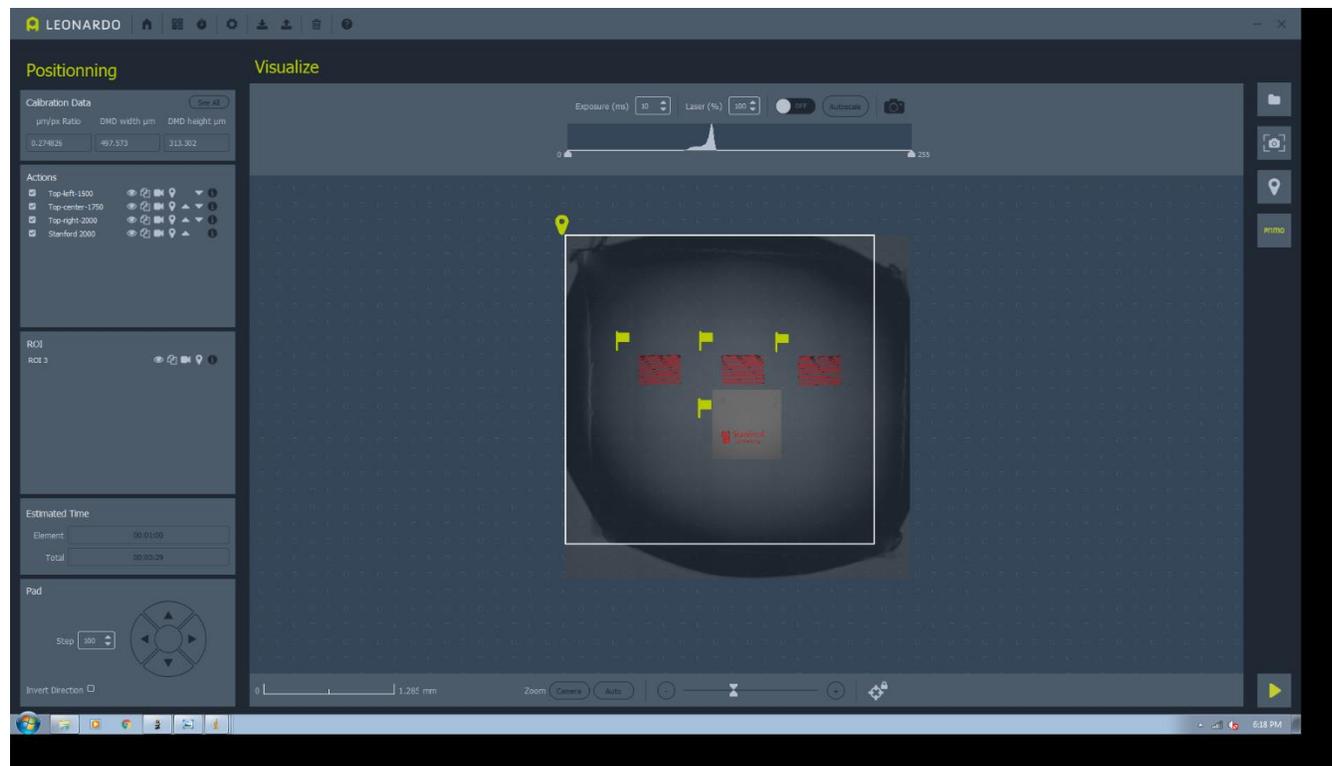


Figure 12: Four different patterns (red) to be generated within a square ROI (square PDMS stencil)

2.4.5 Applying PLPP

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

2.4.6 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 un-patterned 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 is often out of the Z-AFC operating range, but this feature would likely not function properly on PDMS regardless. 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.

2.4.7 Exposure

Make sure the Infinity Port is toggled to the Rear (PRIMO laser module) and that brightfield (BF) mode is selected. **Leave the transmitted TL-shutter open but turn down the light intensity to zero.**

Set the UV exposure for each pattern and press play to begin exposure. We have found that a range of 750 to 1000 mJ/mm² is effective for thin PDMS layers (under 1mm), though this may vary depending on the setup conditions and particular reagents.

After pressing play, verify that the laser light is on by holding a piece of white paper or index card above the condenser lens and looking for a small blue projection of light.

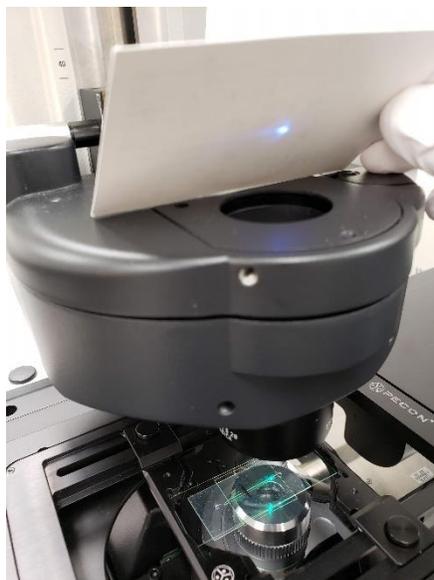


Figure 14: Verify that PRIMO laser is on by identifying blue spotlight on index card held above condenser lens

2.4.8 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 100 ug/ml and found that a 10 minute incubation in the dark at room temperature is sufficient for pattern visualization, though this a point which may vary among users depending on the protein, substrate, and end application. You may wish to incubate longer (e.g. 1 hr. 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. We have found that rinsing with PBS and then DI water before imaging produces cleaner images.

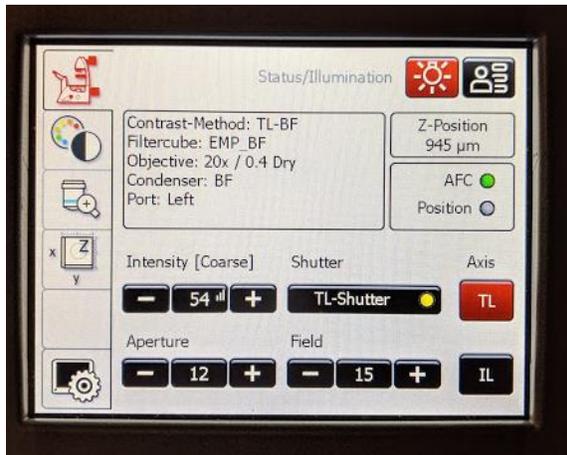
If there are issues with protein clumping (as identified by small bright dots in the fluorescence image), start from a fresh protein stock solution. Micro-centrifuging the solution and taking the supernatant may also help to avoid clumps.

2.4.9 Tool Shutdown

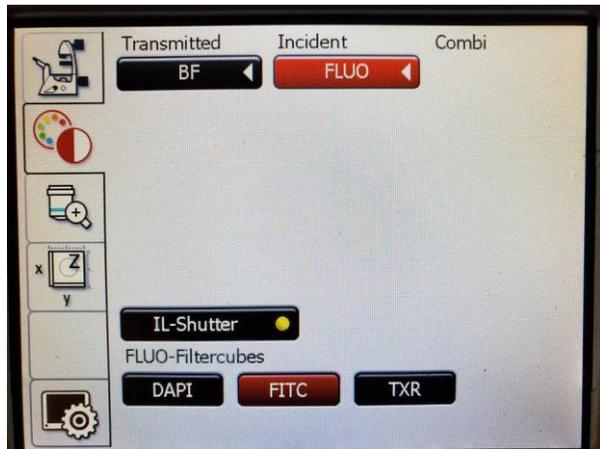
Be sure to shut down the equipment and log out of Badger before leaving.

2.5 Pattern Imaging

Following protein incubation and rinsing, use a fluorescent microscope to image your patterns. If desired, this can conveniently be done on the same microscope used for patterning. Use the CHG RL button on the Leica controller to switch the illumination source from the PRIMO laser module to the Sola Light engine as depicted below.



Default microscope settings for patterning with the PRIMO laser diode.



Step 1: Switch from brightfield (BF) to fluorescence mode (FLUO) and select the proper filter cube to view your protein of interest (e.g. FITC for Gelatin-488).



Step 2: Use the CHG RL button on Leica controller to adjust the Infinity Port setting (incoming light source) from Rear (PRIMO module) to Side (Sola Light Engine). The output port should remain set to Left (camera) or Eyepieces (if viewing manually).



Step 3: Final microscope settings for fluorescent imaging. **As a courtesy to other users, please return the Infinity Port setting to Rear once imaging is completed.**

2.5.1 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.
- 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.