Part C - Patterning biomolecules on 3D nanostructures using PRIMO to allow stable cell adhesion in given regions of desired shapes

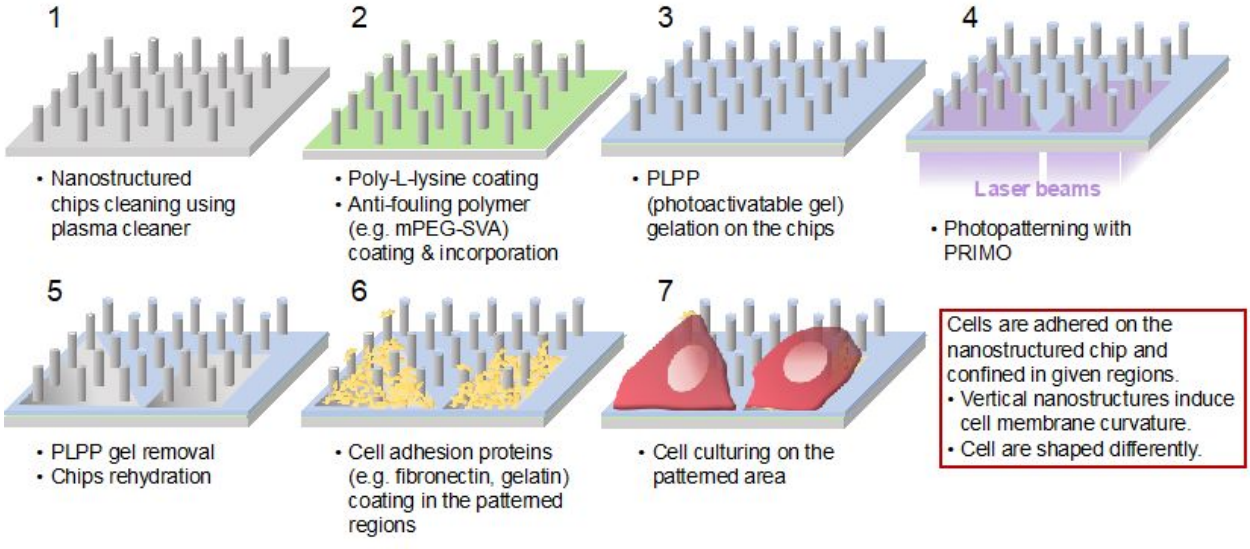

Figure 3. Schematic illustration of the bioprinting process on the nanostructured quartz surface using PRIMO technique.

In this part of experiments, we have employed a new PLPP gel protocol provided by the Alveole company. We especially would like to highlight several advantages of this new protocol which outperforms the previous method:

- The gel formation protocol is compatible with the chip flipping
- It only requires a low-dose exposure for the photo-patterning. (30-100 mJ/mm^2, ~100X lower compared to the previous protocol)(We've optimized the laser dose for the patterning on the chips which ranges from 60-100 mJ/mm^2).
- It takes less time to pattern the chips.
- Samples are more stable, can be stored (in fridge) for a long period of time.

1. PRIMO Patterning for the Biomolecule Coating & Cell Adhesion

1-1. Chip passivation & coating

- Immerse chips in piranha and incubate for 15-30 min.
- Clean the nanostructured chip for 20 min using plasma cleaner.
- Add 50 µL 1% poly-L-lysine (PLL) solution (in ddH_2O or 1X PBS) on the hydrophobic side of parafilm. Flip the cleaned chip on the PLL droplet to face the nanostructured side toward the PLL solution and incubate for 30 min. Wash the chip with ddH_2O to remove free PLL. Gently air flow or use Kimwipe to remove liquid on the chip.
- Add 50 µL 100 mg/mL mPEG-SVA solution on the hydrophobic side of parafilm. Put the PLL-coated side of the chip on the mPEG-SVA droplet and incubate for 1 hr. Wash the chip profusely with ddH_2O to remove free mPEG-SVA. Gently air flow or use Kimwipe to remove liquid on the chip.
  - ★ 100 mg/mL mPEG-SVA solution (in HEPES buffer, pH=8.0-8.5; an anti-fouling agent) has to be freshly prepared.
- Place the mPEG-coated chip on the glass bottom petri dish (Nanostructured side should face up).
- Add 3 µL photoactivatable PLPP gel at the center of the chip.
- Add 20 µL 99% ethanol onto PLPP solution to trigger the gelation process. Manually rotate the petri dish to homogenize the gel solution.
- Dry the PLPP-decorated chip in the air.

1-2. PRIMO patterning

- Open the "Leonardo" software.
- Place the glass bottom petri dish (with the chip) on the microscope.
- Calibrate the microscope for the PRIMO: Use one corner of the chip to find the focus (switch off the bright-field light, turn on the UV laser). Select an optimal magnification and adjust the focus by tuning the exposure time and laser power.

- Launch the desired pattern. Place the patterns on the desired sites of the chip. (The spacing between two adjacent patterns should not be shorter than ~40 μm. Otherwise a single cell would adhere on multiple patterns rather than one single pattern.)
- Pattern the PLPP-coated chip under low-dose exposure (60-100 mJ/mm^2)
- Wash the chip profusely with 1X PBS or ddH_2O for at least 10 min to remove PLPP gel from the surface of the chip.

2. Biomolecule Coating & Cell Culturing on the PRIMO-patterned nanostructured chip

2-1. Biomolecule Coating on the PRIMO-patterned nanostructured chip

- Place the chips on a new glass bottom dish or into the wells of 12- or 24-well plate.
- Incubate the chip with gelatin (0.05%, FITC-labeled) solution for 2.5 hrs at 4℃.
- Wash the chip with 1X PBS solution for three times to remove free proteins.
- Use epi-fluorescence or confocal microscope to detect the fluorescence signal of the surface-bound proteins.

2-2. Cell culture on the PRIMO-patterned nanostructured chip

- Place the chips on a new glass bottom dish or into the wells of 12- or 24-well plate.
- Incubate the chip with 40 μg/mL matrix protein (gelatin: fibronectin = 1:1) for 2.5 hrs at 4℃.
- Wash the chip with 1X PBS solution for three times to remove free proteins.
- Trypsinize the cells and then dilute the cell solution into 1X DMEM growth medium (with 1X antibiotics) to the density of ~$1x10^4$-$5x10^4$ cells/cm^2.
- Put the well plate into the incubator and culture cells at 37℃ for 24 hrs.
- (Fix cells with 4% paraformaldehyde (PFA) for 15 min at room temperature; Wash the cell-adhered chip with 1X PBS solution for three times to remove free PFA.)
- (Permeabilize cells with 0.1% triton-X for 5 min; Wash the cell-covered chip with 1X PBS for three times to remove free triton-X.)
- (Immunostain cells with primary and secondary antibodies of interest for 1-2.5 hrs at 4℃. Stain cells with any counterstain (e.g. DAPI nuclear staining) for 15 min at room temperature. Wash the cell-covered chip with 1X PBS for three times to remove free molecules.)
- Use epi-fluorescence or confocal microscope to detect the fluorescence signal from the antibodies and counterstains. Use bright-field channel to check the morphology and the distribution of cells.