PRIMO PROTOCOL: PLPP Gel

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

1. Reagents and equipment

Equipment

- Plasma cleaner
- PRIMO® setup
- PDMS stencil (optional, *Alvéole*)

Reagents

- mPEG-SVA (mPEG-Succinimidyl Valerate, MW 5 000, *Laysan bio*)
- HEPES 8,3 < pH < 8,5 at 0,1 M
- Parafilm®
- H_2O mQ
- PBS 1 X
- EtOH (70% or more)
- PLPP Gel (*Alvéole*)
- Surfactant Mix (only for soft PDMS, *Alvéole*)
- Protein solution of interest

Some specific reagents are needed depending on the passivation. Please refer to the paragraph 2.1 for advice to choose your passivation.

PLL + PEG-SVA passivation:

- Poly-L-Lysine (PLL, *Merck/ Sigma-Aldrich*, ref: P8920)

Laminin + PEG-SVA passivation :

- Laminin

APTES + PEG-SVA passivation :

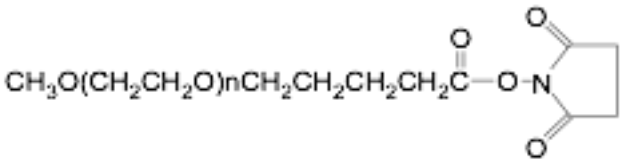
- 3 aminopropyltriethoxysilane (APTES, *Sigma-Aldrich* ref A3648).

2. Passivation

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

1) Coating of the substrate with PLL, APTES or laminin

2) Coupling of the amines of the PLL, APTES or laminin 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.

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2.1. Choosing the right passivation

PLL + PEG-SVA

The passivation by default. Easier and cheaper than the others, it works for most applications.

Laminin + PEG-SVA

This passivation is more suited for patterning cardiomyocytes, induced pluripotent derived stem cells (IPS) and embryonic stem cells (ES).

APTES + PEG-SVA

Recommended for microtubules and purified protein patterning. It also works for classic cell lines and could also be an alternative for patterning cardiomyocytes, IPS and ES.

2.2. PLL or laminin-based passivation

2.2.1. Petri dish

- Place the dish on plasma cleaner (HI for 1 min). Optional, but highly recommended.
- Incubate with 100 µl of PLL at 100 µg.ml^{-1} diluted in mQ water for 30 minutes, or laminin at 100 µg.ml^{-1} diluted in PBS 1X for 30 minutes. The whole surface should be covered. If 100 µl is not enough, add more.
- Wash profusely with HEPES. You should let just enough liquid so your surface is not dry. Do not let the surface dry.
- 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.

- Add 200 µl of the PEG-SVA solution. After adding the solution, you can gently pipet to mix well the PEG-SVA with what liquid was left on your substrate. Incubate 1 hour at room temperature, in humid chamber to avoid evaporation. Be careful not to dry.
- Wash profusely with mQ water. When washed enough, the dewetting of the surface should be fast.
- Dry the petri dish with an air flow or put it vertically and wait.

2.2.2. Glass coverslip

- 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 at 100 µg.ml^{-1} diluted in mQ water or laminin at 100 µg.ml^{-1} in PBS 1 X 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 whole 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 whole 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.

2.3. APTES based passivation

- Place your substrate in the plasma cleaner and start plasma air for 1 minute.
- Transfer your substrate in a plastic box (plastic petri dish for example).

Silanization must be performed under a chemical hood

- Detach the cap of a 1,5 mL Eppendorf and put it in the plastic box near to the substrate.
- Fill the cap with 200 µl of APTES.
- Close the box and seal it with Parafilm®. Wait 1 hour.
- Make a solution of PEG-SVA in 0,1M 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.

2.3.1. Petri dish

- Add 100 µl of the PEG-SVA solution. Incubate 1 hour at room temperature, in humid chamber to avoid evaporation. The whole surface should be covered. If 100 µl is not enough, add more. Be careful not to dry.
- Incubate 1 hour at room temperature, in humid chamber to avoid evaporation. Be careful not to let the surface dry.
- Wash profusely with mQ water.
- Dry the petri dish.

2.3.2. Glass coverslip

- Put a drop of 50 µl of the PEG-SVA solution on a parafilm and put the coverslip on top, silanized coated face in contact with the PEG. The whole surface should be covered. If 50 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.

3. Use of the PLPP Gel photoinitiator

3.1. Rigid substrate (Glass, PDMS, Plastic…)

- Mix well the gel with EtOH 70 % in an Eppendorf. The amount of gel should be around 0,6 µl.cm^{-2}. The ethanol is just here to help the spreading of the gel. Usually, a ratio of 1 µl of PLPP Gel to 10 µl of EtOH gives good results. This ratio, as well as the amount of gel, may need to be optimized.
- Be sure that all your surface is covered.

If working on petri dish (MatTek, Ibidi, Fluorodish, …) the surface/volume ratio may not be the

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same due to PLPP gel lost on the side. For example, for matTek 35 mm (20 mm internal diameter) you will need to use 3 µl of PLPP Gel (1 µl.cm^{-2}).

- Wait for the complete drying. Depending on the conditions and temperature, it can take from 10 to 30 min. You can accelerate the drying using a hotplate at 50 °C

3.2. Soft PDMS

- In an Eppendorf, mix 3 µl of PLPP Gel, 1.85 µl of the Surfactant Mix and 30 µl of EtOH
- Add the necessary amount (approx. 0.6 µl.cm^{-2}) on your substrate. 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.

4. 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 (the brightest reflection). Putting a GFP filter on the way will remove the reflection.
- Launch patterning sequence.
- When finished, rinse profusely with mQ water.

4.1. Petri dishes and chambers coverslip

- If working with chambers coverslip or petri dishes, rinse directly with PBS without drying.
- Rehydrate the substrate with PBS for 5 minutes.
- Incubate 5 minutes with a solution of 20 µg/ml of protein in its dedicated buffer. You can increase the concentration to 100 µg.ml^{-1} and the incubation time to 15 min.

Longer incubation time will lead to higher protein concentration on patterns but also higher background.

- Rinse profusely with PBS without drying.

4.2. Glass coverslips

If working with coverslips, follow the following procedure:

- 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.

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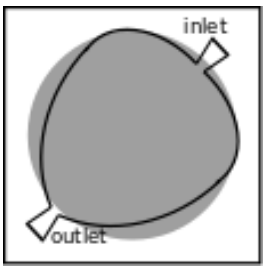

- Rehydrate the slide with PBS for 5 minutes.
- Incubate 5 minutes with a solution of 20 µg/ml of protein in its dedicated buffer. You can increase the concentration to 100 µg.ml^{-1} and the incubation time to 15 min.
- Rinse profusely with PBS without drying.

5. Cell seeding

This step is really cell type dependent and will need some adjustment.

- Rinse your sample with media without dewetting the substrate.
- Add 1.10^4 to 1.10^5 cells per cm².
- Carefully put your sample in the incubator and let them adhere for 15 min to 1 hour (or more) depending on your cell type.
- Rinse gently with cell culture media to remove the non-adherent cells.
- If cells are attaching outside the patterns, wait one more hour and pipet over the cells until the one outside of the pattern detach.

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