This protocol is a guideline and you need to optimize it for each different application.

1. Reagents and equipment

Equipment

- Glass coverslips
- PDMS stencils
- PRIMO Setup

Reagents

- PLPP 1X
- hydrogel: 4-Arm-PEG-Acrylate, PEG-DA, PEG, collagen methacrylate
- Acryl-PEG-SVA (MW 3400 $g.mol^{-1}$,)
- Poly-L-Lysine (MW 15,000-30,000 $g.mol^{-1}$, Sigma Aldrich)
- DMF

2. Z-controlled UV structuration of hydrogels

2.1. principle

The idea of the structuration with PRIMO is to take advantage of oxygen polymerisation inhibition to control the height of hydrogel structure. A porous PDMS ceiling allows oxygen to diffuse at the top of the illuminated area and stops the polymerisation at a given height. Increasing UV power increases oxygen consumption rate leading to a taller structure. Increasing insolation time allows to extend the reticulation leading to more reticulated gels.

PRIMO can interpret grayscale in two different manners that allow the control over height or stiffness:

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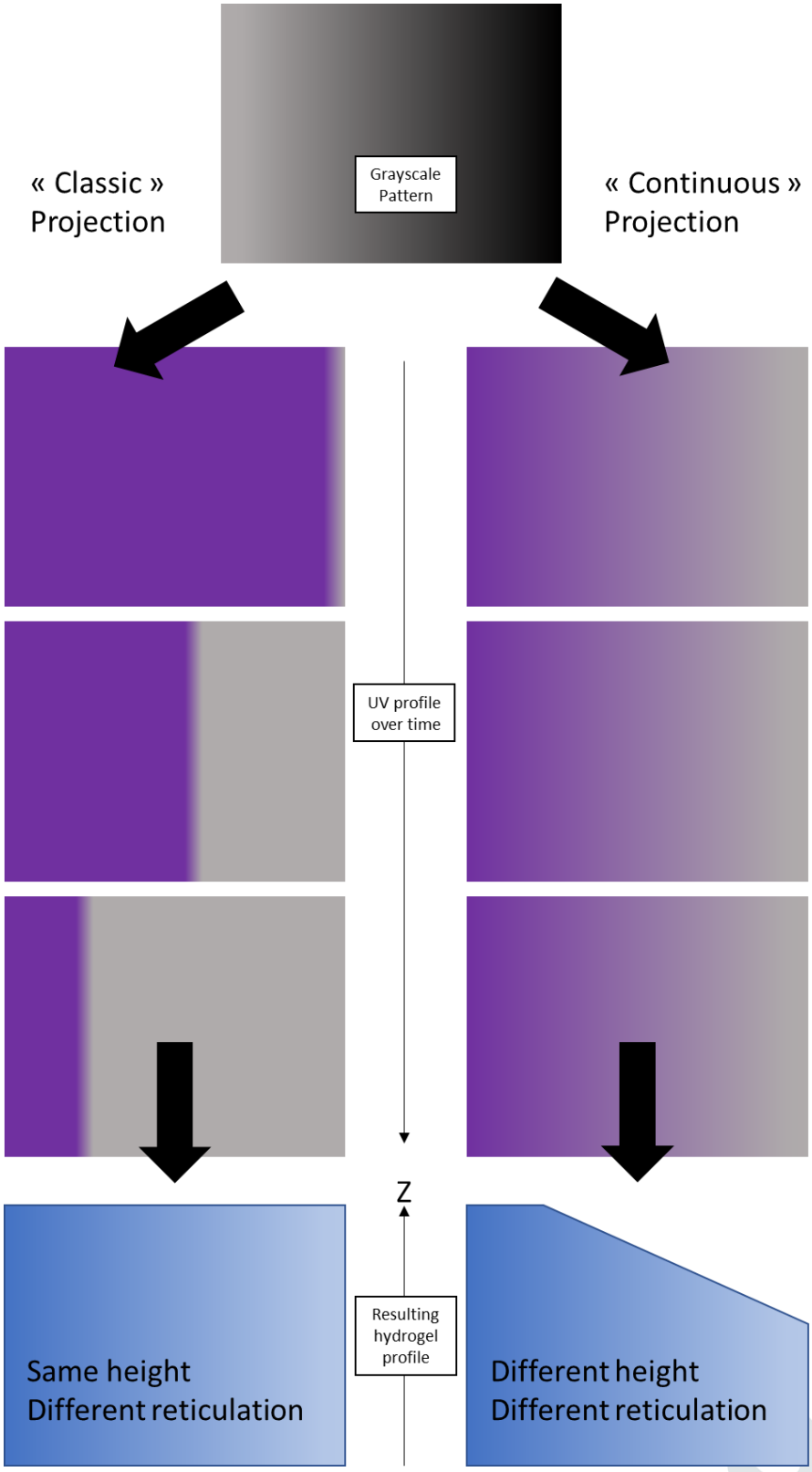

Figure 1: Control of height or stiffness depending on the exposure mode

Here we describe how PRIMO can be used in conjunction with Leonardo software to play on these two parameters and tailor hydrogels structures.

2.1. protocol

- **Assemble a chamber made of a glass coverslip on top of which two PDMS stencils are stacked** (as described in *figure 1*).

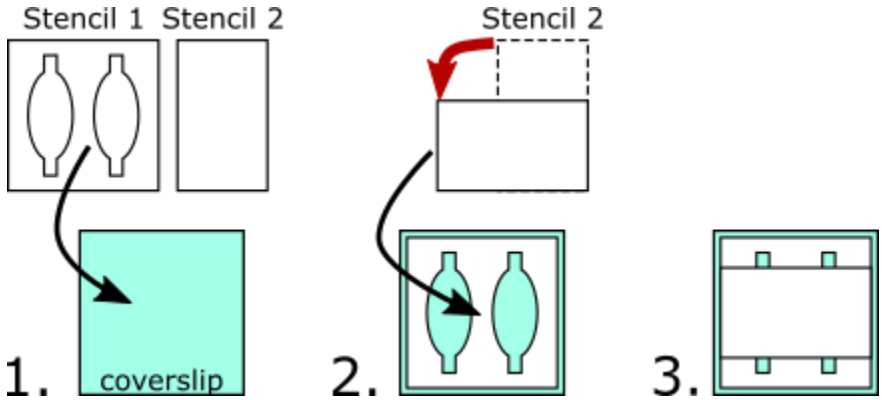

Figure 2: Assembly of the PDMS chamber

- **Inject inside the resulting PDMS chamber a solution of hydrogel with PLPP as photo-initiator.**

The following hydrogel precursors were successfully tested:

- 4-ARM-PEG-Acrylate (5% in PLPP). MW 10K and 20K
- PEGDA (down to 5% in PLPP)
- Collagen MA
  Hydrogels precursors with no acrylate function can also undergo polymerization with PLPP albeit with reduced performance.
- PEG 20K 5% in PLPP
- PEG 8K 5% in PLPP
- Matrigel 5mg/mL in PLPP

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2.2. Hydrogel panel

- Select the hydrogel panel and open the “expert mode” by clicking on it. There is an option that will allow you to control the illumination mode.
- The “Stiffness” button corresponds to the classic illumination and allow you to control the reticulation of your hydrogel.
- The “Thickness” button corresponds to the continuous projection and will allow you to control the height of your hydrogel.

Figure 3 : The hydrogel panel is on the right side 1). You can select in the export your illumination mode depending on your need: 2) Stiffness or 3) thickness control.

2.3. Alternative method: using the live mode of Leonardo

If you do not have a Leonardo that include the hydrogel panel, you can use this alternative method.

- Control of height: use continuous projection, load the pattern in the preferences and save it. Then use the “ON” button. Toggle the “ON” button for the desired insolation time then switch it “OFF” (see figure3).

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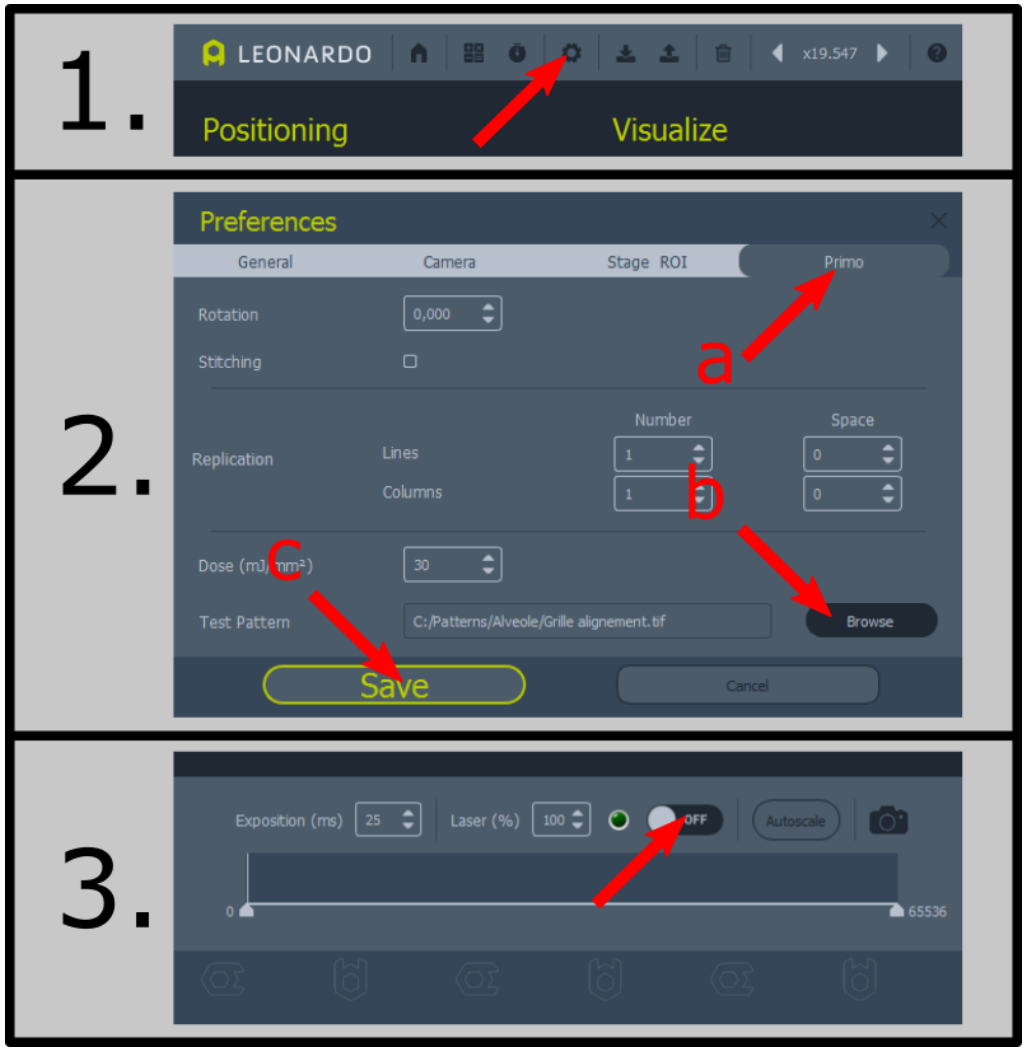

Figure 4: Using continuous mode with Leonardo: 1. Open "preferences" menu in top left corner of Leonardo software. 2. Go in the "Primo" tab (a) and choose your pattern using the Browse button (b) then save your choice with the Save button (c). 3. Start illumination by clicking the ON/OFF button, the chosen pattern will be projected where the camera is looking at. Use the same button to turn off the UV illumination.

Load the grey-scale pattern of interest into LEONARDO and project at maximum power until the lower part of the anticipated photopolymerized hydrogel appears crosslinked.

2.4. UV dose and exposure time

The UVdose/exposure time depends on the power and the model of your PRIMO. And of course, of the hydrogel and PLPP concentration. Here is a table with the recommended parameters for 4-arm-PEG-acrylate (5% in PLPP 1X) using 4X magnification. Because the polymerization depends on the profile of 0_2 depletion, which depends on the projected pattern, it's necessary to optimize the UV dose for each experiment. The grey levels of the pattern also need to be optimized to create the desired structure.

	Primo (laser, 375 nm)	Primo 2 (LED, 365 nm)
Hydrogel panel (UV dose)	30-60 $mJ.mm^{-2}$	15-30 $mJ.mm^{-2}$
Leonardo's live mode (time)	30-60 s	15-30 s

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Rinse extensively with PBS. The structures are ready.

2.5. How to design pattern for increased structure sharpness

As shown in the figure 4, to get structures with sharp edges and small detail a "non-zero" background of illumination can help. Typical grey level for this background are in the range 5 to 15 (grey level total range is 0 (black) to 255 (white)), the optimal value might depend on the specific condition of your experiment. The value must be sufficient to deplete oxygen in solution while remaining low enough so that polymerization does not occur.

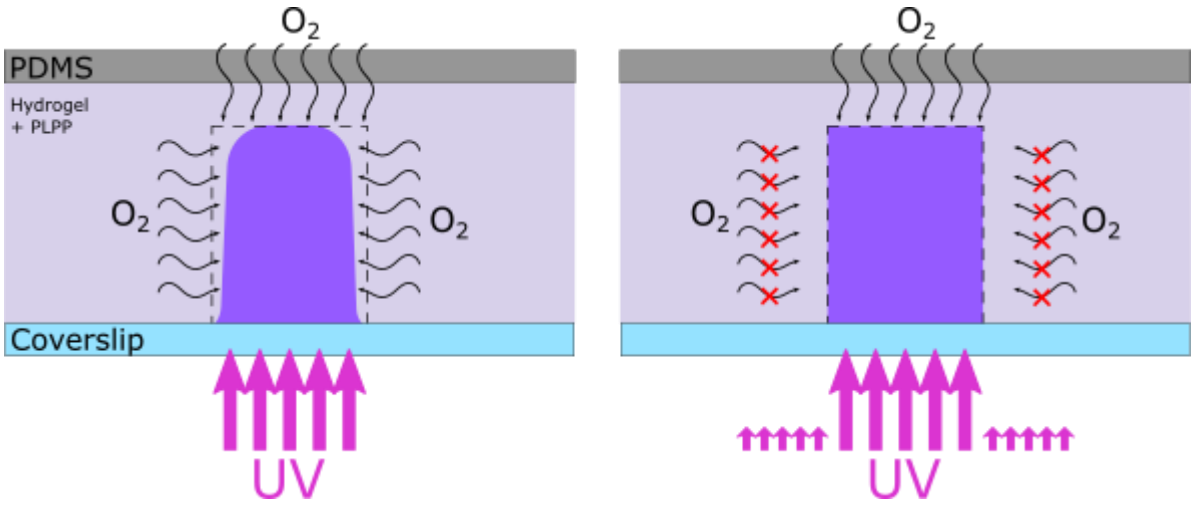

Figure 5: Oxygen in solution inhibits polymerization at the edges of the wanted structure resulting in loss of sharpness. Adding a homogeneous "non-zero" background prevents this effect and increases fidelity of the result.

3. Decoration of an existing hydrogel

Create a hydrogel of interest as described above.

Weigh Acryl-PEG-SVA prior to the decoration process, protect the aliquot and the stock from humidity.

Variant 1:

- For each 0.5 mg of Acryl-PEG-Sva add 1 µL of anhydrous DMF (500 mg.ml^{-1} final concentration)

This solution is stable at 4°C for two weeks.

- Inject 4 µL PLPP 1X in each well of the PDMS chamber, load the pattern of interest into LEONARDO.
- Inject 1 µL of the Acryl-PEG-Sva solution and project with UV dose 60-120 mJ.mm^{-2} with 4x objective.

Variant 2:

- Load the pattern of interest into LEONARDO.
- For each 1 mg of Acryl-PEG-SVA add 5 µL of PBS and 5 µL of PLPP (100 mg.ml^{-1} final concentration).

This solution is single use but does not contain DMF.

- Inject into the PDMS chamber and project with UV dose 60-120 mJ.mm^{-2} with 4x objective.

In all cases:

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- Rinse extensively with PBS.
- Incubate Poly-L-Lysine (100 µg.ml^{-1} in PBS) for 1 hour.

- Rinse extensively with PBS.
- Incubate the biomolecule of interest (such as fibronectin) at 100 µg.ml^{-1} for 15 min.
- Rinse extensively with PBS

Note: Using a fluorescent solution of Poly-L-Lysine helps controlling the quality of the micropatterning process.

Note2: One can skip the PLL incubation if needed: this step is used to enhance cell adhesion. Instead, incubate the biomolecule for 3 hours.

4. Photo-scission of an existing hydrogel

- Create a hydrogel of interest as described above, decorated or not.
- Look in the table below for the required PLPP concentration, laser power and illumination time.
- Inject PLPP in the PDMS channel (check concentration in the table).
- Load the pattern of interest into LEONARDO and project with parameters from the table.
- Rinse extensively with PBS.

GEL	PLPP CONCENTRATION	POWER (AND POWER % FOR OBJECTIVES)		DURATION (SEC)	UV DOSE (mJ.mm^{-1})
MATRIGEL	0.0125 X	6 mW.mm^{-2}	25% @ 20X 100% @ 10X --	7200	43200
4-ARM PEG ACRYLATE	0.0125 X	6 mW.mm^{-2}	25% @ 20X 100% @ 10X --	1200	7200
AGAR-AGAR	1 X	4.3 mW.mm^{-2}	18% @ 20X 71% @ 10X --	7200	31000
PAAM	1 X	1.7 mW.mm^{-2}	7% @ 20X 28% @ 10X --	7200	12000
4-ARM PEG AMINE / 4-ARM PEG SAS (50/50)	1 X	0.036 mW.mm^{-2}	0.15% @ 20X 0.6% @ 10X 3.6% @ 4X	150	5.4

Table 1: Scission parameters for common hydrogels. This table can be used as a starting point but optimal parameters will depend on the specific needs and conditions of your experiment

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