# Bioprinting on 3D nanostructures with Alveole PRIMO

## Final Report
## (Standard Operating Procedures)

ENGR 241 Project
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## Project Objective

Precise manipulation of nanostructures is crucial for understanding how surface nano-topography affects the biological processes of cells. Previous studies have proposed a detailed nanofabrication procedure using electron-beam lithography (EBL). However, the EBL process is time-consuming and costly, and the resultant nanostructures also lack the geometric diversity in the vertical direction. Therefore, this project aims to develop a shape-selective fabrication method to achieve a large-scale synthesis of vertical nanostructures of various shapes and investigate their bio-related applications. The proposed fabrication process incorporates two-stage etching methods with photolithography, to make nanostructures with the resolution down to ~200 nm in diameter without the aid of the EBL process. The other main focus of this project is to develop an efficient, robust bioprinting process to pattern biomolecules on nanostructured chip for cell-based studies using Alveole PRIMO. We aim to combine nanofabrication with biological research to explore more scientific frontiers. This protocol provides an easy-to-use process for the fabrication of large-scale nanostructures with structural diversity as well as their bio-related application, which will benefit the SNF future users.

## SOP Objective

In this protocol, we will describe step-by-step procedures for fabricating various vertical nanostructures for bio-related experiments. The method described here consists of two parts. In the first part, we'll introduce a novel, efficient fabrication process that incorporates a two-stage etching method with the photolithography process. In the second part, we'll demonstrate its capability for bio-related applications with the aid of PRIMO bioprinting technique.

# SOP Descriptions

## Part A - Making Vertical Nanostructures with Various Shapes

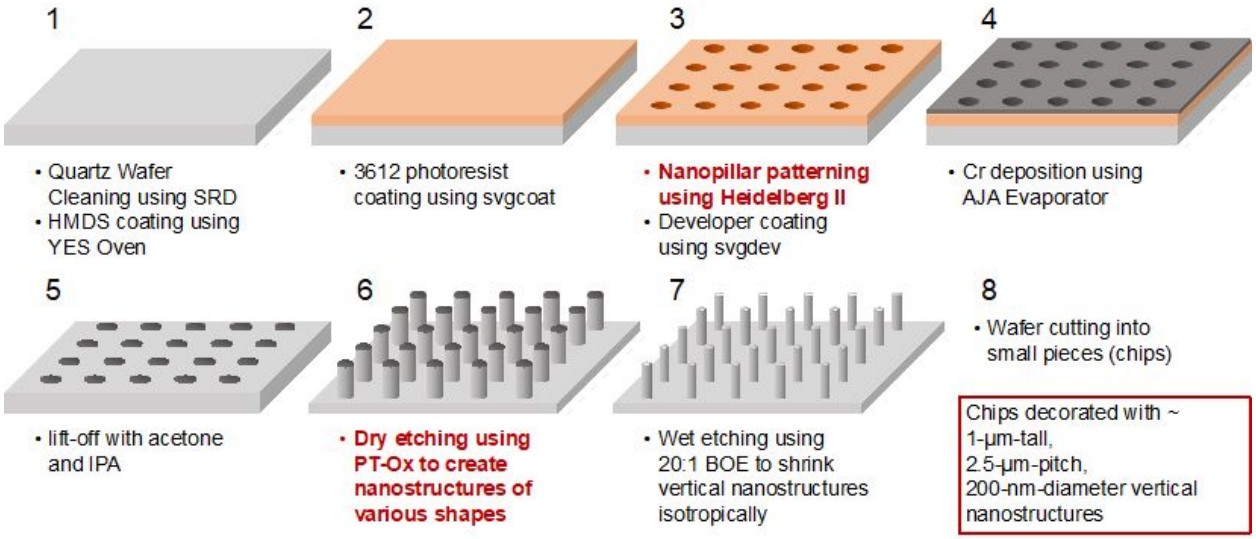



**Figure 1.** Schematic illustration of the fabrication procedure for making vertical nanostructures with different shapes on quartz surface.

## 1. Wafer Cleaning and Nanostructure Patterning

### 1-1. Rinse wafers using SRD

- Apply the recipe 1 to wash (default recipe).

### 1-2. Coat clean wafers with HMDS (HexaMethylDiSilazane) using YES oven

- Apply the recipe 1 to bake (default recipe).

### 1-3. Coat HMDS-covered wafers with photoresist using svgcoat (automatic track)

- Select the program which applies “3612 1μm w/o vp 2mm EBR” photoresist to wafers.

### 1-4. Exposure photoresist-coated wafers with desired patterns using Heidelberg2

- Substrate Template: Wafer 4 inch (Round shape)
- Layer: Only monolayer exposure (FirstExposure only)
- Parameters:
  - Designed pattern: 700-nm-diameter nanopillar array
  - Design Size: 81.8 x 81.4 mm
  - Design Type: Binary
  - Laser wavelength: 375 nm
  - Laser dose: 132 mJ/㎠

    - Defoc: -2
- Select “Expose with substrate angle” and “Auto-Unload the Substrate”.

1-5. Bake and Develop patterned wafers using svgdev

- First-run - Developer program #9 (null) + Oven program #1 (Baking only)
- Second-run - Developer program #3 + Oven program #1 (Developing + Baking)

1-6. Check the patterns on the wafers under microscope

## 2. Nanostructure Fabrication

2-1. Deposit Cr on patterned wafers using AJA evaporator

- Parameters:
    - Rate: 1Å/s
    - Thickness: 120 nm (1.2kÅ)

2-2. Lift off immediately at wetflexsolv (solvent bench)

- Rinse wafers with acetone for 2 min (avoid acetone evaporation!), then sonicate wafers in acetone for 5 min.
- Rinse wafers with isopropyl alcohol (IPA) for 2 min, then sonicate wafers in IPA for 5 min.
- Dry wafers with nitrogen gas.

2-3. Dry etching using PT-Ox

- Before start:
    - Check the badger and enable the tool. If the tool is disabled, “facilities service has failed” alarm will pop up on the plasma-therm control screen.
    - Check the transfer arm has been set to the correct wafer size configuration.
    - On the plasma-therm screen, make sure there are no active alarms on the system. Process state is in “Stand-by” mode.
- Use the dummy wafer to clean the chamber:
    - Choose the “Ws_O2 Clean” recipe to run the cleaning process. Click “Go to recipe temperature”. check AL and PM1 are both in the production mode.Vent the loadlock.
    - Load the dummy wafer. Align the flat bottom with the line marked on the arm, then close the load lock.
    - Make sure the recipe sequence and recipe steps are correct and have the desired settings.
    - Select “Vent after Job”, then click “Start Job”.
    - Click “Jobs”/”Adjust” to monitor the process. Monitor ICP forward, ICP reflected, temperature and pressure.
- Etch sample wafers:

- After the cleaning is done, put the dummy wafer on top of the sensor on the right-hand side of the load arm, and load the sample wafer. Align the flat bottom with the line marked on the arm, then close the load lock.
- Make sure the recipe sequence and recipe steps are correct and have the desired settings.
- Select “Vent after Job”, then click “Start Job”.
- Since quartz wafers are transparent, the sensor is unable to detect the wafer. It’s necessary to ‘trick’ PT-Ox in order to sense the wafer and proceed the etching process. To do so, First click “maintain” button. Choose “AL” and change its status into “Maintenance” mode. Click “Edit material” and delete wafer detected in the loadlock. Go back to the AL chamber status and switch its mode back to the “Production” mode.
- After the process is done, take out the sample wafer and the dummy wafer.
- Pump down the loadlock and disable the tool.

## 2-4. Wet etching at wetflexcorr (corrosive solution bench)

- Incubate dry-etched wafers in Cr etchant for 20 min to remove the Cr mask.
- Incubate the wafers in 20:1 BOE for 5 min to shrink vertical nanostructures isotropically.
- Wash the wafers with dd$H_2O$. Gently air flow oto remove liquid on the wafers.

## 2-5. Cut patterned wafers into small pieces for the biological applications

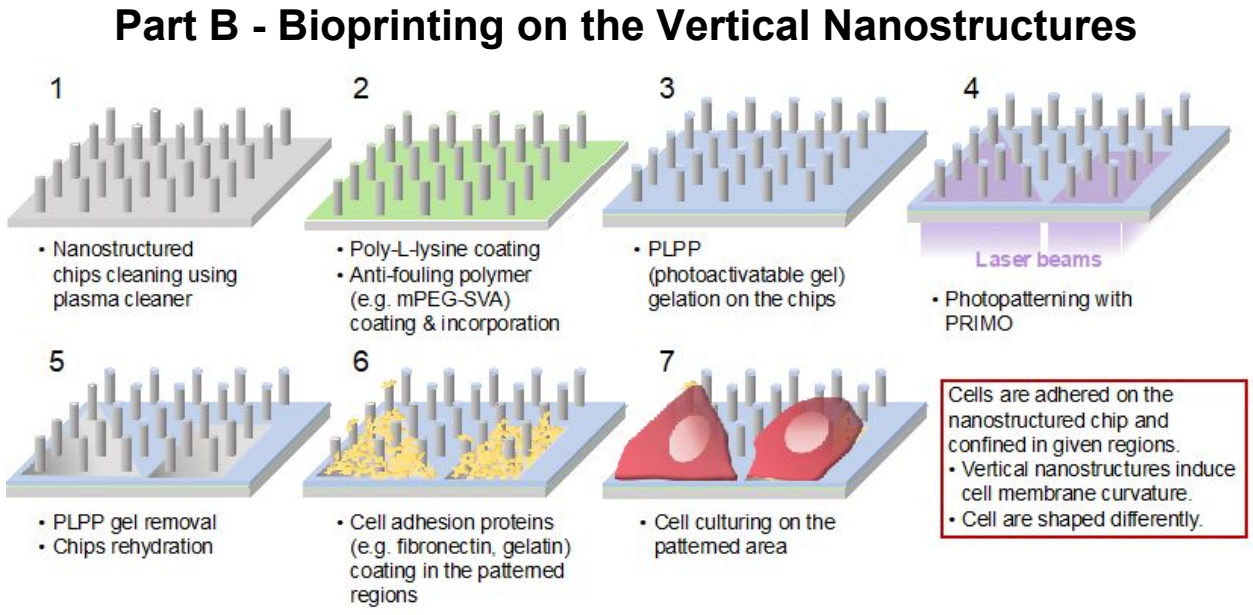



**Figure 2.** Schematic illustration of the bioprinting process on the nanostructured quartz surface using PRIMO technique.

In this part of experiments, we attempt to try 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 $mJ/mm^2$, 100X lower compared to the previous protocol)
- It takes less time to pattern the chips.
- Samples are more stable, can be stored for a longer time.

## 1. PRIMO Patterning for the Biomolecule Coating & Cell Adhesion

### 1-1. Try new PLPP gel protocol

- Clean the nanostructured chip for 20 min using plasma cleaner,
- Add 50 µL 0.1% 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 brigth-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.
- Pattern the PLPP-coated chip under low-dose exposure (30 $mJ/mm^2$)
- Wash the chip profusely with $ddH_2O$ 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) and fibronectin (0.05%) protein solution for 5 min.
- 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 from 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 gelatin (0.05%, unlabeled or fluorescently-labeled) and fibronectin (0.05%) protein solution for 5 min.
- 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 ~$1x10^4$ cells/$cm^2$.
- Put the well plate into the incubator and culture cells at 37 °C for 1 hr - 24 hrs.
- (Fix cells with 4% paraformaldehyde (PFA) for 15 min at room temperature; Wash the cell-covered 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 hr at room temperature. 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.

# Results and Discussion

## A. Photolithography Patterning and Cr Deposition

Cleaned, photoresist-coated 4 inch quartz wafers were decorated with numerous amounts of nanopillar patterns using Heidelberg 2 photolithography. These patterns became clearer after developing process and AJA evaporator-assisted Cr deposition. The feature diameter and the pitch of the nanopillar array are 700 nm and 2.5 μm, respectively (Figure 3).

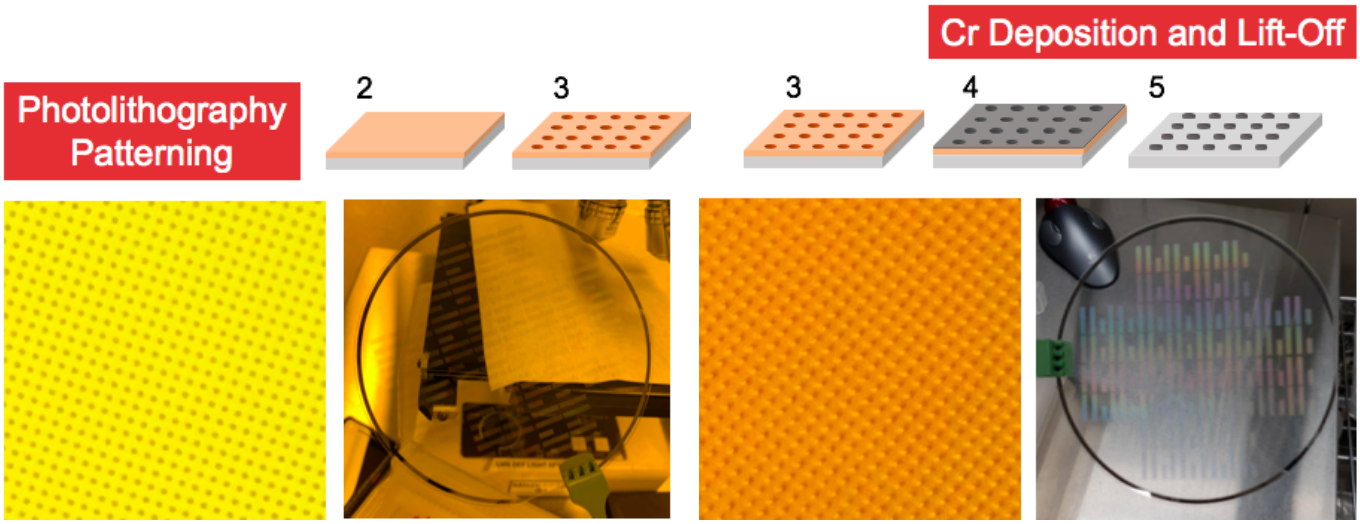
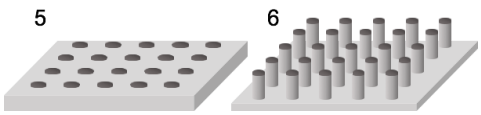


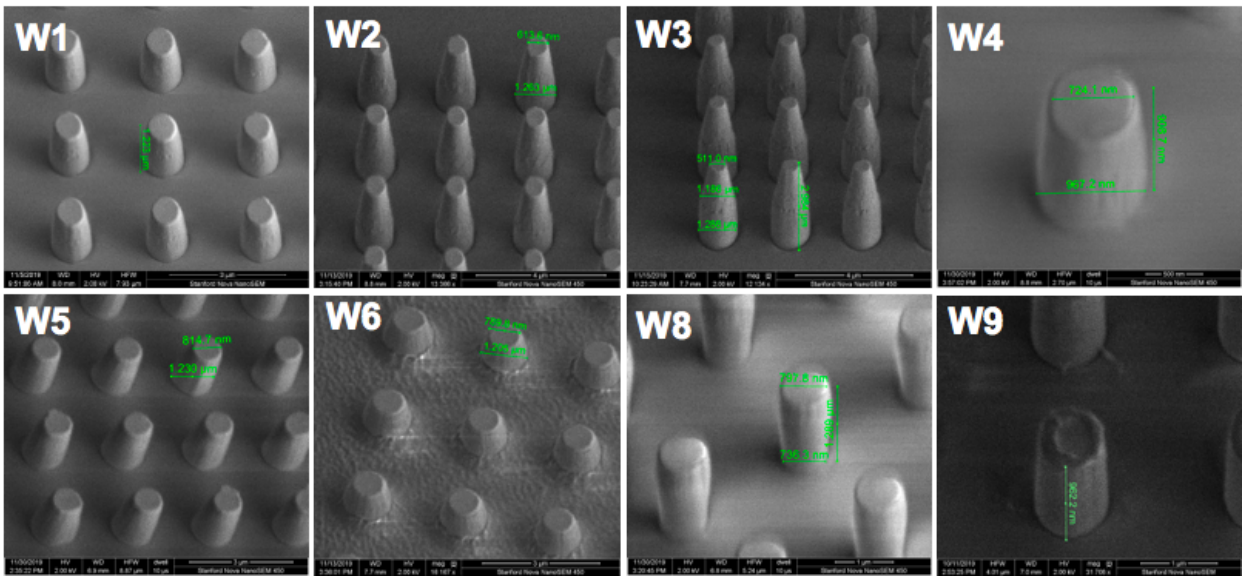

**Figure 3.** 700-nm-diameter, 2.5-μm-pitch nanopillar patterns on the quartz wafers. (Left: After photoresist developing; Before Cr deposition. Right: After Cr deposition and Lift-off)

## B. Dry Etching Process to Shape Nanostructures Differently

### B-1. Images and Data of Dry Etch Experiments

After depositing Cr mask on the patterned wafers, we took advantage of PT-Ox dry etching tool to convert 700-nm-diameter-featured nanopillar pattern into vertical nanostructures of various shapes. We finely tuned the etch gas compositions, bias power and reaction time of dry etch process to find out the conditions for making nanostructures of specific shapes, as shown in Figure 4. The shape diversity ranges from straight pillar (W9), frustum (W1, W2, W5, W6) to bullet (W3) and inverted frustum (W8).






| No. | Pattern | $CHF_3$ Flow Rate (sccm) | $C_4F_8$ Flow Rate (sccm) | $H_2$ Flow Rate (sccm) | Ar Flow Rate (sccm) | Bias power (W) | Etching Time (min) | Height (nm) | Diameter (nm) | Etching Rate (nm/min) | Slant Angle (deg) | Optimized Parameters |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| W1 | +++−+ | 80 | 20 | 40 | 0 | 200 | 3 | ~1223 | ~705 (top)<br>~1031 (bottom) | ~408 | ~82.4 | *$CHF_3$ flow rates:* 80 sccm<br>*ICP Power:* 1500 W<br>*Pressure:* 7 mT |
| W2 | +++−+ | 80 | 20 | 40 | 0 | 200 | 3+3 | ~2150 | ~613 (top)<br>~1263 (bottom) | ~309 | ~81.4 | |
| W3 | +++−+ | 80 | 20 | 40 | 0 | 200 | 3+3+3 | ~2984 | ~511 (top)<br>~1186 (2/3-height)<br>~1288 (bottom) | ~278 | ~66.6 | |
| W4 | +−+−+ | 80 | 0 | 40 | 0 | 200 | 3 | ~909 | ~724 (top)<br>~967 (bottom) | ~303 | ~82.4 | |
| W5 | +−+−− | 80 | 0 | 40 | 0 | 100 | 6 | ~1200 | ~815 (top)<br>~1230 (bottom) | ~200 | ~80.2 | |
| W6 | +−++− | 80 | 0 | 40 | 10 | 50 | 10 | ~566 | ~790 (top)<br>~1205 (bottom) | ~56.6 | ~69.9 | |
| W7 | +−−−+ | 80 | 0 | 10 | 0 | 200 | 3 | ~1009 | ~722 (top)<br>~1103 (bottom) | ~336 | ~79.3 | |
| W8 | +−−−− | 80 | 0 | 10 | 0 | 100 | 6 | ~1293 | ~775 | ~216 | ~90.0 | |
| W9 | −++++ | 0 | 80 | 40 | 20 | 200 | 3.3 | ~1000 | ~780 | ~300 | ~90.0 | *No $CHF_3$* |

**Figure 4.** (Upper) SEM images and (Lower) Measurable quantities of nanostructured quartz wafers from dry etch experiments.

## B-2. The Effects of 4 Parameters on the Shapes of Nanostructures

In this part, we investigated the importance and the effects of 4 parameters on the dry etch experiments for **quartz wafer**. Briefly speaking, we discovered that (Figure 5):

- $C_4F_8$-based chemistry gives straighter side walls (straight pillars) and faster etching rates.

- $CHF_3$-based chemistry gives tapered side walls (smaller slant angles; Frustum or bullet-shaped).
- High $H_2$ level leads to slower etching rates and the formation of more tapered side walls due to the polymer protection on the side walls.
- High bias power brings about faster etching rates and the creation of straighter nanopillars (similar consequences as $C_4F_8$ chemistry does).

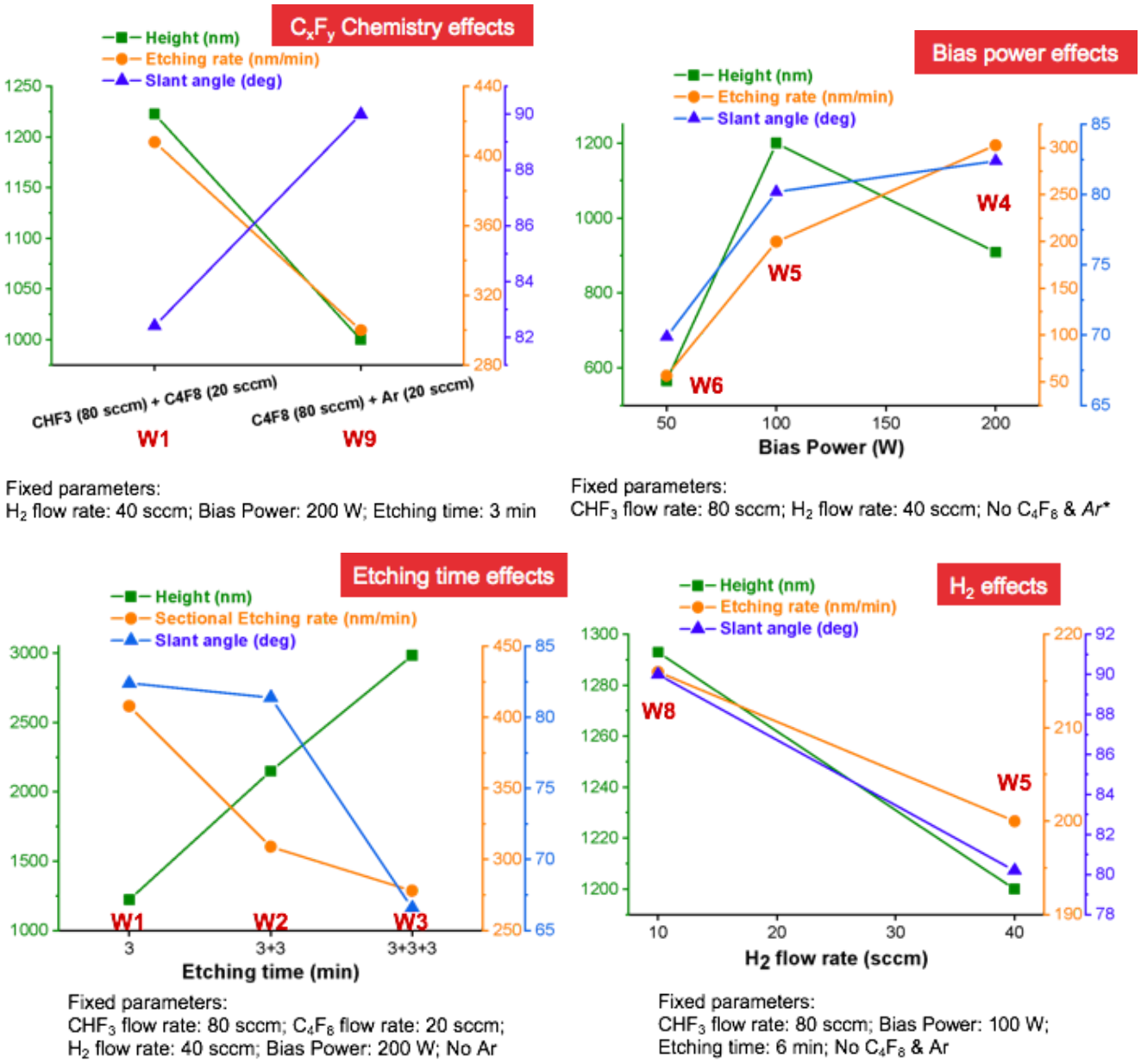



**Figure 5.** The effects of 4 parameters on the dry etching rate, height and slant angle of vertical nanostructures.

## C. Wet Etching Process to Isotropically Shrink Nanostructures

In order to obtain the nanostructures with the diameters down to ~200 nm, we employed wet etching process to make the vertical nanostructures much taller and thinner. Here, we found that 5 min incubation in 20:1 BOE shrunk nanostructures by ~320 nm vertically and horizontally (Figure 6). After two-stage etching processes, the wafers are cut into small chips for the future biological research, such as PRIMO biopatterning experiment and subsequent cell-based studies.

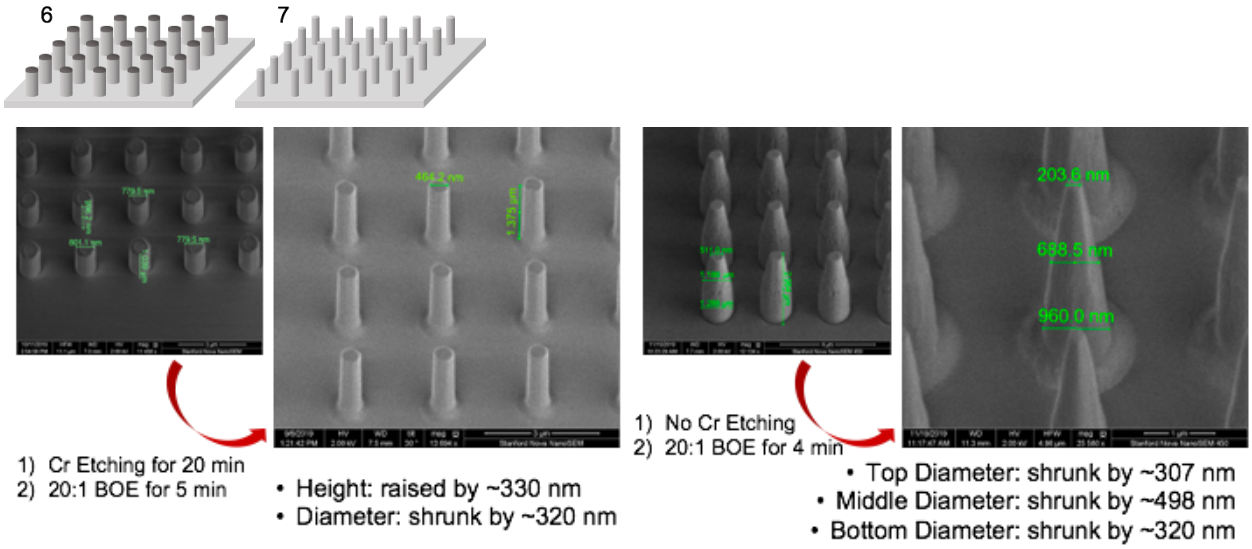



**Figure 6.** Vertical nanostructures were shrunk isotropically via wet etching process.

## D. Bioprinting on Nanostructured Chip

To initiate the PRIMO-assisted bioprinting process, we coated nanopillar chips with poly-L-lysine, mPEG-SVA and photoactivatable PLPP gel sequentially. With the aid of Leonardo software from Alveole PRIMO company, we used low-dose laser exposure (30 mJ/mm$^2$) to decorate the nanopillar chips with various patterns, including squares, rectangles and Stanford Main Quad. These patterned regions are now the docking sites for the biomolecules, as visualized by the fluorescence signals from FITC-labeled gelatin using confocal microscope. Furthermore, cells were also stably adhered on the gelatin-coated, patterned nanopillar chip (Figure 7).

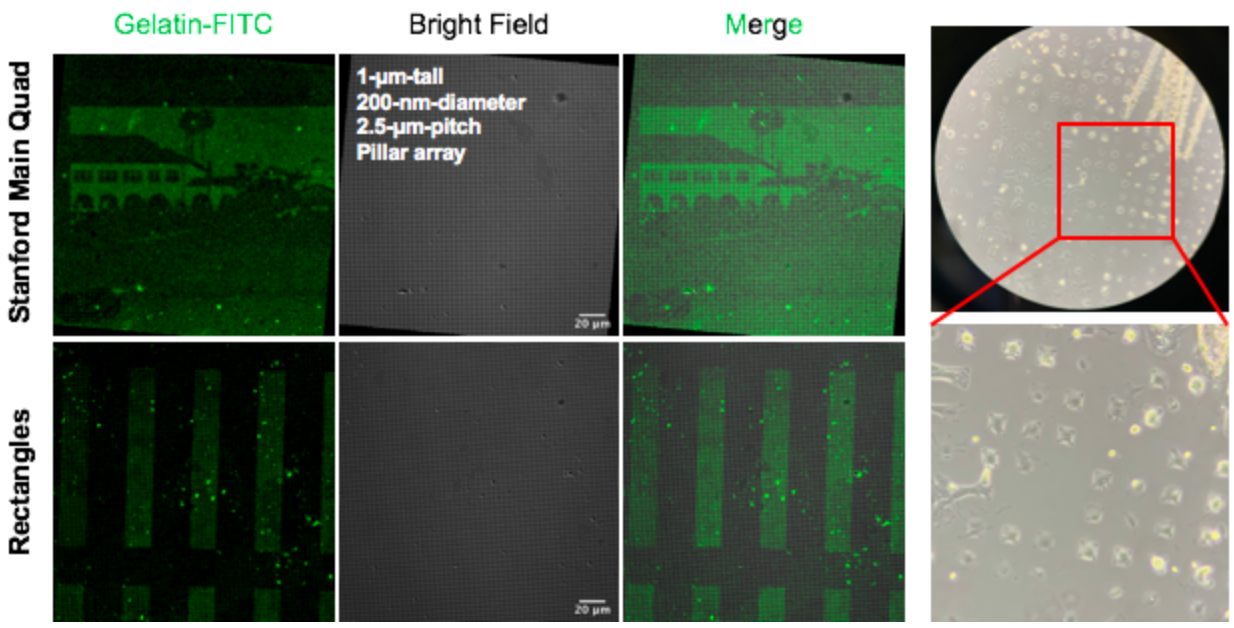
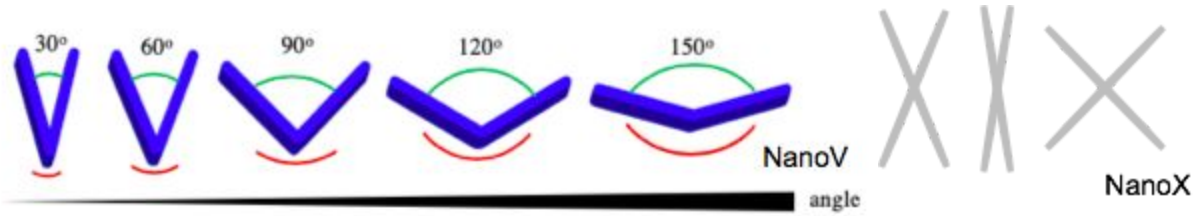



**Figure 7.** (Left) Fluorescently-labeled gelatin coating on the PRIMO-patterned nanopillar chips (rectangle and Stanford main Quad patterns). (Right) Mammalian cell culturing on the gelatin-coated PRIMO-patterned nanopillar chips (square pattern).

## Future Plans

1. Apart from nanopillar-derived patterns, we are also going to fabricate chips decorated with nanobar (various sizes), nanoV, nanoX (various angles) to study how cells respond to various physical perturbations and investigate cell membrane curvature-dependent biological pathways.




2. Design more PRIMO patterns and Optimize PRIMO experimental conditions, such as PLL-mPEG coating density, PLPP gelation protocol, and UV dose.
    - For instance, bioprinting various patterns to induce different cell shapes with the same cell area to investigate how cell shape influences mechanobiological properties of cells

# References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2. Devmalya C, Charbonier FW, Protein photopatterning on PDMS in 3D with the Alveole PRIMO. ***Stanford ENGR241 course*** (2018).
3. PLPP photo-activable reagent for micro-patterning. https://www.alveolelab.com/our-products/plpp-photoactivatable-reagent/


# Acknowledgements


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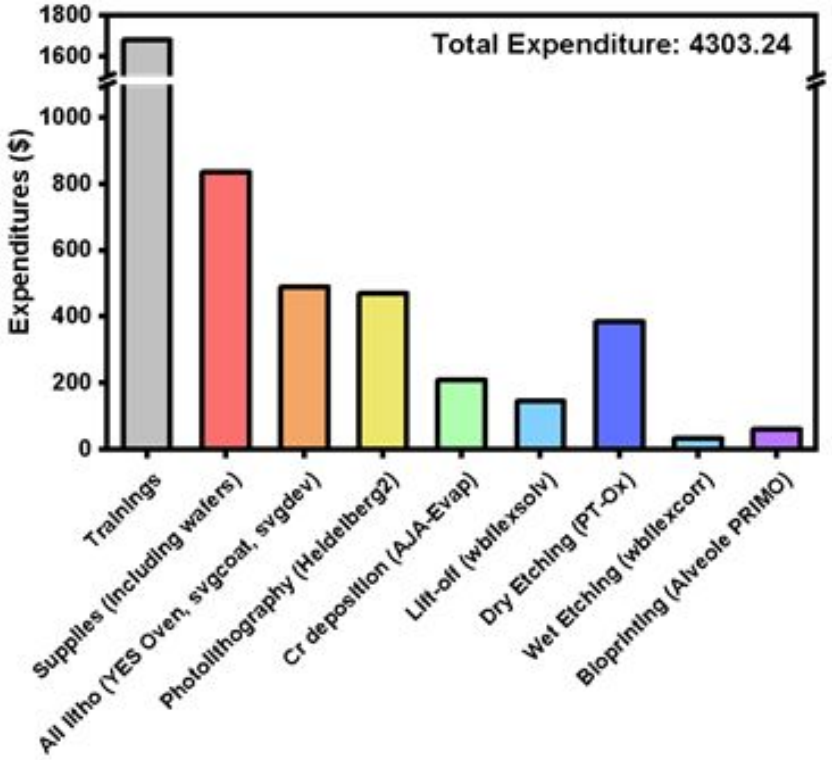
# Quarterly Budget



# Author Contributions

| Task | Contributors |
|---|---|
| Experimental Design | Zeinab, Ching-Ting, Chih-Hao |
| Wafer Cleaning & HMDS Coating/ Photoresist Coating/ Photolithography/ Developer Coating/ Cr Deposition & Lift-off | Ching-Ting, Chih-Hao |
| Etching Experiments | Ching-Ting, Chih-Hao |
| SEM Characterization | Zeinab, Ching-Ting |
| PRIMO Experiments | Ching-Ting, Chih-Hao |
| Fluorescence Imaging/ Cellular Imaging | Ching-Ting, Chih-Hao |
| Oral Presentation | Ching-Ting, Chih-Hao |
| Poster Presentation | Ching-Ting, Chih-Hao |
| Project Report Edition | Ching-Ting, Chih-Hao |
| Winter-Quarter Proposal Edition | Ching-Ting, Chih-Hao |