Grid photopatterning: customized protocol

Responsible person: Raaja
Autors/Illustrations: Carlotta, Šárka

A. Reagents and Equipment

Equipment

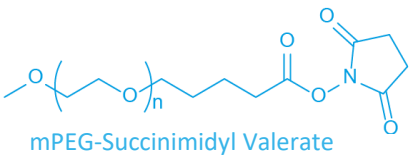
- PRIMO® setup
- TEM grid
- Parafilm
- Glass slides
- Wet chamber
- Plasma cleaner or glow discharge system (optional depending on the passivation)
- Optional: hot plate, Al-foil

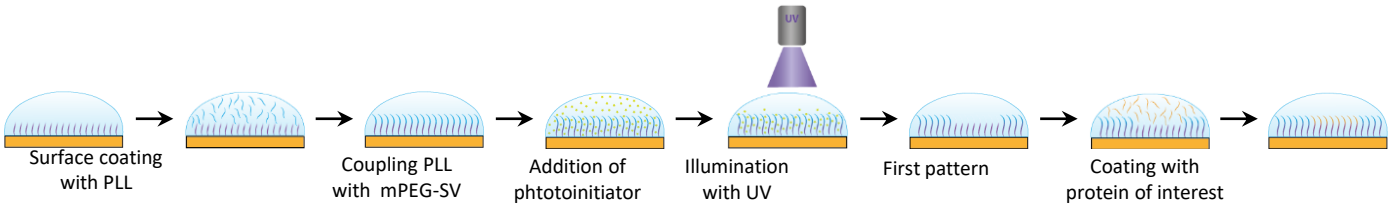
Reagents

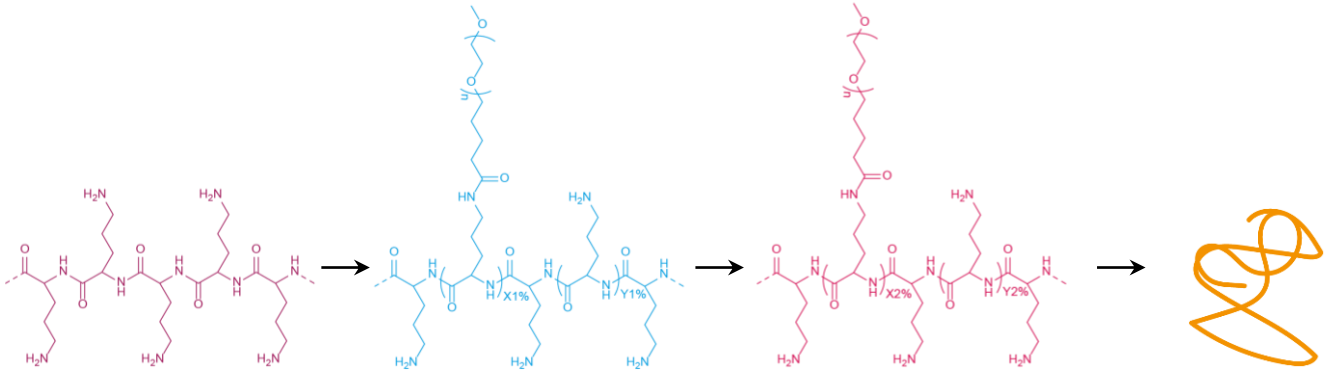
- Protein solution of interest
- 1x PBS, mQ H_2O, MeOH/EtOH
- HEPES pH 8.3 – 8.5 at 0.1 M … ! critical: needs to be check at each exp. !

HEPES 0.1M; pH 8.3 – 8.5 :
preferably freshly prepared
0.59 g … 25 mL mQ H_2O
1.19 g … 50 mL mQ H_2O
2.38 g … 100 mL mQ H_2O
Adjust pH with NaOH

- mPEG-SVA (mPEG-Succinimidyl Valerate, MW 5 000, Laysan bio)
- Poly-L-Lysine (PLL, Merck/ Sigma-Aldrich, ref: P8920)
- oriPLPP … PLPP Gel (Alvéole)
  oursPLPP … 14.5 mg/mL in MeOH
- mPEG-SVA is a PEG (5kDa) coupled with a Succinimidyl Valerate (SVA) ester. This ester is used for PEGylation of primary amine of the polylysine



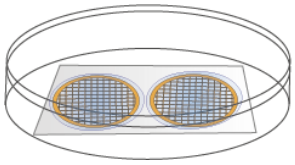




B. Patterning using PLPP Gel photoinitiator and PEG-SVA passivation

before you start – check Leonardo software, if calibration of Primo is alright (viz. Note)

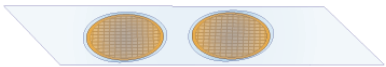
B1. Passivation protocol with PEG-SVA



- Check pH of HEPES (freshly prepared or only a few days in advance)
- Glow discharge TEM grid
- Place grid onto parafilm in wet chamber (carbon side top)
- Add PLL: 100 µg/1 mL of HEPES (10 µL of stock + 90 µL HEPES) , 20 - 30 µl/grid, 30 min
- ! Rinse 3 x 5 min with large volume of HEPES aprox. 200 - 500 µL (pH 8.3 – 8.5)
  – pipet up and down several times during each wash
- Prepare a fresh solution of PEG-SVA 100 µg/mL (70 µg/mL - 100 mg/mL) in HEPES; during the last rinsing
- **Note:** As the half-life of the SVA ester is 10 minutes at pH 8.5, the solution must be prepared just before use.
  . If the passivation is not strong enough, you can increase the concentration up to 100 mg/mL.
- **Recomendation:** Change parafilm (support, I do not do that and works well)
- Add PEG-SVA: 200 - 500 µl/grid, 60 min
- Rinse with 3 x 5 min mQ H_2O; pipet up and down several times during each wash

Stopping point: The passivated grid can be kept dried for few weeks at 4°C. Clean and sealed box on parafilm.

B2. Photopatterning with PRIMO using PLPP photoinitiator



Tip: Drying steps
Glass slide with grids can be placed on heatinng plate on Al-foil: 70°C; aprox. 30 - 45 min

- (***OriPLPP***: Prepare a solution of PLPP gel in mQ H_2O:
  1 µl of PLPP gel /2 µl of mQ H_2O;);
  *Wait for the complete drying of the solution, a transparent gel will form
- ***OursPLPP***: aliquot from Rajaa; add 66 µL of MeOH or EtOH; 3 µl
- Place the grid upside down on the glass slide (gold side top)
- Add a solution of PLPP : 3 µl/grid before patterning

Note: At this point – in the meantime, you can start Primo2 and Leonardo software and proceed calibration on another glass slide (**C1 – C3; detailed protocol page 4**). Briefly:

- Focus on the surface of the glass slide
- Load and lock your pattern on Leonardo software
- Detection functionality or the TEM grid functionality (Check tutorial video at https://www.alveolelab.com/tutorials/micropatterning-tem-grid/)
- The typical UV doses : Primo2 (LED, 365nm): 20mJ/mm^{-2}. It might depend on the density of PLPP Gel, substrate, etc … dose adjustment might be needed.
- Launch patterning sequence – then continue with your grids ....

Tip: Usefull numbers in Leonardo 1
Pixel size of pattern: 200 x 200 px
Laser: 25%
Grid diameter: 3000 µm
View: 36 square (200 mesh)

D. After Patterning ..... **Note:** do not stop, finish until cell seeding

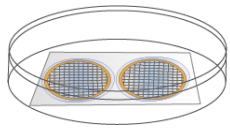
- Rehydrate the slide with PBS for another 5 – 10 minutes (30 min).
- ! Rinse profusely with large volume PBS: 3 x 5min

D1. Coating with protein of interest

- Coat with protein of interest according to its specific procedure/your experience:
  e.g. Incubation time 5 – 60 (120 min), 20 – 100 mg/mL
- ! Rinse profusely with PBS without drying:
  - 3 x 5 min, large volume
  - keep PBS on grids for easier handling, idealy for less than 10 min

D1. Seed cells

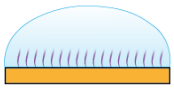
Rinsing is the critical step – do it properly !!!



Grid on to parafilm in wet chamber

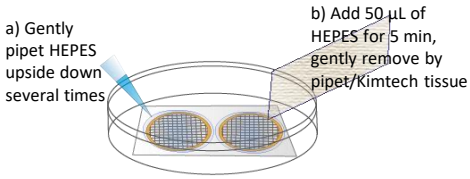
- Glow discharge TEM grid
- Place grid on to parafilm in wet chamber

HEPES pH 8.3 – 8.5

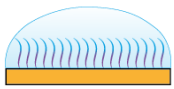


Surface coating with PLL 30 min

- Prepare a solution of PLL in HEPES: 100 µg/mL (10 µL of PLL + 90 µL of HEPES)
- Add PLL: 20 - 30 µl/grid; 30 min
- Rinse 3 x with HEPES (200 - 500 µL)



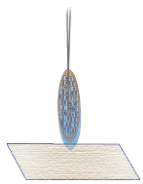
Rinsing options: a) is included in b)
Note: do not let grid dried !!!



Coupling PPL with mPEG 60 min

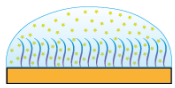
- Prepare a solution of PEG-SVA in HEPES: 100 µg/mL (options: 70 µg/mL – 100 mg/mL)
- Add of PEG-SVA: 200 (500) µl/grid, 60 min
- Rinse 3 x with x mQ H_2O;
- The last drying before PLPP: quickly and very gently dip grid on Kimtech

Flip grids gold side up



The last drying before PLPP: quicly and very gently dip grid on Kimtech

Stopping point: The passivated grid can be kept dried for few weeks at 4°C. Before PLPP:Rehydrate the slide with PBS



Addition of ptotoinitiator

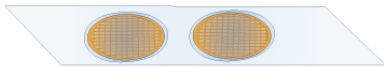
- Place grid upside down on a glass slide

oriPLPP

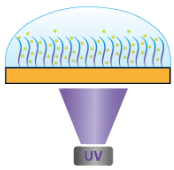
- Prepare solution oriPLPP 1 µl of PLPP gel /2 µl of mQ H_2O;
- Wait for the complete drying of the solution (70°C, 1h, in dark)

oursPLPP:

- aliquot from Rajaa in MeOH/EtOH, directly use 3 µl/grid before pattern, w/o drying step



Grid upside down on a glass slide.
That also protect mesh from breaking during drying.



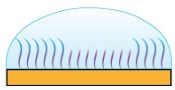
Illumination with UV aprox. 10 min/grid

Calibration:

- use another glass slide
- can be done during PLPP step

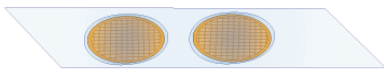
C. Primo2: pages 4 – 5

- Pattern your grids

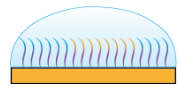


First patern

- Rehydrate the slide with PBS (mQ H_2O) for 5 – 10 min directly on the glass slide (I usually fill glass slide full of PBS and leave it for 30 min)
- After rehydration: you can gently transfer grids somewhere …
- Rinse profusely with PBS: 2 x 5 min



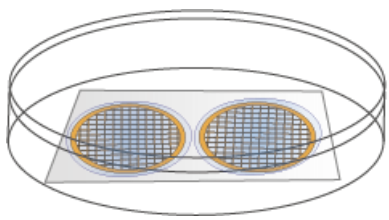
Recommendation: do not store grids after pattering.

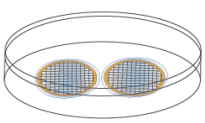


Coating with protein of interest 10 – 120 min

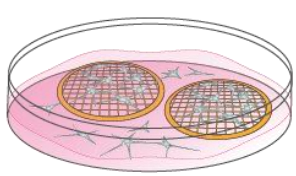
(I do that on Parafilm – on bench; can be done in the cell culture hood)

- Coat with protein of interest according to its specific procedure/your experience
- Rinse profusely with PBS without drying
  Note - washing: 10-cm dish with parafilm: place grids onto parafilm, pour approx.. 10 mL PBS and pipet with pipetboy up and down several times, leave it under PBS for 5 min
  do it twice





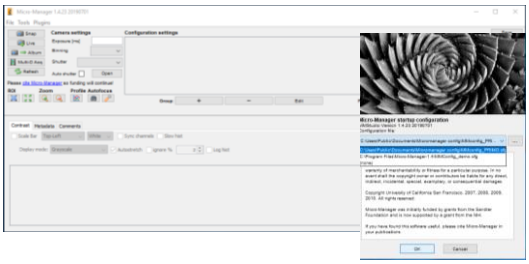
Place into sterile dish
Seeds cells

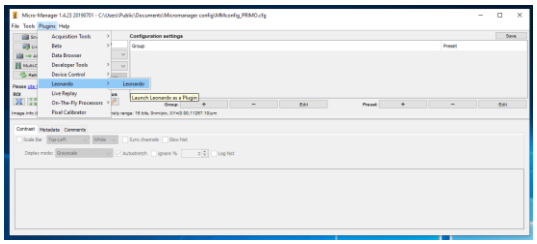


- (sterilization of grids: UV)
- Seed cells

Timing for 12 grids
Leonardo cal. : 30 min
Glow discharge: 30 min
PPL: 30 min
Wash: 20 min
PEG: 60 min
Wash: 20 – 30 min
Primo + PPLP: 10 min/grid
Wash: 45 min
Protein of interest: 30 – 120 min
Wash: 30 min
Seeding: 60 min

C. Primo2



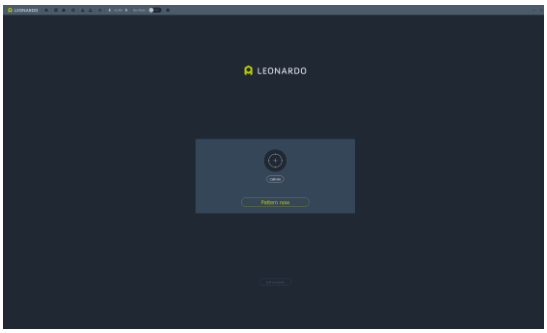


C1.

- Open Micromanager
- Select: PRIMO
- Upper bar → Leonardo → Leonardo

C2. Controlle microscope:

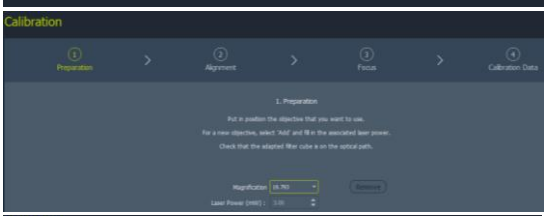
- Magnification for grids: 20x
- EP1: mirror 1
- EPI2: empty/none
- DIA : LED intensity 50
- DIA : shutter OFF
- ZDC: in



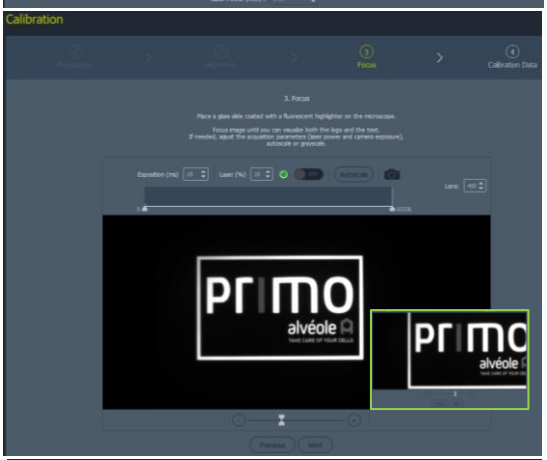
C3. Leonardo software

I. Calibration

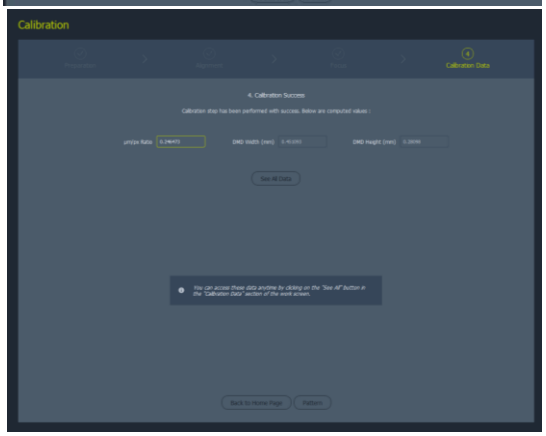
- Preparation
  - Magnification: 19.793
  - Laser power: 3
  - Lens 450

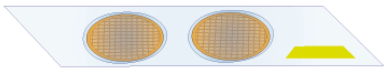


- Aligment
  - Done by Rajaa

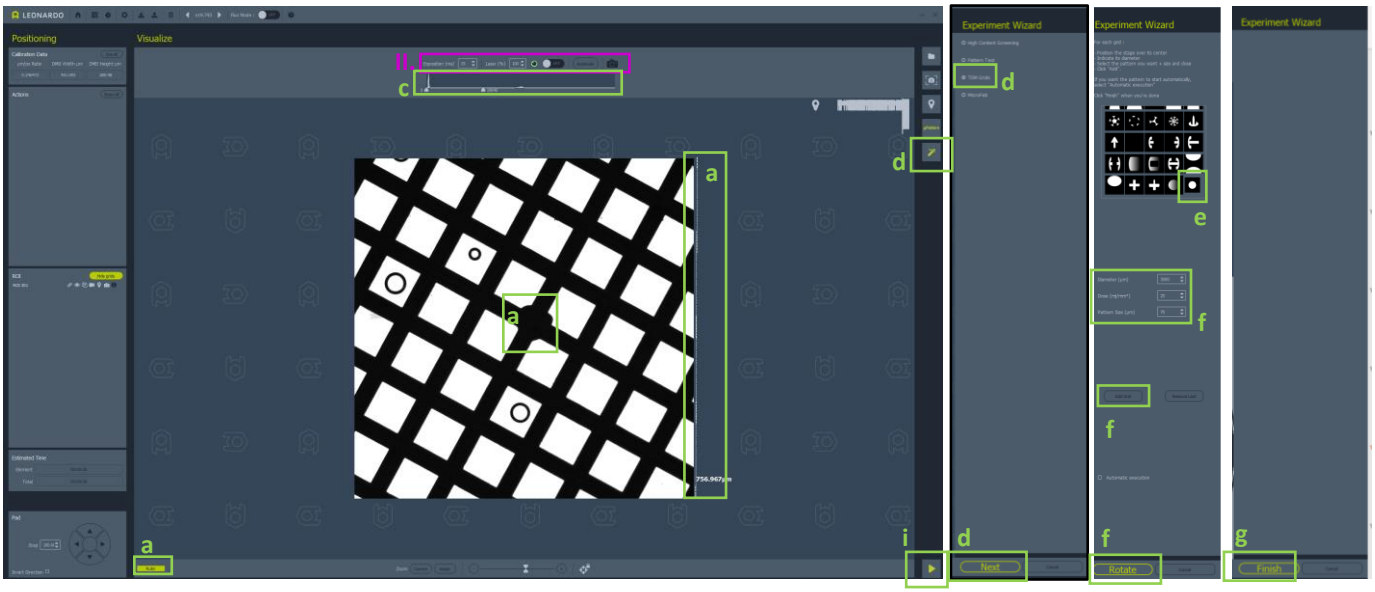


- Focus
  - Start operation
  - Exposure: 25 ms
  - Laser: 25%
  - 'PRIMO‘ logo:
    focus aprox. 5000
    focus on the smallest feature
    (ctrl + mouse wheel)
  - ZDC: in
  - DIA : shutter OFF





Tip: If the calibration was not done during PLPP, it is possible to draw highlighter line next to grids; but ***not recommended;***
the highlighter line can be smudged by PBS



II. Patterning

I. Control panel: DIA: shutter ON

II. Leonardo: upper bar: Laser 100%

a. Find centre of grid – place it in the middle of view (ruler tool)

b. Focus on squares/holes

c. Set bright light at correct intensity (the min/max of the histogram) - to have a “black & white” image: meshes are completely white and their contours black.

d. Wizard → TEM grids → NEXT

e. Select a shape

f. * Diameter of ROI, (dose), shape size
→ add grid →ROTATE→ ☑ keep ratio → LOCK

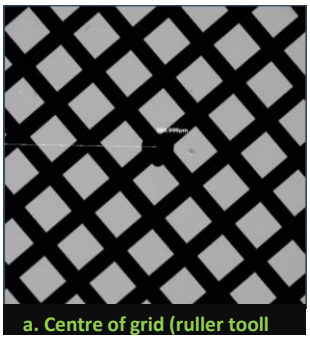
g. FINISH

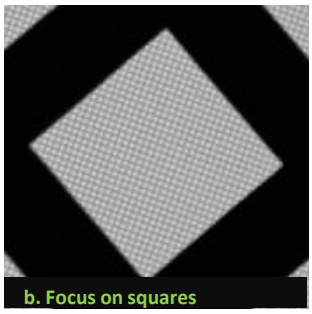
h. Wait for ▶

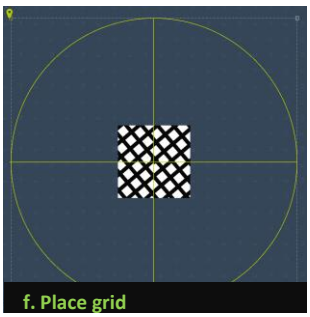
i. Press ▶

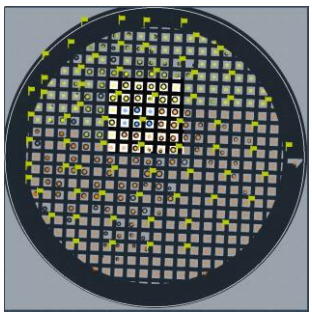
j. Wait until finish

k. Change grid → repeat: a. → j.


a. Centre of grid (ruller tooll


b. Focus on squares


f. Place grid

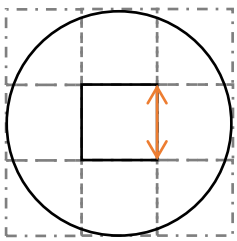


- Orange dots: found after (g) FINISH
- ▶
- Blue dots: in progress
- Yellow dots: done

- *diameter of ROI : variable,
  grid ... 3000 µm
  the smallest area to be pattern = field of view ... square; 750 µm
  → for smaller ROI – it is possible to change
  the ratio (to have rectangle ...)

→ LOCK

dose: during calibration



D. Customized shapes/templates for Leonard

I. Draw shape(s) in suitable software

- e.g. Illustrator, Inkspace, Affinity
  Export in as:
  - Black shape
  - White background
  - TIFF
- Fiji ImageJ
  - Import to Fiji – drag TIFF
  - Edit (upper bar) → Invert
  - Image (upper bar) → Type → 8-bit
  - Image (upper bar) → Scale → Width 200px; Height 200px

II. Transfer to microscope computer

- via your Citrix there: copy/paste
- Note: it doesn't work from Drop-in
  and there is no internet connection

III. Transfer to Leonardo

- Put it into:
  Micromanager 1.4 ´folder´ → Patterns

SaS – calculations

12 grids → 200 µL/each → 2400 µL

PPL:

12 grids: 40 µL + 360 µL

33 µL/grid

PEG:

12 grids: 2.4 mg + 2.40 mL

200 µL/grid

PLPP:

Ours from Rajaa: add 3 µL immediately before each grid

can be add also before start and just continue

Both ways works – there is only one problem – tilting of grids – saturation can help