Fabrication of an Etched Silicon Microdissection Platform with Applications in Organotypic Culture and Personalized Immunotherapy Testing

ENGR 241 Fall Quarter Report

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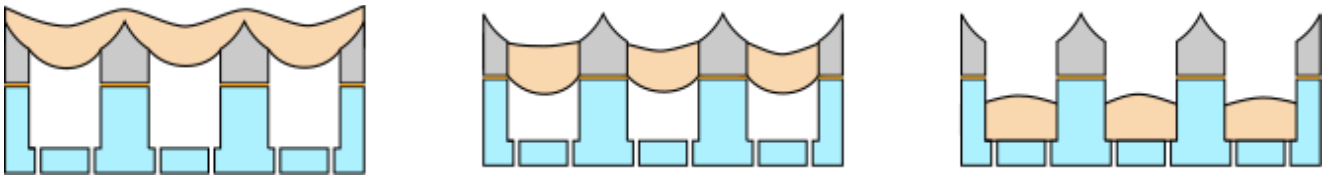


Introduction

Motivation

Background: In 2018 approximately 1.7 million people in the United States were diagnosed with cancer. Cancer is an extremely heterogeneous disease, and one of the key challenges lies in predicting the efficacy of drugs on individual patients. While there are diagnostic methods based on sequencing the cancer genome, these methods are limited because they cannot account for the specific tumor microenvironment (TME). The TME modulates several components of tumorigenesis including initiation, progression, and metastasis. Recent studies show that TME is an increasingly crucial player in modulating the response of cancer cells to chemotherapy [1]. While it would be most useful to test drug assays using tumor biopsy samples directly, samples are often limited in amount and size, demanding the need for model-based methods. Within *ex vivo* models of the TME, patient-derived organoids (PDOs), which are organ-like tumor tissues, have been shown to maintain both the organization and physiological structure of the source tumor. PDOs are thus promising platforms for advancing personalized cancer therapeutics.

Challenges. The use of mechanical dissection of tissue into smaller fragments, instead of enzymatic digestion, is critical to the success of PDO preparation to preserve the TME. However, the current methods of mechanical dissection are inadequate: they are either slow or imprecise, and they are not amenable to multiplexed drug screening. Specifically, PDOs are typically generated by manual mechanical dissection (e.g. mincing) of tissues [2]. Much care is needed in mincing tumor-cell rich nodules for the best chance of success in their isolation. Due to the variability of manual mincing, a board range of fragment sizes (ranging from tens of microns to centimeters) are generated. It is unclear how many of the fragment can recapitulate the actual TME. While methods such as laser-based microdissection of tissue allow more precise control of fragment size, they remain expensive and time-intensive, and still require downstream manual handling of dissected samples for subsequent analysis or drug screening.

Thus, there is a **critical unmet need** for reproducible, high-throughput method to generate uniform-sized tissue fragments for PDOs that can preserve the TME while remaining compatible with subsequent multiplexed drug screening. The overall **objective** of this work is to address this need by developing a microfabricated device, referred to as the "μTissue Dicer," to dissect tissues into uniform-sized sub-millimeter fragments in a reproducible, high-throughput manner, and to interface the μDicer with microfluidics for subsequent multiplex drug screening of organoids.

Our method is expected to accelerate the tissue dissection process for PDOs and subsequent drug screening assays. Achieving this project goal will be **significant,** as it will 1) standardize the preparation for PDOs, which is critical for its wider clinical adoption, 2) accelerate the tissue dissection, analysis, and drug screening process by allowing more PDOs to be tested in a

multiplexed and streamlined fashion, 3) generate fundamental insight into how initial fragment size affects cell type diversity, the tumor microenvironment, and drug response.

Project Concept and Vision

Our aim is to optimize the silicon-etched microdevice for generating both fresh and fixed tissue of various origins (ranging from soft lung tissue to stiffer breast tissue) into uniform sections of 50 μm to 200 μm in their longest dimension. The geometries of the blade will be optimized to ensure minimum loss of intrinsic cells in tumor biopsies having different stiffnesses. The tissue section will be driven through the blades using vacuum (See Figure 1 for a schematic diagram of the device). Alternatively, centrifugal force may be used. Our etched silicon device will then be interfaced with optically clear microwells for downstream drug screening that will be fabricated with 3D printing or soft lithography. We will evaluate the success of our device based on the uniformity of the resultant fragments and their ability to recapitulate the tumor architecture and fibroblast stroma.

Our downstream experiments are to determine the relationship between tissue fragment size, PDO viability, immune diversity in PDOs, and the corresponding drug response. We envision that our μTissue Dicer will enable high throughput, high spatial resolution, and unbiased preparation of tissue fragments and facilitate multiplexed drug screening in PDOs. Our method is expected to be useful not only for PDOs but also for other applications that require organotypic cultures.

This leads us to four primary objectives during our realization of this project:
Objective 1: Optimize etch recipe for fabricating arrays of angled blades by tuning C_4F_8:SF_6 gas ratio, etch time, and bias voltage to produce etch profiles that are not quite isotropic, yet also not crystal plane dependant (termed pseudo-isotropic).
Objective 2: Test etched devices against tissue phantom with compression test to validate cutting ability.
Objective 3: Etch through-holes in wafer to allow for tissue to be extruded through device and strengthen cutting surface by passivation with a robust metal (i.e. platinum).
Objective 4: Integrate bladed array with an extruding force to draw tissue through the device into collection microwells for subsequent biological analysis.

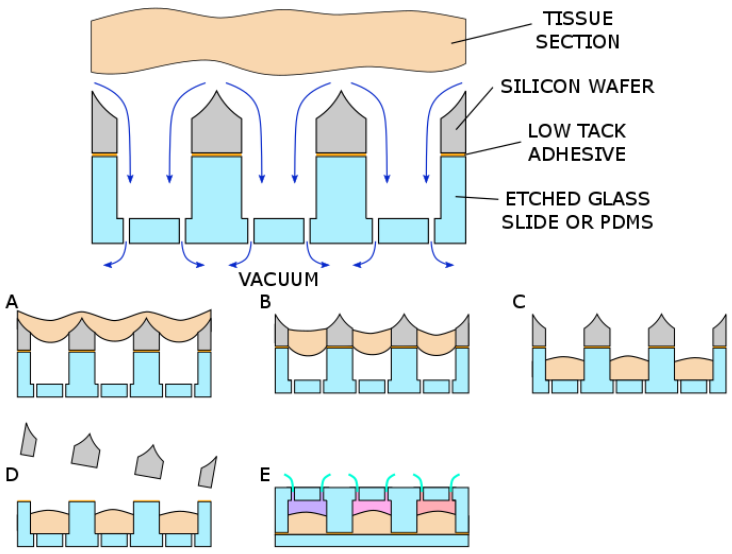


Figure 1. *Cross-sectional schematic of vacuum driven cutting of a tissue sample using the µTissue Dicer.(A-C) Extruding tissue through etched silicon "blades" and collection microwells (D) Removal of silicon "knives" that have been lightly adhered to wells with a low-tack adhesive. (E) Closing wells with another glass/PMDS backing layer and interfacing with microfluidics to incubate individual tissue sections for downstream drug assay testing.*

Benefits to the SNF Community

In the fall quarter, while working towards our project goals of pseudo-isotropic etching of silicon to form "blades" along with some supplementary objectives, we will contribute the following characterizations to the SNF community:

- Characterize the degree of undercut and corresponding blade geometry of an oxide-masked etch of silicon using SF_6 and C_4F_8 gases in the PT-DSE
    - Develop a python script for predicting profile given a gas ratio (*in-progress, cont. second quarter)*
- Characterize oxide etch rate in PT-DSE varying SF_6 and C_4F_8 gasses
- Investigate non-conventional uses of the PT-DSE for pseudo-isotropic etch profiles
- Investigate and report on the potential of using multi-stage PT-DSE recipes for single mask pseudo-isotropic etching and Bosch process etching

In the second quarter, while working towards our project goals of through-etching a silicon wafer, characterizing cutting efficiency, and interfacing with microwells, we will continue to benefit the SNF community by producing SOPs for:

- Through-wafer etching by bulk etching with PT-DSE followed by through-etching with an anisotropic wet etch (KOH).
- 3D printing microwells or using soft-lithography for microfluidics to integrate with etched silicon and the best methods for interfacing the two aspects of such a system
- Best practice for functionalizing silicon for biocompatibility
- Potential methods for extending the life of etched structures by passivating silicon with a metal or fabricating duplicates with cheaper materials

The SOP on how to utilize and develop recipes on the PlasmaTherm Deep Silicon Etcher (PT-DSE) for the purpose of etching pseudo-isotropically is included in the appendix of this report. Additionally, we have characterized oxide etch rate in the PT-DSE for varying C_4F_8: SF_6 gas ratios. All of the processes we develop will be available to SNF users who are interested in fabricating “blade-like” or “pillar-like” structures without the use of wet-bench. SNF users will also be provided with SOPs on interfacing etched silicon with microwells.

Fabrication and Experimental Methods

Overall Process Flow and Tools

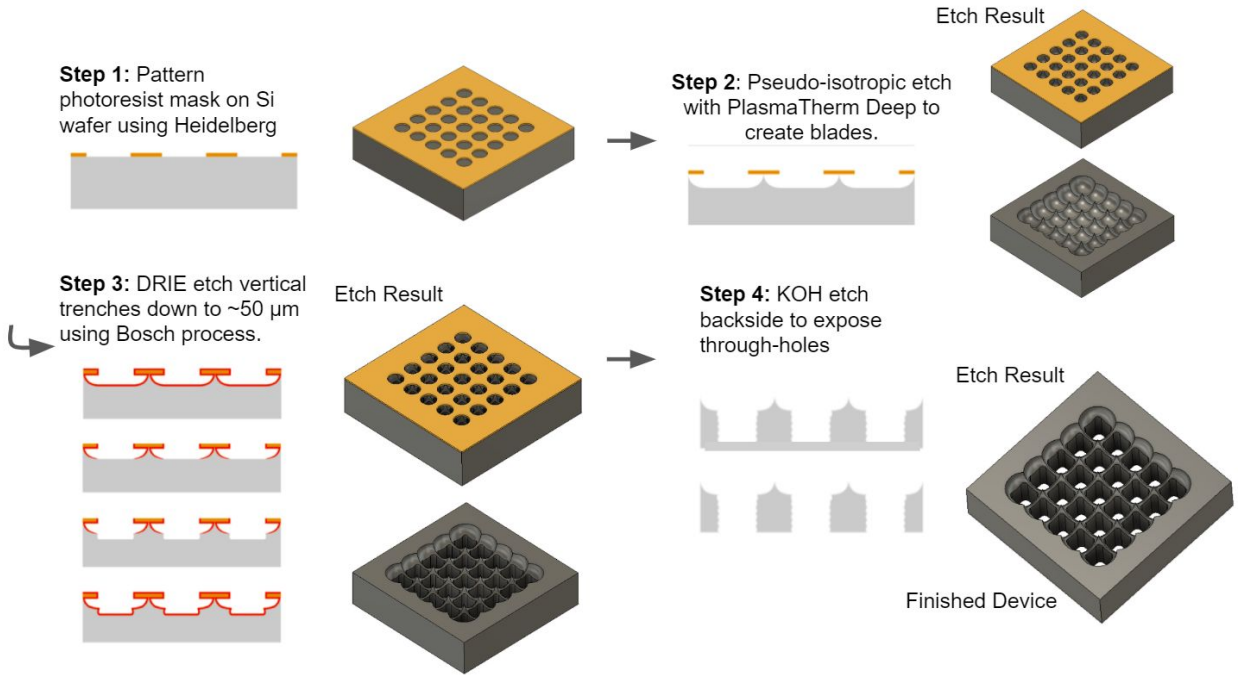


Figure 2. *Side and top view of process flow that predominantly comprises of a two stage dry silicon etching process where a sharp bladed array is first formed followed by a Bosch recipe to fabricate through holes through which tissue will be extruded*

Specific run and recipe details of pertinent processes are outlined in the Appendix. The following tools were used in the fabrication:

- PlasmaTherm Shuttlelock SLR-730-PECVD for capacitively coupled plasma deposition (CCP-DEP) of oxide on silicon.
- YES Prime Oven for dehydrating wafers at 150°C and priming with HMDS (Hexamethyldisilazane) allowing better coverage and adhesion between oxides and resists.
- SVG Resist Coat for resist coating
- SVG Resist Develop for resist development
- Heidelberg 1 for patterning photoresist
- Technics Plasma Asher

- 6:1 BOE (34% Ammonium fluoride (NH_4F), Hydrofluoric acid 7%, 59% water) oxide etch
- Piranha (70% - 90% sulfuric acid (H_2SO_4), hydrogen peroxide(H_2O_2)) photoresist strip
- PlasmaTherm Deep Silicon Etch of silicon using an oxide hard mask
- Apreo Scanning Electron Microscope, Nanospec2, and Keyence Digital Microscope VHX-6000 for characterizing resultant etches
- *Miscellaneous*: Oxford PlasmaPro 80 - Reactive Ion etcher and Matrix Plasma Resist Strip were important told for initial prototyping.

Silicon Dry Etch Recipe for Blade Array

A significant portion of the fall quarter dealt with optimizing and testing various etch conditions using the PlasmaTherm Dry Silicon Etcher (PT-DSE), which is an inductively-coupled-plasma etch system that's traditionally configured for silicon etches using the Bosch process. In our fabrication of blade arrays, we use a continuous etching step and neglect the standard use of cycling between passivation and etching in order to form pseudo-isotropic etches that result in a parabolic-like profile---not quite isotropic and not quite anisotropically conforming to crystal planes. We propose that these coinciding etch fronts will form sharp blades suitable for cutting tissue.

The following is a table containing a column of parameters that were varied to change the etch profile. The second column contains the effect of increasing the parameter.

Parameters	**Effect**
Bias RF Voltage Setpoint (V)	Vertical etch depth
Temperature Electrode Setpoint (℃)	*characterization underway*
C_4F_8 : SF_6 Gas Ratio (or SF_6 : O_2, which was not explored in this project) (sccm:sccm)	Degree of undercut and horizontal to vertical etch rate ratio
Process Time Setpoint (or, equivalently if number of loops if cycling) (s)	Horizontal and/or vertical etch amount
ICP Match Tune Position Setpoint (W)	ICP RF Reflected Power

Our goal was to fabricate blades that were ~20° sharp, which is approximately the radial angle of a surgeon's scalpel. In the appendix, our SOP includes a section on how to debug errors that may arise on the PT-DSE due to changing the parameters mentioned. We have included our SEM images from these etches in the Results and Discussion section.

Experimental Protocol for Evaluating Tissue Cutting Effectiveness

There are some nuances to the strength of biological tissue that stem from their viscoelastic behavior. As viscoelastic materials, biological tissues exhibit strain rate rate dependance, whereby lower loading rates result in the viscoelastic materials behaving more viscous and compliant and higher loading rates lead to a more elastic and rigid response. These are important properties of biological material to keep in mind when designing experiments to test the effectiveness of our blade array and the tissue analogs we are using. Herein lie some crucial considerations to take when designing a method to extrude tissue through our final blade array.

We began developing an experimental protocol for evaluating the effectiveness of tissue cutting using some preliminary etched blade arrays. We used porcine articular cartilage harvested by the Soft Tissue Biomechanics lab as analogs for tumor organoids. Cartilage has an elastic modulus (effectively stiffness) ranging from 0.4 to 27 MPa depending on loading conditions and time scale [3] while tumor organoids exhibit stiffness on the order of 100 Pa to 5 kPa [4]. Thus, in terms of evaluating the blades ability to cut through a tough biological material, cartilage as an analog provides a conservative predictor, however with the properties of viscoelasticity in mind a softer, more compliant tissue could prove to be more challenging to cleanly section. Nonetheless, with a tensile/compression loading machine (Instron) we forced 2 mm by ~600 µm cylindrical plugs of cartilage into a cutting array and against a flat surface for a control. We selected a moderat load rate of 0.02 mm/s up to 0.12 mm of distance pushing the cartilage into the blade array. One critical and noteworthy aspect of mechanical testing on biological material is to keep the sample in a physiologically similar hydrating buffer.

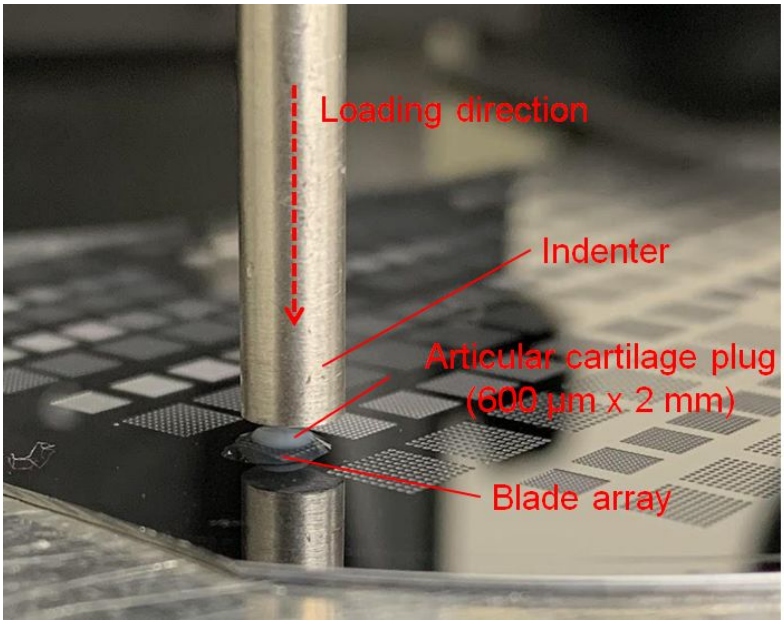


Figure 3. *Cutting experiment setup showing a section of cartilage sitting in a small volume of 1X PBS buffer atop an etched silicon blade array with an indenter from an Instron dropping down to force the cartilage through the micro-blades*

Preliminary Investigation Into Etching Through Holes

One of our proposed benefits to the SNF is exploring non-conventional ways to utilize the PT-DSE. In addition to characterizing the utility of the PT-DSE for pseudo-isotropic etching that produce an array of blades, we propose making use of the same hard mask used in the blade formation and directly adding a second stage to the overall recipe that is a Bosch process for etching straight down into the valleys between blades to form the majority of the through holes through which tissue will be extruded. We conducted a preliminary test of this process showing promising results (Fig. 4). Interestingly the Bosch process conformed very well to the oxide mask considering the mask is far removed (~100 µm) from the surface at the time that the Bosch process begins. We observed some grassing in the bottom of the wells produced by the Bosch process.

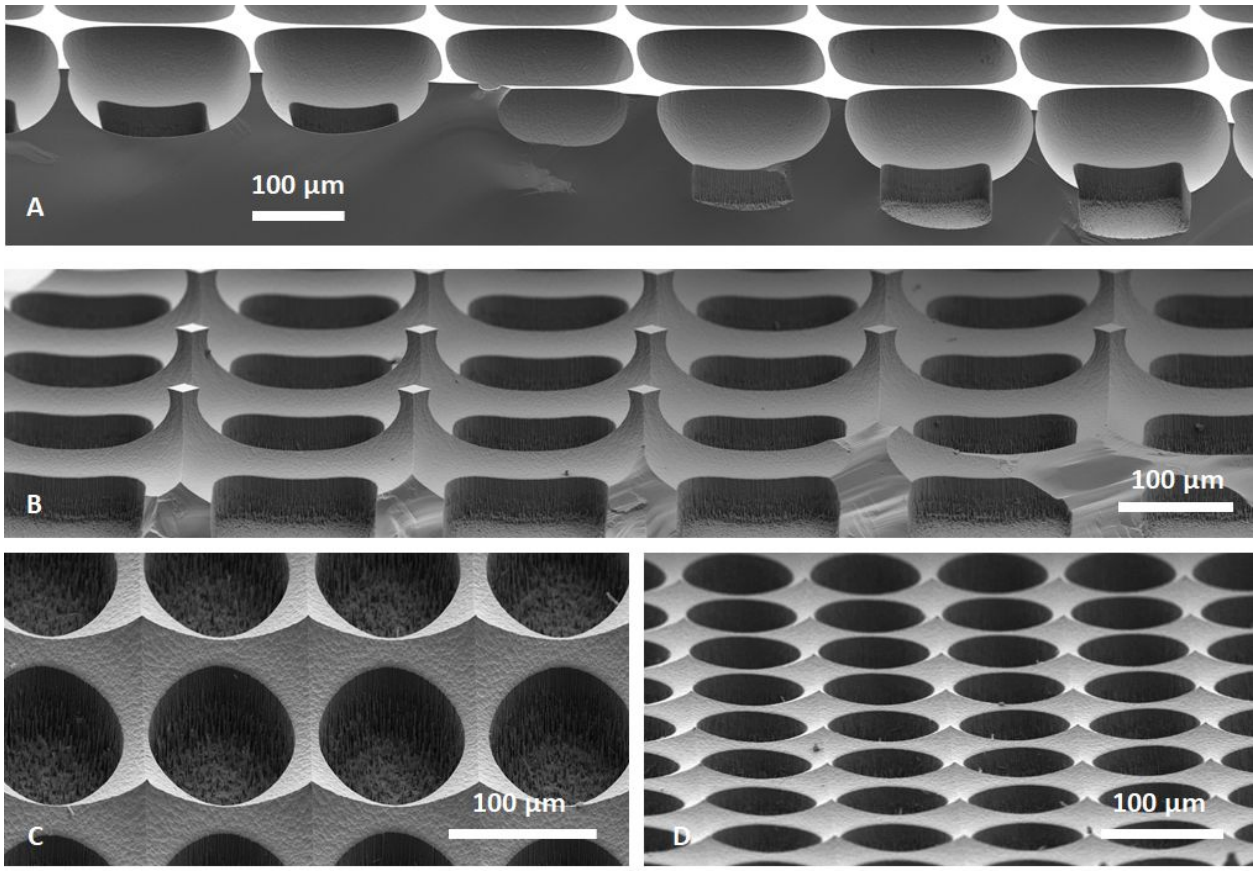


Figure 4. *Results of preliminary attempts to follow the blade etching process with 37 cycles of the Bosch process. A, B, and D are taken at a 75° tilt. C is a 50° view of the array seen in D. It is interesting to note the degree to which the Bosch process followed the shape of the remaining oxide mask.*

Results and Discussion

Silicon etching

Figures 5 and 6 are SEM images of etched silicon using an oxide hard mask. The parameters that were key in our blade fabrication include the bias, etch time, and amount of C_4F_8: SF_6 gas ratio. We noticed, as indicated by Figure 5, that increasing the amount of C_4F_8 appears to decrease the angle of mask undercut as the sidewalls of the parabolic profile appear more vertical, which is more desirable for our blades. Additionally, it appears that C_4F_8 also increases the etch depth of our profile, although characterizing the exact depth is difficult from SEM images alone.

Another unexpected, yet critical realization we came across was the type of silicon wafer etched affected the smoothness of the etched surface. We found that I-test wafers, which a p-type and boron doped (0.1-0.9 Ω) do not have the same roughness apparent in a C-test wafer (insert in Figure 5). In the next quarter, we will further explore the effects of doping type and concentration on the smoothness of etched silicon.

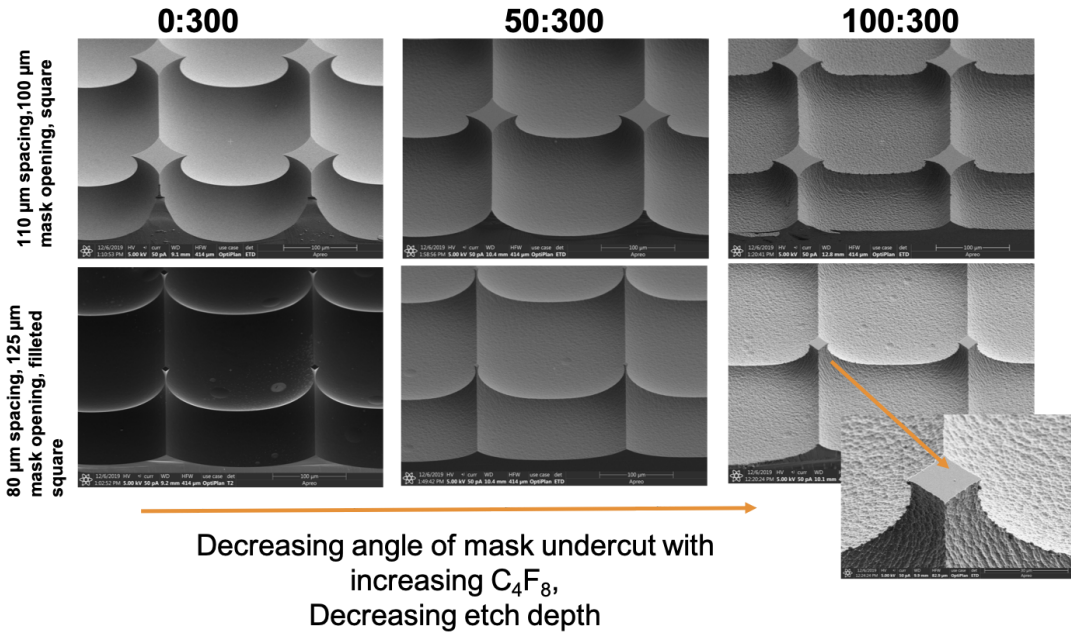


Figure 5. *SEM images of etched silicon using an oxide hard mask. Bias, etch time, amount of SF_6, and electrode temperature are fixed at 10 V, 600 s, 300 sccm, and 15 ℃, respectively. C_4F_8: SF_6 gas ratio is varied from left to right.*

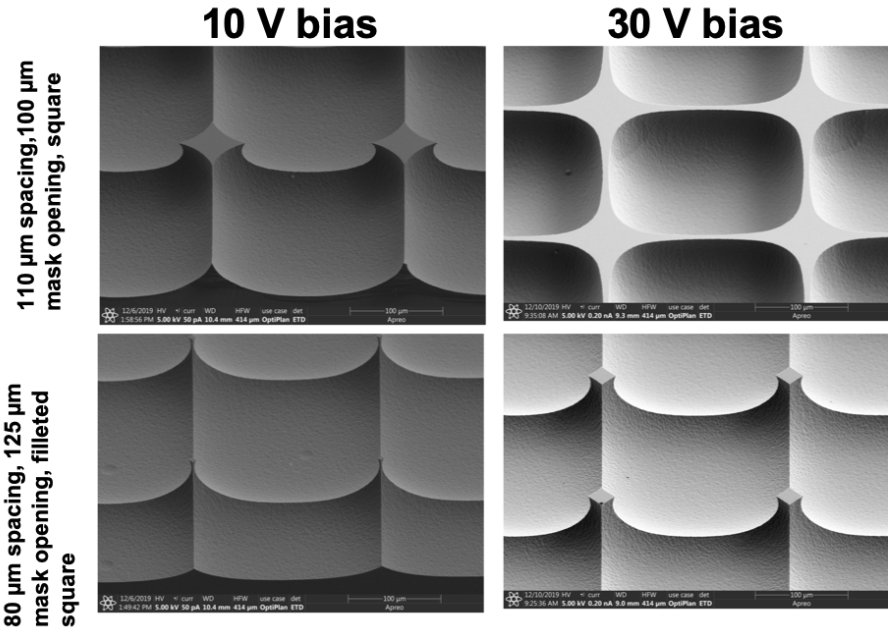


Figure 6. *SEM images of etched silicon using an oxide hard mask. Etch time, amount of C_4F_8: SF_6, electrode temperature are fixed at 600 s, 50:300 sccm, and 15 ^{o}C, respectively. Bias voltage is varied from left to right.*

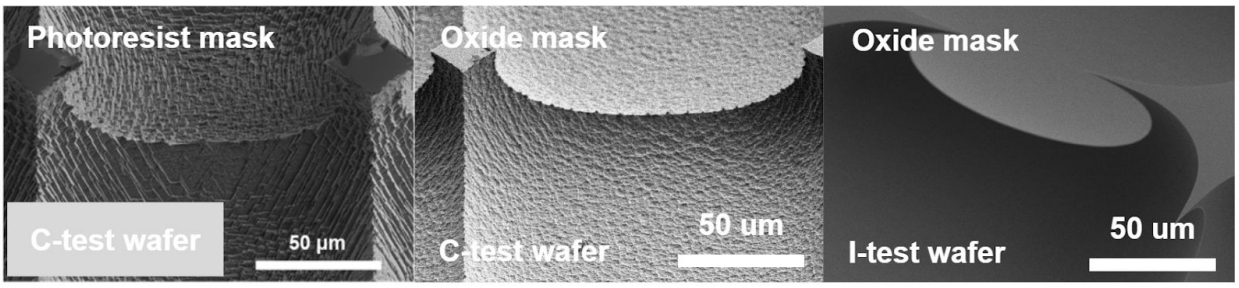


Figure 7.

Cutting efficiency characterization

The resulting force profile indicated that cutting had occurred. In terms of mechanics of materials, the plateau in the force profile is indicative of plastic deformation characterized by slipping along crystal planes. In this case, the plateauing is indicative of the silicon blade slidinging through the articular cartilage at some critical force applied. In this data and in some visually apparent cuts in the cartilage following manually applied force of cartilage onto blade arrays, we see evidence that successful cutting was occuring with these preliminary etches. We should note here that these tested etches were no more than 100 um deep, and thus the cutting depth was limited to ~ 100 um. While this evidence is convincing, we aim to develop other

methods of imaging cut tissue, i.e. fluorescent staining and flash freezing cut pieces for sectioning and microscopy.

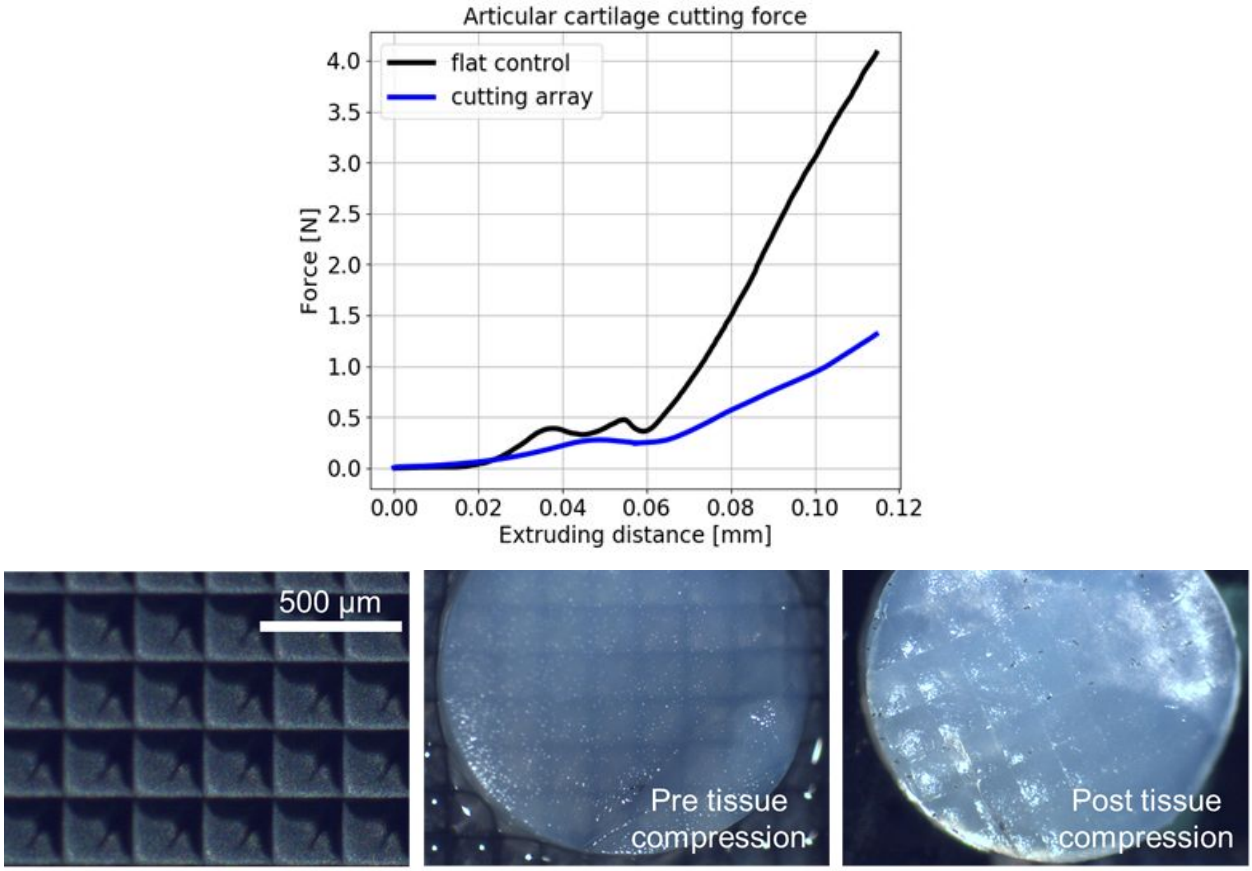


Figure 8. *Resulting load profile for articular cartilage pushed against some preliminary etched silicon blades. The plot shows a plateau in force indicative of slipping or, in this case, cutting of tissue.The bottom left figure shows silhouettes of the peaks between blades that are pressed into the cartilage.*

Future Work

For the second quarter of this course we have several goals to expand on our fall progress. Our first goal is to modify our mask design and etch recipe to improve the blade shape. We will also run more tests with the various etch profiles to determine which shape cuts tissue best. Once we have the blade etch tuned, we will etch the through-holes to complete the silicon device. This will require a BOSCH process to etch most of the material from the wafer (down to ~50 µm) and a KOH wet etch from the backside to finish the through-holes. While we are finishing the silicon devices, we will also fabricate microwells corresponding to the geometry of our wafer for suitable pairing. Ideally, we will have the microwells fabricated by the time we have our silicon device finished so we can then test methods to cut tissue through the device and into the microwells. By the end of the winter quarter, we aim to have a finished device that can dice

tissue and collect the segments in the microwells, a full SOP for fabrication, and a well-characterized recipe for controlling the silicon etch profile in the PT-DSE.

Appendix

Standard Operating Procedures

A. Fabrication of an oxide hard mask for etching silicon

a. Grow oxide mask using PlasmaTherm CCP-DEP PECVD system for oxide on silicon at 350 °C standard recipe for the amount of time corresponding your your desired oxide mask thickness. To grow 2 μm of oxide on silicon, we approximately 30 minutes.
b. Measure oxide mask thickness using the Nanospec2 (silicon on oxide)
c. Put wafer with oxide mask into YES prime oven for 20 minutes following the standard protocol to coat with HMDS (hexamethyldisilazane) for better photoresist adhesion.
d. Using the SVGcoat to spin and soft bake SPR3612 photoresist @1.6μm. This will be patterned and serve as a mask for patterning the oxide layer.
e. Load the desired mask file into the Heidelberg and after running a test exposure, pattern your wafer.
f. Use the SVGdev to develop and hardbake the photoresist.
g. Descum the wafer with the Technics plasma asher to prepare the surface for oxide wet etching.
h. Isotropic wet etch the wafer with 6:1 BOE (34% Ammonium fluoride (NH_4F), Hydrofluoric acid 7%, 59% water) to pattern the oxide, using photoresist as a mask for 22 minutes, followed by 3 DI water rinses.
i. Strip the photoresist with Piranha (70% - 90% sulfuric acid (H_2SO_4), hydrogen peroxide(H_2O_2) for 10 minutes @ 120 °C, followed by 6 DI water rinses.
j. The wafer now has a silicon oxide mask and is ready for dry etching using the PT-DSE

B. Two step etching with single mask using PT-DSE

a. For etching silicon using the PT-DSE, create or adapt a new recipe that includes at least the following main steps will be 1: Gas stabilization, 2: Light, 3: Etch, 4: Pump Detach. An example of reflected power error is below.
    i. If the etch is pseudo-isotropic, the ICP Match Tune Setpoint Position will need to changed to a setpoint of around 72.0. If the etch is anisotropic and requiring cycling, the setpoint is likely around 65.0.
    ii. In either case, test the recipe for a few seconds on a dummy wafer. First, put the tool in maintenance mode, and click to transfer the material

manually from the load lock to the chamber. Then, place the tool back into production mode and monitor the ICP reflected power to ensure that the value is low (< 50W) before deciding on your setpoint. If the reflected power is too high, stop and abort, change the ICP Match Tune Setpoint and process the recipe again.

iii. ICP Match Tune Setpoint can change for a variety of reasons, including the cleanliness of the chamber, parameters of your recipe and the manner in which they interact with other conditions.

b. Parameters that can be changed:
   i. Etch time: change the pseudo-isotropic etch time, holding all variables constant. Loop time: change the number of loops, holding all other variables constant
   ii. Bias Voltage: Change the Bias RF Forward Voltage, holding other values constant.
   iii. Electrode Temperature: Create and save new recipe steps that change the electrode temperature in every step. Manually transfer the material to the load lock and wait until the electrode setpoint temperature has been reached. Failure to do so will result in reflected/delivered ICP power errors.

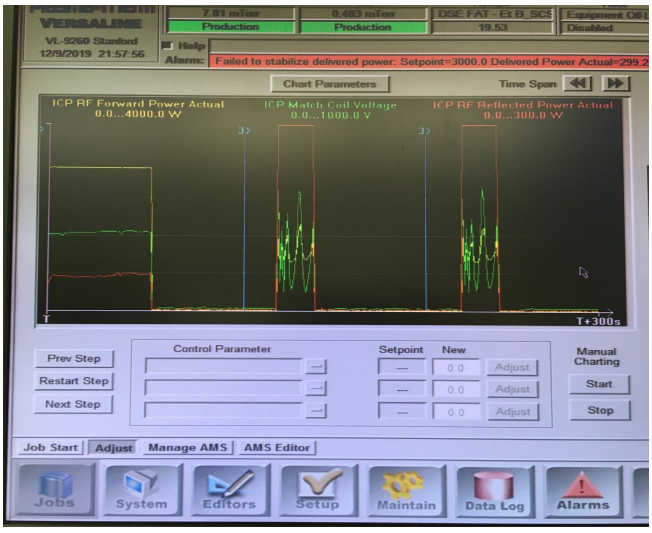
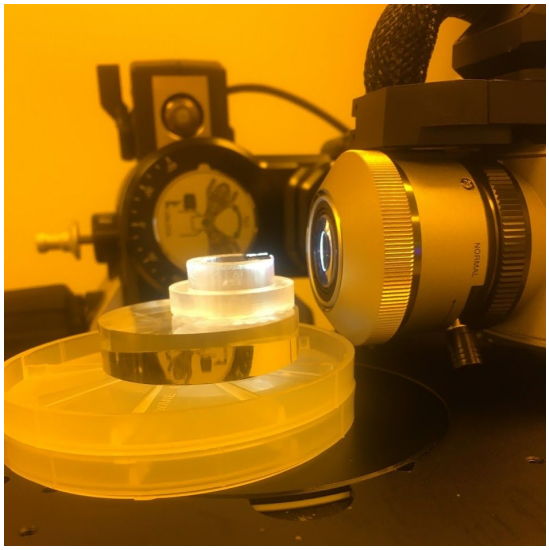


Figure 9. *Example of failure to stabilize delivered and reflected power. Issues with ICP Power mayl arise as demonstrated in this picture.*

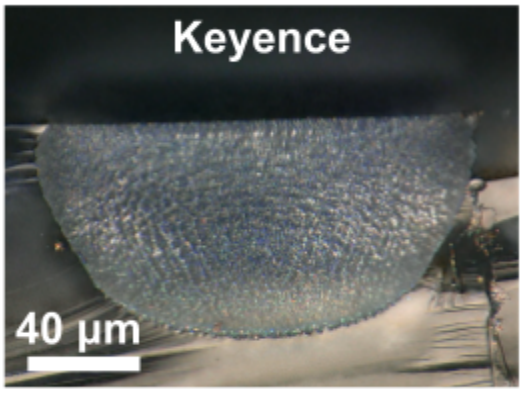
Keyence Imaging to Supplement SEM

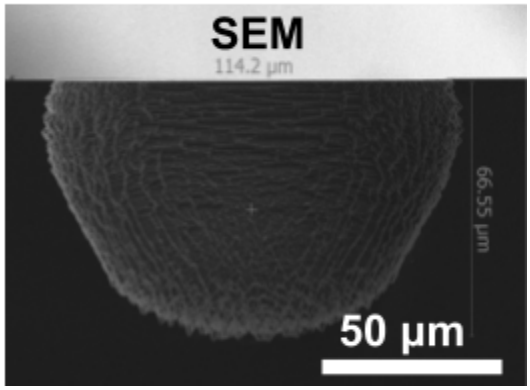
We used the Keyence Digital microscope to image wafer pieces. This was to help us determine which samples we wanted to look at on the SEM and allows us to look at a larger number of

samples relatively quickly. It is cheaper than the SEM and can image a larger number of samples in the same amount of time.



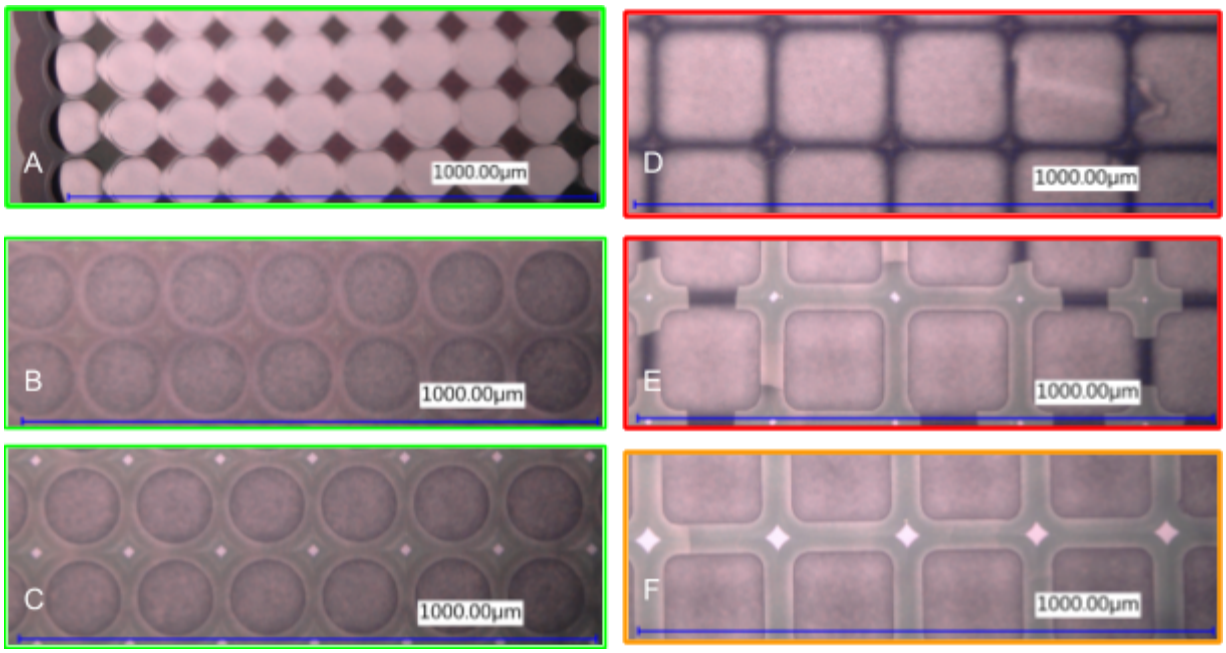
The Keyence digital microscope is rotated 90degrees to take cross-sectional images cleaved wafer pieces. Acrylic blocks and wafer cases are used to hold the piece in front of the optics. Fine-tuning the height can be done with the stage, being mindful not to raise the stage into the optics.





Representative image of a cross-sectional image using the Keyence Digital Microscope (left) and SEM (right) on two different samples.

Oxide mask insights

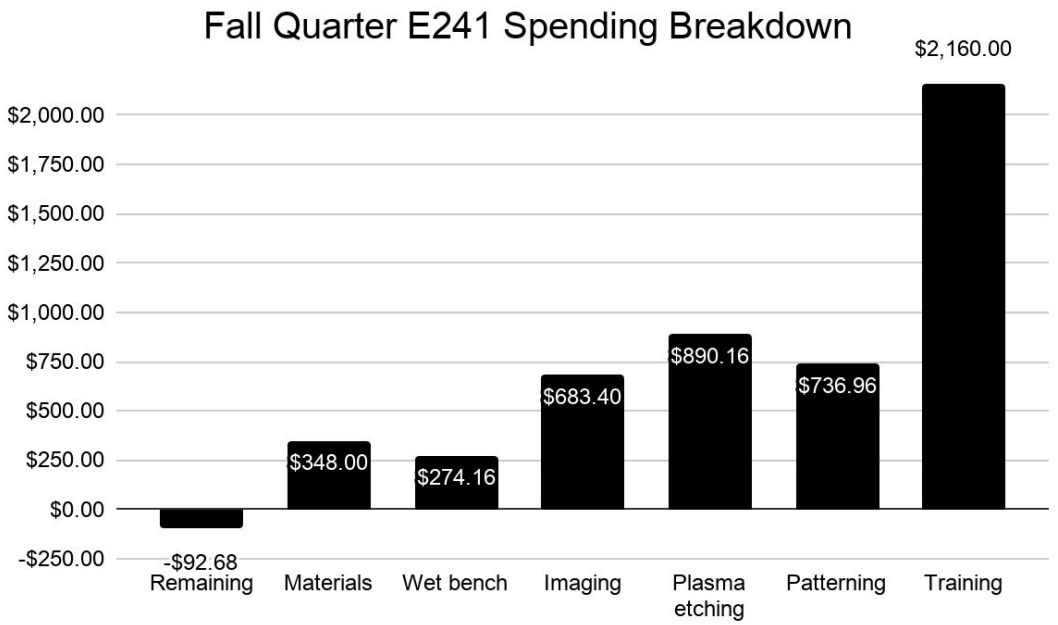


Square oxide mask (D-F) tends to crack/flake once the mask is undercut significantly from etching. This problem did not occur with circular mask openings (A-C) and the mask even remained intact after all supporting substrate was etched (A). This is likely due to stress concentrations in the corners of the square mask. This is an important insight for future mask design when we want to etch under silicon oxide. Using a thicker oxide mask, adding a larger radius whenever possible, or reducing the amount of undercut beneath the mask should prevent this from happening.

References

[1] Nature Medicine, 18, 1359-1368 (2012).
[2] Nature Medicine, 20, 769-777 (2014).
[3] Biophys J., 86, 3269-3283 (2004).
[4] Cell: Trends in Cancer, 4, 281-291 (2018).

Budget



$5,092.68	TOTAL EXPENSES	-$92.68	REMAINING BUDGET		
Training	**Quantity**	**# Trainees**	**Cost per trainee**	**Total**	
General Safety	1	2	80	$160.00	
All-Litho	2	3	80	$480.00	
PTDSE	1.5	3	80	$360.00	
Heidelberg	2	1	80	$160.00	
Keyence Digitial	1	2	80	$160.00	
SVG Coater/ Developer	1	3	40	$120.00	
Thermco4	0.5	1	40	$20.00	
Matrix	0	1	0	$0.00	
Apero SEM	5.5	1	80	$440.00	
Oxford-RIE	1	1	80	$80.00	
Wetbench Flex Corr	2.25	1	80	$180.00	

			TOTAL	**$2,160.00**	
Materials					
Clean Room Notebook	1		8	$8.00	
Wafer - C-test (100 mm)	9		17	$153.00	
Cassette (storage) - 100 mm	1		17	$17.00	
Wafer - C-test (100 mm)	5		17	$85.00	
Wafer - I-test (100 mm)	5		17	$85.00	
			TOTAL	**$348.00**	

Tool usage	**User**	**Amount**	**Cost**	**Date**	
Heidelberg	Nic	67	$39.08	10/23	
SVG coater	Nic	88	$73.33	10/23	
SVG dev	Nic	13	$10.83	10/23	
Yes oven	Nic	34	$28.33	10/23	
SEM (Helios)	Sai (w/ David)	60	$75.00	10/26	
PT-DSE	Sai + Seth	277	$230.00	10/25	
Yes oven	Nic	27	$22.50	10/27	
SVG coater	Nic	16	$13.33	10/28	
SVG dev	Nic	20	$16.67	10/28	
Heidelberg	Nic	51	$29.75	10/28	
Keyence	Nic	118	$68.83	10/28	
Keyence	Nic	63	$36.75	10/29	
Yes oven	Nic	24	$20.00	10/31	
SVG coater	Nic	14	$11.67	10/31	
SVG dev	Nic	15	$12.50	10/31	
Heidelberg	Nic	55	$32.08	11/1	
Keyence	Nic	74	$43.17	11/3	
SEM (Helios)	Nic (w/ David)	45	$56.25	11/3	
PT-DSE	Sai (last	93	$77.50	11/3	

	week)				
Matrix	Sai (last week)	13	$10.83	11/3	
PT-DSE	Sai	90	$75.00	11/10	
Matrix	Sai	21	$17.50	11/10	
SEM (Helios)	Sai (w.David)	30	$37.50	11/10	
Keyence	Seth	56	$74.40	11/13	
Oxford-RIE	Sai	74	$143.33	11/19	
Yes oven	Nic	64	$53.30	11/23 and 11/25	
wbflexcorr-3	Nic	153	$127.50	11/23 and 11/25	
wbflexcorr-2	Nic	79	$65.83	11/23 and 11/25	
SVG dev	Nic	39	$32.50	11/23 and 11/25	
SVG coater	Nic	56	$46.67	11/23 and 11/25	
heidelberg	Nic	132	$110.25	11/23 and 11/25	
CCP-dep	Sai	221	$184.17	11/23	
SEM (Apreo)	Nic	150	$112.50	12/6	
wbflexcorr-3	Nic	67	$55.83	12/6	
Keyence	Seth	114	$66.50	12/6	
SEM (Apreo)	Nic	150	$112.50	12/10	
PT-DSE	Sai	403	$336.00	12/6 and 12/10	
wbflexcorr-3	Nic	30	$25.00	12/10/2019	
		TOTAL	**$2,584.68**		
GRAND TOTAL	**$5,092.68**				