Bioprinting on 3D nanostructures with Alveole PRIMO

Final Report

ENGR 241 Project
Win 2020

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Project Objective, Motivations & Contributions to SNF community

Precise manipulation of nanostructures is crucial for understanding how nano-topography affects the cellular processes. 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.

1. **Hollow pillars fabrication for potential electrophysiological Measurements**

   Nanoelectrodes have been proposed as a way to record action potential with a high signal-to-noise ratio. One early idea was that these nanoelectrodes could potentially lead to membrane ruptures. Based on this observation, we have designed and fabricated electrically-conductive hollow pillar chips for potential electrophysiological measurements. With the aid of the hollow electrodes, we anticipate the signals can be detected with much higher sensitivity.
2. **Create large amounts of vertical nanostructures of various shapes using photolithography & two-stage etching processes**

   The proposed fabrication process incorporates two-stage etching methods with the photolithography to make nanostructures precisely with the resolution down to ~200 nm in diameter without the aid of the Electron-Beam Lithography process. In this quarter, we especially focused on the fabrication of the vertical nanostructures which can physically induce different types of cell membrane curvatures.
3. **Pattern biomolecules on 3D nanostructures using PRIMO to allow stable cell adhesion in given regions of desired shapes.**

Micro-fabrication is a complicated process which makes the prototyping of a chip for bio-related applications both time- and money-consuming. In this two-quarter-long project, we've used PRIMO, a maskless on-demand UV projection system with facile sample preparation, to create patterns on nanostructured chips for biological applications. Moreover, since the surface of nanostructures are not flat, the biomolecules adsorbed on the surface might not be evenly distributed. PRIMO bioprinting technique enables us to create a platform for the uniform and stable biomolecule coating and cell adhesion.

- **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.**

Results and Discussion

A. Fabricating hollow pillar chip for high throughput electrophysiological measurements

Strategy & Process Workflow

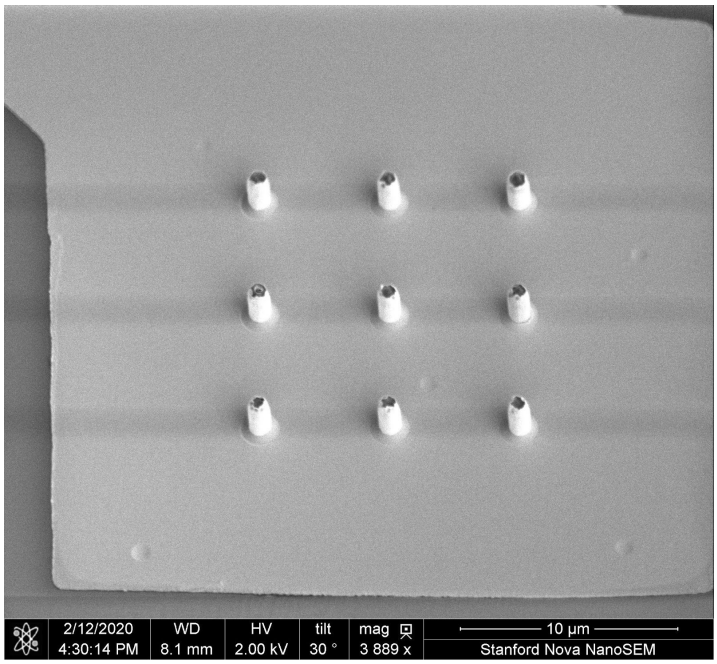


Figure 1. Demonstration of the hollow electrodes fabrication

In hope to make hollow electrodes, we need to get access to the interior of the nanoelectrode (SiO_2). The biggest challenge is remove the Pt on top since it is very inert. Therefore, we want to use resist as a mask to protect the bottom of the nanoelectrodes. Since it's difficult to find an etchant to remove the platinum but keep the resist intact, we incorporate the dry etching strategy. The Ar RIE etching allows us to remove the metal coating by collision. After the dry etching process, the BOE etching allows us to engineer nanoelectrodes with any depth.

B. Making large amounts of nanostructures of various shapes using photolithography & two-stage etching processes

- **Pattern Design**

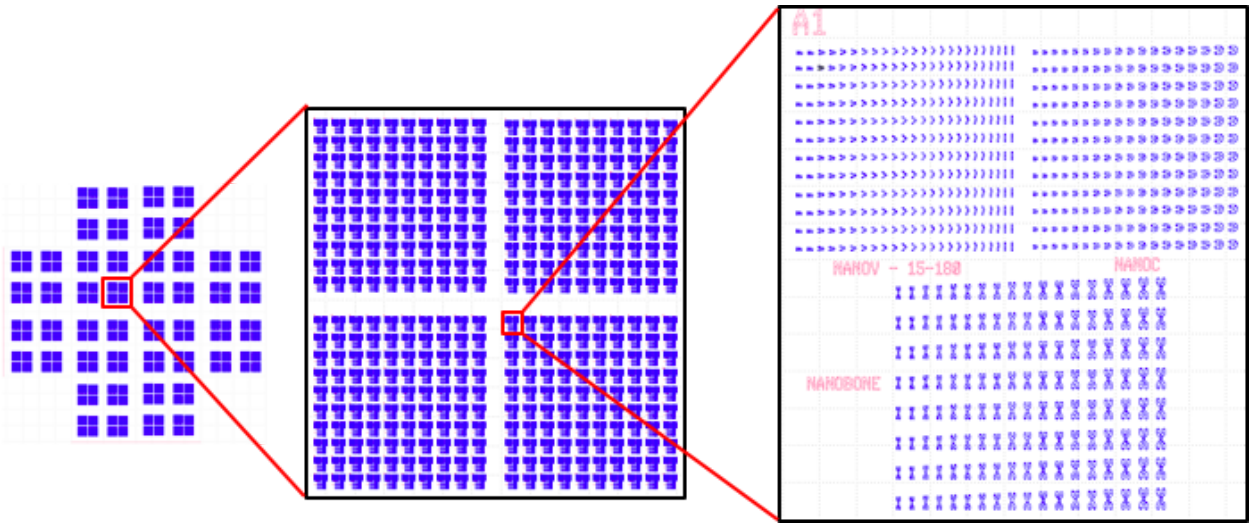


Figure 2. Pattern design on a 4 inch quartz wafer.

There are 48 chips available from a 4 inch quartz wafer. 400 patterned regions are present on each single chip (Figure 2). A large numbers of patterns allow for numerous data acquisitions in an individual experiment. If zooming in on each single pattern, there are multiple gradient arrays of various shapes, including V, C, X and Bones. For the NanoV array, the included angle ranges from 15^{o} to 180^{o}, as shown in Figure 3. The width of all NanoV patterns is 700 nm, which corresponds to the resolution limit of Heidelberg photolithography. For the NanoC and NanoBone arrays, the widths and concaves are both varying from 700 to 1500 nm. The varying widths, angles and concaves of these nanostructures enable us to create different sizes and types (positive, negative or no curvatures) of cell membrane curvatures.

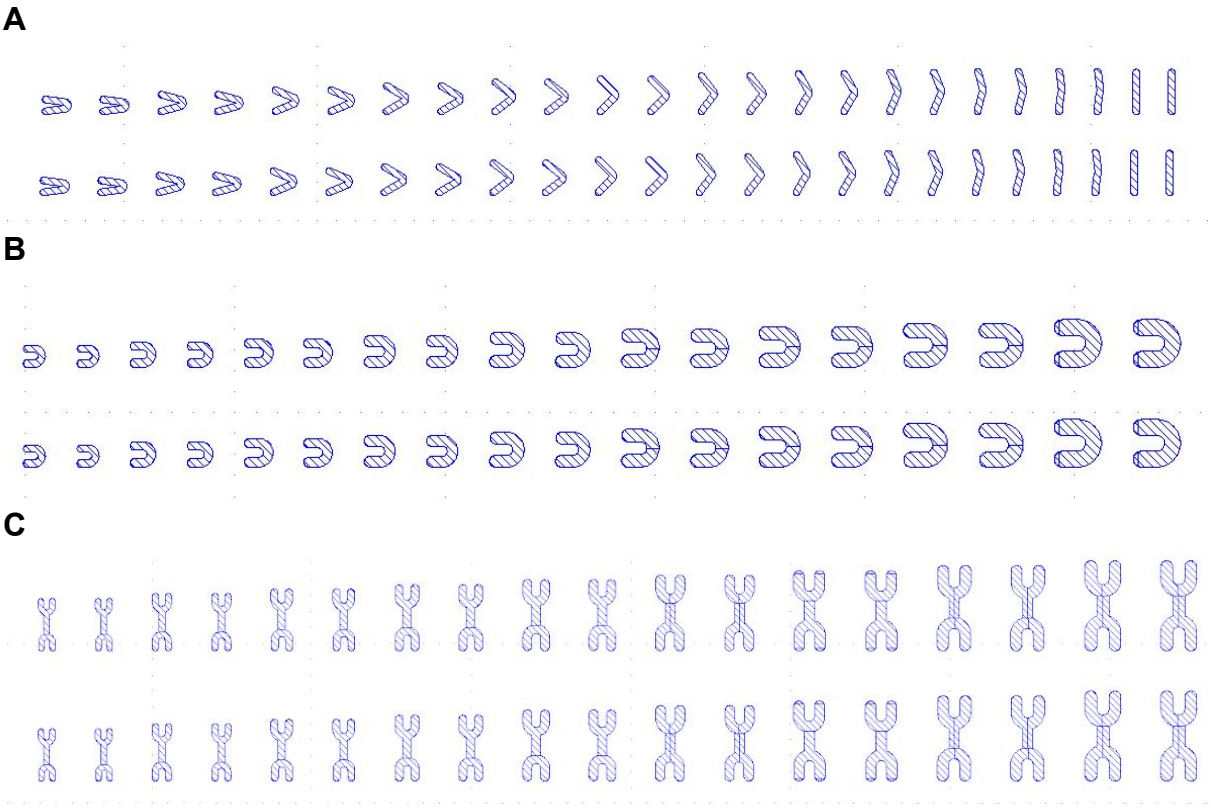


Figure 3. Various gradient array patterns. **(A)** NanoV; **(B)** NanoC; **(C)** NanoBone.

- **Photolithography Patterning**
  Cleaned, photoresist-coated 4 inch quartz wafers were decorated with numerous amounts of NanoC/V/X/Bone array patterns using Heidelberg 2 photolithography. These patterns became clearer after developing process and AJA evaporator-assisted Cr deposition. The feature width of the pattern array ranges from 700 to 1500 nm (Figure 4).

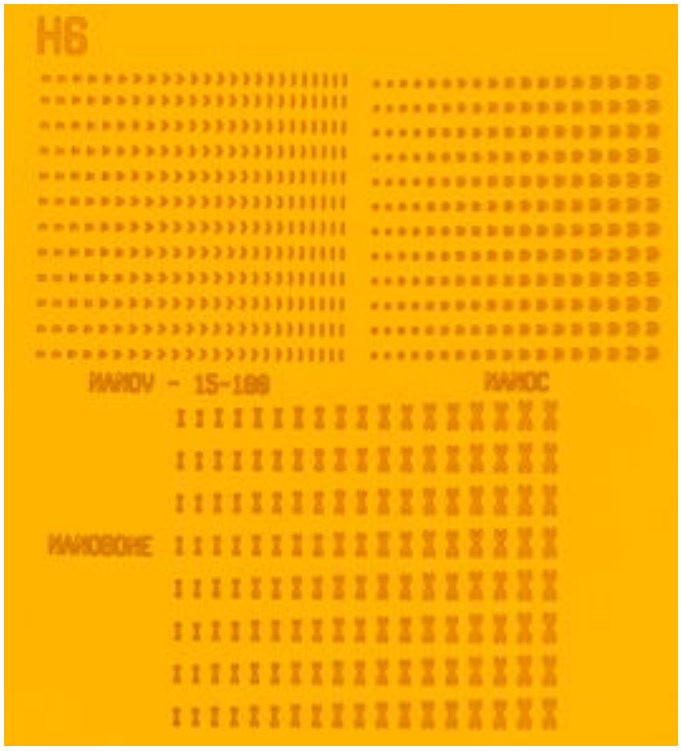
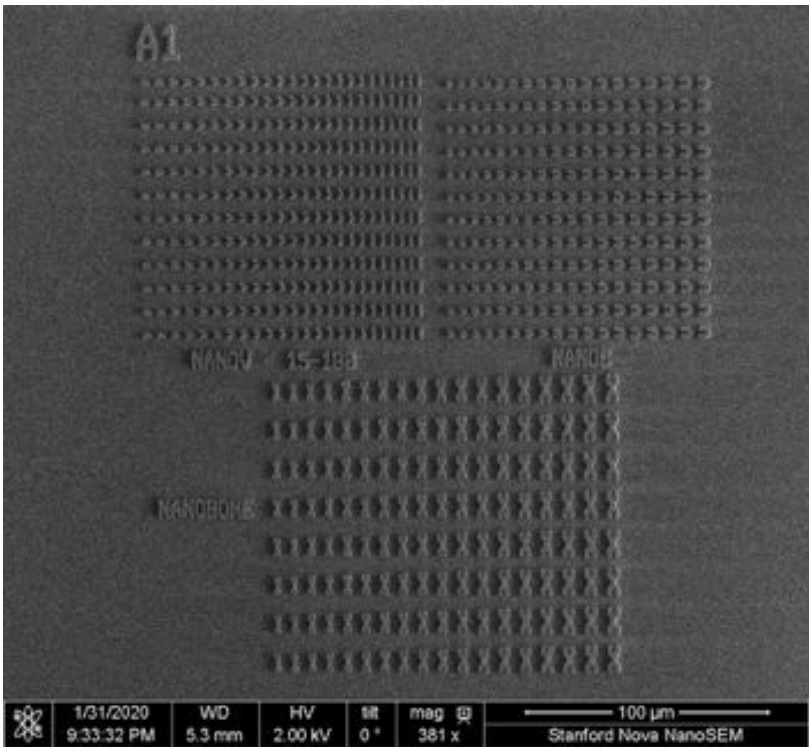


Figure 4. Gradient pattern array on the quartz wafers. (After photoresist developing; Before Cr deposition)

- **Dry Etching Process to Shape Nanostructures Anisotropically**
  After depositing Cr mask on the patterned wafers, we took advantage of PT-Ox dry etching tool to convert 700-nm-diameter-featured patterns into vertical nanostructures of various shapes. We took advantage of previously optimized condition to make either ~1.9 or ~3.2-μm-tall structures (Table 1, Figure 5).

Condition	C_4F_8 Flow Rate (sccm)	H_2 Flow Rate (sccm)	Ar Flow Rate (sccm)	Bias power (W)	Etching Time (nm)	Height (nm)	Etching Rate (nm/min)	Optimized Parameters
A	80	40	20	200	3	~1900	~633	*ICP Power:* 1500 W
B	80	40	20	200	6	~3200	~533	*Pressure:* 7 mT

Table 1. Dry etch condition used for the fabrication of NanoC/V/X/Bone arrays.



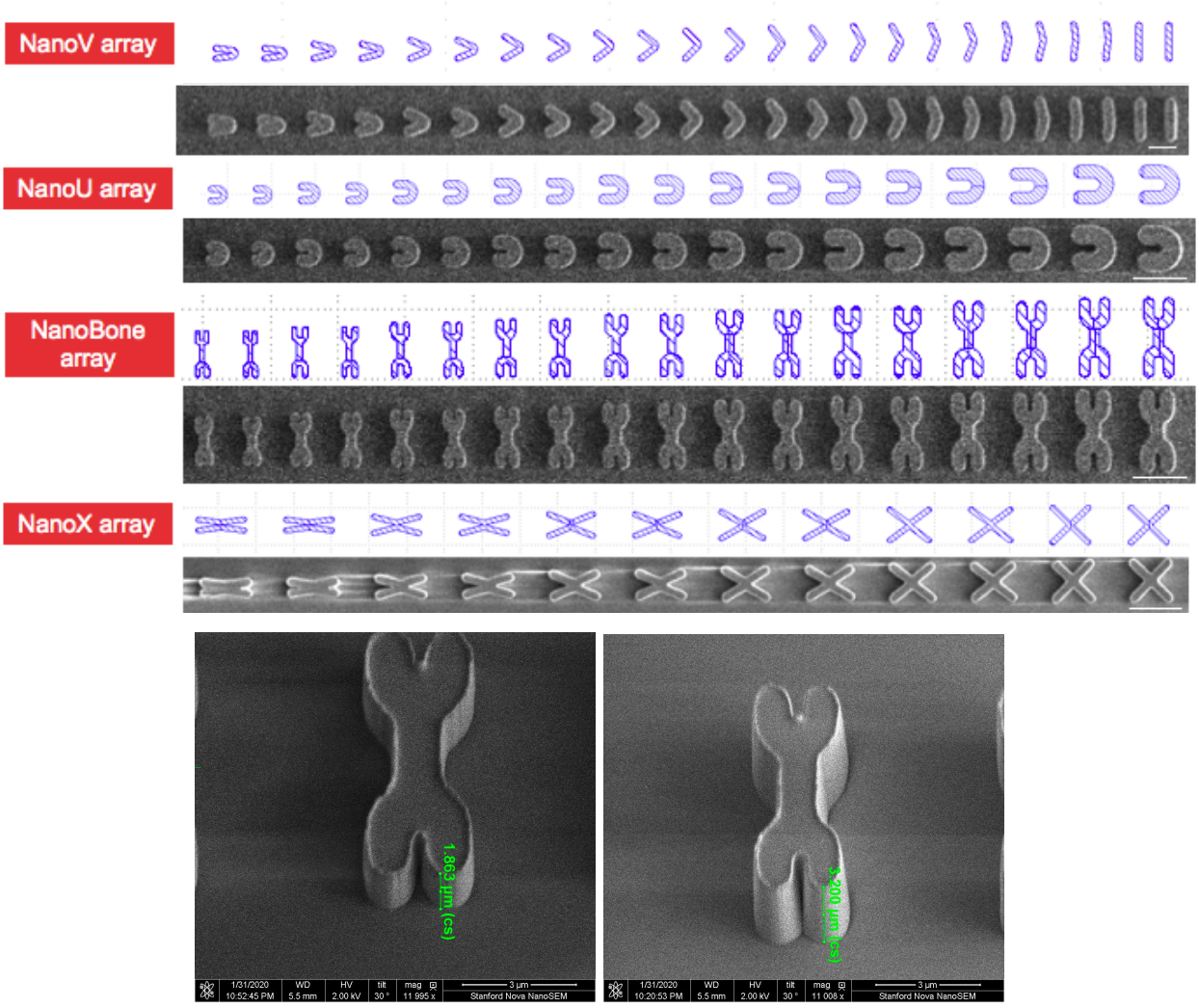


Figure 5. SEM images of various nanostructure arrays on 4 inch quartz wafer. Bottom-left image: A NanoBone structure which was etched under the condition A. Bottom-right image: A NanoBone structure which was etched under the condition B.

- **Wet Etching Process to Isotropically Shrink Nanostructures**
  In order to obtain the nanostructures with the diameters down to ~200 nm, we exploited wet etching process to make the vertical nanostructures much taller and thinner. Here, we found that 10 min incubation in 20:1 BOE shrunk nanostructures by ~300 nm vertically and horizontally (Figure 6). After two-stage etching processes, the wafers will be cut into small chips for the future biological research, such as PRIMO biopatterning experiment and subsequent cell-based studies.

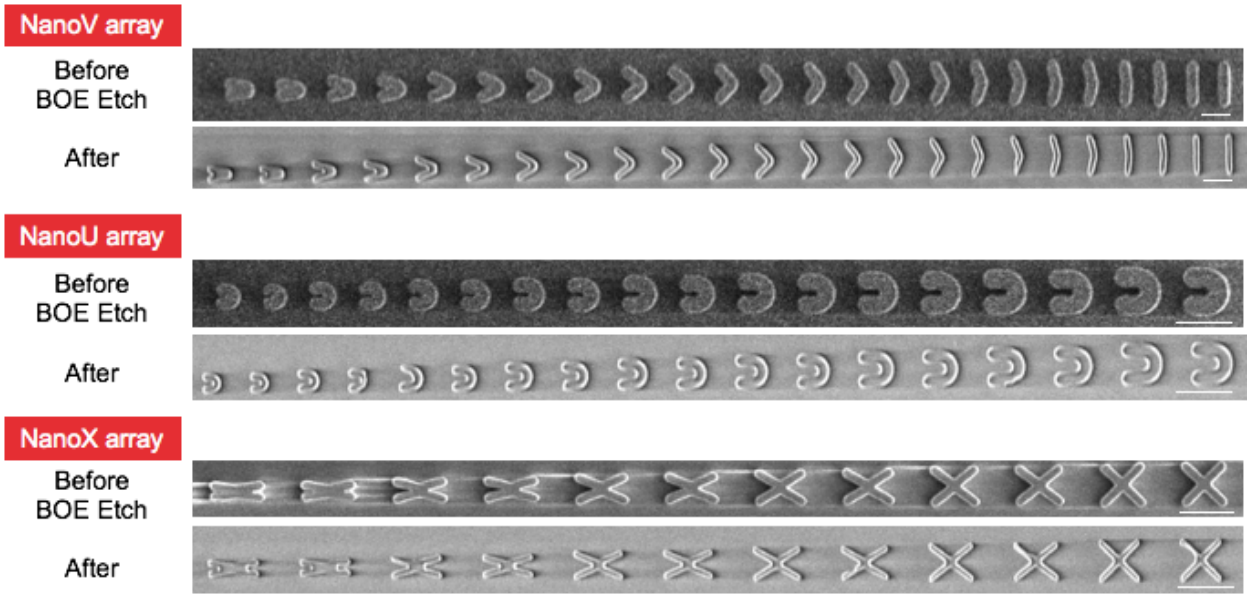


Figure 6. Vertical nanostructures were shrunk isotropically via wet etching process.

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

- **PRIMO Condition Optimization**

  To initiate the PRIMO-assisted bioprinting process, we coated nanopillar chips with poly-L-lysine, mPEG-SVA and photoactivatable PLPP gel sequentially. Afterwards, the PLPP-coated chip is subject to laser exposure to create desired patterns. These patterned regions are now the docking sites for the biomolecules, as visualized by the fluorescence signals from FITC-labeled gelatin using the confocal microscope. With the aid of Leonardo software from Alveole PRIMO company, we have optimized low-dose laser exposure for our experiments. We found that 60-100 mJ/mm^2 laser doses provide the best signal-to-noise ratio under the confocal microscope (Figure 7). This indicates that a lower dose of the laser might not successfully degrade the PLPP gel and completely remove the antifouling agents on the desired regions, making the matrix protein coating less feasible and specific. However, using a much higher dose may damage the microscope, laser and the sample.

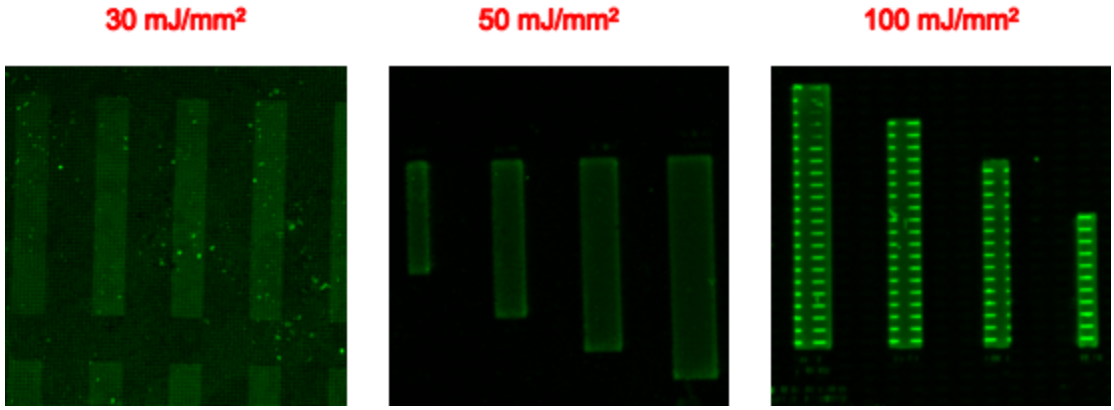


Figure 7. PRIMO patterning at different laser doses.

We also discovered that two poly-L-lysine concentrations (0.1% and 1% w/v), which are much less than the recommended concentration, also work very well for the PRIMO experiments.

For the matrix protein coating on bio-patterned chips, we found that long incubation time (such as 1 day) leads to severe non-specific binding of proteins even though the non-patterned regions have been passivated by mPEG (Figure 8). Incubation for 2.5 hrs at 4℃ gives a better surface protein coverage and significantly reduces non-specific binding.

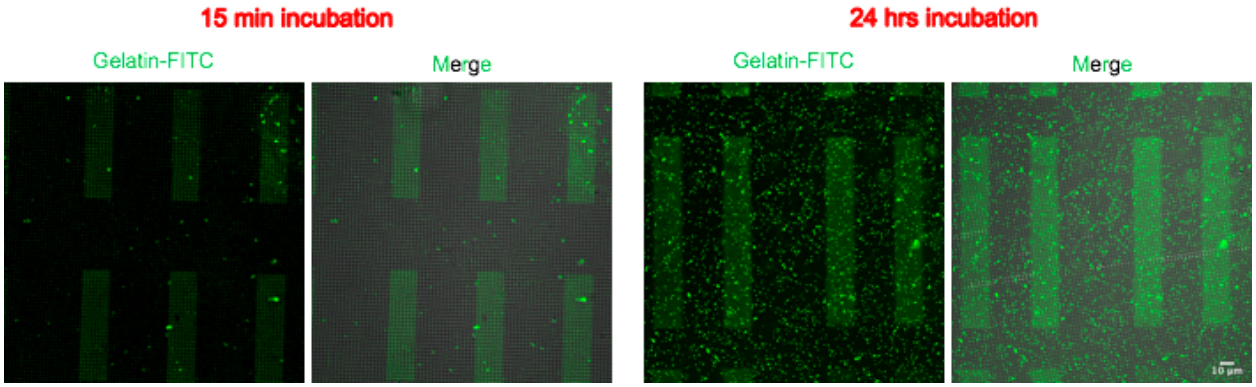


Figure 8. Long protein incubation time compromises the binding specificity on the patterned regions.

With the aid of the 3D reconstruction using the confocal microscope, we examined whether or not the matrix protein was not only distributed on the flat region but on the nanostructures. As expected, fluorescently-labeled matrix proteins are also coated on either nanopillar or nanobar, as shown in Figure 9. Based on this observation, we firmly believe that cells also adhere stably on the vertical nanostructures.

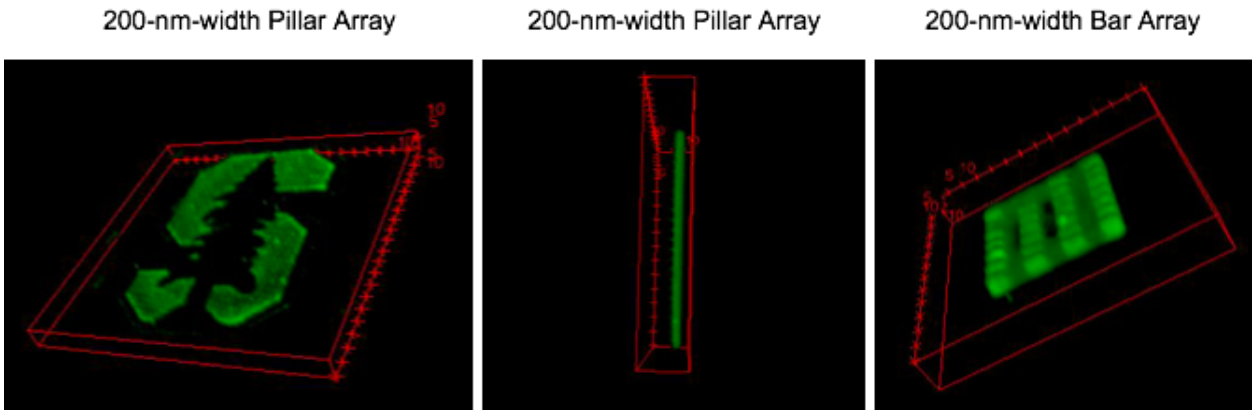


Figure 9. 3D reconstruction of confocal images of FITC-labeled gelatin-coated, PRIMO-patterned chips.

- **Cell Culturing on the Bioprinted chip and Imaging**

  After optimizing the PRIMO experimental condition, we decorated the nanostructured chips with various bioprinted patterns, including squares, rectangles, circles, T, gradient rectangles and Stanford Badge. we subsequently cultured U2OS cells on those patterned chips. Cells are stably attached on the matrix protein-coated, patterned nanostructured chip (Figure 10). Nonetheless, it's required to achieve the "single-cell in single-pattern" for the meaningful electrophysiological measurement. Consequently, we've optimized the condition to reduce multi-cell adhesion into single-cell adhesion (Figure 11), including:

  ➔ Print more patterns on a nanopillar chip.

  ➔ Increase cell adhesion proteins concentration: 40 µg/mL (Originally 1.5 µg/mL).

  ➔ Increase cell adhesion proteins incubation time to ensure high matrix coverage: for 2.5 hours at 4°C (Originally 5 min at room temperature).

  ➔ Reduce the numbers of cells to prevent multiple cell attachment onto one pattern: ~$1x10^4$ - $5x10^4$ cells per well (Originally ~$5x10^4$-$1x10^6$ per well).

  ➔ Increased cell incubation time to allow cell to thoroughly spread in the patterns: 2.5 days (Originally 2 days).

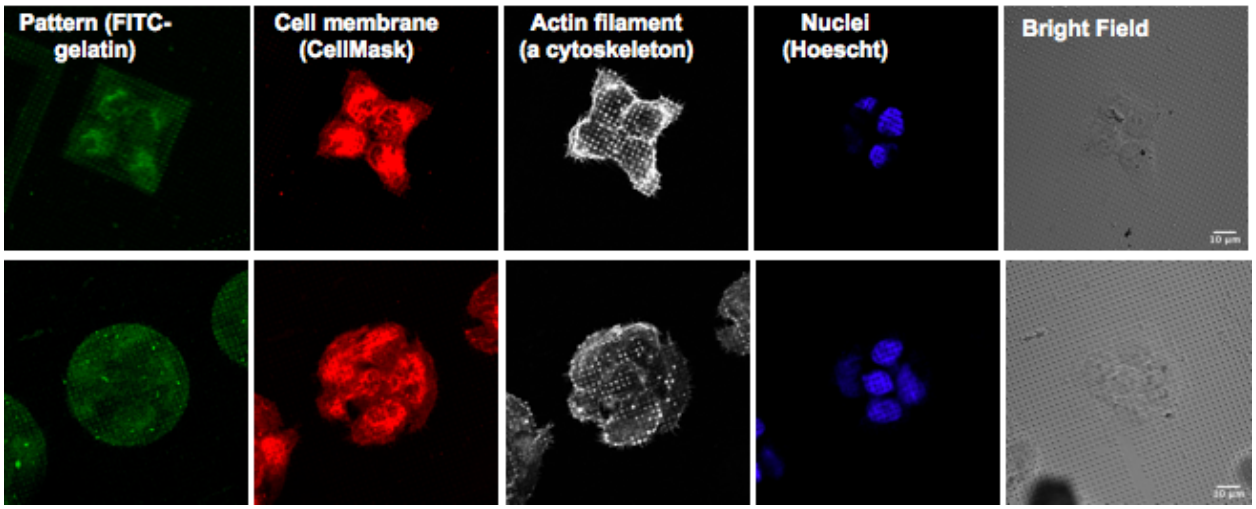


Figure 10. Multiple cells adhesion on a single pattern.

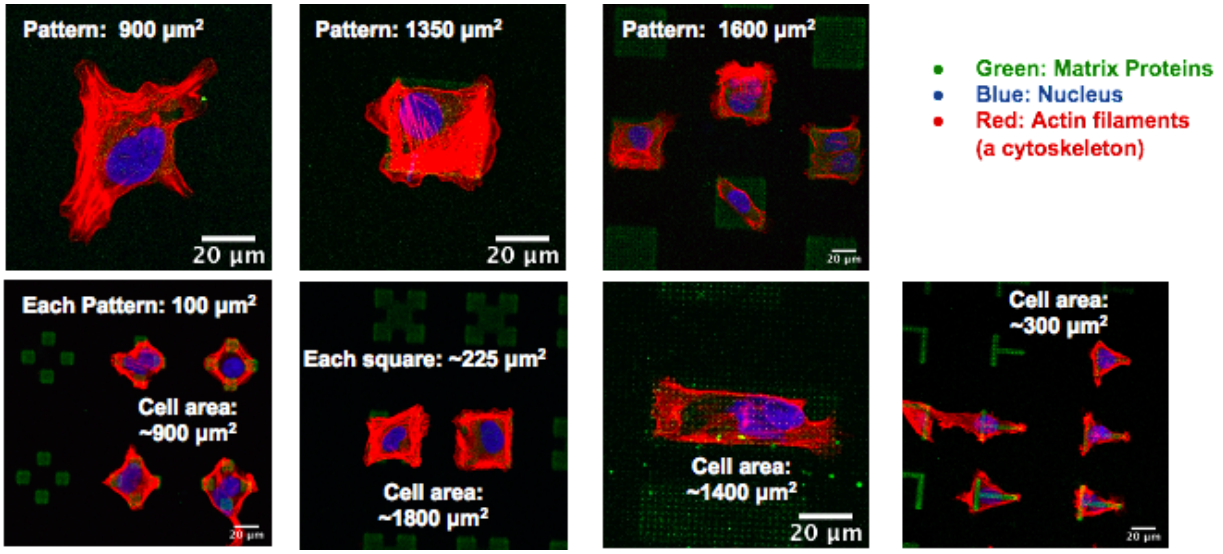


Figure 11. Single cell adhesion on a single pattern.

Eventually, we also discovered that the pattern spacing affects the cell spreading. A single cell is capable of adhering on multiple patterns if the patterns are spaced at a distance shorter than ~ 40 μm (Figure 12).

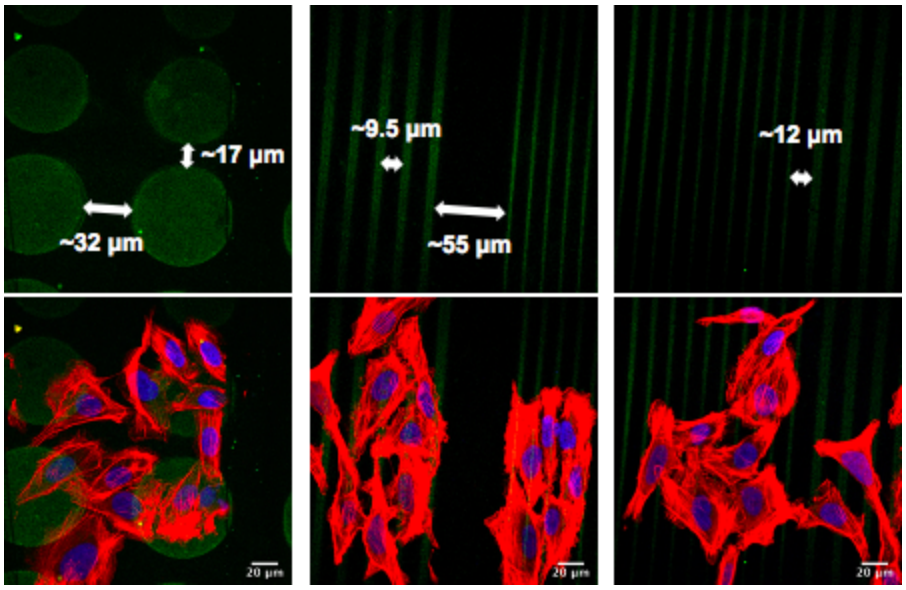
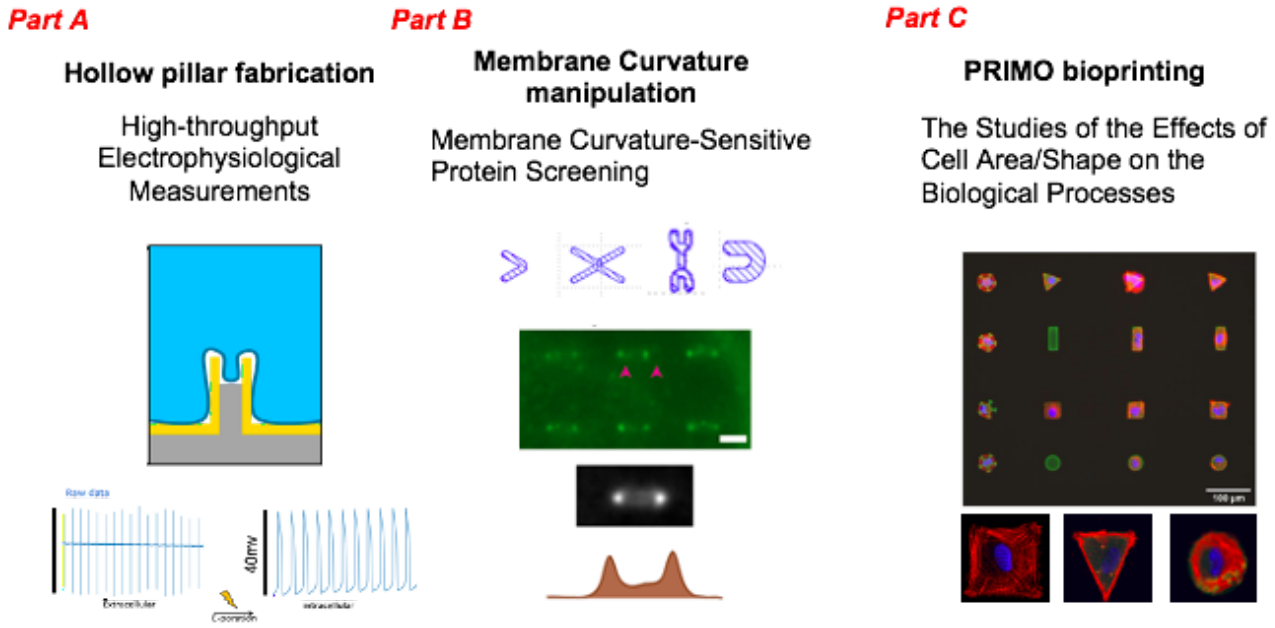


Figure 12. Single cell adhesion on multiple patterns.

Future Applications



References

1. Li X, Matino L, Zhang W, Klausen L, McGuire AF, Lubrano C, Zhao W, Santoro F, Cui B, A nanostructure platform for live-cell manipulation of membrane curvature. ***Nat. Prot.*** (2019).
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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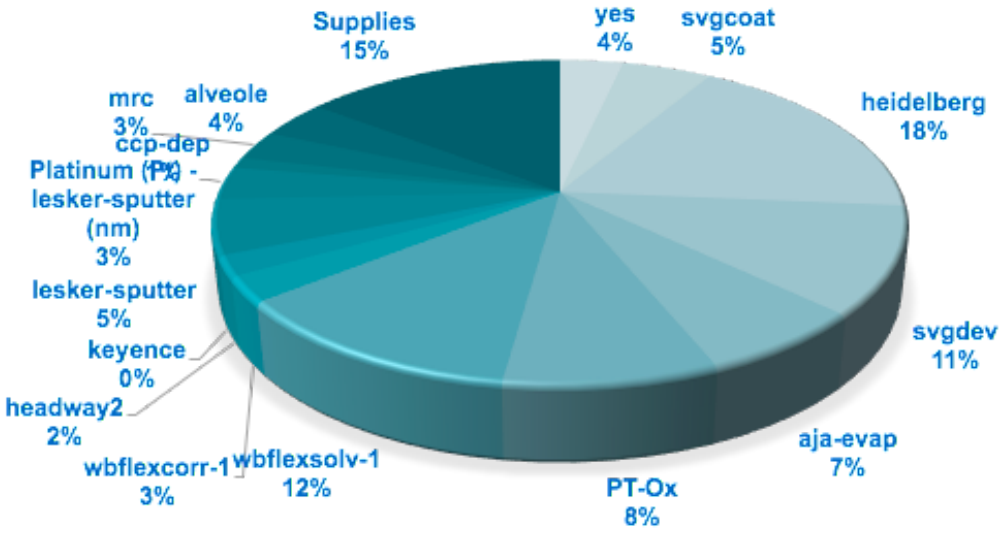
Quarterly Budget



Winter quarter Expenditure: $4314

(2-quarter Expenditure: $8617)

Author Contributions

Task	Contributors
Experimental Design	Zeinab, Ching-Ting, Chih-Hao
Hollow Pillar Fabrication	Ching-Ting
Fabrication of NanoC/V/X/Bone array	Ching-Ting, Chih-Hao
SEM Characterization	Ching-Ting
PRIMO Experiments	Chih-Hao
Fluorescence Imaging/ Cellular Imaging	Chih-Hao
Simulation	Csaba, Ching-Ting
Oral Presentation	Ching-Ting, Chih-Hao
SOP Edition	Ching-Ting, Chih-Hao
Final Report Edition	Ching-Ting, Chih-Hao