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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 (optional depending on the passivation)
- PRIMO® setup
- PDMS stencil

Reagents

- PLPP 1X
- PBS 1X
- Protein solution of interest (typical concentration: 10-100 µg.ml^{-1})

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

PLL-g-PEG passivation:

- PLL-g-PEG (*Alvéole,* PLL(20)-g[3.5]-PEG(2) 0,1mg.ml^{-1} in PBS 1x, storage : -20°C, 10x solution)

PEG-SVA passivation:

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

PLL + PEG-SVA passivation:

- Polylysine (PLL, *Merck/ Sigma-Aldrich*, ref: P8920)

Laminin + PEG-SVA passivation :

- Laminin

APTES + PEG-SVA passivation :

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

2. Passivation protocol

2.1. Choosing the right passivation

PLL-g-PEG

The easiest way to passivate. **Works only on glass substrates and EM grids**. Using a plasma cleaner (or a super clean coverslip) is mandatory. The passivation is less strong than the following ones. Suitable for 24 hours to 48 hours experiments.

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PEG-SVA based passivation

These kinds of passivation are stronger and are more stable in time. Your cells will be confined on the patterned area for days.

PLL + PEG-SVA

The PLL + PEG-SVA passivation by default. Easier and cheaper than the other PEG-SVA passivation, it works for most applications all substrates.

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. Passivation with PLL-PEG

This passivation works only on glass substrates and EM grids

Prepare the PDMS stencil by removing areas corresponding to the well.

- Introduce your substrate in the plasma cleaner. Remove any lid that could prevent the plasma cleaning process.
- Plasma air 1 minute.
- Place the PDMS stencil on the glass surface.
- Add 10-15 µL of PLL-g-PEG at 100 µg.ml^{-1} in the wells.

Important: from this point, never let the surface dry or dewet.

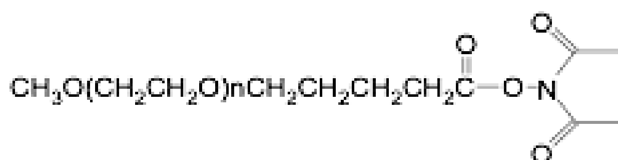
- Incubate for 1 hour at room temperature. Cover your substrate or put it in a humid chamber to avoid evaporation. Be careful of drying.
- Rinse the wells 4 to 5 times with PBS 1x.
- Leave 10-15 µL of PBS 1x in each well.

2.3. Passivation with PEG-SVA

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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2.3.1. PLL or laminin-based passivation

Prepare the PDMS stencil by removing areas corresponding to the well.

- Plasma clean your substrate for 1 min. Optional, but highly recommended.
- Put the PDMS stencil on the surface of your substrate.
- Add 15 µl of PLL at 100 µg.ml^{-1} diluted in mQ water for 30 minutes or laminin at 100 µg.ml^{-1} in PBS 1 X in the wells.

Important: from this point, never let the surface dry or dewet.

- Incubate 1 hour at room temperature. Cover your substrate or put it in a humid chamber to avoid evaporation.
- Rinse the wells 3 to 4 times with HEPES.
- 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 10-15 µl of the PEG-SVA solution. Incubate 1 hour at room temperature, in humid chamber to avoid evaporation. Be carefull of drying.
- Rinse the wells 4 to 5 times with PBS 1x.
- Leave 10-15 µL of PBS 1 X in each well.

2.3.2. APTES-based passivation

- Plasma clean your substrate for 1 min.
- 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 1,5 mL tube 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.
- Put the PDMS stencil on the surface of your substrate.
- 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.

- Incubate with 15 µl of the PEG-SVA solution for one hour. Cover your substrate or put it in a humid chamber to avoid evaporation.

Important: from this point, never let the surface dry or dewet.

- Rince the wells 4 to 5 times with PBS 1x.
- Leave 10-15 µL of PBS 1x in each well.

3. Use of the PLPP photoinitiator

- Remove the solution from the well without drying the surface.
- Fill the wells with 5-15 ml of PLPP.

4. Photopatterning with PRIMO

- Place your substrate on the microscope.
- Check that the PRIMO UV filter is on the optical pathway.
- Turn on the PRIMO set up.

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- 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)
PLL-g-PEG	600 mJ.mm^{-2}	300 mJ.mm^{-2}
PEG-SVA	1200 mJ.mm^{-2}	600 mJ.mm^{-2}

- Focus on the surface you want your pattern to be. This step is crucial to have nice and sharp pattern. You can use your substrate imperfection like dusts to focus.
- Launch patterning sequence.
- Rinse the wells 3 times with 20 µL of PBS 1x.
- Remove the solution from the wells but leave a few microliters so the surface will not dry out.
- 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 the well 4-5 times with 20 µL of PBS 1 X
- Leave 10-15 µL of PBS 1 X in the well.

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 cm^2
- 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.

6. Related publications

LIMAP technology:

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

Classical proteins photopatterning (glass with PLL-g-PEG):

- *Altered microtubule dynamics and vesicular transport in mouse and human MeCP2- deficient astrocytes, Delépine et al., Hum Mol Genet., 2016.*
- *Collagen assembly and turnover imaged with a CRISPR-Cas9 engineered Dendra2 tag, Pickard et al., biorxiv, 2018.*
- *The cytoskeleton as a smart composite material: A unified pathway linking microtubules, myosin-II filaments and integrin adhesions, Rafiq et al., biorxiv, 2018.*

Patterning on soft PDMS:

- *Extracellular matrix stiffness regulates force transmission pathways in multicellular ensembles of human airway smooth muscle cells, Polio et al., biorxiv, 2018.*

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Proteins photopatterning on 3D structures:

- *A new approach to design artificial 3D micro-niches with combined chemical, topographical and rheological cues, Stoecklin et al., Adv biosys, 2018.*
- *Controlling the topography and biochemistry of cell culture substrates with PRIMO® photopatterning system, Strale et al., Nature Methods, 2018 (Application note).*

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