PRIMO PROTOCOL: Micropattern transfer onto PAA hydrogels

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

PAA = Polyacrylamide

Caution: Please read the MSDS on 3-(Trimethoxysilyl)propyl methacrylate, alcrylamide and bis-acrylamide. These reagents must be manipulated under a chemical hood until the gel has polymerized.

1. Reagents and equipment

Equipment

- PRIMO® setup
- Glass coverslips (if possible of two different sizes)
- PDMS stencil
- Tweezers
- Beaker
- Vacuum chamber
- Plasma cleaner
- Oven

Reagents

- mPEG-SVA
- Polylysine (PLL)
- HEPES 8,3 < pH < 8,5 at 0,1 M
- Silanization solution (3-(Trimethoxysilyl)propyl methacrylate, M6514, Sigma, USA)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (HN 77.5, Carl Roth, Germany)
- Acrylamide solution (01697, Fluka Analytical, USA)
- N, N0-Methylenebisacrylamide solution (66675, Fluka Analytical, USA)
- TEMED (T9281, Sigma, USA)
- Ammonium persulfate (APS) (A3678, Sigma, USA)
- Parafilm®
- Acetic acid
- PBS 1X solution pH = 7,4
- Ethanol 70 %
- PLPP Gel
- Solution of protein of interest

2. Coverslip silanization for PAA polymerisation

Protocol from Vignaud et al. 2014.

The silanization treatment is necessary to ensure a good attachment of the PAA gel to the glass coverslips.

- Start the oven at 120°C and let it warm up.

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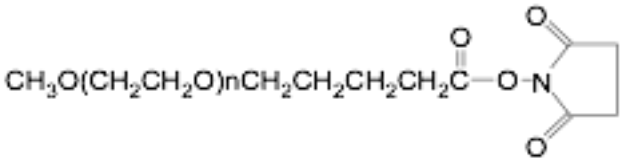
- Put the glass coverslips in your plasma cleaner either horizontally or in a specific holder that will allow both sides of coverslips to be in contact with the ionized gas during the plasma treatment. Plasma clean for 1 to 3 min.
- If the coverslips were horizontal in the reactor, flip them and repeat the plasma process.
- Put the coverslips in a box that can be hermetically closed (e.g. an empty tip's box hermetically closed with parafilm). Add a centrifuge tube cap in the box.
- Put 40 µl of 3-(trimethoxysilyl)propyl methacrylate in the cap then close hermetically the box. Let the coverslips be silanized for 1 hour.
- Cure for 1 h at 120°C.
- Blow off dust with pistol airflow and store at room temperature. This treatment is stable for a few weeks, you can prepare many coverslips at the same time.
- Store the coverslip until gel transfer.

3. New coverslip passivation for photopatterning

The strategy for passivation with PEG-SVA is the following:

1) Coating of the substrate with PLL.

2) Coupling of the amines of the PLL with the SVA ester.



mPEG-SVA is a PEG (5kDa) coupled with a Succinimidyl Valerate (SVA) ester. This ester is used for PEGylation of primary amine.

The silanized coverslip will be used for the gel polymerization. Do not use this one here. Use a new coverslip. This one will be used for patterning only.

- Put a droplet of water on the bench, and put a parafilm on it. Check that the parafilm is flat.
- Place the glass coverslip in a glass petri dish and place it on plasma cleaner (HI for 1 min)
- Put a 50 µl droplet of PLL (0,1 % (w/v) diluted in MQ water) or laminin (100 $\mu g.ml^{-1}$ in PBS) on the parafilm.
- Take the coverslip with tweezers and flip it on the droplet in order to have the plasma-activated side of the coverslip facing the PLL or laminin solution and let incubate for 30 min. The hole surface should be covered. If 50 µl is not enough, add more.
- Wash with MQ water. Afterwards, gently lift up the coverslip from the side using tweezers and put it vertically. Let the liquid run off by gravity. You can gently air flow to remove all the liquid or use a Kimtech.
- Make a solution of PEG-SVA in 0,1 M HEPES with $8,3 < pH < 8,5$ at 70 $mg.ml^{-1}$. If the passivation is not strong enough, you can increase the concentration up to 100 $mg.ml^{-1}$.

As the half-life of the SVA ester is 10 minutes at pH 8,5, the solution must be prepared just before use.

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- Put a drop of 50 µl of the PEG-SVA solution on a parafilm and put the coverslip on top, PLL (or laminin) coated face in contact with the PEG-SVA. The hole surface should be covered. If 100 µl is not enough, add more.
- Incubate 1 hour at room temperature, in humid chamber to avoid evaporation. Be careful not to dry.
- Wash profusely with MQ water.
- Dry the coverslip.

4. Use of the PLPP Gel photoinitiator

- Put your coverslip on a plastic petri dish.
- Deposit 3 µl of PLPP Gel (for a coverslip of 22x22 mm (0,62 µl.cm^{-2}), adjust it for different size) at the center of the glass slide.
- Add 50 µl of EtOH at the center of the coverslip, homogenize by manual rotation of the petri dish. A gel will form. The EtOH is here only to help the PLPP to spread. If you have trouble covering the surface, add more. Be careful of the spreading of the gel.
- Wait for the complete drying. Depending on the conditions and temperature, it can take from 10 min to 30 min.

5. Photopatterning with PRIMO

- Place your substrate on the microscope.
- Load and lock your pattern on Leonardo software. The typical UV doses are listed below. It might depend on the density of PLPP Gel, substrate, etc… **dose adjustment might be needed.**

Primo (laser, 375 nm)	Primo 2 (LED, 365 nm)
30 mJ.mm^{-2}	15 mJ.mm^{-2}

- Use one corner of your slide to make the focus: switch off the brightfield light, switch the UV laser on, adjust the exposure time and histogram. Make the focus on the UV reflection. Be careful, there are reflections on both side of the glass coverslip, be sure that you are on the top one.
- Launch the patterning sequence.

5.1. Working with coverslip chambers

- If working with coverslip chambers, rinse directly with PBS without drying.
- Rehydrate the slide with PBS for 5 minutes.
- Incubate 5 minutes with a solution of 20 µg/ml of protein.

This protein concentration and incubation time is an indication. You may need to increase them if you have a bad protein transfer. You can go up to 100 µg.ml^{-1} for 15 min

- In the meantime, aliquot the desired amount of acrylamide solution and allow it to degas in a vacuum chamber.
- Rinse profusely with PBS without drying.

5.2. Glass coverslips

If working with coverslips, follow the following procedure:

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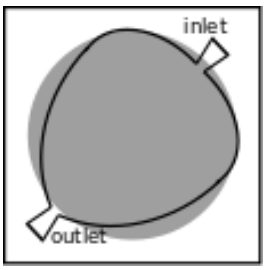
- Dry the coverslip and add a PDMS flow chamber stencil:

  - Stick the flow chamber to the glass coverslip

  - Place a 15 mm round glass coverslip over the center of the stencil (vwr ref 631-1579 for example). Let the entrances free of access.

  - Add the liquid on the entrance located on the larger side of the chamber (inlet). You can use a Kimtech paper on the outlet to flow the liquid. Be careful not to dewet.



- Rehydrate the slide with PBS for 5 minutes.
- Incubate 5 minutes with a solution of 20 µg/ml of protein.

This protein concentration and incubation time is an indication. You may need to increase them if you have a bad protein transfer. You can go up to 100 µg.ml^{-1} for 15 min

- Rinse profusely with PBS.

6. Gel formulation and pattern transfer

Protocol from Vignaud et al. 2014.

In this step, the PAA gel will be sandwiched between the patterned coverslip and the silanized coverslip for polymerization.

Make sure that the polyacrylamide precursors are thermally equilibrated at room temperature before starting.

- Prepare an Acrylamide/Bis-acrylamide solution. The ratio between the two solutions will give you a gel of a defined stiffness. To find the ratio you need, you can use the table from Tse and Engler (2001) which covers a wide range of stiffnesses. For instance, for an 8 kPa gel, mix 62,5 µl of 40% Acrylamide with 56,25 µl of Bis-acrylamide in 381 %l of mQ water to obtain 500 µl of solution.
- Degas the solution in a vacuum chamber or in a desiccator for 20-30 min.
- During this time, prepare the coverslips. Put the larger of the two coverslips on a parafilm with the side of interest facing up. If both are of the same size, put a small drop of water on the parafilm and cover it with the patterned coverslip with the side of interest facing up. The small drop of water will prevent the acrylamide solution from sliding under the patterned coverslip.
- Rinse profusely the patterned coverslip with MQ water.
- Remove the PDMS stencil or the coverslip chamber and wash again with MQ water. Afterwards, gently lift up the coverslip from the side using tweezers and put it vertically. Let the liquid run off by gravity. You can gently air flow to remove all the liquid or use a Kimtech.
- Collect the Acrylamide/Bis-acrylamide solution from the vacuum chamber/desiccator and keep the container closed.

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Optional: If you want to add some fluorescent beads to your gel for force measurements, they should be added at this stage of the process in the acrylamide solution and the solution should be sonicated for 5 min to destroy any bead aggregates that could have formed during storage.

- Prepare TEMED, APS, and other coverslips. You will add TEMED and APS solution to the acrylamide with the following proportions: 1 µl of TEMED and 1 µl of APS 10% for 165 µl of acrylamide solution. You should proceed as fast as possible in the next steps since the polymerization will occur rapidly.
- Add TEMED then APS to the Acrylamide/Bis-acrylamide solution and mix while avoiding adding bubbles.
- Put a drop of 7 $\mu l.cm^{-2}$ of the acrylamide polymerization mix on each glass coverslip previously placed on parafilm.
- Place the other coverslip of interest on top while taking care to avoid bubbles.
- Put a cap (to prevent evaporation) and let the gel polymerize for 30 min. Keep the rest of the acrylamide in a closed container as a control of gel polymerization.
- Once the polymerization is finished (you should check it by detaching the remaining acrylamide from the tube, it should have the shape of the container and be elastic if you try to pinch it with a pipette tip), immerse the sandwiched coverslips in PBS.
- Detach carefully the two coverslips using a scalpel and/or tweezers, making sure that they remain fully immersed during the entire detachment process. The PAA gels should be attached onto the silanized coverslips.
- Rinse the silanized coverslip with the PAA gel in PBS.
- Control quality with fluorescence microscopy if possible.
- Store at 4°C and use as soon as possible.

Tips:

- For a better transfer efficiency, the protein patterning and the transfer onto PAA gel should be performed in succession.
- If possible, use a silanized coverslip and patterned coverslips of two different sizes because it will then be much easier to detach them from each other.

7. Cell seeding

This step is really cell type dependent. You will have to adapt it, but here is how we do.

- Detach and collect cells using EDTA-Trypsin or EDTA (0,02% in PBS). With some cell lines it seems to be really important not to use trypsin (HeLa cells for example, ref: Micropatterning on glass with deep UV, Carpi et al., Nature protocol exchange).
- Resuspend cells in pre-warmed medium and centrifuge.
- Remove supernatant and resuspend cells in pre-warmed medium (FBS does not affect patterning) at the desired density. For most of applications, you can seed your cells in large excess in order to fill entirely your patterns.
- Typical densities are around $1x10^4$ to $10x10^4$ cells/cm2.
- Seed cell suspension drops in the wells (for a 5 mm round well add 20 µL) and put the petri dish at 37°C for 20 minutes without moving it to allow cell adhesion (time might depend on the cell type).

When seeding cells on PDMS or polyacrylamide gel, the adhesion time might be longer.

- You can check cell adhesion with a microscope.

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- Rinse with medium (either each PDMS well one by one or the entire dish) and flush gently to remove cells that do not have adhered.

8. Related publications

LIMAP technology:

- *Multiprotein Printing by Light-Induced Molecular Adsorption, Strale et al., Advanced materials, 2015.*

Gel transfer:

- *Polyacrylamide Hydrogel Micropatterning, Vignaud et al., Methods in Cell Biology, Volume 120*

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