BME Design-Spring 2024 - ELIJAH DIEDERICH
Complete Notebook

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ANURAAG SHREEKANTH BELAVADI

on

May 01, 2024 @08:15 PM CDT

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Nick Herbst - Mar 07, 2024, 3:45 PM CST

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Project description

Nick Herbst - Jan 26, 2024, 4:26 PM CST

Course Number: BME 402

Project Name: Tissue Model of the Epithelial Mesenchymal Trophic Unit

Short Name: Tissue Model

Project description/problem statement: A multitude of chronic lung diseases such as pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) can cause damage to epithelial tissues of the lungs. This presents a problem because when this tissue is damaged, a fibrotic response is triggered in sub-epithelial fibroblasts that results in further disease and fibrosis. There are currently no tissue models that accurately recreate the lung extracellular matrix and its changes due to cell injury. Such a model would need to have tunable mechanical stiffness and porosity, as well as be cell adhesive and degradable. Dr. Brasier of the UW School of Medicine and Public Health requires a scaffold that meets these criteria to be fabricated with a bioprinter. The scaffold must have a uniform and replicable composition that allows for epithelial cell culture in an air-liquid-interface (ALI) so that his lab can study the effects of fibrosis on small-airway lung epithelial cells.

About the client: Dr. Allan Brasier is the Executive Director of the UW-Madison Institute for Clinical and Translational Research. His research focuses on the inflammation and its role in advancing pulmonary and cardiovascular disease.

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01/25/2024 Advisor Meeting

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WILLIAM ONUSCHECK - Jan 30, 2024, 12:24 PM CST

Title: January 25th Advisor Meeting

Date: January 25th 2024

Content by: William Onuscheck

Present: Team, Dr. Tracy Puccinelli

Goals: To establish goals for the semester

Content:

- Set up regular meeting schedule with client.
- The main 402 difference is a journal article in addition to the standard report.
- Standard report and journal article can / will be similar
- Planning presentation rather than preliminary presentation
    - Similar to what we did in the Fall since we went off of 402 guideline
    - Only with advisor
    - Informal
    - “Very detailed plan”
    - Some rubric items may not be applicable
    - Add other things that are relevant
    - Soft 10 min
- Do things concurrently while working on presentation
- Journal article
    - Identify appropriate journal
    - Look at journals where we have been pulling our resources from
    - Shoot for a moderate impact factor (3 - 6 range)
    - Option to submit if motivated
- New progress report
    - To reflect industry level of detail
    - Current protocols (lead with up front) rather than drawings
    - More detailed timeline
        - Gantt chart
- Show and tell
    - Going to give advice to 300 level
- Reschedule advisor meeting with full team if conflicts come up
    - Feb 16
    - Feb 23
    - Mar 22
- Methodical staining and viewing

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    - Use the Nikon
- Body temperature rheometry testing
    - May want to look into making a new protocol on the rheometer to bypass the 5 minute normal force test
- Methodical
- Dry weight for swelling

Conclusions/action items:

Begin working on preliminary presentation. Find literature supporting drying times for swelling ratio.

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02/02/2024 Advisor Meeting

Nick Herbst - Feb 02, 2024, 12:30 PM CST

Title: Advisor Meeting

Date: 02/02/2024

Content by: Nick Herbst

Present: Team, Prof. Puccinelli

Goals: Update advisor on weekly activities

Content:

- Told advisor about rheometry of pipette-based hydrogels
  - Rheometer plate missing so found a substitute that thought would work but stiffness came out very very low
    - Likely due to equipment issues (wrong plate or installed improperly)
  - Need to reach out to someone to talk about set up
  - Dr. P says that the plates were scratched and **the coolant fluid was low**
- Wet weight of pipette-based hydrogels went down from the pre-swelling weight
  - Very strange since this should not have happened
  - Either errors with pre-weighing or errors with wet weight
    - using wipe to remove surface water possibly could have been too forceful but unlikely
- Told advisor that we set up meetings for bioprinter demo and for client meeting

Conclusions/action items:

- Re-make new pipette-based gels and do rheometry testing and weighing
  - Look for rheometer manual and for the normal head used
  - Before using it again, reach out to original graduate student who taught Elijah and/or reach out to a PI who regularly uses
- Reorient with bioprinter
- Make bioprinted gels and do rheometry
- Meet with the client to give demo on bioprinter
- Work on preliminary presentation for next week

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03/01/2024 Advisor Meeting

Nick Herbst - Mar 01, 2024, 12:22 PM CST

Title: Advisor Meeting

Date: 03/01/2024

Content by: Nick

Present: Nick, Carley, Anuraag, Elijah, Caitriona, Prof. Puccinelli

Goals: Update our advise on the current status of our project and begin to discuss preliminary deliverables

Content:

- Cells look good
- Need to email client the discussion notes from meeting that was missed on Thursday
- Bioprinting went well last time, more extrusion vs dispensing, *much* better structure, stiffness is in between the ranges, going to go back Sunday
- Tong lecture in 2 weeks
    - Meet with advisor afterwards
- Show and Tell is in 3 weeks
    - Meet in ECB atrium to advise 301 teams
    - If going to miss it, email Tracy to get put on list of people who will do Zoom advice with 301 teams
- First comments on preliminary report
    - Really weird that biomaterials science doesn't have manuscript formatting requirements (spacing, font, etc.)
    - Tracy says typically in a manuscript you'd see all figures/tables at the end with captions, and that methods should be before results

Action items:

Continue to work to meet project goals

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2-7-2024 - WIMR Meeting

Caitriona Treacy - Feb 14, 2024, 5:45 PM CST

Title: GelMA bioprinting trial - with Brasier and Dianhua

Date: 2-7-2024

Content by: Elijah Diederich

Present: Carley, Elijah, Caitriona

Goals: To showed Dr. Brasier an introduction to the bioprinter and showcase our learning

Content:

Parameters that will stay the same:

- Infill density of 35%

- Dimensions 3 x 0.6mm (for 3D Structure print)

- UV crosslinking time - 30 seconds, 405nm, 4cm distance away

- Temp set at 37 degrees, waiting 30 min for GelMA to thaw

- Pre-flow = -100 ms

Trial 1:Structure Print

- Print temperature: 29 degrees

- Extrusion Pressure: 22 kPa

** GelMa did not print during run ** --> Started to troubleshoot, unclogging etc.... brasier not impressed. Carley removed the bubble with excess flicking of the dispensing nozzle.

Trial 2: Structure Print

Print temperature: 31 degrees

Extrusion Pressure: 24 kPa

*** GelMa also did not print during run ***

Trial 3: Structure Print

Pre-flow delay: -130 ms

Print Temperature: 31 degrees

Extrusion Pressure: 22 kPa

**** GelMA not printing during run **** Attempted to do test flow, nothing happens @ 22 kPa

Went to 24 kPa, no extrusion achieved

26kPa - No extrusion

28 kPa - No extrusion

After this, used a syringe (provided by Dr. Brasier) to potentially remove obstruction, no success.

Trial 4: Droplet Bioprinting test run - Switched to 6 well plate

Print Temperature: 30 degrees

Extrusion Pressure: 15 kPa

Extrusion time: 1sec

UV: 30sec, 405, 4 cm away

** No pre-flow delay on the droplet bioprinting **

*** Recalibration needed because bioprinter is confused as to where the UV light and what well it is printing in ***

Droplet bioprinting also did not work

Trial 5: Back to Structure Based Bioprinting - Petri Dish

Pre-flow delay: -150 ms

Print temperature: 29 degrees

Extrusion Pressure: 25 kPa

*** No print achieved ***

** Test flow, achieved a blob on the petri - 35 kPa used for the test flow **

Bumped pressure down - 33 kPa and a large blob came out

Trial 6: Structure Based Bioprinting

Extrusion Pressure: 28 kPa

Temperature: 29 temperature

Pre-flow delay: -150 ms

** Were able to achieve a printed structure **

Trial 7: Structure Based

Extrusion Pressure: 21 kPa

Temperature: 29 degrees

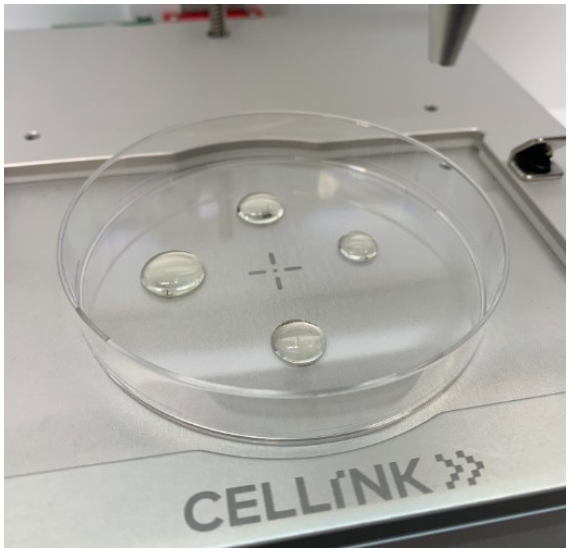
Pre-flow delay: -150 ms

Trial 8: Structure Based

Extrusion Pressure: 23 kPa

Temperature: 29 degrees

Structure Printed



Conclusions/action items:

1. Do rheology on bioprinted gels (placed in incubator)

2. Practice final presentation for Friday

02/13/2024 Client Meeting

WILLIAM ONUSCHECK - Feb 27, 2024, 3:34 PM CST

Title: Client Meeting

Date: 02/17/2024

Content by: William

Present: Elijah

Goals: Establish the need for communication with field science, and establish a rationale for caclien use

Content:

- Response from tech support: Check in of equipment virtually at thursday at 11, detect remotely, check for internet connection on printer for software, have GelMA warmed up for remote testing. Details forwarded to Carley.
- Ordering new GelMA cartridges, for
  Rheology testing, gels in the fibrotic range, kPa, consistency, soft gels have already been produced. Explanation of X-Linking. Increase throughput.
- Calcein staining approved, follow up with sending ordering link.

Action items:

Work on preliminary deliverables

2-15-2024 - CELLINK Meeting

Caitriona Treacy - Feb 19, 2024, 10:10 AM CST

Title: Cellink Troubleshooting Meeting

Date: 2-15-24

Content by: Caitriona and Elijah

Present: Carley, Elijah, Caitriona, Will

Goals: To meet with the client as well as representatives from Cellink to further Troubleshoot the bioprinting process with GelMA

Content:

- Suggested to preflow delay -100 ms and heating to 37˚ in an exterior waterbath for 5-10 minutes while also maintaining 37˚ temperature in the temperature controlled printhead.

- 22-27 kPa. There will be +/-

- Bottom row cannot be printed because of door interference with temperature controlled printhead. With 6-well plate, you need to rotate.

- Photocrosslinking print head gets closer.

- Distance between the wells. Turn inside lights off inside to avoid ambient light effects. Manual moving.

- This material is very sensitive to temperature changes; a 0.3˚ temperature difference changes print when working with GelMA. 27 guage can help with structural integrity. Always test flow before doing a print.

1. Turn light off

2. Put well plate inside

3. Print for first 3 well plates and then can go to the move command (move UV to on top of back left well plate)

4. Go to tools--> modules--> Turn on UV at 405 nm

5. Use calipers to measure distance from UV module to plate

Test Print w/ GelMA using protocol that we have:

1. Preloaded protocol

- Change light to 20%

- Extrusion:

-Pre-flow:

- Diameter 6mm

*** Use 27g instead of 22g so that there can be a more precise construct ***

Ambient light = Clogging; that is why we turned it down to 20%

- Changed to 10 seconds of UV light just for demo purposes

-Pre-flow @ -100ms

- Temperature kept at 27 degrees celcius

- Extrusion pressure: 40 kPa

2. Went to layers --> Preview (zoomed in) **Said construct shouldn't fill in at these settings,** said we should change pressure and temp for better printability.

3. Kimwipe under nozzle, changed to 32 kPa and clicked on extrude, 33kPa, nothing extruded, 34kPa nothing, 35 kPa nothing, 36 kPa nothing. Declogged and then it came out.

4. 35 kPa worked, 34 kPa worked, 33 kPa worked (very watery at these pressures, too high) kept here @ went to 25 degrees celcius (thinks it is a temperature issue). Should be integrity to construct coming out of the nozzle. Waited for the temperature to go down. Wait 3–5 minutes for homogeneity temperature change. **0.3 degree change to where it changes from gel to watery fluid. GelMA will slightly change during these settings (no more than 10%)**

5. Once the temperature reached 26 degrees celcius, did a test flow. Still at 33 kPa (nothing out). 34 kPa nothing, 35 kPa (strarting to extrude), 36 kPa (didn't come out), 37 kPa (nothing), 38 kPa-40 (nothing came out). Went to 26.5 degrees celcius for temperature. **Should be between 26–27 degrees, determined by steps above.**

6. At 26.5 degrees, declogged nozzle and then went to 33 kPa (comes out). GelMA comes out like a gel, but field scientist said it was too liquid. **Went to 26.3 degrees Celsius.** Wants the GelMA to come out like a filament. We're dispensing material (not extruding it) with previous trials.

- Rounding on top --> initial calibration (go down 300 microns instead of 400 microns) - Flat surface

7. Unclogged and then tried to extrude at 33 kPa (watery), 30 kPa (extruded), 31 kPa (still runny). **Went down to 26.1 degrees celcius.** 30 kPa (liquid). **Went to 25.5 degrees Celsius. ***25.7-26.5 degrees Celsius is ideal for GelMA*****

8. Unclogged at then went to 30 kPa (more gel like) very promising, 32 kPa (very gel like). 34kPa too high. Declogged 33 kPa (gel like), 34 kPa (gel like but a bit more watery)

9. 35 kPa test print, still at 25.5 degrees Celsius. Test print. Idea to start going from low temp and low pressure to minimize wasting material. Then did calibration. Automatic and manual.

10. Doing manual, unscrewed the syringe so it could move up and down. Went to center of well that we are going to print in. Tightened knob. Using 22 guage, move down 400 microns and then clicked calibrate. 25 guage - 300 microns - 27 gauge - 250 microns.

11. After calibration, went to extrude (35 kPa) - nothing, 36 kPa - nothing. Clogged, so went to calibrate and went 1.5 mm down twice (3mm) so that we had access to nozzle to unclog. Went back up 3mm. Did a manual recalibration and just fixed Z-Axis calibration. Then moved down 400 microns --> Calibration acheived.

12. Test extrude at 36 kPa (came out), big delay. 37 kPa (better, but curling around tip), 38 Kpa (curling). 39 Kpa - Test print - Nothing extruded. Went to speed of 8 mm/s for tool 2 speed (8-14 mm/s) good range. 40 kPa - test extrusion (curling around tip).

14. Increased temperature to 25.7 degrees celcius and 42 kPa. Waited one minute. Test print - Looks much better, but not great?. Curling around nozzle. 45 kPa - collecting at top of nozzle. Tried again, same result. Went to 47 kPa - curled. 50 kPa - curled around nozzle. Field scientist said that consistency was good though. 50 kPa - curled. **55 kPa - looked fantastic. 57 kPa (curled). 60 kPa (still curling). Curling of filament means pressure is too high and expanding in all direction.**

15. Test Print - 60 kPa, increased temp to 25.8 degrees Celsius. Results: 3 structures were printed (one in each well). Note: UV calibration was very good. Better diameter - ideal in #2. Need to optimize pressure and use a 27 gauge needle. Also, decreasing the speed can also help to keep material steady in the tube. **If decreasing speed, decrease pressure. If increasing speed, increase pressure.**

Need to optimize printing parameters such as pressure and speed. Temperature range should be optimized, though. Higher molecular weight GelMA's are better for printing, but have worse cell attachment.

******Low speed is always good******

Material sterility practices: Will asked someone in production team and fill in team via email. Storing GelMA in biosafety cabinet and bringing out.

Using bacterial hood, could move bioprinter so that it would be in a clean environment.

- Better way to solve clogging: 2 syringes in a water bath and use thin needle

*** Delay can be fixed if we add in G code ***, these preflow delays come into play too. Change with these or can code.

**** Dr. Brasier mentioned that he wants size to be in a 12 well plate to fit ALI. ****

Conclusions/action items:

1. Do rheology on bioprinted gels (placed in incubator)

2. Practice final presentation for Friday

02/27/2024 Client Meeting

Nick Herbst - Feb 27, 2024, 12:02 PM CST

Title: Client Meeting

Date: 02/27/2024

Content by: Nick

Present: Nick, Anuraag, ~~Dr. Brasier~~

Goals: Update client on current status of the project

Content:

- Discussion Plan
    - Told Dr. Brasier that bioprinting is going better after starting to implement the advice from the field scientist
        - Better printed structure (flat rather than globs)
        - Higher variability but getting better
    - Want to soon do epithelial cell culture on gel surface (end of this week/beginning of next week)
        - To reduce contamination can the bioprinter be moved closer to BSC?
            - other idea would be to just cover printed dish when moving and clean entire interior of bioprinter with 70% ethanol between uses
        - Does Dianhua have flask of epithelial cells going and if not then can that get started?
            - planning on printing gels for him Friday or Sunday for him to seed cells on Monday
    - Currently working on our preliminary report
- Dr. Brasier notified team last minute about a conflict so he couldn't make it to the meeting
    - This discussion plan was communicated to the client via email

Action items:

Work on preliminary deliverables

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03/12/2024 Client Meeting

Nick Herbst - Mar 12, 2024, 12:16 PM CDT

Title: Client Meeting

Date: 03/12/2024

Content by: Nick

Present: Nick, Anuraag, Elijah, Dr. Brasier

Goals: Update client on current status of the project

Content:

- Asked about status of materials that Dianhua ordered
  - LIVE/DEAD Cell Viability Kit was ordered 2 weeks ago, no news from Thermofisher on order status
  - 3 new GelMA bioink cartridges were requested 3 weeks ago but Dianhua missed an email from CELLINK so the order was not actually placed until now
- Updated client on status of bioprinting trials
  - Attempting to make thicker hydrogels
  - Dr. Brasier said that Dianhua showed him the seeded bioprinted gels and it appeared as goo
  - Dr. Brasier suggested that Dianhua add an MMP-inhibitor when he seeds the epithelial cells if the thicker hydrogels still "melt"
- Asked if Dianhua could take pictures of the hydrogels seeded with epithelial cells regardless of how they turned out
  - We need the images for our deliverables
- Dr. Brasier said that CELLINK field scientist says that GelMA bioink is their most "finnicky" bioinks and he mentioned possibly switching off of GelMA
  - At this point we can't do that

Action items:

Troubleshoot LIVE/DEAD staining

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02/09/2024 Product Design Specifications

Nick Herbst - Feb 28, 2024, 1:27 PM CST

Title: Product Design Specifications

Date: 02/09/2023

Content by: Everyone

Present: Everyone

Goals: Update the PDS

Content:

- We need to update the PDS from last semester

- See the attached file for the full PDS

Action items:

- Work on preliminary deliverables

Nick Herbst - Feb 28, 2024, 1:28 PM CST

Tissue Model of The Epithelial Mesenchymal Trophic Unit

BME Design

Date: February 9th, 2024
BME 402

Product Design Specification

Client: Dr. Allan Brasier
Advisor: Dr. Tracy Jane Puccinelli

Team Members:
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William Ormecheck ormecheck@wisc.edu (BSAC)
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Nick Herbst nherbst2@wisc.edu (BPAG)

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BME_402_PDS.pdf (221 kB)

Design of Troubleshooted LIVE/DEAD Staining

WILLIAM ONUSCHECK - May 01, 2024, 10:44 AM CDT

Title: Design of Troubleshooted Live Dead Staining

Date: March 13, 2024

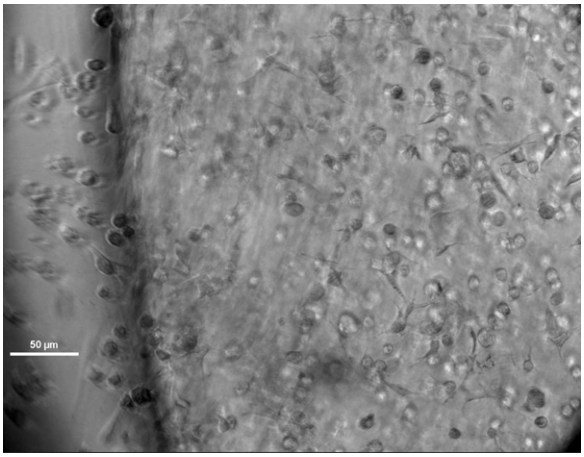
Content by: William

Present: William

Goals: To establish what methods could be changed to obtain live / dead images that are not washed out

Content:

On March 4th, 2024, an initial batch of cell laden hydrogels were fabricated for the purpose of validating the live dead staining protocol. Last semester, there was an issue where imaging under the FITC filter was resulting in completely washed out images. Brightfield image of these cells indicated that cells were indeed, imbedded in the gels, and although not super fibroblastic in their morphology, were in tact, and should be staining as live or dead:



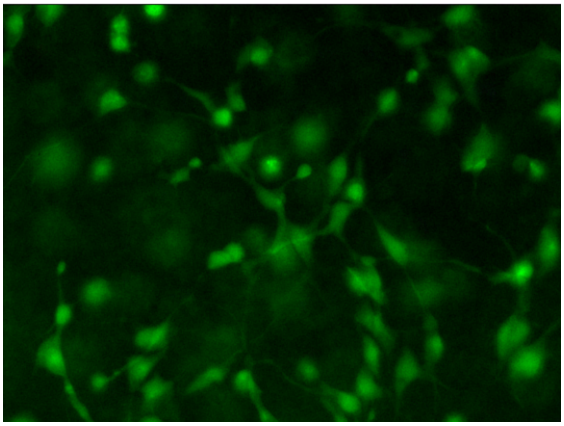
This image from March 4th is representative of that. LIVE/DEAD staining was performed on these March 4th gels using .53 µM calcien-AM and 2 µM ethidium homodimer which are concentration in line with both the literature and the manufacturer's recommendation's. 3 washes were performed consecutively using PBS. Of note, the March 4th gels were not allowed to rest in the PBS wash, and a pink hue from their culture media persisted through the washes. The March 4th gels were then treated with the staining solution, and allowed to incubate at room temperature for 30 minutes. The LIVE/DEAD images from the gels fabricated on March 4th, stained on March 5th were still (staining occurred on march 5th) remained washed out:

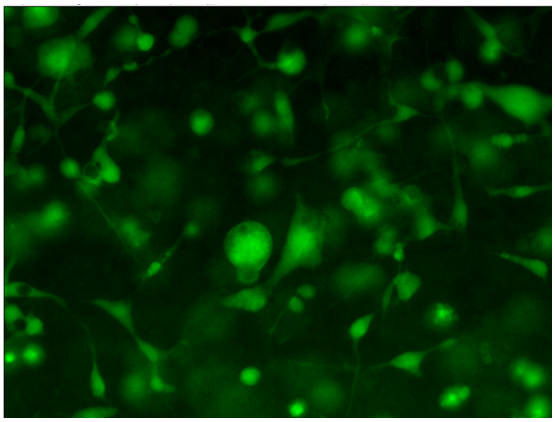
https://drive.google.com/file/d/19EoH5ZSseY0wllor_wi_iBRyXLR8k64F/view?usp=drive_link

↑ Link to video of washed out gels, LabArchives does not support insertion of non-URL video.

From this, there was that esterases were persisting in the extracellular space of the gels, resulting in the cleaving of calcien-AM into calcien, enabling its fluorescence. The following variables were identified as variables which may improve the imaging quality: an increased number of PBS washes, increased PBS wash time, and decreased incubation time in staining solution.

In an effort to troubleshoot using these variables the gels fabricated on march 12th (LIVE/DEAD imaged on March 13th) were split into two groups. One group would be incubated for 15 minutes in the staining solution, and one group would be incubated for 30 minutes in the staining solution. Both groups would be washed 5 times, being allowed to sit in the wash for 5 minutes. We figured that longer wash times could not hurt.





Both groups that were incubated for 15 and 30 minutes yielded fairly clear images, and we concluded that the number of washes and the incubation time in wash were responsible for improved imaging.

Conclusions/action items:

Fabricate a batch of cell laden gels for final imaging over a longer period of time (2 weeks). Moving forward, wash gels 5x for 5 minutes prior to LIVE/DEAD imaging. Update imaging protocol to reflect this change.

02/27/2024 Preliminary Materials List

Nick Herbst - Feb 27, 2024, 11:12 AM CST

Title: Preliminary Materials List

Date: 02/27/2024

Content by: Nick

Present: Nick

Goals: List current materials and expenses

Content:

See attached spreadsheet for materials list

Conclusions/action items:

work on preliminary deliverables

Nick Herbst - Feb 27, 2024, 11:25 AM CST

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402_Materials_-_Sheet1.pdf (62.4 kB)

01_30_2024PipetteGelMaking

ANURAAG SHREEKANTH BELAVADI - Jan 30, 2024, 12:35 PM CST

Title: Making GelMA Hydrogels for Initial Rheometry and Swelling Tests

Date: 01/30/2024

Content by: Anuraag Belavadi

Present: Anuraag, Will

Goals:

Content:

Conclusions/action items:

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2024/30/01-Bioprinter Advanced Protocol

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Nick Herbst - Mar 08, 2024, 3:31 PM CST

Title: Bioprinter protocol (extreme detail)

Date: 01/30/24

Content by: Carley Schwartz

Present: Self

Goals: To create a very detailed protocol for other group members

Content:

Materials:
1. GelMA in amber cartridge from fridge
2. CELLINK Bioprinter
2a. Temperature controlled printhead attachment
2b. Temperature maintaining nozzle attachment
3. 22 gauge nozzle attached to cartridge (Many incase of clog)
3a. Smallest needle for declogging
4. Pressure hose
5. Luer lock connections
6. Petri dish

Method:
1. Place the GelMA cartridge into the connected temperature controlled print head
2. On the bioprinter interface select the type of print you would like to conduct, in our scenario we will be doing a structured print
2a. Go to bioprint -> Simple shapes -> scroll down to cylindrical and select the 3x06mm.stl
2b. Go to surface -> select your surface (for us petri dish)
2c. Printer -> tool 1 (or whichever you are using/what the print head is connected to) -> tool type: temperature controlled ; temperature: 37 degrees to make a liquid
2d. At this point place the GelMA cartridge with a 22 gauge nozzle and the tube attached to the machine
2e. Once the time has passed pull the cartridge out and move it up and down to make sure it is a liquid
2f. Bioink profile: Cellink GelMA 1
2g. Pre-flow: -100 ms
2h. Photocrosslinking: 405 nm
3. Layers
3a. Select concentric for infill pattern
3b. For infill density select 35%
4. On the Print screen
4a. Press the settings symbol and go to Tools - for temperature change to printing temp (27) and wait for 15 minutes for it to equilibrate
5. Once in the print screen -> go to the print option which will then take you to calibration
6. Calibrate first with be leveling and the probe in the back
7. Next manual calibration to provide the printer information on how far you want the nozzle to be from the dish while printing and in what region of the dish
7a. UV light requires it to be in the back half
8. Now the machine should be ready to print but you should check to make sure there are no bubbles in the cartridge nozzle by flicking and make sure the tip isn't clog with one of the cell link needles
9. Then you press start to do the print trial
9a. If issues arise adjust the temperature, extrusion pressure, unclog the nozzle or flick the tube
9b. If continual issues you can test the extrusion pressure with test flow but avoid doing this as it wastes a lot of product!!!

Conclusions/action items:

As the semester continues more details will be added based on UV time and distance for fibrotic and healthy states.

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02/02/2024 Bioprinting Trials 1

Nick Herbst - Feb 28, 2024, 1:29 PM CST

Title: Bioprinting Trial 1

Date: 02/02/2024

Content by: Caitríona

Present: Everyone

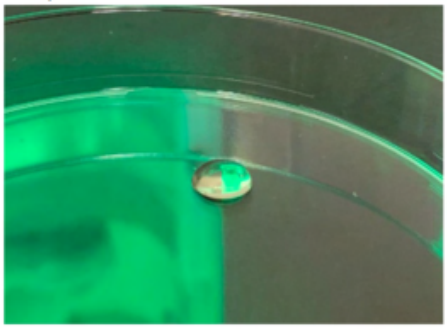
Goals: Reorient ourselves on the bioprinter and establish concrete protocol

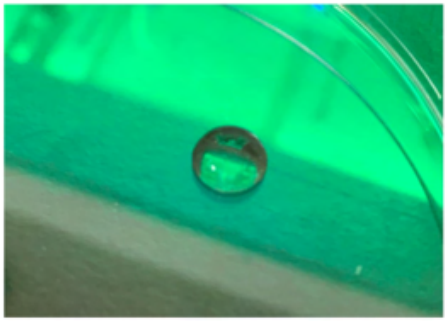
Content:

- GelMA heated to 37˚ for 45 minutes. It was confirmed that the GelMA was liquid by removing the cartridge and turning it side to side. The cartridge was put back into the temperature controlled printer head. The temperature was then decreased to 27˚ and a 15-minute interval passed to allow the GelMA to undergo the temperature change.
- Concentric cylindrical geometry, 3 mm diameter. Height of 0.6 mm. 35% infill density. 1 layer. UV time set to 50 sec. 405 nm UV wavelength.
- Successful test flow at 22 kPa. Nozzle de-clogging to remove stuck GelMA.
- First print unsuccessful - nothing came out. More de-clogging of the nozzle.
- Temperature increased to 28˚. Pressure increased to 25 kPa. Unsuccessful.
- Temperature maintained at 28˚. Pressure increased to 33 kPa. GelMA was too liquidy and the gels exceeded the desired diameter.
- UV cross-linking time changed to 15 s. Nozzle replaced. Pressure adjusted to 36 kPa. Unsuccessful, no GelMA was dispensed
- See attachment for pictures

Conclusions/action items: Two gels were printed successfully. Carley and Caitríona will return to WIMR on Monday to conduct another bioprinting trial.

Nick Herbst - Feb 28, 2024, 1:29 PM CST





Download

gel_2_2.png (290 kB)

02/05/2024 Pipette Based Hydrogel Fabrication

WILLIAM ONUSCHECK - Feb 05, 2024, 2:05 PM CST

Title: 02/05/2024 Pipette Based Hydrogel Fabrication

Date: 02/05/2024

Content by: Will

Present: Will

Goals: To fabricate non -cell laden hydrogels at healthy and fibrotic stiffness

Content:

9 full gels were produced at each stiffness condition from (950µL media, 50 µL LAP, and 50mg GelMA per batch).

3 min fridge, 5 min UV and 5 min fridge, 5 min UV were used for healthy and fibrotic gelling and swelling conditions.

Post Fab masses were taken of four gels per condition.

8 Gels per condition were stored in 1 mL media at 37 °C.

1 Gel per condition were stored in media at 4 °C for the sake of "Validating" the rheometer when testing gels.

Conclusions/action items:

Perform rheology on gels, record 24H weight weight, allow gels to begin drying.

02/05/2024 Bioprinting Trials 2

Nick Herbst - Feb 28, 2024, 1:47 PM CST

Title: Bioprinting Trial 2

Date: 02/05/2024

Content by: Caitríona

Present: Carley & Caitríona

Goals: Fabricate bioprinted gels whose stiffness values can be tested tomorrow.

Content:

- GelMA heated to 37˚ for 30 minutes. GelMA cartridge removed to verify that GelMA was liquid before decreasing the temperature to 27˚ and waiting 15 minutes. Constant cylinder dimensions of 3x0.6mm. 35% infill density. UV crosslinking for 30 s at 405 nm wavelngth, 4 cm distance.
- Temperature 27˚. Pressure 25 kPa. Pre-flow delay 1000 ms. Print did not work. The printer tip was severely clogged, so we switched to a new 22 gauge tip.
- Temperature increased to 37˚ for 3 minutes. Temperature decreased to 33˚ for 2 minutes. Pressure 27 kPa. Pre-flow delay 1000 ms. Print did not work. GelMA is visibly liquid in the tip and seems ready to print. Immediately followed this trial with the next one.
- Temperature 33˚. Pressure 25 kPa. Pre-flow delay 1000 ms. Print did not work.
- Temperature 33˚. Pressure 32 kPa. Pre-flow delay 1000 ms. Print did not work. Declogged the tip using a needle before the next trial, even though it seemed to be liquidated in the tip.
- Temperature 33˚. Pressure 32 kPa. Pre-flow delay 1000 ms. Print did not work.
- Temperature 33˚. Pressure 32 kPa. Pre-flow delay 1000 ms. Print did not work. A possible faulty variable could be that the Tool 1 of the printer is not applying pressure correctly. We switched the print head to Tool 2 which has a separate insertion point for the pressure tube of the temperature controlled print head.
- Temperature decreased to 29˚ for 4 minutes. Pressure 23 kPa. Pre-flow delay 1000 ms. Print did not work.
- Temperature 29˚. Pressure 35 kPa. Pre-flow delay 1000 ms. Print did not work. The tip was declogged using a needle, despite no bubbles or obvious clogging.
- Temperature 29˚. Pressure 40 kPa. Pre-flow delay 1000 ms. Print did not work. Declogged.
- Temperature 29˚. Pressure 50 kPa. Pre-flow delay 1000 ms. Print did not work. Declogged.
- Temperature 29˚. Pressure 25 kPa. Pre-flow delay 2000 ms. Print did not work. Declogged. Checked to verify that GelMA was, in fact, liquidated.
- Temperature 29˚. Pressure 35 kPa. Pre-flow delay 2000 ms. Print did not work. Declogged.
- Test flow at 25 kPa revealed that drop printing was functional. No issues with extrusion. Indicates that the pressure source is working.
- Temperature 29˚. Pressure 25 kPa. Pre-flow delay 2000 ms. Print did not work.
- Switched from three-dimensional structure to droplet printing with extrusion time of 1.5 s. Temperature of 29˚. Pressure15 kPa. UV crosslinking time of 30s at 405nm wavelength.
    - see attachments for photo 1
- Too much material extruded during last trial. Still droplet printing. Temperature 29˚. Pressure 20 kPa. 0.75 s extrusion. The drop that can be seen in the right-most gel was a drop that fell off of the printer tip, not an additional print trial. UV crosslinking did not occur in the correct position.
    - see attachments for photo 2
- Switched back to three-dimensional cylindrical structure printing. Temperature 29˚. Pressure 22 kPa. Pre-flow delay 5000 ms. Print did not work. Gel could be seen liquidated in the tip prior to print attempt. Declogged anyway.
- Temperature 29˚. Pressure 22 kPa. Pre-flow delay -5000 ms. Way too much GelMA was dispensed. Cancelled the print as soon as we could.

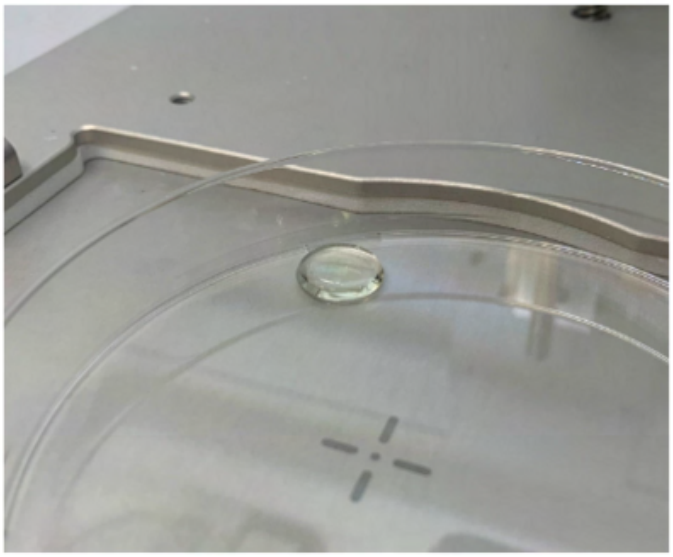
- Temperature 29˚. Pressure 22 kPa. Pre-flow delay -100 ms. Print did not work. Declogged.
- Temperature 28˚. Pressure 22 kPa. Pre-flow delay -100 ms. Dimensions too large.
    - see attachments for photo 3
- Temperature 26˚. Pressure 18 kPa. Pre-flow delay -100 ms. In the picture below, this new print is located to the right of the previous print. Size is of more accurate dimensions.
    - see attachments for photo 4

- Temperature 26˚. Pressure 18 kPa. Pre-flow delay -100 ms. Print did not work. We noticed significant bubbles. Took the cartridge out and flicked it to try to eliminate these before the next print.
- Temperature 26˚. Pressure 18 kPa. Pre-flow delay -100 ms. The resulting gel contained significant bubbles. Nozzle replaced. see attachments for photo 5 showing the new print situated between the last two structures (bubbles can be seen in this middle gel).

- Temperature 26˚. Pressure 18 kPa. Pre-flow delay -100 ms. Print did not work.

Conclusions/action items: The three gels in the final photo will be transported to the ECB Teaching Lab so that their stiffness can be tested in 24 hours. The main gel of interest is the gel with smaller dimensions and no bubbles. This was considered our most successful print, and we need stiffness values so that UV crosslinking time can be adjusted in future iterations with the same temperature and pressure values, 26˚ and 18 kPa, respectively.

Nick Herbst - Feb 28, 2024, 1:48 PM CST



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2-5_gel_1.png (120 kB)

Nick Herbst - Feb 28, 2024, 1:48 PM CST

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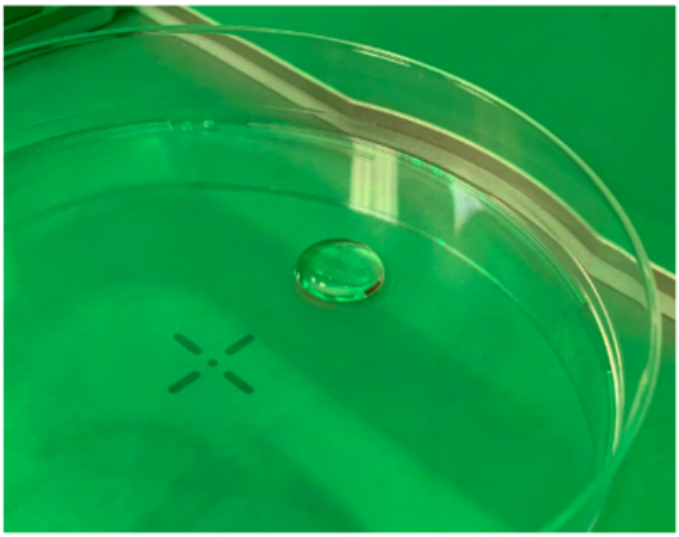
2-5_gel_2.png (228 kB)

Nick Herbst - Feb 28, 2024, 1:48 PM CST



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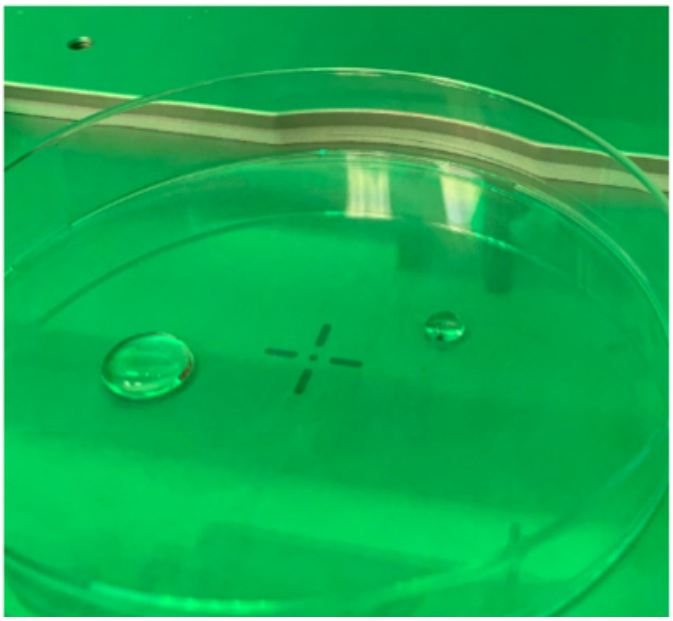
Nick Herbst - Feb 28, 2024, 1:48 PM CST



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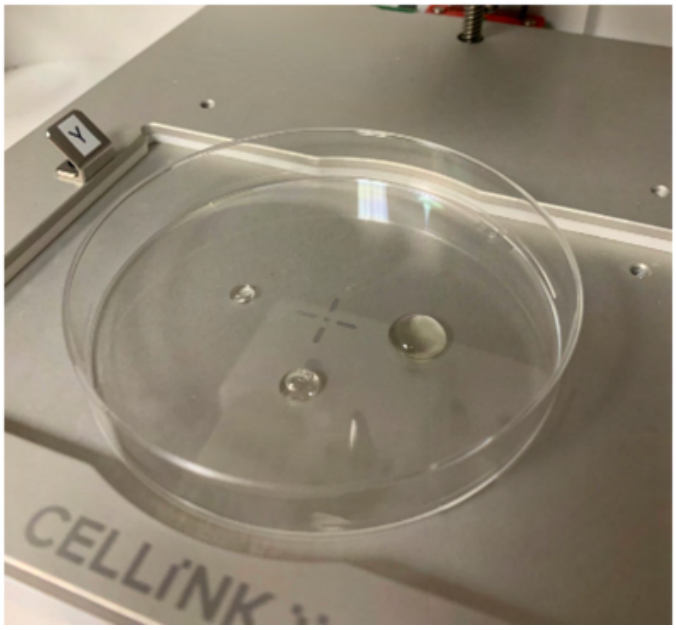
Nick Herbst - Feb 28, 2024, 1:48 PM CST



Download

2-5_gel_5.png (155 kB)

02/14/2024 Bioprinting Trials 3

Nick Herbst - Feb 28, 2024, 1:41 PM CST

Title: Bioprinting Trial 3

Date: 02/14/2024

Content by: Caitríona

Present: Caitríona

Goals: Fabricate bioprinted gels whose stiffness values can be tested tomorrow.

Content:

Unchanged Parameters for this print trial: Cylinder dimensions of 3x0.6mm. 35% infill density. UV crosslinking for 30 s at 405 nm wavelength, 4 cm distance.

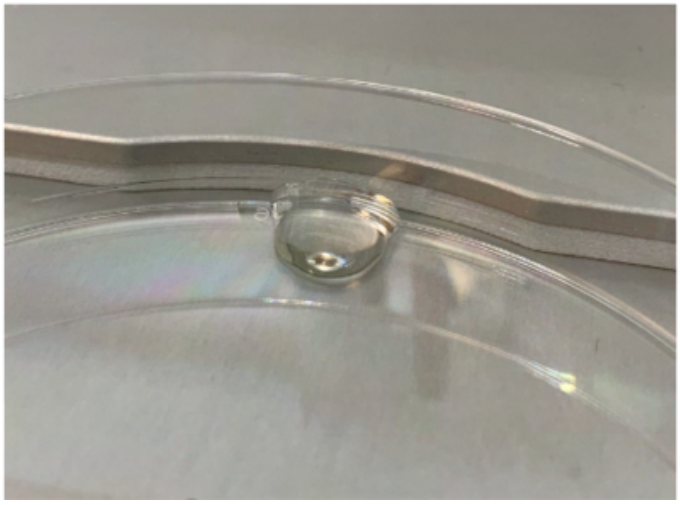
- GelMA heated to 37˚ for 30 minutes in temperature controlled printer head – tool 2. The cartridge was then removed from the printer and vortexed for 1 minute on speed 1. The cartridge was returned to the temperature-controlled printer head, where the heat was maintained at 37˚ for an additional 15 minutes. Temperature reduced to 26˚ and the cartridge was left to sit for 15 minutes. Again, the cartridge was removed from the printer and vortexed for 30 seconds on speed 1.
- Selected print setting. Calibrated.
- Temperature 26˚. Pressure 22 kPa. Pre-flow delay -100 ms. Print did not work. Declogged.
- Test flow (26˚ held constant):
  - 20 kPa – Nothing
  - 21 kPa – Success
- Temperature 26˚. Pressure 21 kPa. Pre-flow delay -100 ms. Print did not work.
  - Test flow at 22 kPa successful
- Temperature 26˚. Pressure 22 kPa. Pre-flow delay -200 ms. Print did not work.
  - Test flow 25 kPa successful. Declogged
- Temperature 26˚. Pressure 25 kPa. Pre-flow delay -300 ms. Print did not work.
- Temperature 26˚. Pressure 32 kPa. Pre-flow delay -400 ms. Print did not work.
- Temperature 26˚. Pressure 42 kPa. Pre-flow delay -400 ms. Print did not work, but a small drop of GelMA came out, staying on the tip (~1 mm in diameter). The problem seems to be related to the GelMA being too viscous and staying adhered to the GelMA in the cartridge. The temperature was increased to 29˚ for 5 minutes before attempting the next print.
- Temperature 29˚. Pressure 42 kPa. Pre-flow delay -400 ms. Print worked, but GelMA was too liquidy. Printhead temperature was decreased to 27˚, and 5 minutes passed before declogging and conducting the next print.
  - see attachments for photo 1
- Temperature 27˚. Pressure 42 kPa. Pre-flow delay -400 ms. Print was successful. Still larger than intended dimensions. Extrusion pressure should be lower.
  - see attachments for photo 2
- Temperature 27˚. Pressure 40 kPa. Pre-flow delay -400 ms. Print was successful. Dimensions still too large. Decreasing the pre-flow delay should help.
  - see attachments for photo 3
- Temperature 27˚. Pressure 40 kPa. Pre-flow delay -300 ms. Print was successful, but again lacking the correct structure.
  - See attachments for photo 4

- See attachments for photo 5: Top-down photo included of all prints for size comparison and to show the lack of cylindrical structure:

  Note: Prints are shown in order, with print number 1 on the left and print number 4 on the far right.

Conclusions/action items: Transport gels to ECB so that their stiffness values can be measured tomorrow. Attend the virtual meeting with a Cellink representative tomorrow, Dr. Brasier, and a couple members of the team. Today will serve as a documentation of the current state of the project.

Nick Herbst - Feb 28, 2024, 1:42 PM CST



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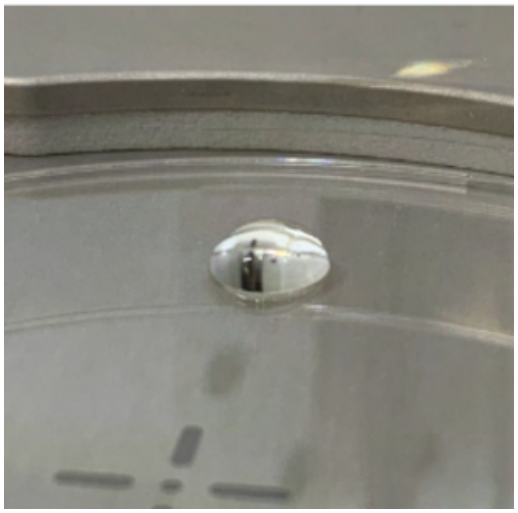
2-14_gel_1.png (176 kB)

Nick Herbst - Feb 28, 2024, 1:42 PM CST



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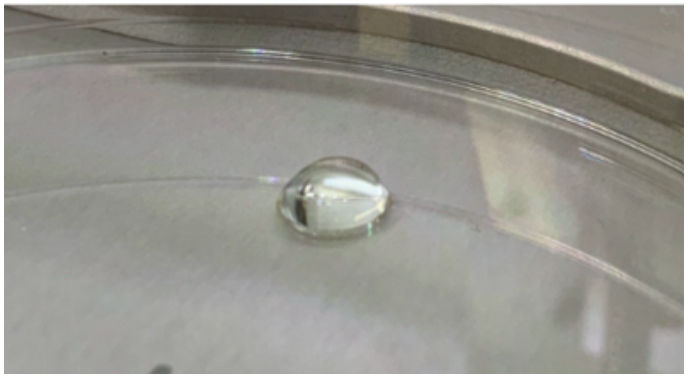
Nick Herbst - Feb 28, 2024, 1:42 PM CST



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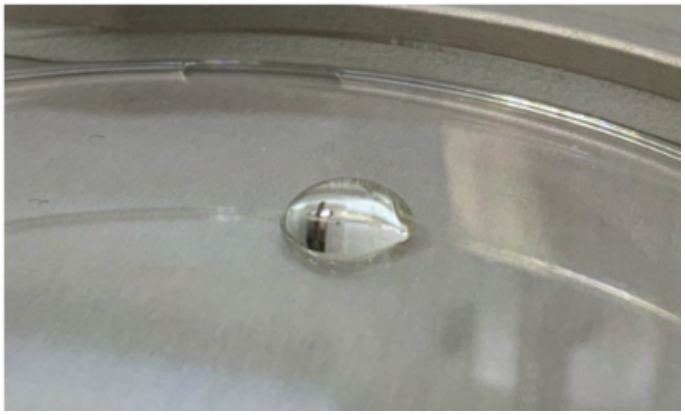
Nick Herbst - Feb 28, 2024, 1:42 PM CST



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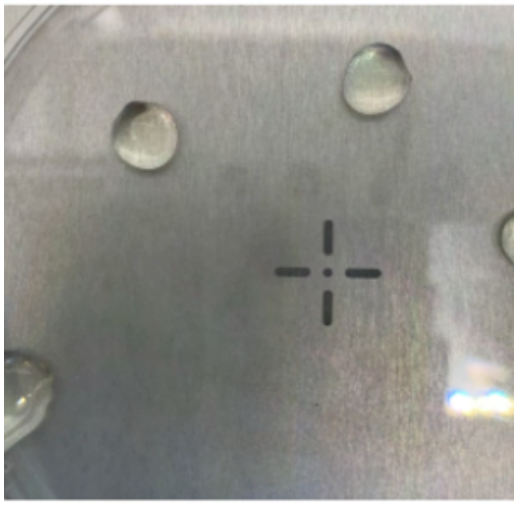
2-14_gel_4.png (166 kB)

Nick Herbst - Feb 28, 2024, 1:42 PM CST



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2-14_gel_5.png (140 kB)

02/16/2024 Bioprinting Trials 4

Nick Herbst - Feb 28, 2024, 1:36 PM CST

Title: Bioprinting Trial 4

Date: 02/16/2024

Content by: Caitríona & Carl `

Present: Caitríona, Carley & Elijah

Goals: Fabricate bioprinted gels through the application of what was learned at the meeting with Cellink Bioprinting Field Scientist yesterday.

Content:

**Today's print attempt started using a 27G nozzle (instead of 22G). This means calibration height between nozzle and printing surface was 250 microns. Infill density was therefore increased to 60%. Pre-flow delay of 100 ms. UV crosslinking for 10 s at 405 nm wavelength, 4 cm separation. Printing surface was a 6 well plate. Speed at 8 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 25.8˚ and 15 minutes passed to allow for thorough cooling.

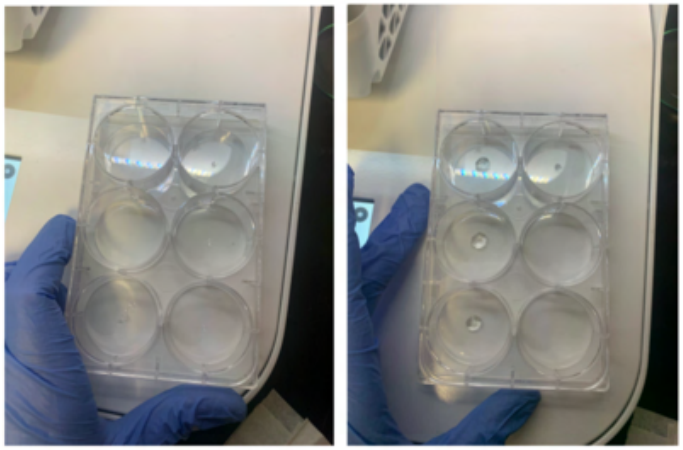
- Temperature 25.8˚. Pressure 60 kPa. The first print extruded well, but lacked shape. Infill density is too high, started forming a second layer. The next two wells did not print, suspected clogging in the nozzle. We switched to the original 22G nozzle. Specifications required re-calibration with nozzle size change; new height between nozzle and printing surface 400 microns.
- Temperature 25.8˚. Pressure 60 kPa. Infill density returned to 35%. All three prints were extruded, but again there was a lack of structural integrity. Too much material being dispensed; need to reduce extrusion pressure to be more compatible with the 8 mm/s print head speed.
- Temperature 25.8˚. Pressure 46 kPa. Infill density returned 35%. All three prints extruded, but the needle was dragging in the material again, causing more structural variation. Too much material is dispensed, but diameters are tiny.
- See attachment for photos

Now with our ability to form a structure that does not lose shape and extrude outward we will be trying a larger size cylinder: 10 mm diameter with 1 mm thickness (10x1) all previous parameters will be maintained.

- Temperature 25.8˚. Pressure 60 kPa. Small portions of GelMA were extruded but lacked structure, ran out of GelMA on the print.

Conclusions/action items: Four gels were extruded that can be tested tomorrow for stiffness values (10 second crosslinking time).

Nick Herbst - Feb 28, 2024, 1:36 PM CST



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Screenshot_2024-02-28_133547.png (427 kB)

02/21/2024 Bioprinting Trials 5

Nick Herbst - Feb 28, 2024, 1:35 PM CST

Title: Bioprinting Trial 5

Date: 02/21/2024

Content by: Caitríona

Present: Carley & Caitríona

Goals: Fabricate bioprinted gels.

Content:

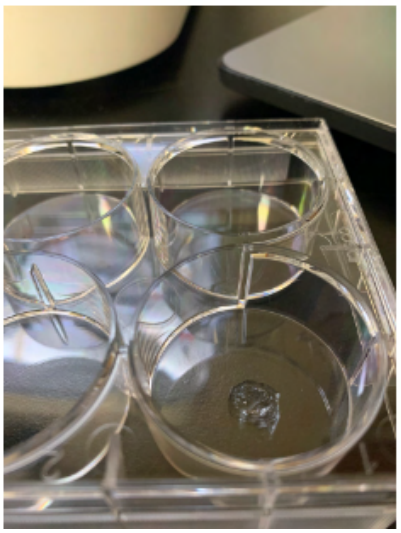
**Today's print attempt used a 22G nozzle (400 micron distance between nozzle and printing surface). Pre-flow delay of 100 ms. UV crosslinking for 10 s at 405 nm wavelength, 4 cm separation. Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 8 mm/s for all print trials. 10 mm diameter with 1 mm thickness.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 25.8˚ and 15 minutes passed to allow for thorough cooling.

- Temperature 25.8˚. Pressure 28 kPa. 35% infill density. Structure printed, but there were inconsistencies in extrusion and low infill density resulted in empty pockets in the structure.
    - See attachments for photo 1
- Temperature 25.8˚. Pressure 35 kPa. 35% infill density. The structure printed with fewer inconsistencies in the extrusion. The increased GelMA flow due to the increased pressure resulted in better a better ratio between extrusion speed and print head speed.
    - See attachments for photo 2
- see attachments for photo 3 (all 4 gels)
- see attachments for photo 4 (Close-up of surface) Surface structure is much flatter than previous prints have been. This is an advantage for future cell culture.

Conclusions/action items: Perform stiffness testing on the printed gels tomorrow.

Nick Herbst - Feb 28, 2024, 1:34 PM CST



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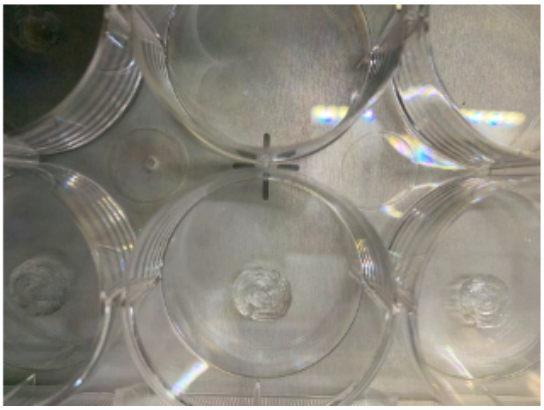
gel_2_21_1.png (133 kB)

Nick Herbst - Feb 28, 2024, 1:34 PM CST



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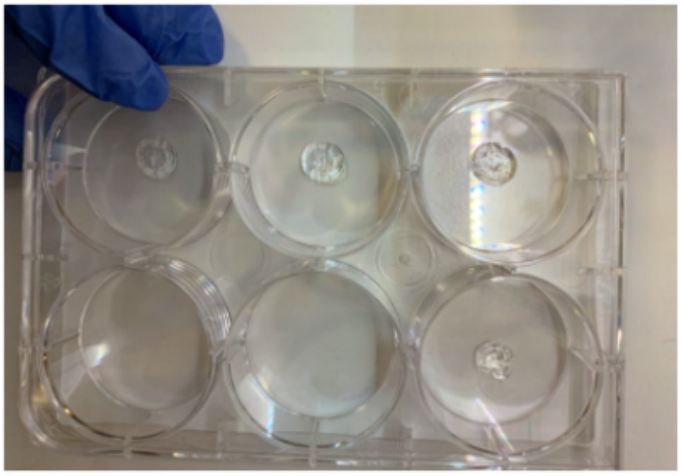
gel_2_21_2.png (124 kB)

Nick Herbst - Feb 28, 2024, 1:34 PM CST



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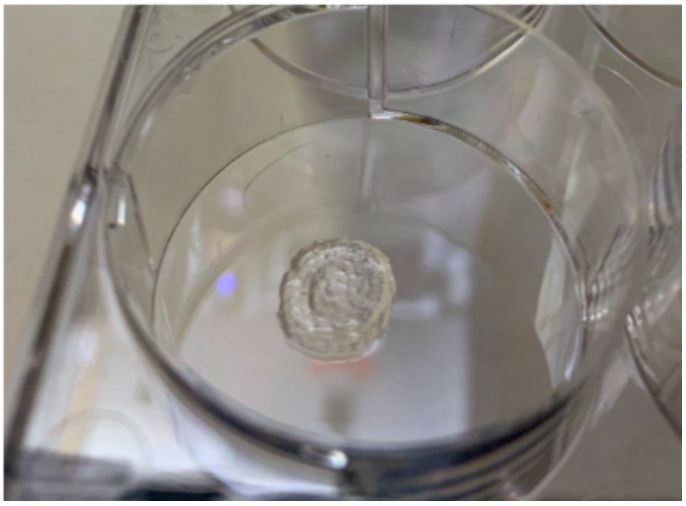
gel_2_21_3.png (176 kB)

Nick Herbst - Feb 28, 2024, 1:34 PM CST



Download

gel_2_21_4.png (185 kB)

03/3/2024 Bioprinting Trials 6

Caitriona Treacy - Apr 30, 2024, 3:31 PM CDT

Title: Bioprinting Trial 6

Date: 03/3/2024

Content by: Caitríona

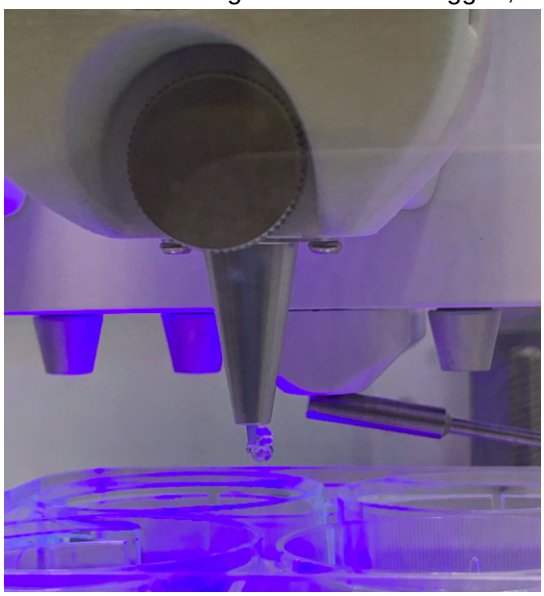
Present: Elijah, Nick, and Caitríona

Goals: Fabricate bioprinted gels.

Content:

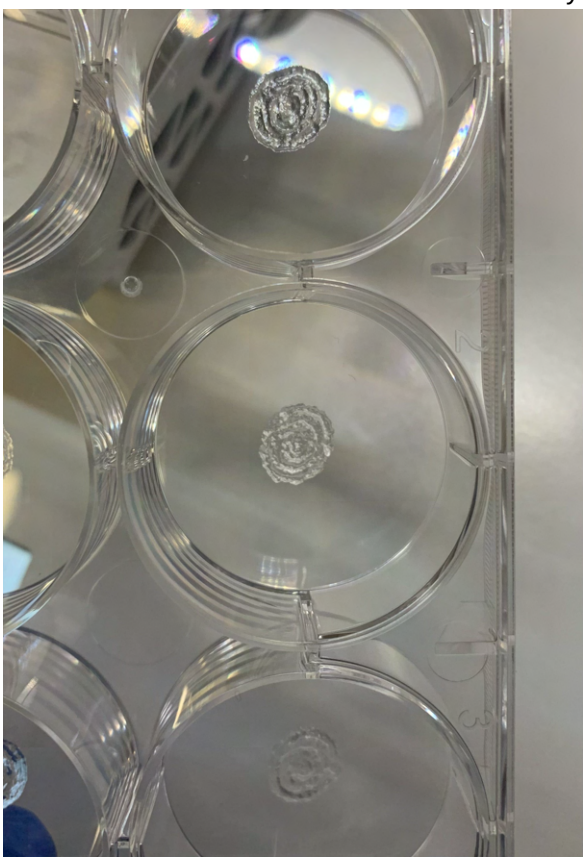
**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for 13 s at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness. Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 8 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 25.8˚ and 15 minutes passed to allow for thorough cooling.

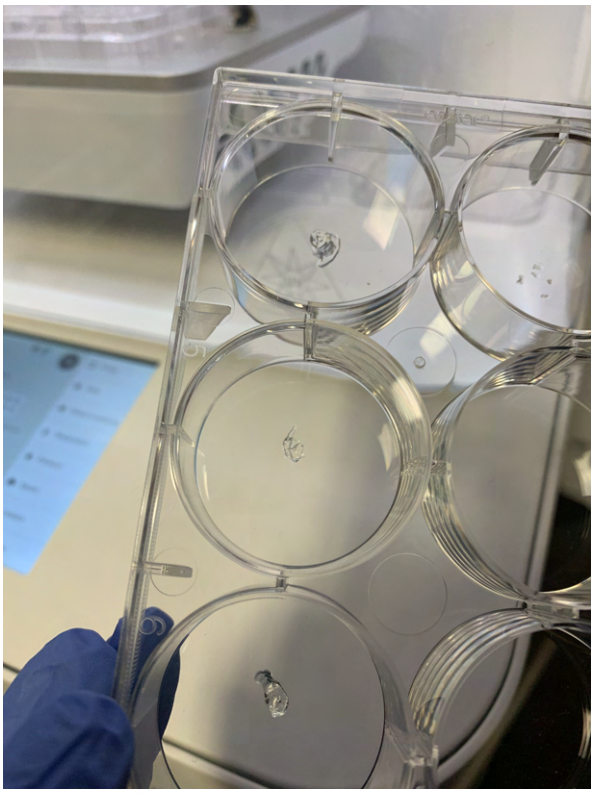
- Temperature 25.8˚. Pressure 30 kPa. 35% infill density. Pre-flow of 100 ms. First two structures printed, but with inconsistencies in the first part of the first layer. Pre-flow density will be increased during the next trial to address this challenge. The nozzle clogged, and the third print did not extrude.
- 
- Temperature 25.8˚. Pressure 30 kPa. 35% infill density. Pre-flow of 200 ms. Unsuccessful.
- Temperature 25.8˚. Pressure 30 kPa. 35% infill density. Pre-flow of 200 ms. Unclogged the nozzle and did not change any print parameters because test flow functioned properly. Unsuccessful.
- Temperature 26.1˚. Pressure 36 kPa. 35% infill density. Pre-flow of 200 ms. Extreme curling, nothing extruded onto the surface. Unsuccessful.
- Temperature 26.1˚. Pressure 35 kPa. 35% infill density. Pre-flow of 200 ms. Surface extrusion was extremely blotchy and inconsistent. Unsuccessful.

- Temperature 26.4˚. Pressure 35 kPa. 35% infill density. Pre-flow of 200 ms. Gel was globbling onto the printer tip and had a liquid character to it. Print was unsuccessful. We are recalibrating and returning to original temperature of 25.8˚ for the next print. Temperature changed, and 8 minutes passed.
- Temperature 25.8˚. Pressure 39 kPa. 35% infill density. Pre-flow of 200 ms. Same globbing problem, all GelMA collected on the tip. Tip replaced for the next trial.
- Temperature 25.8˚. Pressure 52 kPa. 35% infill density. Pre-flow of 200 ms. First gel structure looked good. The second two had large gaps and were largely incomplete.

- Temperature 25.8˚. Pressure 54 kPa. 35% infill density. Pre-flow of 200 ms.



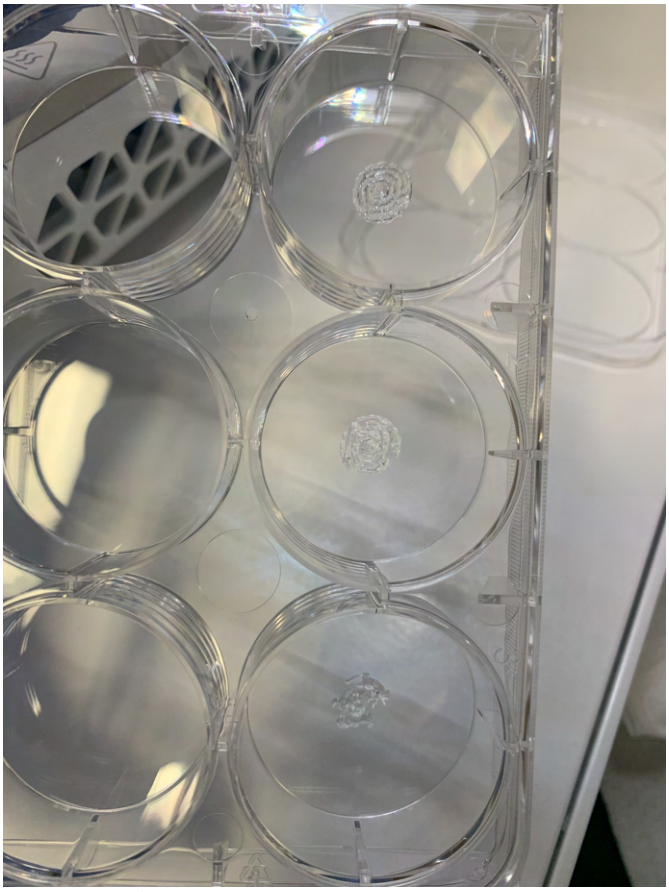
- Temperature 25.8˚. Pressure 55 kPa. 35% infill density. Pre-flow of 200 ms. First two gels were extremely blotchy, barely any extrusion. Unsuccessful.



- Temperature 25.8˚. Pressure 55 kPa. 35% infill density. Pre-flow of 200 ms. Nothing extruded. Unsuccessful.
- Temperature 26.0˚. Pressure 60 kPa. 35% infill density. Pre-flow of 200 ms. Inconsistent structures for all three gels. Filament kept fragmenting as the nozzle turned. Nozzle speed decreased for the next trial.

- Temperature 26.0˚. Pressure 58 kPa. 35% infill density. Pre-flow of 200 ms. Print head speed decreased to 7.0 mm/s. All three gels printed. Structure integrity deteriorated with each. Fragmenting of filament still

seems to be the main concern, increased temperature to address this issue and waited 8 minutes before the next print.

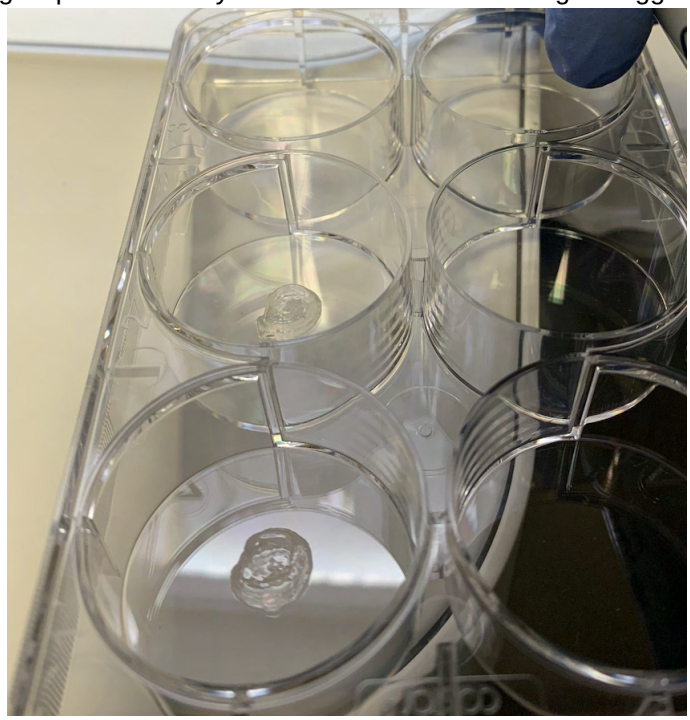


- Temperature 26.2˚. Pressure 50 kPa. 35% infill density. Pre-flow of 200 ms. Print head speed 7.0 mm/s. Terrible structures, random fragments on the surface. The filament is not maintaining continuity throughout the print. Temperature increased again, to 26.4˚, and 8 minutes passed before the next print.

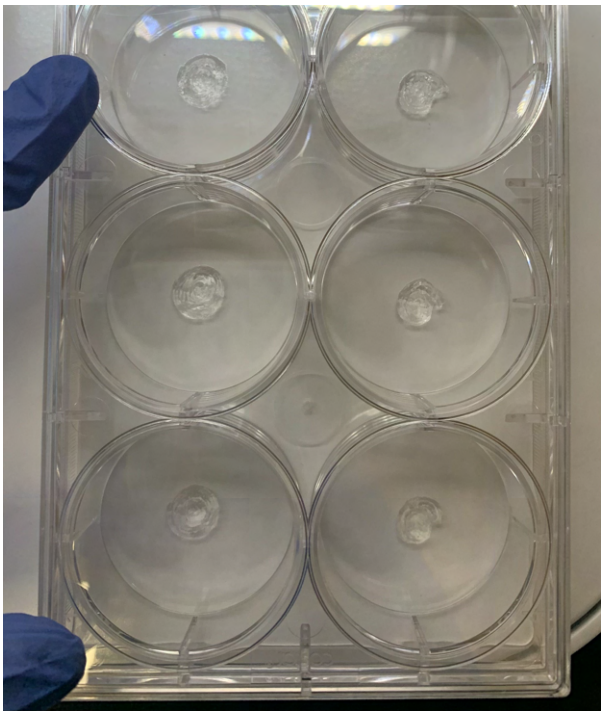
- Temperature 26.4˚. Pressure 50 kPa. 35% infill density. Pre-flow of 200 ms. Print head speed 7.0 mm/s. Fragmented structures again. Stopped print after 2 gels. Temperature increased again, and 8 minutes passed before the next print.

- Temperature 26.6˚. Pressure 60 kPa. 35% infill density. Pre-flow of 200 ms. Print head speed 7.0 mm/s. Improvement in all three structures compared to last print, still with some fragmenting happening. Increased the temperature again and allowed 8 minutes to pass before the next print.

- Temperature 26.8˚. Pressure 60 kPa. 35% infill density. Pre-flow of 200 ms. Print head speed 7.0 mm/s. All three gels printed with cylindrical structure. Small ridges suggest need for higher infill density.



- Temperature 26.8˚. Pressure 61 kPa. 40% infill density. Pre-flow of 200 ms. Print head speed 7.0 mm/s.

- 
- These six willl be left for Dianhua to perform cell seeding on.

Conclusions/action items: Perform cell seeding on the final 6 gels that were printed and use today's fabrication to update bioprinter protocols.

03/04/2024 3T3 Encapsulated Gel Fabrication Protocol

Nick Herbst - Mar 08, 2024, 3:31 PM CST

Title: 3T3 Encapsulated Gel Fabrication Protocol

Date: 03/03/24

Content by: Carley

Present: Will and Carley

Goals: Create cell encapsulated gels

Content:

GelMA	Media uL	LAP uL	Seeding Media uL	Number of Cells (million)	Number of Gels
25	425	25	50	10 (1,000,000 per gel)	4
25	425	25	50	5 (500,000 per gel)	4
25	425	25	50	2.5 (100,000 per gel)	4

cells/mL - 28.8 million cells

Hydrogel Fabrication Protocols

1. Set water bath to 50 ℃
2. Measure out 50 mg of GelMA and place in 5ml sterile tube
3. Add 850µL of embedding media and place in water bath [PBS if not using embedding media]
4. While GelMA is dissolving, prepare a 20 million cells/ml stock solution [Can vary based on cell type to be encapsulated]
5. Sanitize molds with ethanol and UV light [Maximum 5 minutes, can deform the molds]
6. Press silicone molds to a petri dish, making sure that the seal is tight to prevent polymer solution from leaking
7. Get dissolved GelMA solution from water bath
8. Moving quickly to prevent temperature dependent gelation of the GelMA solution, add 50 µL of LAP and 100 µL cell solution and mix well
9. Place 100 µL of solution into each 9mm silicone mold
10. Place gels in 4 ℃ fridge for 15 minutes [Can vary]
11. Place gels under UV light for 5 minutes
12. Place gels in 24 well plate with 400 µL of media

Conclusions/action items:

03/08/2024 Bioprinting Trials 7

Caitriona Treacy - Mar 08, 2024, 2:37 PM CST

Title: Bioprinting Trial 7

Date: 03/8/2024

Content by: Caitríona

Present: Elijah, Nick, Carley and Caitríona

Goals: Fabricate bioprinted gels.

Content:

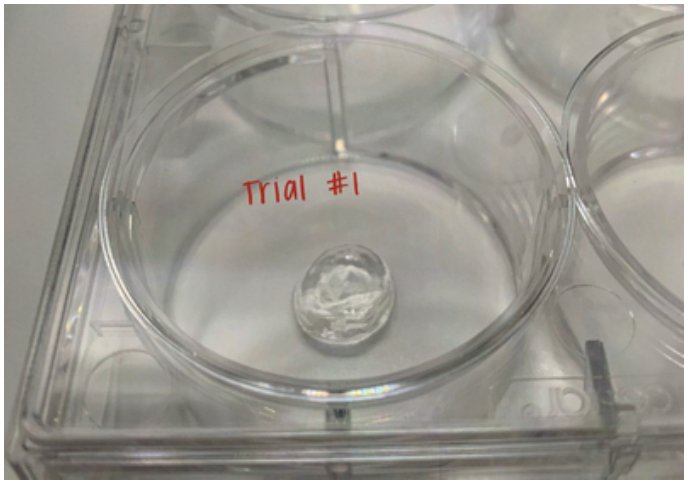
**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for 13 s at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 7.0 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 26.1˚ and 25 minutes passed to allow for thorough cooling.

- Temperature 26.1˚. Pressure 40 kPa. 45% infill density. Pre-flow of 100 ms. The first structure printed, but with inconsistencies in the first part of the first layer. The nozzle clogged, and the second/third print did not extrude.
- Temperature 26.1˚. Pressure 40 kPa. 45% Infill density. Pre-flow of 100 ms. The first structure printed the second half of the first layer and all the second layer. This specific gel appears to be a little crater shaped in the middle, but is similar in size to the first gel.
- Before this print, we recalibrated the printhead so that the 22G nozzle was not protruding as much from the insulating nozzle cover. The goal was to conceal more of the nozzle to block UV light. Temperature 26.1˚. Pressure 46 kPa. 45% Infill density. Pre-flow of 100 ms. First two structures with variable structure. That is, the final gels are filled in, but filament was breaking, leaving gaps over the duration of the print. The third gel was extremely thin and variable. Upon inspection, the GelMA cartridge was emptied completely. This should be noted as a factor that likely affected all three of these structures.

Conclusions/action items: Gels will be transported to ECB where they will be put into media and incubated for 24 hours prior to stiffness testing.

Caitriona Treacy - Mar 08, 2024, 2:30 PM CST



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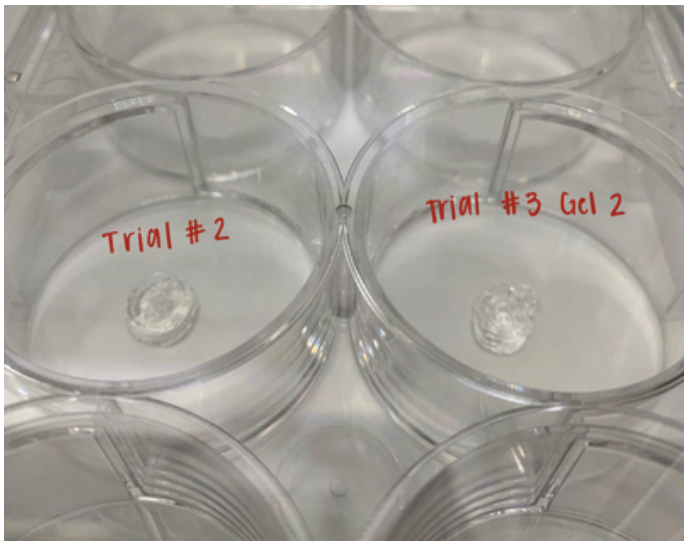
Caitriona Treacy - Mar 08, 2024, 2:31 PM CST



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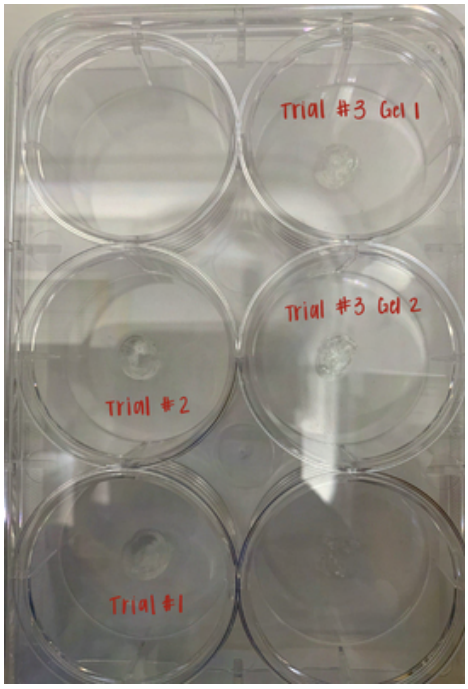
Caitriona Treacy - Mar 08, 2024, 2:31 PM CST



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04/05/2024 Bioprinting Trials 8

ELIJAH DIEDERICH (ediederich@wisc.edu) - Apr 30, 2024, 8:46 PM CDT

Title: Bioprinting Trial 8

Date: 04/05/2024

Content by: Elijah

Present: Elijah & Caitríona

Goals: Fabricate bioprinted gels.

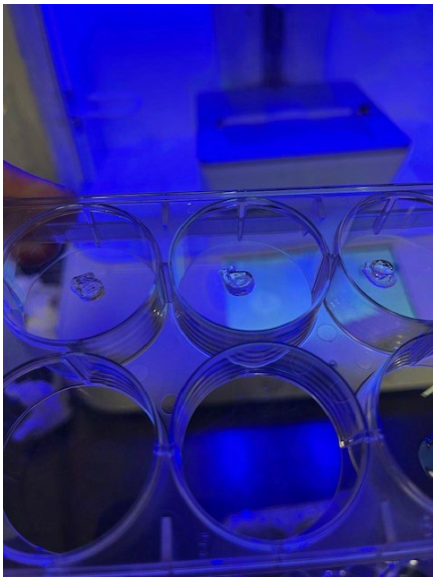
Content:

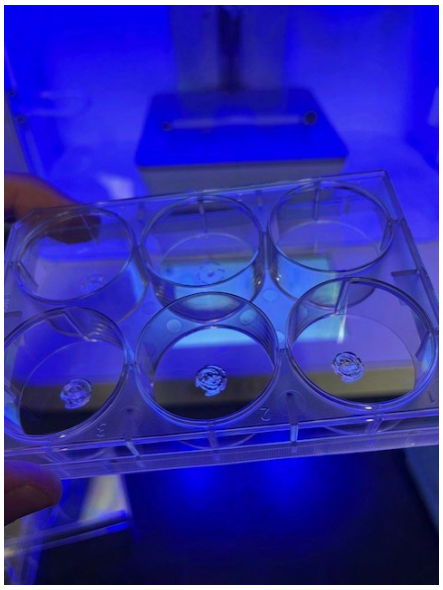
**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for 13 s at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 7.0 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 40 minutes. Temperature reduced to 26.1˚ and 25 minutes passed to allow for thorough cooling.

- Temperature _26.1_˚. Pressure _25_ kPa. _45%_ infill density. Pre-flow of _-200_ ms. Speed of __7.0 mm/s___** Test flow was very liquidy, not a gel-like consistency. Switch to 25.8 degrees celcius to fix this issue.
- Temperature __25.8__ Pressure _25_kPa. _45%_ infill density. Pre-flow of _-200_ms. Speed of __7.0mm/s_ ** First two structures have decent shape. Printer still fails to complete full circle leaving "tail" of GelMA on the first layer. Third structure first layer was very bad but second layer filled in some of the gaps. Will run one more trial with an increased pressure to try and fix problem. First two gels appear to be testable.
- In between these 2 trials, the printer refused to recalibrate. Restarted printer.
- Temperature __25.8__ Pressure _42_kPa. _45%_ infill density. Pre-flow of _-200_ms. Speed of __7.0mm/s_ ** Test flow looked ok. Not as liquidy as trial 2. Structures 1-3 on this print trial look similar to gels that we have previously printed. Very thin and the infill doesn't appear to be making a complete structure.
- Temperature __25.8__ Pressure _42_kPa. _50%_ infill density. Pre-flow of _-200_ms. Speed of __7.0mm/s_ ** Infill density increased to 50% to combat problem of gel not printing complete structure. Allowed structure 1 to print and it did not look good. Cancelled print and went home due to worries of wasting GelMA. Printing will resume soon.

After thoughts: The trial 2 structures were much better than the other trial structures in terms of infill. I am thinking this is due to the GelMA being at a more liquidy appearance as mentioned above. This allowed the infill to be greater than the set value due to greater amounts of GelMA exiting the nozzle. Trouble shooting appears to becoming much easier for the groups in terms of getting the printer to extrude. The structure however still seems to be causing us issues. I think that this is an infill density problem and we need to structure our following prints on this idea.





Conclusions/action items: Gels will be transported to ECB where they will be put into media and incubated for 24 hours prior to stiffness testing.

Cell Encapsulated Gel Fabrication 04-08-24

CARLEY SCHWARTZ - Apr 08, 2024, 5:10 PM CDT

Title: Encapsulated Gel Fabrication

Date: 04-08-24

Content by: Carley

Present: Carley & Will

Goals: To create fibroblast encapsulated hydrogels

Content:

Gel Types	24 hr, 48 hr, 7 day, and 14 day	14 day controls	Total Gels	Cells per gel
Healthy	3 +1	3 +1	10	250,000 cells
Fibrotic	3 +1	3 + 1	10	250,000 cells

We will make 10 gels in total for each condition

Conclusions/action items:

Gels were made following previously described protocols.

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04/10/2024 Bioprinting Trials

Caitriona Treacy - Apr 30, 2024, 7:03 PM CDT

Title: Bioprinting Trials

Date: 4-10-2024

Content by: Elijah and Caitriona

Present: Elijah and Caitriona

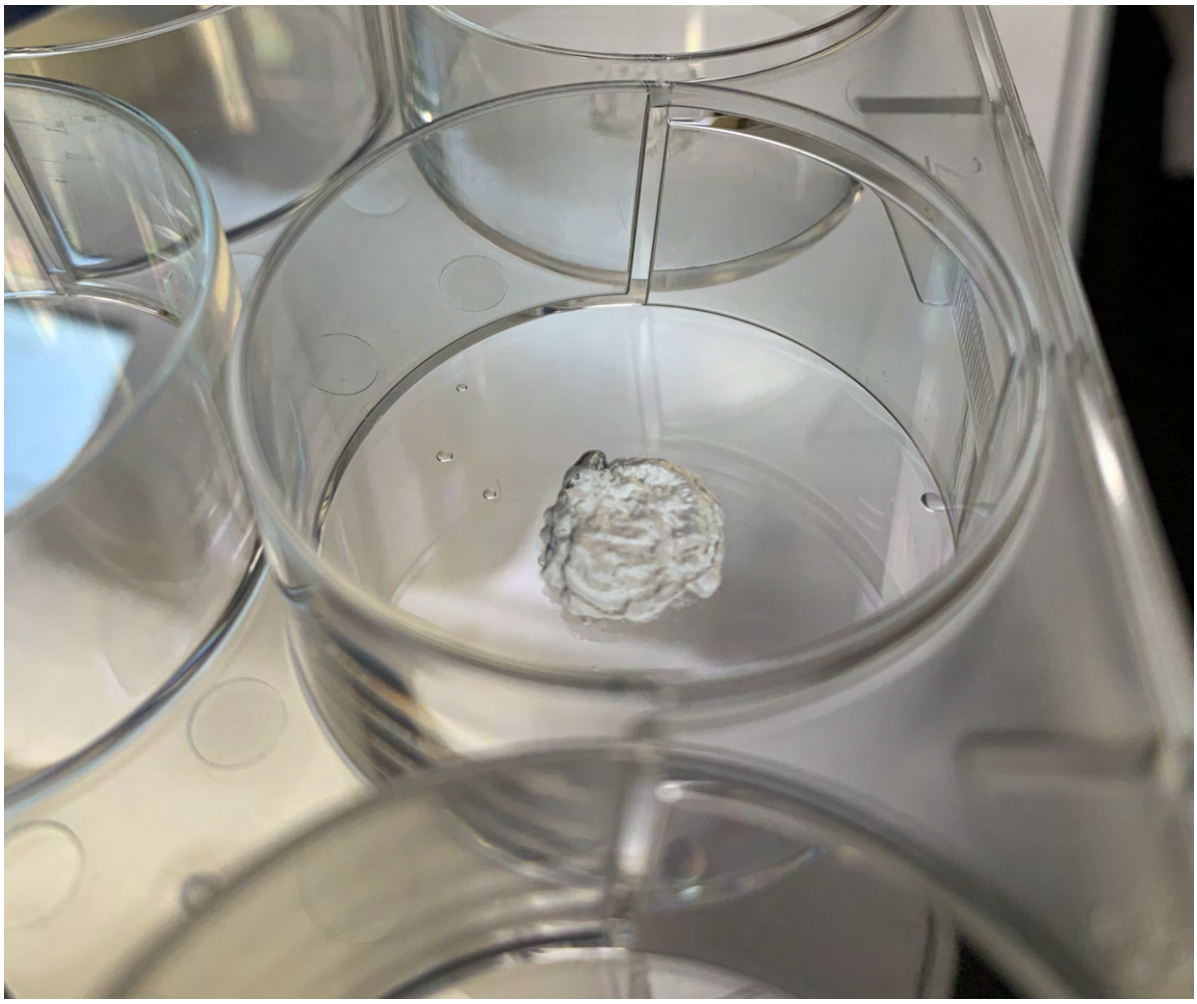
Goals: To bioprint gels and continue to try and optimize their structures

Content:

**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for 6 s at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 7.0 mm/s for all print trials.

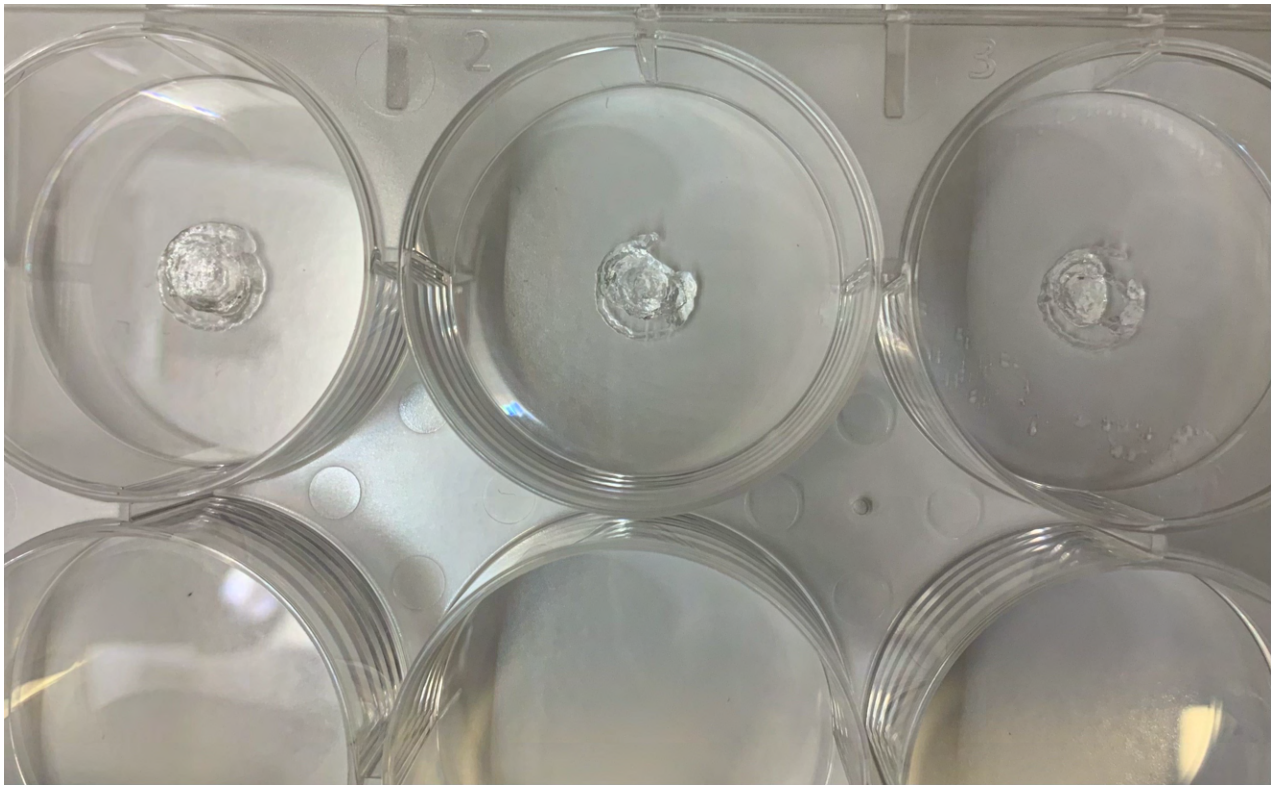
Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 26.1˚ and 25 minutes passed to allow for thorough cooling.

- Temperature 25.9˚ C Pressure _42_ kPa. 55% infill density. Pre-flow of -200 ms. ** 3 structures printed, nozzle dragged, but the gels had decent structure. Convinced that this may be due to the increased infill density, as the test flow filament looked very good (gel-like, not liquidy). Next trial will have a decreased infill density



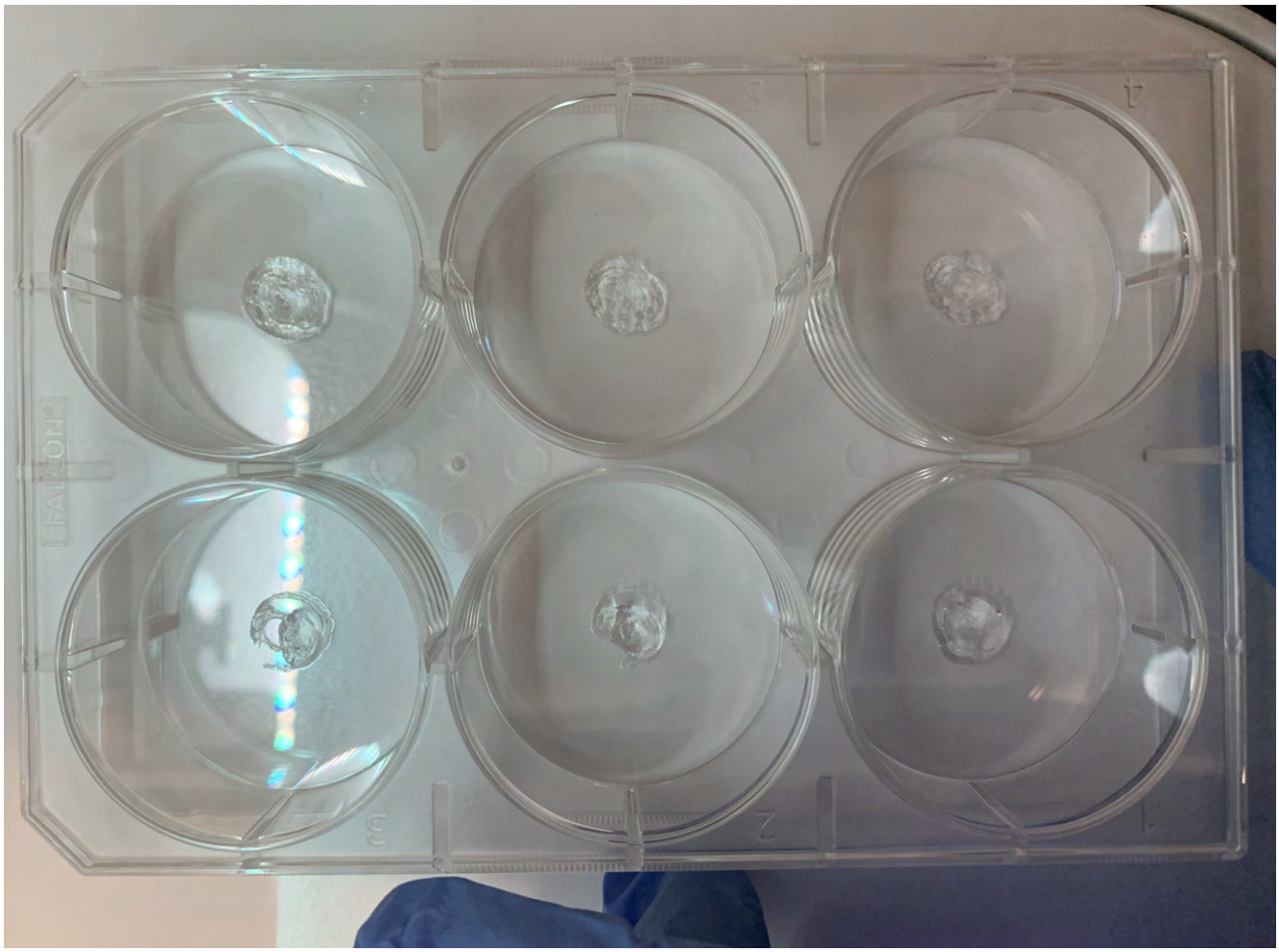
- Temperature 25.9˚ C Pressure _38_ kPa. 52% infill density. Pre-flow of -200 ms. ** First structure printed looked very good, no gaps in circle structure (maybe best structure ever printed). Second and third gel of the trial did not look great. Many gaps in structure (outer and inner rings). We think this may be due to a temperature issue where the GelMA is too thick as the printing progresses. **

- Temperature 26.1˚ C Pressure _42_ kPa. 52% infill density. Pre-flow of -200 ms. **First structure looked pretty good, had minimal gaps in the circular printing structure but worse than last trial. Second and third structure showed much more gaps and are not testable at all. GelMA appears to be gathering near the middle circles and "piling up", getting caught on previous circles printed. This causes gaps between the circular layers and untestable gels to print. Will do one more trial by decreasing the print speed, hoping to fix this issue.



For this trial below, the print speed was decreased to 6.5 mm/s.

- Temperature 26.1˚ C Pressure _42_ kPa. 52% infill density. Pre-flow of -200 ms. ** All three of these prints came out VERY thin (<1mm), but the concentric pattern was strong overall. The first one had no visible gaps in the structure, whereas there were some visible inconsistencies in the second and third, but the structure looked continuous overall in all three prints. Might think about increasing the temperature next time to keep the filament from clumping and to extrude more volume per print.



Conclusions/action items:

1. Prepare for ImageJ analysis next week

2. Finish up LIVE/DEAD imaging

04/12/2024 Bioprinting Trials

Caitriona Treacy - Apr 30, 2024, 6:30 PM CDT

Title: Bioprinting Trial Pre-Advisor Meeting

Date: 4-12-24

Content by: Elijah and Caitriona

Present: Elijah and Caitriona

Goals: To further work on optimization of structure and printing parameters

Content:

**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for _6_ seconds at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 6.5 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 26.1˚ and 25 minutes passed to allow for thorough cooling.

- Temperature 26.1˚ C Pressure _32_ kPa. 52% infill density. Pre-flow of -200 ms. ** GelMA looked a tad bit liquidy on the testflow before the print, decreased the pressure to combat this issue. First structure was very large due to temperature being too high. Print cancelled. Temperature reduced.

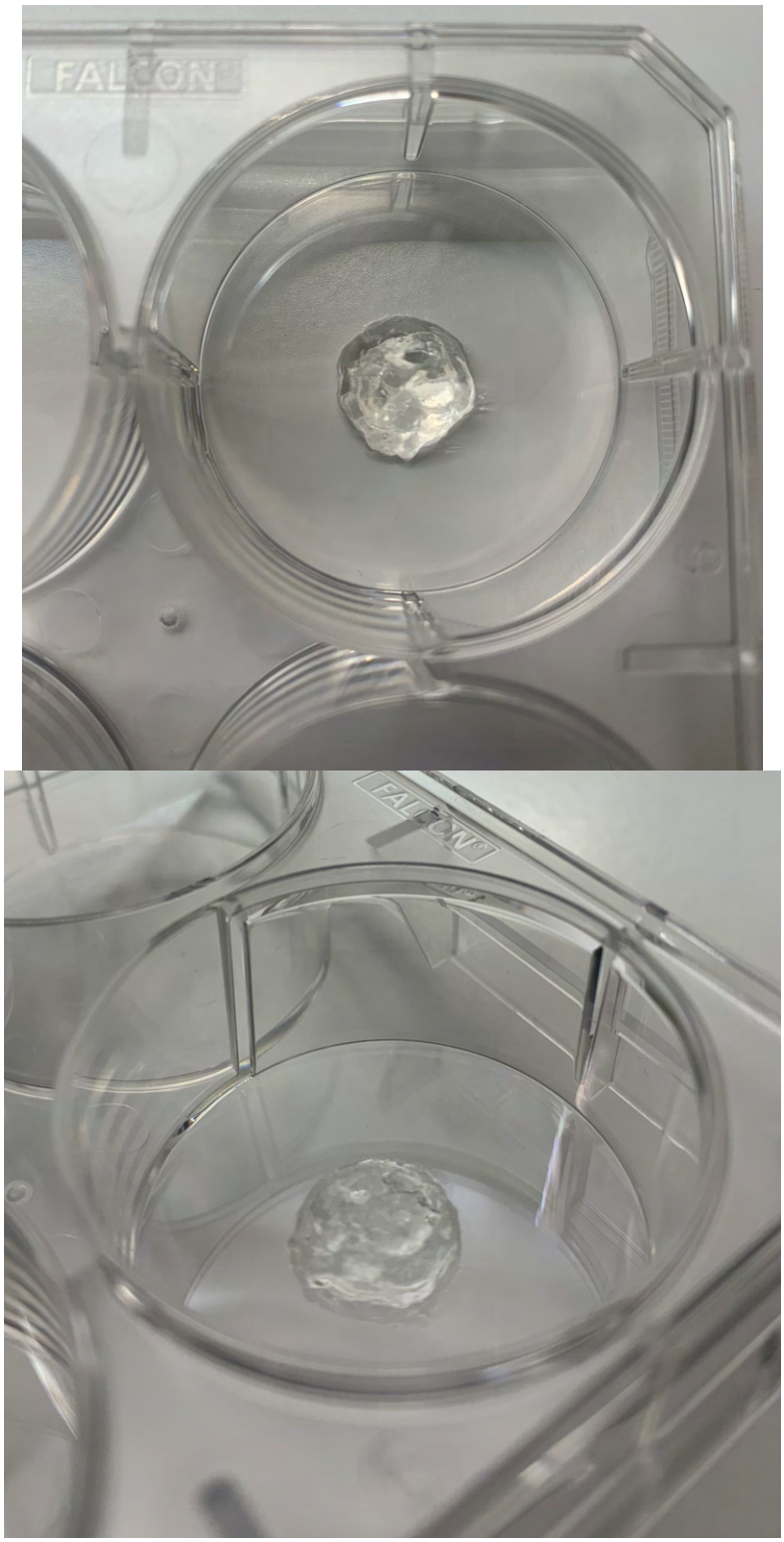


Figure 1: Top view (left) and side view (right) of the first print trial. Lacking precise structure, it looks as though the printer dispensed liquid GelMA rather than extruding filament.

- Temperature _25.9__˚ C Pressure __43_ kPa. 52% infill density. Pre-flow of -200 ms. ** Gels looked absolutely fabulous if I say so myself. I think these may be the best gels we have ever printed. Structure looked very similar throughout all three gels printed. Will try to replicate in next trial for an N=6 value for rheometry testing.

Figure 2: Top view of print 2 of the day. All three gels printed with consistent structure.

- Temperature _25.9__˚ C Pressure __43_ kPa. 52% infill density. Pre-flow of -200 ms. ** Very good trial print again, these gels appeared to be little bit thinner than the previous trial even though all parameters were maintained. We will try to print another trial with a small increase in temperature because the opening and closing of the bioprinting door may be affecting the temperature of the GelMA.

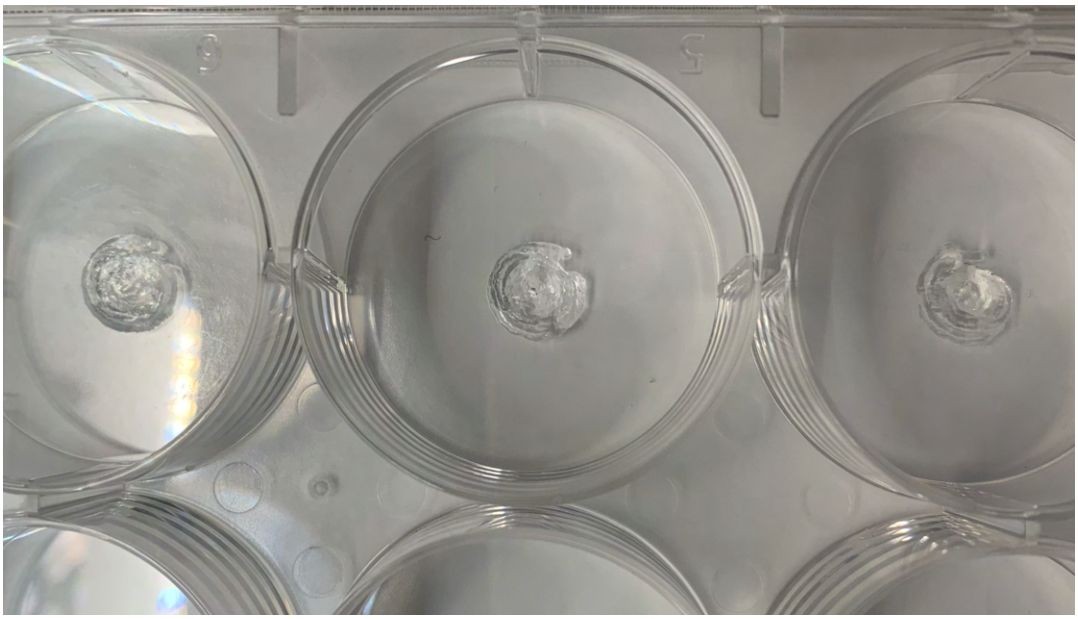


Figure 3: Top view of print 3 of the day. All three gels have consistent structure in the middle, but start to lack volume on the outer edges. There appears to be gapping occurring between concentric filaments.

- Side view of trial 2 (left) and trial 3 (right) in an effort to show the gel thickness:

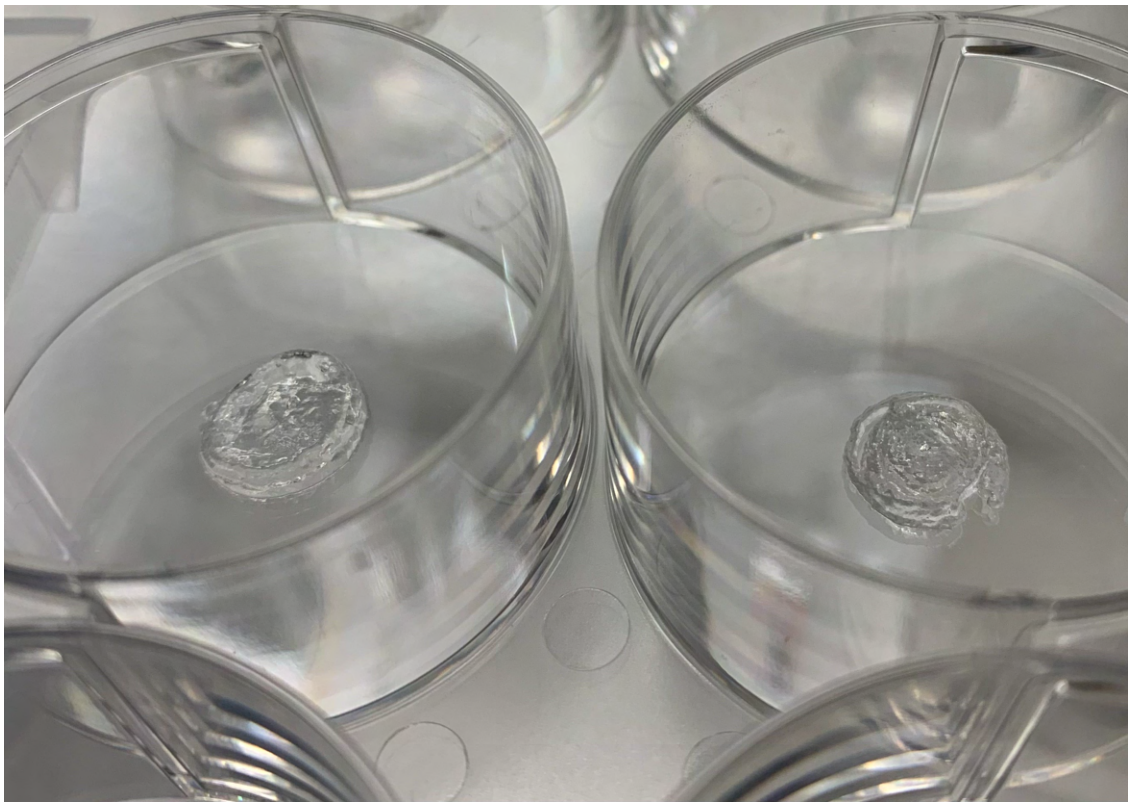


Figure 4: A side view of print 2 (left) and print 3 (right). As can be seen in the image, the gels from print 2 had a higher average thickness than those from print 3, despite the print settings being the same.

- Temperature _26.0__˚ C Pressure __42_ kPa. 52% infill density. Pre-flow of -200 ms. ** Gels did not print very well. Their structures have significant gaps for all three gels in between the circular printed rings. Temperature was increased again back to 26.1 to see if this makes a difference in structural integrity.

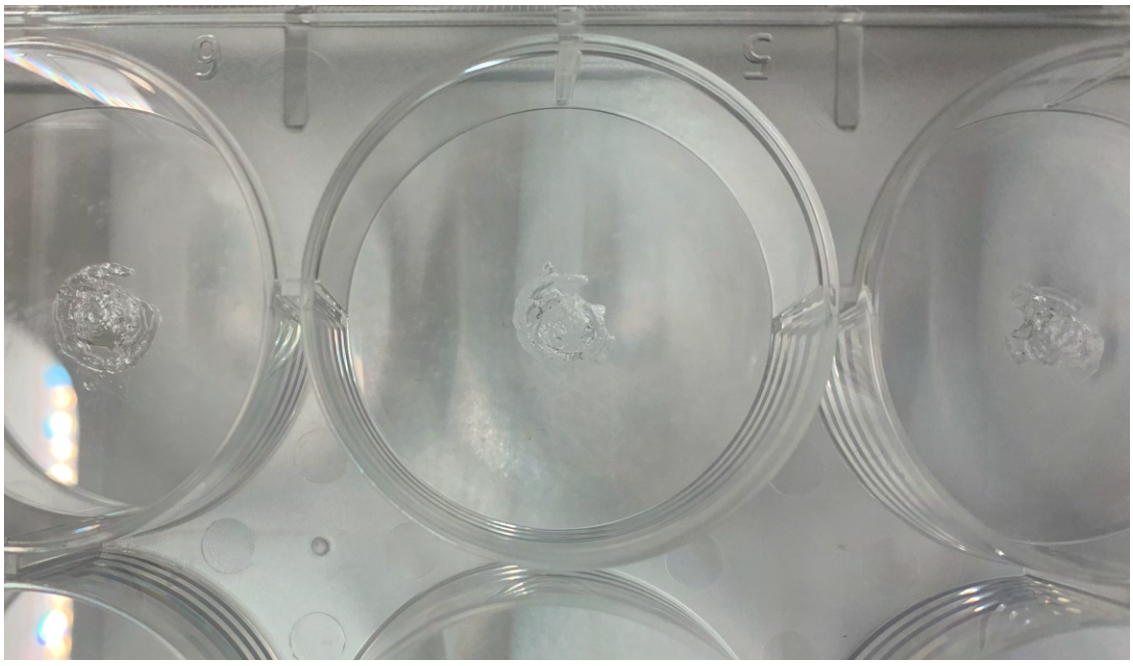


Figure 5: Top view of the gels from print 4 of the day. The structural integrity of these gels continues to decline with each print. These three gels have too much gapping between filaments to be able to run mechanical testing on them.

- Temperature _26.2__˚ C Pressure __44_ kPa. 52% infill density. Pre-flow of -200 ms. ** Did a test-flow at 26.1 degrees C and the GelMA looked thick. Increased to 26.2 degrees C. Not due to lack of effort, the 3 structures printed

were very subpar. GelMA failed to dispense from the nozzle at times, leaving holes in the circular printed structure. Upon removal of the cartridge, it was noted that very little GelMA was left in the cartridge. This likely had an influence on the final print trial and could be why so little material was dispensed. This last print resulted in one tube of GelMA being used up out of the previous 3 ordered. 2 left in the refrigerator.

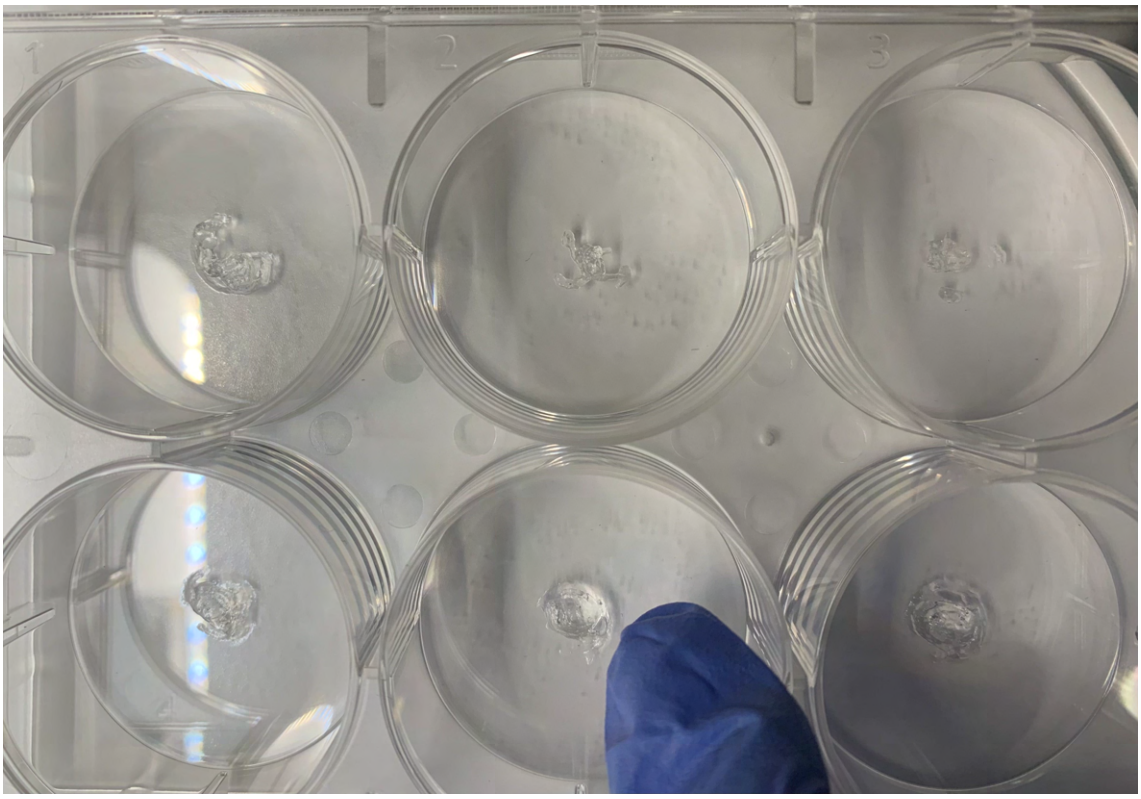


Figure 6: Top view of gels from print 5 (top row) and print 4 (bottom row). Extreme lack of volume, likely caused by low GelMA supply in the cartridge, as described above.

**** All gels printed today brought to client meeting to show advisor the inconsistencies that this printer along with the difficult reproducibility of this material ****

Conclusions/action items:

1. Advisor Meeting at 12:05, bring these new gels with to discuss latest prototypes.

2. Need to start developing multiple protocols that will lead to the greatest success

04/17/2024 Bioprinting Trials

Caitriona Treacy - Apr 30, 2024, 6:50 PM CDT

Title: Bioprinting Trials - 4/17/24

Date: 4/17/2024

Content by: Elijah and Caitriona

Present: Elijah and Caitriona

Goals: To continue to optimize bioprinter settings for the final report

Content:

**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for _4_ seconds at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 6.5 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 26.3˚ and 25 minutes passed to allow for thorough cooling.

- Temperature _25.7˚_ C Pressure __36_ kPa. _52%__ infill density. Pre-flow of -200 ms. **Test-flow was very liquidy, so the temperature was backed down to 26.1 degrees C and left to cool for 5 minutes. Test Flow #2 - Test flow #2 was also very liquidy. Temperature was bumped down again to 25.9 degrees C and left to cool for 5 minutes. Test flow #3 - still looked very liquidy. Temperature bumped down again to 25.7 degrees C and left to cool for 5 minutes. After adjusting the pressure, the test flow appeared to be more gel-like and the print commenced. All 3 gels printed had pretty decent structure and will all be able to be rheologically examined.

Figure 1: Top view of print 1. The outer ring of structure on the last two prints shows inconsistencies.

- Temperature _25.7˚_ C Pressure __36_ kPa. _52%__ infill density. Pre-flow of -200 ms. ** Test-flow with the same settings at trial 1 was very similar. The print was then started. Similar to what has been happening in the previous bioprinting sessions, the gels were visibly thinner than the first trials despite having the same settings. The first gel printed had a complete structure but was thinner. The 2nd and 3rd gel of this print lacked structure, including gaps in the circular printed rings. The first and second gels will be saved for rheology testing, while the third was discarded.

Figure 2: Top view of print 2. Structural degradation with each consecutive print.

- Temperature _26.0˚_ C Pressure __38_ kPa. _52%__ infill density. Pre-flow of -200 ms. Temperature of this print was increased to 26.0 because of the lack of structure in the previous trial. Test-flow looked like a gel-filament and the print was started. Gels are still noticeably thinner compared to trial #1. Preliminary results from previous rheology testing shows that this will have an impact on the Young's Modulus. 3 very thin structures were printed and transferred to ECB for swelling. Rheology testing will be performed 24 hours post-printing to obtain Young's Modulus.

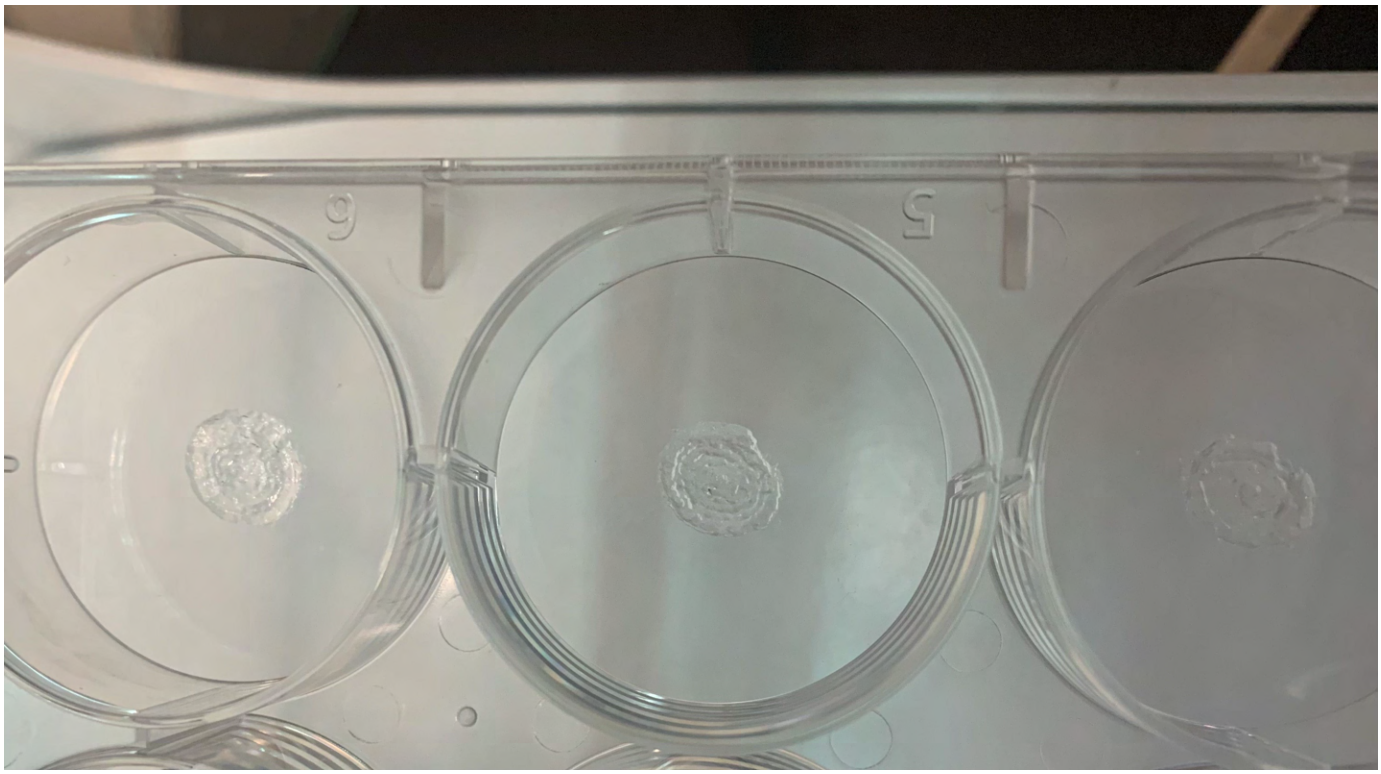


Figure 3: Top view of print 3. The concentric pattern structure looks good, but the thickness of these gels suggests that insufficient volume was being dispensed during this print (see figure 4).

- Side view of print 3:

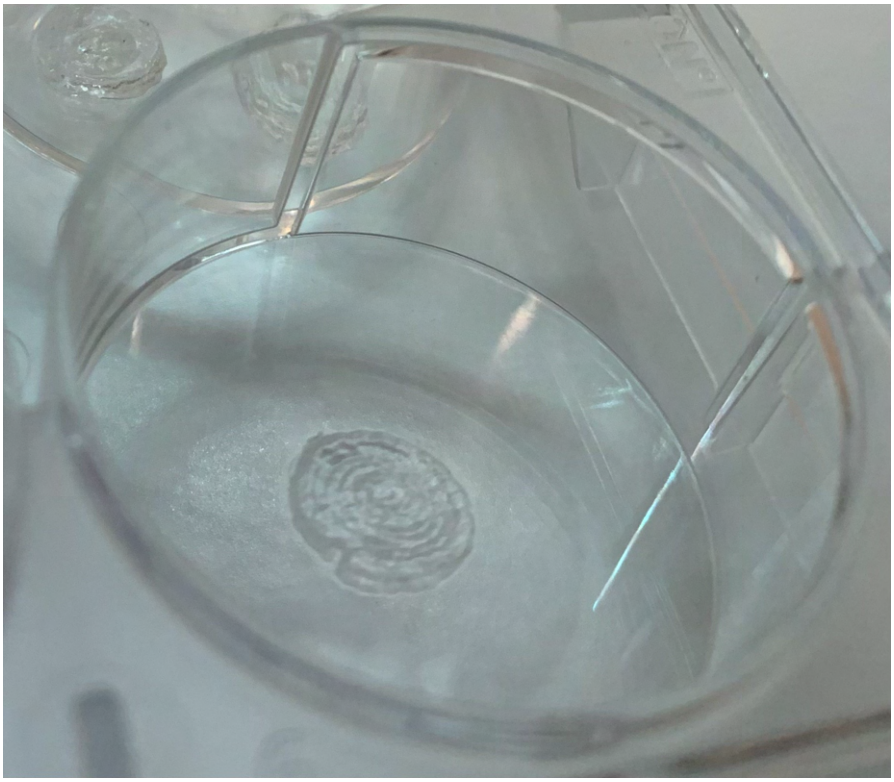


Figure 4: Side view of print 3 in an effort to show how thin this print trial was. Despite the structure thickness being set to 1 mm, these gels had a thickness of about 0.75 mm.

Conclusions/action items:

1. **Perform Rheology on printed gels**

2. **Begin to work on poster for 4/26**

04/19/2024 - Final Bioprinting Trial

Caitriona Treacy - Apr 30, 2024, 7:00 PM CDT

Title: Pre-Advisor Meeting Bioprinting

Date: 4-19-24

Content by: Elijah and Caitriona

Present: Elijah and Caitriona

Goals: To print gels in the healthy lung tissue range for poster

Content:

**Print attempt using a 22G nozzle (400 micron distance between nozzle and printing surface). UV crosslinking for _2 (Not for trial 1; see below)_ seconds at 405 nm wavelength, 4 cm separation. Cylinder structure dimensions: 10 mm diameter with 1 mm thickness (layer thickness = 0.51 mm). Two layers are included in this print structure thickness. UV crosslinking occurred only once, at the end of the print, rather than between layers. Printing surface was a 6 well plate. Speed at 6.5 mm/s for all print trials.

Printer overhead light reduced to 20%. GelMA cartridge placed in temperature controlled print head, set at 37˚ for 45 minutes. Temperature reduced to 26.3˚ and 25 minutes passed to allow for thorough cooling.

- Temperature _25.9˚_ C Pressure _35_ kPa. _52%__ infill density. Pre-flow of -200 ms. ** Test flow looked good at 25.9˚ C compared to 26.1˚ C. Three gel structures were able to get printed but were pretty large. This may be due to a temperature issue, so we will compensate for this during the next trial. The UV time also appeared to be __6__ seconds for this trial, even though the UV time was set to _2_ seconds. This was reflected as being 6 seconds and the print was exited to hopefully solve this issue.

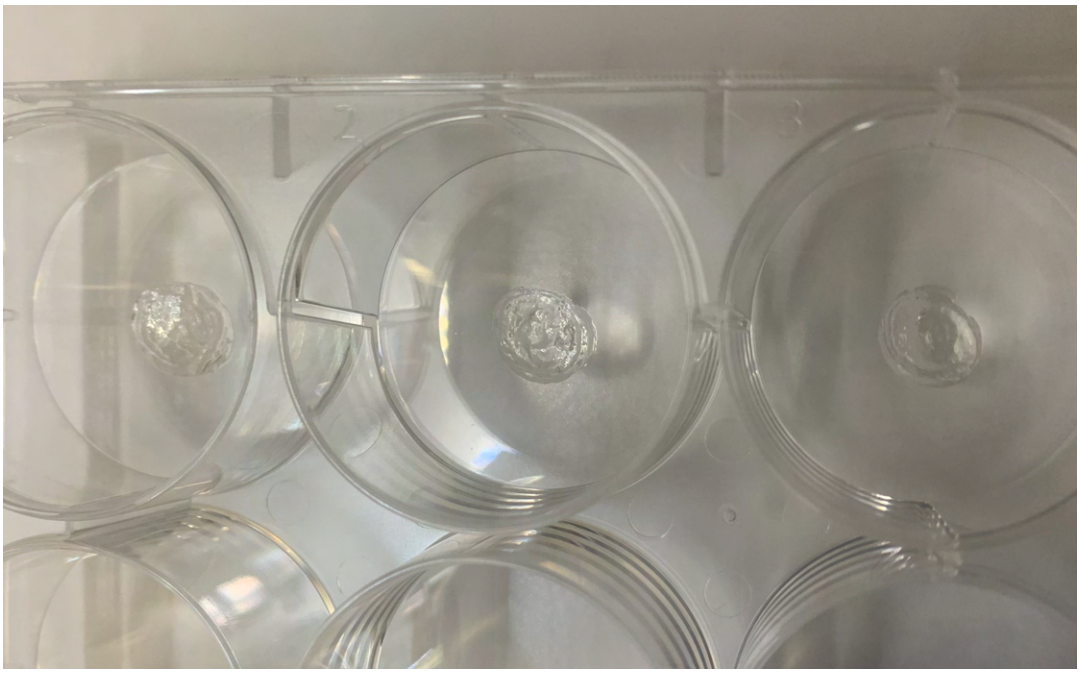


Figure 1: Top view of print 1. All three gels printed with structures lacking gaps, but diameters exceed 10 mm.

- Temperature _26.0˚_ C Pressure _46_ kPa. _52%__ infill density. Pre-flow of -200 ms. ** Test flow looked great, first 2 gels printed looked great, but the last one appeared to be getting thicker. Temperature increased by 0.1 degrees C for next trial. This UV time appeared to be 2 seconds. We will do one more trial so that we have more data for this UV time.

Figure 2: Top view of print 2. Loss of structural integrity on the third gel off this print, observed to likely be due to an increase of viscosity in the GelMA as the print progressed.

- Temperature _26.3˚_ C Pressure _45_ kPa. _52%__ infill density. Pre-flow of -200 ms. ** After a bit of tinkering with the pressure and temperature, the test-flow looked good (gel-like filament) and the print was started. First gel printed looked good, all the circular printed motions looked filled in with no gaps. The second and third gel were a bit worse, having some gaps in the circular printed layers.

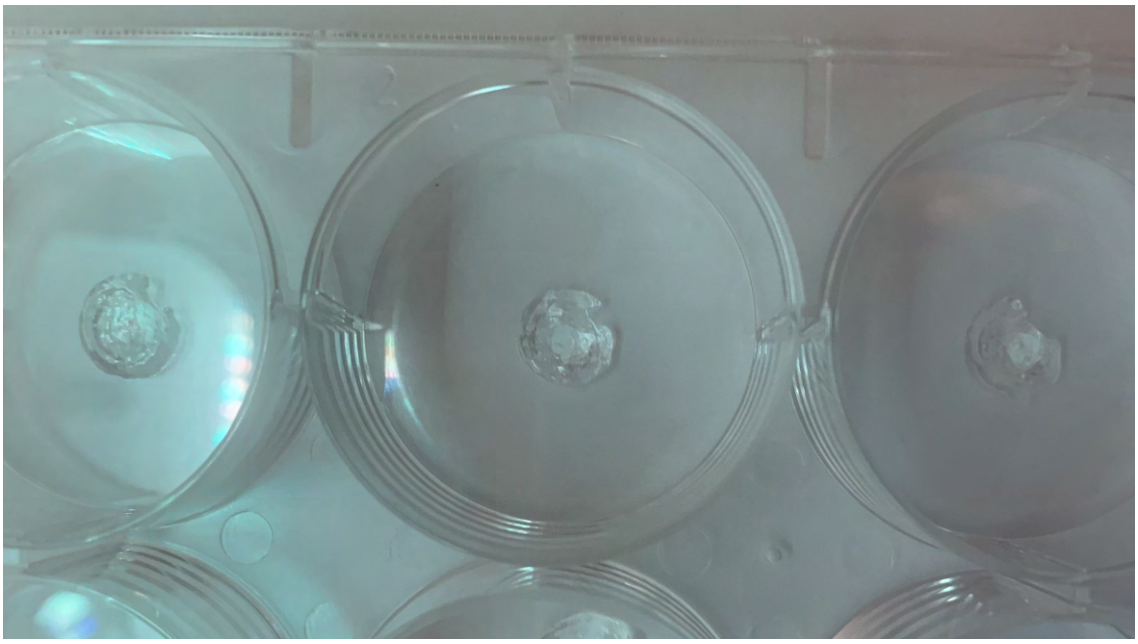


Figure 3: Top view of print 3. Gaps between GelMA filaments started to become apparent in gels 2 and 3 of this trial.

Conclusions/action items:

1. Work on poster presentation

2. Gather rheometry results for 2 seconds UV

03/12/2024 Cell Encapsulation for LIVE/DEAD troubleshoot

WILLIAM ONUSCHECK - May 01, 2024, 11:01 AM CDT

Title: Cell Encapsulation for LIVE/DEAD troubleshoot

Date: March 12th, 2024

Content by: William

Present: William

Goals: To fabricate gels to troubleshoot LIVE/DEAD staining's washout problem

Content:

Cells

Conclusions/action items:

Use gels to troubleshoot LIVE/DEAD Staining protocol as described in "Design of Troubleshooted LIVE/DEAD Staining Protocol"

02/09/2024 Preliminary LIVE/DEAD Staining Protocol

Nick Herbst - Mar 08, 2024, 3:28 PM CST

Title: Preliminary LIVE/DEAD Staining Protocol

Date: 02/09/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Establish protocol for LIVE/DEAD staining

Content:

Materials:
1. LIVE/DEAD Viability Kit (Thermofisher) containing calcein AM and ethidium homodimer-1
2. 15mL conical tube
3. 20µL pipette + tips
4. 1000µL pipette + tips
5. 10mL serological pipette
6. Phosphate-buffered saline (PBS)
7. Fluorescence microscope

Method:
1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add 5µL calcein AM and 20µL ethidium homodimer-1 to the PBS
a. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cells and wash twice with PBS
4. Add 200µL of staining solution directly to cells
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
a. Use the FITC filter for calcein-AM (live cells)
b. Use the RFP filter for ethidium homodimer-1 (dead cells)

Conclusions/action items:

Use this protocol to image the fibroblasts encapsulated in GelMA hydrogels. Cell viability can be determined by comparing the number of calcein AM positive cells to the number of ethidium homodimer-1 positive cells

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02/27/2024 Rheology Protocol

Nick Herbst - Feb 27, 2024, 10:42 AM CST

Title: Rheology Protocol

Date: 02/27/2024

Content by: Elijah Diederich

Present: Myself

Goals: To inform fellow notebook readers about the rheology protocol used

Content:

Frequency Sweep Rheometry Protocol

1. Once GelMA hydrogels have been formed and allowed to set and swell for approximately 24 hours, rheometry testing may be performed.
2. Carefully remove 3-4 hydrogels of each type; healthy lung ECM and fibrotic lung ECM, keeping the gels of the same type in their respective petri dish.
3. Once gels are in two separate weighing dishes, make your way over to the rheometer testing machine (Malvern Rheometer - Kinexus Ultra+)
4. Make sure that the bottom plate is locked on the rheometer by pushing the level, located on the front of the machine below the bottom parallel plate, all the way to the right
5. Open rSpace application on the computer and when prompted to select a certain test, select the 0035 test; Frequency Sweep Strain controlled.
6. When this specific test is selected, the user will then be prompted to enter a Gap value. This value will pertain to the thickness (mm) of the hydrogel being tested. Center the hydrogel on the bottom parallel plate. Measure the thickness (mm) of the hydrogel and enter the gap value. The upper plate will then move to this gap value.
7. Once making sure that the upper plate makes contact with the top of the hydrogel and the thickness is the correct value, enter values for various testing parameters such as room temperature, start frequency, end frequency, shear strain %, and samples per decade. In this specific test, the values were as follows: Start Frequency = 0.1 Hz, End Frequency = 10 Hz, Room Temperature = 25 ℃, shear strain = 1%, and 10 samples per decade.
8. Once the various testing parameters are entered, the user will then be able to start the test. A 5 minute calibration will be performed before the actual test begins. Once this calibration has been completed, the frequency sweep test will take approximately 10 minutes.
9. When the test is completed, the results table can be copied into an excel spreadsheet. Enter a gap value that is greater than the thickness of the hydrogel to remove the hydrogel from the machine. Clean upper and lower parallel plate surfaces with ethanol.
10. Repeat steps 5-9 for remaining hydrogels. In this specific test, 3-4 hydrogels of each type were tested for a total of 6-8 separate frequency sweeps.
11. When testing is completed, results can be interpreted in Excel.

Conclusions/action items:

1. Proofread prelim report sections

03/12/2024 Improved LIVE/DEAD Staining Protocol

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Nick Herbst - Mar 12, 2024, 2:16 PM CDT

Title: Improved LIVE/DEAD Staining Protocol

Date: 03/12/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Rewrite protocol for LIVE/DEAD staining with more in-depth information

Content:

Materials:

- 15mL conical tube
- 20µL pipette + tips
- 1000µL pipette + tips
- 10mL serological pipette
- Phosphate-buffered saline (PBS)
- Fluorescence microscope
- LIVE/DEAD Viability Kit (Thermofisher) containing calcein AM and ethidium homodimer-1 (EthD-1)
    - Store in a -20 ºC freezer protected from light
        - Either keep the reagent vials in a box or wrap them in foil
    - Before using the stock solutions, allow them to thaw to RT and centrifuge them briefly before opening them
        - Fluorophores can aggregate overtime
    - Calcein AM hydrolyzes when exposed to moisture, so be sure to keep it away from water (stock solution is in anhydrous DMSO)
    - Aqueous working solutions of calcein AM should be prepared immediately prior to use and must be used within one day
    - Ethidium homodimer-1 is not sensitive to moisture; Aqueous working solutions of it can be stored at -20 ºC for up to one year
    - Before refreezing stock solutions, seal all vials tightly

Fluorescent Dye Concentration Optimization:

1. Prepare samples of live and dead cells
    1. Dead cells can be obtained by killing cells with 70% methanol for 30min
2. Use samples of dead cells to optimize the EthD-1 concentration
    1. Dilute the EthD-1 in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting EthD-1 to 0.1 - 10µM
    2. You want the lowest EthD-1 concentration which stains the dead cell nuclei bright red with minimal cytoplasmic staining
3. Use a sample of dead cells to start to optimize the calcein AM concentration
    1. Dilute the calcein AM in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting calcein AM to 0.1 - 10µM
    2. You want the lowest calcein AM concentration which gives minimal cytoplasm staining
4. Use a sample of live cells to confirm the optimization of the calcein AM concentration
    1. Dilute the calcein AM in PBS to the same concentration that used in the prior step, add it to live cells, incubate for 30min, then image
    2. See if the used concentration gives a sufficient green stain in live cells
        1. If not, try a higher concentration on another sample of live cells

LIVE/DEAD Staining:

1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add enough calcein AM and ethidium homodimer-1 to the PBS to achieve the concentrations determined in optimization process
    1. Anecdotally, 20µL of EthD-1 and 5µL of calcein AM in 10mL of PBS gives concentrations of 4µM and 2µM, respectively, and is suitable for 3T3 cells
    2. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cell-laden hydrogels and wash with PBS

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4. Add 200µL of staining solution directly to the hydrogels
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
    1. Dim the lights in the working space and keep the gels covered whenever not imaging
    2. Use the FITC/GFP filter for calcein-AM (live cells)
    3. Use the TRITC/RFP filter for ethidium homodimer-1 (dead cells)

Tips:

- If you have extracellular fluorescence, **do additional and/or longer washes with PBS** prior to staining because media can have esterases which hydrolyze the calcein AM to cause fluorescence
    - Calcein AM is calcein acetoxymethyl ester, and it functions by permeating live cell plasma membranes and then getting hydrolyzed by intracellular esterases which releases the fluorescent calcein
- Be sure to dim the lights of the work space and have cells covered in foil when not actively adding something or imaging
    - The fluorescent dyes are photosensitive

Conclusions/action items:

Use this protocol to image the fibroblasts encapsulated in GelMA hydrogels. Use the Cell Viability Image Analysis protocol to analyze the captured fluorescent images

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03/20/2024 Cell Viability Image Analysis Protocol

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Nick Herbst - Apr 09, 2024, 11:37 AM CDT

Title: Cell Viability Image Analysis Protocol

Date: 03/20/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Write step-by-step protocol for using ImageJ to analyze the LIVE/DEAD staining images to quantify cell viability

Content:

Materials:

- Computer
- ImageJ software with Bio-Formats plugin package
- Fluorescent LIVE/DEAD staining images

Methods:

1. Capture a fluorescent image and save the file with both the green (live) and the red (dead) channels
    1. If a Nikon fluorescence microscope was used, the file will save as a .nd2 \
2. Open the file in ImageJ
    1. File > Open > select the .nd2 file
    2. In the Bio-Formats Import Options window, set “View stack with:” to “Hyperstack” and “Color mode:” to “Default”
    3. Click “OK”
3. Split the image such that each channel is its own image
    1. Image > Color > Split Channels
    2. Close the blue channel since only the green and red channels will be used
4. Change the images’ type
    1. Image > Type > 8-bit
5. Eliminate some background noise by adjusting the brightness and contrast
    1. Image > Adjust > Brightness/Contrast
    2. A window will pop up with a pixel intensity histogram
        1. Move the “Minimum” slider to the right until the image looks cleaner without losing any important signal
        2. If necessary, move the “Maximum” slider to the left to increase the intensity of the staining
        3. When done, click “Apply”
    3. Repeat B/C adjustment for the other image
    4. **Keep B/C settings constant between different images of the same channel**
        1. All green images should have same settings and all red images should have same settings, but green and red images don't have to have the same settings
6. Convert the gray-scaled images into binary images via Thresholding
    1. Image > Adjust > Threshold
        1. Make sure dropdown menus say “Default” and “Red” and that “Dark background” is checked
    2. Move the top slider (the left bound of the the threshold) so that only the stained cells are highlighted in red
    3. Thresholding is a *critical* step
        1. Too high of a threshold results in losing a lot of signal
        2. Too low of a threshold will fuse cells close to each other and include background noise, making counting more difficult
    4. Click “Apply” to get a binary thresholded image
    5. Repeat thresholding for the other image
7. On both images, separate fused cells
    1. Process > Binary > Watershed
8. Set up the measurements window
    1. Analyze > Set Measurements
    2. Make sure “Area” is checked

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3. Click “OK”

9. Establish inclusion criteria for analysis
    1. Analyze > Analyze Particles
    2. Check “Add to Manager” and “Include holes”
    3. Set “Size” to “0-Infinity” and “Circularity” to “0.00-1.00”
    4. Click “OK”
    5. With the ROI Manager open, click on a ROI that is the smallest but is still clearly a stained cell, then click “Measure” in the window to get its area in μm^2
    6. Record this area, then click “Delete” to delete all ROIs from the manager
    7. Repeat this process for the other image
        1. Dead cells are smaller than live cells, so you cannot use the same minimum area for both images
10. Obtain stained cell counts for each image
    1. Once again, go to Analyze > Analyze Particles
    2. Check “Summarize” and “Include holes”
    3. Set “Circularity” to “0.00-1.00”
    4. Set “Size” such that the minimum is slightly smaller than the area that was recorded from the previous step and the maximum is “Infinity”
    5. Click “OK”
    6. In the Summary window that pops up, record the value in the “Count” column
        1. This is the number of stained cells for that particular channel
    7. Repeat this process for the other image
11. Calculate the percentage of live cells to obtain cell viability
    1. % Live = $(Count_{green} / (Count_{green} + Count_{red})) * 100$
        1. $Count_{green}$ = number of live cells stained with calcein AM in the green channel
        2. $Count_{red}$ = number of dead cells stained with ethidium homodimer-1 in the red channel

Conclusions/action items:

Use this protocol to analyze the captured fluorescent images

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04/30/2024 - Final Bioprinting Protocol

Caitriona Treacy - Apr 30, 2024, 1:27 PM CDT

Title: Final Bioprinting Protocol

Date: 04/30/24

Content by: Elijah & Caitríona

Goals: Develop a comprehensive protocol for the bioprinter that is clear and can be easily followed by the client and others who wish to replicate the design.

Content:

Bioprinting Protocol without Cells:

1. Turn on the bioprinter and reduce lighting to 20% using the printer control options in the upper right corner of the home screen. Click on “Select Protocol” and then select “cait10mmx1mm”. Editing the protocol will allow the user to change settings for subsequent steps. This protocol currently has the following settings:
    - Dimensions: Cylindrical structure with 10 mm diameter and 2 mm thickness (2 layers)
    - Infill density: 52%
    - Printer head speed: 6.5 mm/s
    - Pre-flow delay: -200 ms
    - UV crosslinking once at the end (rather than between layers)
    - Print surface: 6-well plate.
    - UV crosslinking wavelength: 405 nm
    - UV crosslinking distance: 4 cm

    These print settings can be easily changed and saved in the protocol using the “edit protocol” tools before beginning the print.

2. Remove GelMA cartridge from 4℃ fridge. Attach a 22G nozzle and the upper pressure hose to the cartridge and place it in the temperature-controlled print head. Place the desired printing surface into the lower print bed. Most likely this will be a 6- or 12-well plate. Set the temperature to 37˚C and let the GelMA heat up for 45 minutes.
3. Once the GelMA has heated up for 45 minutes, reduce the temperature to 26.1˚C (printing temperature) and allow it to cool for 30 minutes.
4. Calibrate the printer. This can be done using the “move” option on the right side of the print screen. The metal calibration probe must be put in the vertical position for bed-leveling, and the printer door must be left open. Within the calibration menu, click “Automatic bed leveling” and follow the instructions on the screen. Once this is done, push the metal probe back into its neutral position and continue to “manual calibration” in the calibration menu. You will be prompted to move the print nozzle to the front left well of the print surface. To properly calibrate the height:
    - Ensure that the screw of the print head that secures the cartridge is completely loose.
    - Adjust the height of the print head using the calibration controls until the nozzle makes contact with the print surface. Increase the height of the print head until the nozzle is mostly covered by the temperature-controlled print head. Leave ~1 mm of the nozzle exposed to avoid GelMA residue buildup in the metal nozzle cover. Open the printer door and tighten the screw to secure the position of the cartridge in the print head.
    - With a 22G nozzle, the printer bed height should now be set 400 microns below the nozzle. If using a 27G nozzle, this distance should be 250 microns. Finalize the calibration.

    Note: After calibration, it can be useful to hit “print” followed promptly by “cancel print”. This will leave the print head in an elevated position, which makes unclogging and test flow more convenient before beginning the first print.

5. A test flow will now be performed to determine if the GelMA is ready to print. Navigate to the pressure screen and hold a KimWipe underneath the print head nozzle.
    - Starting at 35 kPa, perform a test flow. The test flow button must be held continuously throughout the test flow period. The filament coming out of the nozzle should appear gel-like without a viscous appearance.
    - If the test flow has a low viscosity and appears to be liquid, the print temperature is too high. Reduce the print temperature by 0.1-0.2˚C and wait for 10 minutes. If multiple test flows at higher pressures are unsuccessful, the GelMA may be too viscous and the temperature should be increased by 0.1-0.2˚C and 10 minutes should pass

before doing another test flow. **Unclogging the nozzle between test flows and before beginning the print is crucial.**

- If the filament curls around the tip of the nozzle to one side, the pressure is too low. Increase pressure in increments of 2 kPa. Continue to test flow until the filament does not curl around the nozzle. If the GelMA coming out of the nozzle expands around the tip in all directions upon leaving the nozzle, the pressure is too high and should be reduced. On average, successful prints happened within the range of 35-46 kPa.
- Once the filament is gel-like and not curling around the nozzle, the user is ready to begin the print. Be sure to unclog before checking to make sure the UV light time is set to either fibrotic (13s) or normal (2s) range. Return to the printing screen.
  Note: Sometimes adjusting the UV crosslinking time in the print does not actually change how long the crosslinking occurs for. If this seems to be happening, exit the print and re-enter the protocol menu from the beginning. Click “edit protocol” and adjust the UV settings there before re-entering the print window.

6. Click on the “Print” button on the printing screen to begin the print.
7. The gels will print in the back 3 wells of the 6 well plate, and each gel will be subsequently crosslinked after being printed.
8. Once the print is done (3 gels), rotate the 6 well plate 180˚ about the z-axis so that the other wells can be printed in during the next printing trial.
9. Repeat step 5 to make sure the gel is still in the optimal printing range. Temperature and pressure must continuously be monitored throughout the fabrication process.
10. Click the “Print” button again to run another printing trial.
11. Place gels in hSAEC growth media for 24 hours to swell.

Conclusions/action items: Send this final protocol to the client so that it can be used to fabricate gels in his lab.

MATLAB CODE for T-tests

WILLIAM ONUSCHECK - May 01, 2024, 10:47 AM CDT

Title: MATLAB CODE for T-tests

Date: April 29th

Content by: William Onuscheck

Present: William Onuscheck

Goals: Compare cell viability between stiff and healthy groups for each day LIVE/DEAD imaging was performed

Content:

```
importdata("ForTTest.xlsx")

% Sample data for two experimental groups and four timepoints

Healthy24 = (table2array(ForTTest(1:3,2)))'

Healthy72 = (table2array(ForTTest(4:8,2)))'

Healthy168 = (table2array(ForTTest(9:13,2)))'

Healthy336 = (table2array(ForTTest(14:18,2)))'

Fibr24 = (table2array(ForTTest(19:21,2)))'

Fibr72 = (table2array(ForTTest(22:26,2)))'

Fibr168 = (table2array(ForTTest(27:31,2)))'

Fibr336 = (table2array(ForTTest(32:35,2)))'

[h24,p24] = ttest2(Healthy24, Fibr24)

[h72,p72] = ttest2(Healthy72, Fibr72)

[h168,p168] = ttest2(Healthy168, Fibr168)

[h336,p336] = ttest2(Healthy336, Fibr336)
```

Conclusions/action items: Add to report.

Rheometry 2-2-24

ANURAAG SHREEKANTH BELAVADI - Feb 28, 2024, 2:34 PM CST

Title: Rheometry testing of bioprinted hydrogels

Date: 2-2-24

Content by: Elijah

Present: Will and Anuraag

Goals: To determine preliminary stiffness of bioprinted hydrogels

Content:

Gel	Freq Dep Shear Modulus(kPa)	Youngs modus (kPa)
1	13	39
2	0.514	1.542

- Rheometry protocol followed can be found in the protocols portion of lab archives.

Conclusions/action items:

1. Continue to bioprint gels

2. Schedule meeting with field scientist

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Rheometry 2-5-24/2-6-24

ELIJAH DIEDERICH (ediederich@wisc.edu) - Feb 27, 202

Title: Rheometry testing - Pipette hydrogels

Date: 1-30-2024

Content by: Elijah Diederich

Present: Will

Goals: To determine gel stiffness for pipette based hydrogels

Content:

Date Made	Geling Conditions	Purpose	Well Label	Freq Dependent shear modulus at .1 Hz (Pa)	Youngs Modulus (kPa)	Status	0H PostFab mass (mg)	24H Wet Mass (Wet)	24H
Febuary 5th	3 min fridge, 5 min UV, stored in incubator	For Rheometry	A1	778.5	2.3355	Kept			
			A2	732.2	2.1966	Kept			
			A3	680.5	2.0415	Lost			
			A4	996.6	2.9898	Kept			
		For Swelling	B1			Kept	112	54	
			B2			Kept	121	59	
			B3			Kept	113	56	
			B4			Kept	109	55	
	5 min Frige Time, 5 min UV, Stored in incubator	For Rheometry	C1	484.2	1.4526	Kept			
			C2	622	1.866	Kept			
			C3	753.1	2.2593	Kept			
			C4	655.4	1.9662	Kept			
		For Swelling	D1			Kept	87	39	
			D2			Kept	95	46	
			D3			Kept	90	39	
			D4			Kept	91	46	
	Healthy, 3 min fridge, 5 min UV, stored at 4°C	Control at 4°C for Rheometry	Petri	1193	3579	Disposed of			
	Fibrotic, 5 min Frige Time, 5 min UV, Stored in 4°C	Control at 4°C for Rheometry	Petri	971.1	2913.3	Disposed of			
Feburary 6th	5 min fridge, 10 min UV, Stored in incubator	For Rheometry	Petri	329.6	0.9888	Kept			
				364.8	1.0944	Kept			
				751	2.253	Kept			
				736.9	2.2107	Kept			
	10 min fridge, 5 min UV, Stored in incubator	For Rheometry	Petri	1396	4.188	Kept			
				920.9	2.7627	Kept			
				2245	6.735	Kept			
				945.6	2.8368	Kept			
Feburary 6th	Bioprinted Gel 2/2	For Rheometry	Petri	623.5	1.8705	Kept - 4mm in diameter			
				-	#VALUE!	Kept - 8mm in diameter			
	Bioprinted Gels 2/4	For Rheometry	Petri	12510	37.53	Kept - 10mm diameter			
				1512	4.536	Kept - 8mm in diameter	This one looked sus		
Febuary 14	Biobrinted With UV time of 30 sec	For Rheometry	Petri	1532	4596				
				2250	6750				
				1780	5340				
				7460	22380		massive dude		

- The protocol used can be seen in the rheometry protocol section of lab archives, further investigation of stiffness values will be included in further entries

Conclusions/action items:

1. Continue to make pipette based hydrogels and finish protocol

2. Work on bioprinter optimization protocol

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Rheometry 2-8-24

ELIJAH DIEDERICH (ediederich@wisc.edu) - Feb 27, 2024, 3:52 PM CST

Title: Rheometry testing 2-8-24

Date: 2-8-24

Content by: Elijah

Present: Anuraag and Elijah

Goals: To determine gel stiffness of bioprinted hydrogels

Content:

Sample #	Date Made	Geling Conditions	Storage Modulus (G*)	Young's Modulus	Extrusion Pressure	Size(Dia. x Height)
1	2-7	30 Sec UV	5.855	17.565	35 kPa	14mm x 3.6mm
2	2-7	30 Sec UV	5.836	17.508	28 kPa	11.8mm x 3.1mm
3	2-7	30 Sec UV	4.99	14.97	26 kPa	10.67mm x 2.9mm
4	2-7	30 Sec UV	6.938	20.814	23 kPa	8.67mm x 1.8mm
			Mean	17.71425		
			Std. Deviation	2.394728429		

** Very variable gel sizes and printing parameters ** The team will look into optimizing these parameters as they continue their bioprinting journey

Conclusions/action items:

1. Figure out how to make similar structure gels and sizes

2. Meet with field scientist

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Rheometry 2-22-24

ELIJAH DIEDERICH (ediederich@wisc.edu) - Feb 27, 2024, 3:55 PM CST

Title: Rheometry 2-22-24

Date: 2-22-24

Content by: Elijah Diederich

Present: Anuraag and Elijah

Goals: To determine stiffness of bioprinted hydrogels

Content:

Sample #	Date Made	Geling Conditions	Storage Modulus (G*)	Young's Modulus	Extrusion Pressure	Size(Dia. x Height)
1	2-21	10 Sec UV	3.107	9.321	35 kPa	10mm x 0.75mm
2	2-21	10 Sec UV	5.301	15.903	35 kPa	10mm x 0.80mm
3	2-21	10 Sec UV	4.195	12.585	35 kPa	10mm x 1.0mm
4	2-21	10 Sec UV	3.807	11.421	35 kPa	10mm x 1.0mm
			Mean	12.3075		
			Std. Deviation	2.75134458		

Conclusions/action items:

1. Continue to bio-print hydrogels with notes from field scientist

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04/08/24 to 04/22/24 Cell Encapsulation & LIVE/DEAD testing

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CARLEY SCHWARTZ - Apr 24, 2024, 1:44 PM CDT

Title: Fibroblast gel encapsulated LIVE/DEAD staining

Date: 04-09-24

Content by: Carley

Present: Will and Carley

Goals: To assess live/dead fibroblasts at the 24 hour mark

Content:

Following the protocol detailed below, we did the 24 hour staining on 3 of the gels, these gels will be saved and repeated at the other time points. At each new timepoint two new gels will also be stained as a means to gauge if the repeated staining or time with stain effects cell growth. Table below details what has been described

Time Point	Gels to be stained	Gel notes
24 hours	3 gels	Bright field images of fibroblasts in healthy gels appeared fibroblastic throughout the gel Bright field images of fibroblasts in fibrotic gels appeared slightly longer an average but still fibroblastic morphology Still experiencing some issues where due to the gel being 3D there is always some background fluorescence
72 hours	OG 3 gels + 2 new	Bright field images of both healthy and fibrotic gels had cells with fibroblastic appearance There seems to be some cracking in the gel structure that show up in the bright field images.
1 week	OG 3 gels + 2 new	Bright field images looked good once again Seemed like cell viability was getting lower Hydrogels are very fragile at this point in the culture
2 weeks	OG 3 gels + 2 new	When doing the staining in the fibrotic is appeared that there was a bit higher cell death compared to healthy and this was later confirmed with ImageJ analysis.

Materials:

- 15mL conical tube
- 20µL pipette + tips
- 1000µL pipette + tips
- 10mL serological pipette
- Phosphate-buffered saline (PBS)
- Fluorescence microscope
- LIVE/DEAD Viability Kit (Thermofisher) containing calcein AM and ethidium homodimer-1 (EthD-1)
    - Store in a -20 ºC freezer protected from light
        - Either keep the reagent vials in a box or wrap them in foil
    - Before using the stock solutions, allow them to thaw to RT and centrifuge them briefly before opening them

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        - Fluorophores can aggregate overtime
    - Calcein AM hydrolyzes when exposed to moisture, so be sure to keep it away from water (stock solution is in anhydrous DMSO)
    - Aqueous working solutions of calcein AM should be prepared immediately prior to use and must be used within one day
    - Ethidium homodimer-1 is not sensitive to moisture; Aqueous working solutions of it can be stored at -20 ºC for up to one year
    - Before refreezing stock solutions, seal all vials tightly

Fluorescent Dye Concentration Optimization:

1. Prepare samples of live and dead cells
    1. Dead cells can be obtained by killing cells with 70% methanol for 30min
2. Use samples of dead cells to optimize the EthD-1 concentration
    1. Dilute the EthD-1 in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting EthD-1 to 0.1 - 10μM
    2. You want the lowest EthD-1 concentration which stains the dead cell nuclei bright red with *minimal* cytoplasmic staining
3. Use a sample of dead cells to start to optimize the calcein AM concentration
    1. Dilute the calcein AM in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting calcein AM to 0.1 - 10μM
    2. You want the lowest calcein AM concentration which gives minimal cytoplasm staining
4. Use a sample of live cells to confirm the optimization of the calcein AM concentration
    1. Dilute the calcein AM in PBS to the same concentration that used in the prior step, add it to live cells, incubate for 30min, then image
    2. See if the used concentration gives a sufficient green stain in live cells
        1. If not, try a higher concentration on another sample of live cells

LIVE/DEAD Staining:

1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add enough calcein AM and ethidium homodimer-1 to the PBS to achieve the concentrations determined in optimization process
    1. Anecdotally, 20μL of EthD-1 and 5μL of calcein AM in 10mL of PBS gives concentrations of 4μM and 2μM, respectively, and is suitable for 3T3 cells
    2. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cell-laden hydrogels and wash with PBS
    1. Do 3-5 5min washes
4. Add 200μL of staining solution directly to the hydrogels
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
    1. Dim the lights in the working space and keep the gels covered whenever not imaging
    2. Use the FITC/GFP filter for calcein-AM (live cells)
    3. Use the TRITC/RFP filter for ethidium homodimer-1 (dead cells)

Tips:

- If you have extracellular fluorescence, **do additional and/or longer washes with PBS** prior to staining because media can have esterases which hydrolyze the calcein AM to cause fluorescence

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    - Calcein AM is calcein acetoxymethyl ester, and it functions by permeating live cell plasma membranes and then getting hydrolyzed by intracellular esterases which releases the fluorescent calcein

- Be sure to dim the lights of the work space and have cells covered in foil when not actively adding something or imaging

    - The fluorescent dyes are photosensitive

Conclusions/action items:

Overall the staining protocol derived here works well and following updates will be made as staining progresses, note relevant to each timepoint and the respective gels is noted in the table above.

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Rheometry 4-04 and 4-11

ANURAAG SHREEKANTH BELAVADI - Apr 24, 2024, 4:05 PM CDT

Title: Rheometry data for two bioprinting sessions

Date: 04-04 and 04-11

Content by: Carley

Present: Anuraag

Goals: To test both rounds of bioprinted gels

Content:

Date	UV (sec)	Thickness (mm)	Shear (Ksi)	Youngs (Ksi)	Average	Dev
04/04	13	2.5	7.297	21.891	18.52033333	3.591310253
04/04	13	2.5	6.309	18.927	11.8475	
04/04	13	2.3	4.914	14.743		
04/11	6	1.8	3.037	9.112		
04/11	6	2.1	4.391	13.173		
04/11	6	2.1	4.45	13.349		
04/11	6	2.4	5.976	17.929		
04/11	6	1.3	1.579	4.738		
04/11	6	2.1	4.261	12.784		

Conclusions/action items:

The thirteen second gel trial had an average of 18.5 which is perfect for our fibrotic but the 6 second was above the healthy range.

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Rheometry 04/19

ANURAAG SHREEKANTH BELAVADI - Apr 24, 2024, 4:05 PM CDT

Title: Rheometry Testing

Date: 04-09-24

Content by: Carley

Present: Anuraag

Goals: To test bioprinted hydrogel stiffness

Content:

Gel Trial	UV (Sec)	Thickness(mm)	Young's (MPa)	Ave	STD
1	3	1.8	5.044	4.817	0.571
2	3	1.7	5.286		
3	3	1.55	4.242		
4	3	1.6	4.657		
5	3	1.55	4.737	15.024	1.922
1	6	2.3	16.965		
2	6	2.1	14.102		
3	6	2.1	13.837		

Conclusions/action items:

Both gel trials at 3 and 6 seconds showed good values but the variability in thickness does seem to be influencing the youngs modulus.

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Rheometry 04-18

ANURAAG SHREEKANTH BELAVADI - Apr 24, 2024, 4:05 PM CDT

Title: Rheometry data for bioprinted gels

Date: 04-18

Content by: Carley

Present: Anuraag

Goals: To test bioprinted gels

Content:

Gel	UV Time (sec)	Thickness (mm)	Shear Modulus (Pa)	Young's Modulus (kPa)	Average	Dev
1	3	2.25	3.286E+03	9.858E+00	6.025E+00	3.969700127
2	3	2.20	3.672E+03	1.102E+01		
3	3	1.55	1.320E+03	3.960E+00		
4	3	1.6	2.42E+03	7.25E+00		
5	3	1.5	384	1.152		
6	3	1.2	9.71E+02	2.91E+00		

Conclusions/action items:

3 second UV time this round seemed to be at a higher average than previously at 6 kPa for the average but there were two gels of higher thickness that had much higher youngs moduli that might be pulling as outliers in the average.

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04/22/2024 Cell Viability Data Analysis

Nick Herbst - Apr 27, 2024, 1:18 PM CDT

Title: Cell Viability Data Analysis

Date: 04/22/2024

Content by: Nick

Present: Nick

Goals: Analyze the data from Will's LIVE/DEAD staining images using ImageJ and the previously described protocol

Content:

See attached pictures for raw data, calculations, and a summarizing plot

Conclusions:

Over a 2 week time course, both conditions of GelMA hydrogels maintained high encapsulated cell viability up until the drop-off at the 2 week mark.

Nick Herbst - Apr 27, 2024, 1:18 PM CDT

Gel (Time)	Live Cell Count	Dead Cell Count	% Cell Viability
Healthy Gel 1 (24H)	28	3	90.3
Healthy Gel 2 (24H)	31	3	91.2
Healthy Gel 3 (24H)	30	2	93.8
Healthy Gel 1 (72H)	32	5	86.5
Healthy Gel 2 (72H)	30	6	83.3
Healthy Gel 3 (72H)	69	4	94.5
Healthy Gel 4 (72H)	93	5	94.9
Healthy Gel 5 (72H)	68	9	88.3
Healthy Gel 1 (168H)	135	18	88.2
Healthy Gel 2 (168H)	46	6	88.5
Healthy Gel 3 (168H)	57	19	75.0
Healthy Gel 4 (168H)	132	14	90.4
Healthy Gel 5 (168H)	153	14	91.6
Healthy Gel 1 (336H)	207	37	84.8
Healthy Gel 2 (336H)	205	54	79.2
Healthy Gel 3 (336H)	56	32	63.6
Healthy Gel 4 (336H)	227	28	89.0
Healthy Gel 5 (168H)	136	16	89.5
Fibrotic Gel 1 (24H)	35	5	87.5
Fibrotic Gel 2 (24H)	36	3	92.3
Fibrotic Gel 3 (24H)	24	2	92.3
Fibrotic Gel 1 (72H)	59	9	86.8
Fibrotic Gel 2 (72H)	54	10	84.4
Fibrotic Gel 3 (72H)	41	4	91.1
Fibrotic Gel 4 (72H)	62	10	86.1
Fibrotic Gel 5 (72H)	53	7	88.3
Fibrotic Gel 1 (168H)	37	14	72.5
Fibrotic Gel 2 (168H)	29	5	85.3
Fibrotic Gel 3 (168H)	69	11	86.3
Fibrotic Gel 4 (168H)	136	16	89.5
Fibrotic Gel 5 (168H)	67	6	91.8
Fibrotic Gel 1 (336H)	234	117	66.7
Fibrotic Gel 2 (336H)	255	228	52.8
Fibrotic Gel 3 (336H)	56	35	61.5
Fibrotic Gel 4 (336H)	220	80	73.3

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Nick Herbst - Apr 27, 2024, 1:18 PM CDT

Condition	Average % Cell Viability	% Cell Viability Standard Deviation
Healthy Gels (24H)	91.7	1.8
Healthy Gels (72H)	89.5	5.1
Healthy Gels (168H)	86.7	6.7
Healthy Gels (336H)	81.2	10.7
Fibrotic Gels (24H)	90.7	2.8
Fibrotic Gels (72H)	87.3	2.5
Fibrotic Gels (168H)	85.1	7.5
Fibrotic Gels (336H)	63.6	8.7

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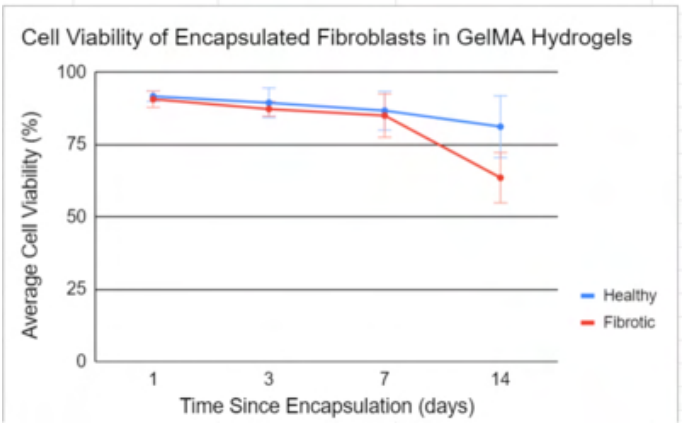
calculations.png (8.49 kB)

Nick Herbst - Apr 27, 2024, 1:18 PM CDT



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02/08/2024 - Preliminary Presentation

Nick Herbst - Feb 28, 2024, 1:49 PM CST

Title: Preliminary Presentation

Date: 02/08/2024

Content by: Everyone

Present: Everyone

Goals: Present our design project progress thus far to our advisor

Content:

- See attachment for full presentation slides

Action items:

- Work to meet project goals

Nick Herbst - Feb 08, 2024, 11:31 AM CST

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02/28/2024 - Preliminary Report

Nick Herbst - Feb 28, 2024, 1:50 PM CST

Title: Preliminary Report

Date: 02/28/2024

Content by: Everyone

Present: Everyone

Goals: Compile our work thus far into a journal article manuscript draft and outline areas that will be filled in later

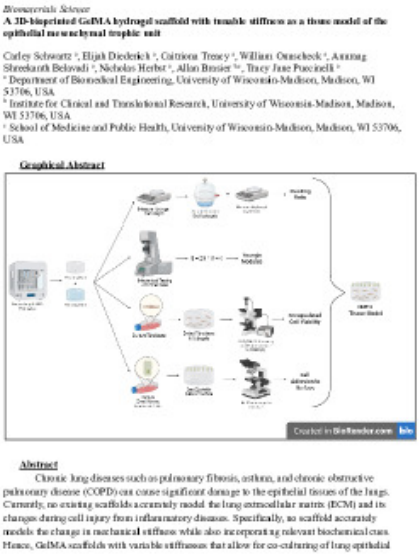
Content:

- See attachment for full report

Action items:

- Work to meet project goals

Nick Herbst - Feb 28, 2024, 8:08 PM CST



Biomaterials Science

A 3D-bioprinted GelMA hydrogel scaffold with tunable stiffness as a tissue model of the epithelial mesenchymal trophic unit

Carley Schwartz [a], Elijah Diedrich [a], Caitriona Treacy [a], William Ormiston [a], Anurag Shreekanth Belavadi [a], Nicholas Herbst [a], Allan Brasier [b,c], Tracy Jane Puccinelli [a]
[a] Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI 53706, USA
[b] Institute for Clinical and Translational Research, University of Wisconsin-Madison, Madison, WI 53706, USA
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Graphical Abstract

Abstract

Chronic lung diseases such as pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) can cause significant damage to the epithelial tissues of the lungs. Currently, no existing scaffolds accurately model the lung extracellular matrix (ECM) and its changes during cell injury from inflammatory diseases. Specifically, no scaffold accurately models the change in mechanical stiffness while also incorporating relevant biochemical cues. Hence, GelMA scaffolds with variable stiffnesses that allow for co-culturing of lung epithelial

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02-19-2024 DAPI Cell Adhesion assay in GelMA hydrogels

CARLEY SCHWARTZ - Feb 19, 2024, 12:44 PM CST

Title: Cell Adhesion Assay

Date: 02-19-24

Content by: Carley

Present: Self

Goals: To look at cheaper cell adhesion assay options

Content:

Nichol, J. W., Koshy, S. T., Bae, H., Hwang, C. M., Yamanlar, S., & Khademhosseini, A. (2010). Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials*, *31*(21), 5536–5544. https://doi.org/10.1016/j.biomaterials.2010.03.064

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2878615/

For cell adhesion studies, square hydrogel sheets (1 cm (w) × 1 cm (l) × 750 μm (h)) were prepared in a similar manner as that used for mechanical testing onto TMSPMA glass slides. Slides were covered with a HUVEC suspension containing 2.5×105 cells/mL to a depth of approximately 1 mm above the surface of the GelMA hydrogel and incubated for 12 h prior to washing twice with DPBS. Media was changed every 12 h for 5 days. GFP fluorescence was visualized using an inverted fluorescence microscope (Nikon TE 2000-U) equipped with a GFP filter cube. GFP images were used to quantify total cell area using NIH ImageJ software. After 5 days, cells were fixed and stained with rhodamine-phalloidin (Invitrogen) and DAPI to visualize F-actin filaments and cell nuclei respectively. Total cell number was quantified using ImageJ by counting DAPI stained nuclei.

NIH 3T3 fibroblasts were trypsinized and resuspended in GelMA macromer containing 0.5% (w/v) photoinitiator at a concentration of 5×106 cells/mL. Microgel units (500 μm × 500 μm) were fabricated as previously described [15] following exposure to 6.9 mW/cm2 UV light (360–480 nm) for 15 s on TMSPMA treated glass. The glass slides containing microgels were washed with DPBS and incubated for 8 h in 3T3 medium under standard culture conditions. A calcein-AM/ethidium homodimer Live/Dead assay (Invitrogen) was used to quantify cell viability within the microgels according to the manufacturer’s instructions.

Conclusions/action items:

It seems that if we want to look at the cell adhesion on the surface we could use DAPI to visualize cell nuclei but doing embedded cell encapsulation measurements may require a calcein based live/dead assay

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2024/02/26 Introduction research for paper

CARLEY SCHWARTZ - Feb 28, 2024, 6:43 PM CST

Title: Introduction research for paper

Date: 02/26/24

Content by: Carley

Present: Self

Goals: To provide better motivation and conceptualize the purpose of the project

Content:

Nizamoglu, M., Joglekar, M. M., Almeida, C. R., Larsson Callerfelt, A. K., Dupin, I., Guenat, O. T., Henrot, P., van Os, L., Otero, J., Elowsson, L., Farre, R., & Burgess, J. K. (2023). Innovative three-dimensional models for understanding mechanisms underlying lung diseases: powerful tools for translational research. *European respiratory review : an official journal of the European Respiratory Society*, *32*(169), 230042. https://doi.org/10.1183/16000617.0042-2023

- Developing appropriate treatments for chronic lung diseases can be bolstered through thorough understanding of disease mechanisms. In spite of the abundance of and advancement in knowledge, the pathogenesis and progression of most chronic diseases remains unclear. Much knowledge has been obtained from rodent studies, which often do not completely recapitulate human diseases.
- Traditional two-dimensional (2D) *in vitro* cell culture approaches have played a fundamental role in advancing current knowledge of cell behaviour and fate. However, these approaches lack a range of essential cell–cell and cell–extracellular matrix (ECM) interactions that have been shown to define cell signalling and function.

Conclusions/action items: Wanted to review some literature to aid in the motivation section of the our paper, specifically I wanted to read some material on the motivation behind a 3D model.

2024/28/02 - Human lung physiology

CARLEY SCHWARTZ - Feb 28, 2024, 6:50 PM CST

Title: Reviewing human lung physiology

Date: 02/28/24

Content by: Carley

Present: Self

Goals: Understanding the physiology of the lung and adding more to the introduction of our paper

Content:

Miller, A. J., & Spence, J. R. (2017). In Vitro Models to Study Human Lung Development, Disease and Homeostasis. *Physiology (Bethesda, Md.)*, *32*(3), 246–260. https://doi.org/10.1152/physiol.00041.2016

- Animal models have been instrumental in our understanding of lung development and disease. However, given many differences between animal and human lung physiology, there is an unmet need for human lung model systems that can complement animal models to improve our understanding of human lung physiology.
- Mechanistically, influences such as biomechanical forces, availability of oxygen, morphogen signaling, and molecular-level regulation of gene and protein expression work together to regulate lung development, homeostasis, and regeneration.
- In the alveolar lung-on-a-chip, structural, functional, and mechanical elements of the interface between the alveoli and the capillary network were modeled by micro-fabricating a microfluidic device that contains two channels separated by a thin flexible membrane coated with ECM proteins. Vascular endothelial cells were cultured in one chamber, and alveolar epithelial cells in the other, which could also be exposed to air.

Conclusions/action items: This article gave a great review for why disease models are necessary and how engineered scaffolds are useful to replicate this.

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2024/30/01-Bioprinter Advanced Protocol

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CARLEY SCHWARTZ - Feb 08, 2024, 11:20 AM CST

Title: Bioprinter protocol (extreme detail)

Date: 01/30/24

Content by: Carley Schwartz

Present: Self

Goals: To create a very detailed protocol for other group members

Content:

Materials:

1. GelMA in amber cartridge from fridge
2. CELLINK Bioprinter
    1. Temperature controlled printhead attachment
    2. Temperature maintaining nozzle attachment
3. 22 gauge nozzle attached to cartridge (Many incase of clog)
    1. Smallest needle for declogging
4. Pressure hose
5. Luer lock connections
6. Petri dish

Method:

1. Place the GelMA cartridge into the connected temperature controlled print head
2. On the bioprinter interface select the type of print you would like to conduct, in our scenario we will be doing a structured print
    1. Go to bioprint -> Simple shapes -> scroll down to cylindrical and select the 3x06mm.stl
    2. Go to surface -> select your surface (for us petri dish)
    3. Printer -> tool 1 (or whichever you are using/what the print head is connected to) -> tool type: temperature controlled ; temperature: 37 degrees to make a liquid
        1. At this point place the GelMA cartridge with a 22 gauge nozzle and the tube attached to the machine
        2. Once the time has passed pull the cartridge out and move it up and down to make sure it is a liquid
    4. Bioink profile: Cellink GelMA 1
    5. Pre-flow: 100 ms
    6. Photocrosslinking: 405 nm
3. Layers
    1. Select concentric for infill pattern
    2. For infill density select 35%
4. On the Print screen
    1. Press the settings symbol and go to Tools - for temperature change to printing temp (27) and wait for 15 minutes for it to equilibrate

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5. Once in the print screen -> go to the print option which will then take you to calibration
6. Calibrate first with be leveling and the probe in the back
7. Next manual calibration to provide the printer information on how far you want the nozzle to be from the dish while printing and in what region of the dish
    1. UV light requires it to be in the back half
8. Now the machine should be ready to print but you should check to make sure there are no bubbles in the cartridge nozzle by flicking and make sure the tip isn't clog with one of the cell link needles
9. Then you press start to do the print trial
    1. If issues arise adjust the temperature, extrusion pressure, declog the nozzle or flick the tube
    2. If continual issues you can test the extrusion pressure with test flow but avoid doing this as it wastes a lot of product!!!

Conclusions/action items: As the semester continues more details will be added based on UV time and distance for fibrotic and healthy states.

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Hydrogel Drying Methods

ELIJAH DIEDERICH (ediederich@wisc.edu) - Jan 31, 2024, 12:30 PM CST

Title: Drying and Storage Effects on Poly(ethylene glycol) Hydrogel Mechanical Properties and Bioactivity

Date: 1-31-24

Content by: Elijah Diederich

Present: Myself

Goals: To determine methods of drying hydrogels that are widely accepted to apply to our design

Content:

Citation: [1] P. T. Luong, M. B. Browning, R. S. Bixler, and E. Cosgriff-Hernandez, “Drying and storage effects on poly(ethylene glycol) hydrogel mechanical properties and bioactivity,” Journal of biomedical materials research. Part A, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3972368/ (accessed Jan. 31, 2024).

Abstract:

- Note that PEG-diacrylate hydrogels are being used in this study exploring the mechanical properties and swelling effects using different hydrogel drying processes.
- The 3 drying procedures used in this article include vacuum-drying, lyophilizing, and hydrating-->vacuum drying

Methods:

- First method of drying that was introduced was a hydration in water followed by vacuumdrying at -30 psi.
- Second method of drying was strictly a vacuum drying without the hydration process in the beginning
- Third method of drying was flash-freezing in liquid nitrogen followed by vacuum drying

- Swelling ratio was calculated by taking the equilibrium swelling mass and dividing it by the dry polymer mass (post-vacuum drying)

- Ws/Wd is the equation for what is described above

- Ws (in this particular study): Discs were processed, soaked in water for 24 hrs and weighed to measure the equilibrium swelling mass (Does this have be water? Or can we use PBS, which is what we are currently using)

- Wd: After obtaining Ws weight, gels were then vacuum-dried for 24 hrs and a Dry polymer mass was taken.

Conclusions/action items:

1. Look into vacuum drying (what setup, availability etc...)

2. Continue to look for articles about leaving the hydrogel in the open over extended periods of time.

3. Rheology measurements for made gels

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02/26/2024 - Existing Research Models

Title: 3D in vitro hydrogel models to study the human lung extracellular matrix and fibroblast function

Date: 02/26/2024

Content by: Caitríona

Present: N/A

Citation: Phogat, S., Thiam, F., Al Yazeedi, S., Abokor, F. A., & Osei, E. T. (2023). 3D in vitro hydrogel models to study the human lung extracellular matrix and fibroblast function. *Respiratory research*, *24*(1), 242. https://doi.org/10.1186/s12931-023-02548-6

Goals: To document research on existing models to be included in the report. This article is a review of various studies that used 3D models to assess the regulation of the ECM on lung fibroblast phenotype and function in altered lung ECM homeostasis in health and in chronic respiratory disease.

Content:

- "During lung injury and in chronic lung diseases such as asthma, idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD), an abnormal feedback between fibroblasts and the altered ECM disrupts tissue homeostasis and leads to a vicious cycle of fibrotic changes resulting in tissue remodeling."
- Lung ECM is recognized as having a bioactive role in physiological and pathological processes. The lung ECM supports the phenotype and function of fibroblasts, among other cells, via biochemical and micro-architectural cues that create signaling niches and provide positional instructions. The lung fibroblast-ECM interactions become abnormal in various lung diseases.
- In most traditional research, two-dimensional monolayer cell-culture systems which are simple and high throughput allow for the study of mechanisms behind increased expression of ECM proteins by fibroblasts in various pathological conditions. These models, however, restrict and do not accurately represent the complex cellular-ECM lung microenvironment. Data is deceptive, but used to inform animal studies.
- In order to mimic the complexities of the 3D relationship between pulmonary ECM and lung fibroblasts, it is essential to establish 3D hydrogel models which embed cells in configurations characteristic of the temporal, spatial and cell type specific connections found in the in vivo lung environment.
- Advancements in 3D hydrogel models have enabled the assessment of the effect of the lung's 3D ECM microenvironment on fibroblast phenotype and function in tissue homeostasis and disease.
- 3D Hydrogel Culture Models: Hydrogels are crucial for building 3D in vitro models due to their similarities to the native ECM in the human body. They are an interconnected 3D network of hydrophilic polymers with the capacity for cellular encapsulation or surface seeding on top of the microfilaments to form a micro-gel. Collectively, they offer new routes to study and experiment with cellular mechanisms in four dimensions (cellular functions in three dimensions against time as the fourth). Through 3D hydrogels, it was established that the lung ECM fundamentally affects cellular behavior and function. In lung research, the two main types of hydrogel scaffolds are natural hydrogels and synthetic hydrogels.
    - Natural polymer hydrogels: Prominent bioactive features that allow them to interface favorably with cells. Within natural hydrogels, the two main types are protein and polysaccharide-based hydrogels. Collagen is the most widely used natural polymer for building 3D hydrogel systems. Most collagen scaffolds are prepared using type I collagen and can be easily modifies by cross-linking. Matrigel is another commonly used natural hydrogel, composed mainly o collagen IV, glycoproteins and various growth factors in the basement membrane protein. It is thermo-sensitive and used widely for cell (co-)cultures, bioprinting and tissue engineering to support independent epithelial cell culture and their co-culture with other cell types such as fibroblasts.

Gelatin methacrylate (GelMa) is another natural ECM mimicking polymer commonly used for building 3D hydrogel models. It is a protein-based polymer manufactured by reacting methacrylic anhydride (MA) with gelatin (naturally occurring hydrolyzed derivative of collagen). Hyaluronic acid (HA) is another important component of connective tissues. HA can be chemically modified by radical polymerization into soft or stiff hydrogel scaffolds. Alginate is another natural polysaccharide that is commonly used in 3 hydrogel system construction because of its rapid cross-linking property (through exposure to calcium chloride). Another form of natural hydrogel scaffolds are those sourced from the ECM through allogeneic or xenogenic lung decellularization (usually through perfusion of detergents or salt solutions). Although this method has the advantage of cellular environment retention to allow for inherent biological activity of the natural matrix, cellular growth and constructive tissue remodeling, a major disadvantage is the challenge of ECM structure damage due to decellularization detergents. **A major advantage of natural polymer application to 3D hydrogel models is their bio-active features (cell-adhesion motifs, non-immunogenicity, non-inflammatory, biodegradability). They also make it easy to integrate peptide ligands and cell membrane receptors through covalent bonding that stimulates adhesion of cells with subsequent spreading and proliferation. However, natural polymer hydrogels have poor mechanical strength and low stability when compared to synthetic polymer-based hydrogels.**

- Synthetic polymer hydrogels: Offer better mechanical properties (PEG and PA), such as strength and stability of the native lung tissue, but tend to lack biological active features. They often offer better control over structure and property that help to ensure replicability of lung tissue architecture. PEG (polyethylene glycol) and PA (polyacrylamide) are two of the most commonly used synthetic molecules for 3D cell culture. PEG had minimum protein absorption, is hydrophobic, and has cross-linking chemistry that is easily modified. Its main applications are in studying changes in cell behavior due to changes in matrix stiffness, as is the case in fibrotic vs healthy tissue. PA hydrogels are popular in cell mechanical studies due to the ability of high-resolution cell imaging through their transparent structure as well as the ability to customize their stiffness and surface functionality. **Synthetic polymers are inert and do not have any effect on cellular activity. Therefore, they are often combined with a bioactive material in order to increase biocompatibility parameters.**
- When recreating a three-dimensional environment, it is often not enough to use a single polymer hydrogel. In some tissue models, it is necessary to use a composite or hybrid hydrogel with more than one polymer to fully meet the functional and structural characteristics of the native ECM. These composites overcome the weak mechanical properties of natural scaffolds while at the same time providing bioactive components to the ECM to modulate cell behavior. **Common composite hydrogels: GelMa/PEG, alginate-PA, collagen-PA.** Hydrogel models have successfully mimicked the physical and mechanical properties of the healthy vs diseased ECM, allowing for exploration of how lung fibroblasts and other cells are affected by changes that occur in their 3D environment.

Specific models that have been developed:

- Marinkovic et al. studied how mechanical properties of the matric impacted fibroblast contractility. PA hydrogels of stiffnesses ranging from 0.3-20 kPa were prepared and conjugated with fluorescent microsphere beads. Gels were seeded with IMR-90 lung fibroblast cell lines, either in the presence or absence of TGF-β1 fibrotic mediator treatment. Traction measurements determined that lung fibroblasts exert lower forces on softer matrices as opposed to greater forces on stiffer matrices. Differences in smooth muscle actin expression on stiffer matrices also suggested an interaction between stiff lung ECM environment and fibrotic mediators, in fibroblast force generation, and FMT.
- Liu et all. engineered a collagen I functionalized PA 3D hydrogel matrix system with a 1D gradient of shear modulus ranging from 0.1 to 50 kPa. CCL-151 lung fibroblast cell lines were seeded and significant changes in CCL-151 lung fibroblast morphology (attenuated round cells at lower stiffness to spindle shaped with dendrites on intermediate, and parallel swirls of spindle-shaped cells at higher stiffnesses). Reported a

stiffness-dependent suppression of cyclooxygenase-2 (COX-2) expression and synthesis of prostaglandin E2 during fibrogenesis.

- Brown et al. found contradicting results through the use of a PA model that showed that increased epithelial cell contractility on stiff matrices causes integrin-mediated TGF-β activation and EMT and that abnormal EMT-derived mesenchymal cells have the potential to revert back to their normal phenotype if the fibrotic stimulant is reversed.
- In 2019, Fu et al. engineered a novel protein-based hydrogel with cyclic and reversible mechanics that tuned a large range of stiffnesses (6 kPa and 20 kPa) via glutathione. Cell culture medium was switched between the oxidizing and reducing state in a cyclic manner to study how lung fibroblasts respond to continuously changing hydrogel mechanics. In oxidizing conditions, human lung fibroblasts (HLF) changed morphology and went from a high cell spread state to a low cell spread state, leading to a significant increase in the cell area, accompanied by a slight decrease in cell roundness. The opposite was observed when conditions returned to a reducing state. This suggests that HLFs have the ability to continuously sense hydrogel stiffness and produce a fully reversible and dynamic mechanoresponse through behaviors such as cell morphology and phenotype.

Conclusions/action items: Use the above information to contribute to the 'existing research' portion of the preliminary draft of the journal article.

02/28/2024 ADDITION: 01/06/2024 Synthesis and Properties of Gelatin Methacryloyl

ANURAAG SHREEKANTH BELAVADI - Feb 28, 2024, 2:56 PM CST

Title: Synthesis and Properties of Gelatin Methacryloyl (GelMA) Hydrogels and Their Recent Applications in Load-Bearing Tissue

Date: 01/03/2024, 02/28/2024

Content by: Anuraag

Present: Self

Goals: Understand why the pipette-based hydrogels exhibited shrinking after being submersed in culture media or an aqueous solution.

Content:

- Evaluation of Swelling Property: The swelling property of GelMA hydrogels was assessed using a weighing method. Samples were prepared, freeze-dried, and then soaked in a solvent (such as ultrapure water or pH 7.4 PBS) at intervals. The swelling ratio was calculated using a specific formula, allowing for analysis of the swelling behavior.
- Influence of GelMA Concentration and Degree of Substitution: Studies, including one by Nichol JW et al., explored how GelMA concentration and the degree of substitution affect swelling ratios. Results indicated that higher degrees of substitution led to decreased swelling ratios, and increasing GelMA concentration resulted in decreased swelling, likely due to increased crosslink densities.
- Role of Hydrogels in Biomedical Applications: Hydrogels, possessing three-dimensional hydrophilic polymer networks, have tunable properties crucial for various biomedical applications such as tissue engineering, regenerative medicine, and drug delivery. Their similarity to the natural extracellular matrix makes them favorable for in vitro cell culture.
- Types of Hydrogels: Hydrogels are categorized into natural and synthetic types. Natural hydrogels offer better biocompatibility compared to synthetic ones, enhancing cellular viability, multiplication, differentiation, and locomotion.
- Limitations of GelMA Hydrogel in Bone Tissue Engineering: Despite its advantages, GelMA hydrogel's mechanical strength limits its application in bone tissue engineering. Various strategies, such as incorporating rigid materials like gellan gum methacrylate (GGMA) or hydroxyapatite (HAP), have been explored to improve its mechanical properties.
- Strategies for Enhancing Mechanical Strength: Strategies include preparing double-network (DN) hydrogels, mixing GelMA with HAP, or combining GelMA with PEGDA. Additionally, using microfibre networks or poly(ε-caprolactone) (PCL) fiber scaffolds, and covalent binding between gel and scaffold, have been shown to enhance mechanical strength.

Reasons for Observing Shrinking:

- Material Composition: Changes in the composition of hydrogels, such as altering GelMA concentration or degree of substitution, can influence their swelling behavior.
- Crosslink Density: Increased crosslink densities, as seen with higher GelMA concentrations, can lead to decreased swelling ratios.
- Environmental Factors: Exposure to different solvents or solutions can induce swelling or shrinking behavior in hydrogels, impacting their overall properties.

Sun M, Sun X, Wang Z, Guo S, Yu G, Yang H. Synthesis and Properties of Gelatin Methacryloyl (GelMA) Hydrogels and Their Recent Applications in Load-Bearing Tissue. *Polymers*. 2018; 10(11):1290. https://doi.org/10.3390/polym10111290

Yin H, Zhu M, Wang Y, Luo L, Ye Q and Lee BH (2023) Physical properties and cellular responses of gelatin methacryloyl bulk hydrogels and highly ordered porous hydrogels. *Front. Soft. Matter* 2:1101680. doi: 10.3389/frsfm.2022.1101680

Conclusions/action items: Adjusting the cross-linking time may contribute to better swelling behavior as cross-linking density is a factor that can be addressed by the group when making more gels in the future.

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UPDATED_05/01/2024_04/22/2024_Welch's T-test

ANURAAG SHREEKANTH BELAVADI - May 01, 2024, 7:23 PM CDT

Title: Welch's T-Test

Date: 04/22/2024

Content by: Anuraag

Present: N/A

Goals: Find a way to show that there is no difference between the two groups to quantify LIVE/DEAD.

Content:

- Validity of t-test:
  - The t-test is suitable when data are normally distributed or when there are at least 50 measurements and the distribution is reasonably symmetric.
  - Formal testing for normality, such as the Shapiro-Wilks test, is not necessary, especially with large sample sizes, as the t-test is robust against departures from normality if the distribution is not heavily skewed.
- Equal standard deviations:
  - The original Student's t-test assumes equal standard deviations between groups, but the Welch t-test is preferred as it allows for different standard deviations.
  - The Welch t-test is practical because it's often difficult to ensure equal variances in real-world data, and it provides similar power to Student’s t-test.
- Non-parametric test:
  - The Mann–Whitney U-test (or Wilcoxon rank-sum test) can be used if there's doubt about the symmetry or normality of the data.
  - This test is distribution-free and compares the ranks of measurements rather than the measurements themselves.
- Example:
  - An example provided in the article demonstrates the application of both the t-test and the Mann–Whitney U-test on two groups with different means and standard deviations.
  - It shows how the choice of test can affect the resulting p-values and emphasizes the importance of selecting an appropriate test based on data characteristics.
- Summary:
  - The Welch t-test is preferred over Student’s t-test when assumptions of normality are met or nearly met, while the Mann–Whitney U-test is a robust alternative when normality assumptions are violated.
  - The Welch t-test tests for differences in means, whereas the Mann–Whitney U-test tests for differences in medians.

Source:
West RM. Best practice in statistics: Use the Welch t-test when testing the difference between two groups. Annals of Clinical Biochemistry. 2021;58(4):267-269. doi:10.1177/0004563221992088

Conclusions/action items:

Move forward with Welch's t-Test as we are measuring differences in means with an assumption of normality.

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02/28/2024 Lab Safety Documentation

ANURAAG SHREEKANTH BELAVADI - Feb 28, 2024, 2:40 PM CST

WISCONSIN
UNIVERSITY OF WISCONSIN-MADISON

This certifies that Anuraag Shreekanth Belavadi has completed training for the following course(s):

Course	Assignment	Completion	Expiration
Biosafety 102: Bloodborne Pathogens for Laboratory and Research	Biosafety 102: Bloodborne Pathogens Safety in Research Quiz 2023	11/28/2023	
Biosafety Required Training	Biosafety Required Training Quiz	10/18/2021	10/18/2026
Chemical Safety: The OSHA Lab Standard	Final Quiz	10/23/2021	

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Screenshot_2024-02-28_143836.jpg (73.9 kB)

03/15/2024 Tong Lecture

ANURAAG SHREEKANTH BELAVADI - Mar 15, 2024, 12:34 PM CDT

Title: Tong Lecture

Date: 03/15/2024

Content by: Anuraag Shreekanth Belavadi

Present: N/A

Goals: N/A

Content:

Exact Sciences

- 1995: Stan Lapidus in partnership with Ed Kania at Flagship Ventures

- Idea of screening for colon cancer from a stool sample

2002: Went public and raised 56M in IPO

- Entered exclusive agreement with LabCrop to distribute the test

2004: Poor efficacy as results of a large prospective clinical trial were published in the New England Journal of Medicine

2009: Clinical Problem, enormous market opportunity

- 80 million Americans over 50, 60% unscreened

Numerous Business Considerations were identified in conjunction with technology development

- Prospective clinical data

- FDA approval

- Commercial Launch

- CMS reimbursement

- Guidelines

Keys to Success

- Clinical Utility

- Regulatory landscape, reimbursement, economic activity

- Team Culture

Conclusions/action items:

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01/26/2024 Identifying Potential Journals

Nick Herbst - Jan 26, 2024, 5:48 PM CST

Title: Identifying Potential Journals

Date: 01/26/2024

Content by: Nick Herbst

Goals: Look for potential journals we should select for our final report

Content:

- *Biomaterials Science*
  - link: https://www.rsc.org/journals-books-databases/about-journals/biomaterials-science/
  - Impact Factor: 6.6
  - "journal exploring the science of biomaterials and their translation towards clinical use"
- *Biomaterials*
  - link: https://www-sciencedirect-com.ezproxy.library.wisc.edu/journal/biomaterials
  - Impact Factor: 14
  - "...journal covering the science and clinical application of biomaterials..."
- *ACS Biomaterials Science and Engineering*
  - link: https://pubs-acs-org.ezproxy.library.wisc.edu/page/abseba/about.html
  - Impact Factor 5.7
  - "...journal scope...characterization, synthesis, and modification...new biomaterials...biomimetic approaches to biomaterials..."
- *Tissue Engineering Part A*
  - link: https://home.liebertpub.com/publications/tissue-engineering-part-a/263
  - Impact Factor: 4.1
  - "...journal for groundbreaking research on all aspects of tissue growth and regeneration including broad-ranging coverage that spans bioengineering..."

Conclusions:

I found these journals by looking at the journals our sources are from. Any of these would likely work, but we will need to discuss it as a team to select one.

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02/03/2024 Biomaterials Science Journal

Nick Herbst - Feb 07, 2024, 12:33 PM CST

Title: Biomaterials Science Journal

Date: 02/03/2024

Content by: Nick Herbst

Goals: Learn more about the journal we chose

Source: https://www.rsc.org/journals-books-databases/about-journals/biomaterials-science/

Content:

- *Biomaterials Science* is the journal we chose to "submit" our project to
- Impact factor of 6.6
- The journal has no specific guidelines for submitted papers because they have professional editors that edit and typeset the paper to match their "house style" for the final publication
- They provided a template that has the following sections
    - Title
    - Authors
        - Have superscript with affiliations for all authors
    - Abstract
        - single paragraph that summarizes paper content
    - Body
        - up to 3 levels of headings
        - "A" headings are largest and for primary heading types
            - Introduction
            - Results and Discussion (one section)
            - Experimental
        - "B" headings are subordinate to "A" headings
            - Materials under Experimental
            - Methods under Experimental
            - Specific things like Hydrogel Characterization under Results and Discussion
        - "C" headings are subordinate to "B" headings
            - Specific things like Bioprinted Hydrogel Fabrication under Methods under Experimental
    - Conclusions
    - Author Contributions
        - They have a link for authorship guidelines embedded in the template
    - Conflicts of Interest
        - if no conflicts of interest, state so
            - Would we have a conflict of interest because we are doing this for a grade????
    - Acknowledgements
    - Footnotes and References
        - Citations should be in this format
            - A. Name, B. Name and C. Name, *Journal Title*, 2020, **35**, 3523
        - The journal prefers citations from primary research over review articles
- See attached for the template

Conclusions:

After meeting, the team decided on the *Biomaterials Science* Journal. Since our final manuscript must be in the format of the journal's standards, I felt it prudent to learn what these standards are. I will inform the team that we should look at papers from this journal to get a feel for the guidelines because the journal has no guidelines since they have in-house editors.

Nick Herbst - Feb 03, 2024, 12:28 PM CST

ARTICLE

Title

Author Full Name,** Author Full Name* and Author Full Name †

A Headings are the primary heading type e.g. Introduction, Results and discussion, Experimental

B Headings should always be subordinate to A headings e.g. Synthetic procedures, Materials and methods, Crystallography.

C headings should always be subordinate to B headings e.g. General procedure for synthesis of compound X. The main paragraph text follows directly on here.

The main text of the article should appear here with headings as appropriate.

Conclusions

The conclusions section should come in this section at the end of the article, before the acknowledgements.

Author Contributions

We strongly encourage authors to include author contributions and recommend using CRediT for standardised contribution descriptions. Please refer to our general author guidelines for more information about authorship.

Conflicts of interest

In accordance with our policy on Conflicts of interest please ensure that a conflicts of interest statement is included in your manuscript here. Please note that this statement is required for all submitted manuscripts. If no conflicts exist, please state that "There are no conflicts to declare".

Acknowledgements

The acknowledgements come at the end of an article after the conclusions and before the notes and references.

Notes and references

1 Footnotes relating to the main text should appear here. These might include comments relevant but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

‡

§

¶

etc.

1 Citations should appear here in the format A. Name, B. Name and C. Name, Journal Title, 2000, 35, 3520; A. Name, B. Name and C. Name, Journal Title, 2020, 46, 3520.

2 ...

We encourage the citation of primary research over review articles, where appropriate, in order to give credit to those who first reported a finding. Find out more about our commitments to the principles of San Francisco Declaration on Research Assessment (DORA).

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02/03/2024 Cell Viability Assays in 3D Hydrogels

Nick Herbst - Feb 07, 2024, 12:33 PM CST

Title: Cell Viability Assays in 3D Hydrogels

Date: 02/03/2024

Content by: Nick Herbst

Goals: Re-review methods of determining the viability of cells embedded in hydrogels

Source: J. J.-M. Su, C.-H. Lin, H. Chen, S.-Y. Lee, and Y.-M. Lin, “Biofabrication of Cell-Laden Gelatin Methacryloyl Hydrogels with Incorporation of Silanized Hydroxyapatite by Visible Light Projection,” *Polymers (Basel)*, vol. 13, no. 14, p. 2354, Jul. 2021, doi: 10.3390/polym13142354.

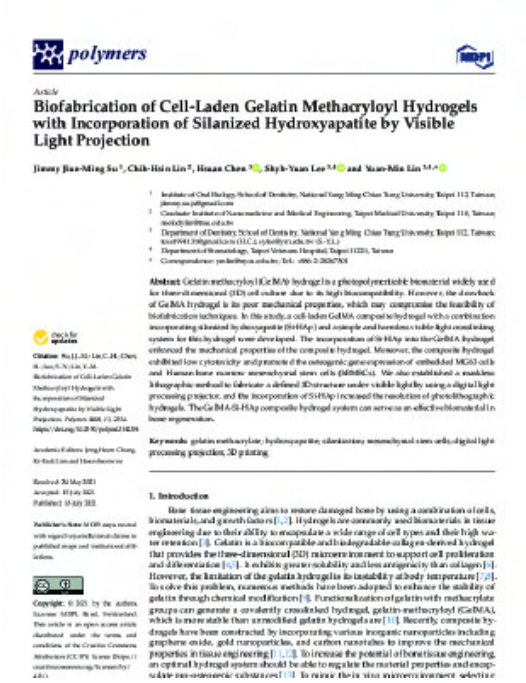
Content:

- This paper made a GelMA-hydroxyapatite composite hydrogel to increase the mechanical properties of the gel and embedded osteoblasts and MSCs into the hydrogel for bone tissue engineering
    - I focused on how they quantified cell viability
    - Of important note, they used 1% VA-086 (visible blue light) as the photoinitiator instead of 0.25% LAP (UV photocrosslinking) to reduce cytotoxicity
- This group use LIVE/DEAD staining for cell viability
    - Gels had a diameter of 6.4mm and a height of 5mm (almost 3 times the volume of our gels) and were seeded with 1e6 - 1e7 cells/mL
    - 5uL of calcein AM and 20uL of ethidium homodimer-1 were added to 10mL of PBS to make the staining solution
    - The cell-laden gels were washed with PBS twice then 200uL of the staining solution was added to each well with a gel in the culture plate
    - The gels were incubated with the staining solution for 30min and then fluorescent images were taken under a fluorescence microscope
- See attachment for the full paper

Conclusions:

One of the key takeaways from the end of last semester was that we needed to find another method for quantifying cell viability since the LIVE/DEAD (calcein/ethidium) staining gave unusable results. After reviewing many papers, I have found that the method we used is the most common method of quantifying cell viability for cells embedded in hydrogels. I *very* strongly believe that this method did not work for us last semester due to errors in methodology. No concrete/established protocol was followed for the calcein/ethidium staining. I believe that we need to try again using this method and follow a protocol. Also, we potentially need to a get new/fresh LIVE/DEAD kit from Thermofisher (catalog no. R37601, $300 for a kit with 10 of each dye)

Nick Herbst - Feb 03, 2024, 1:05 PM CST



polymers

Article

Biofabrication of Cell-Laden Gelatin Methacryloyl Hydrogels with Incorporation of Silanized Hydroxyapatite by Visible Light Projection

Jimmy Jiun-Ming Su [1], Chih-Hsin Lin [2], Hsuan Chen [3], Shyh-Yuan Lee [3,4] and Yuan-Min Lin [3,4,*]

Abstract: Gelatin methacryloyl (GelMA) hydrogel is a photopolymerizable biomaterial widely used for three-dimensional (3D) cell culture due to its high biocompatibility. However, the drawback of GelMA hydrogel is its poor mechanical properties, which may compromise the feasibility of biofabrication techniques. In this study, a cell-laden GelMA composite hydrogel with a combination incorporating silanized hydroxyapatite (Si-HAp) and a simple and harmless visible light crosslinking system for this hydrogel were developed. The incorporation of Si-HAp into the GelMA hydrogel enhanced the mechanical properties of the composite hydrogel. Moreover, the composite hydrogel exhibited low cytotoxicity and promote d the osteogenic gene expression of embedded MG63 cells and Human bone marrow mesenchymal stem cells (hBMSCs). We also established a maskless lithographic method to fabricate a defined 3D structure under visible light by using a digital light processing projector, and the incorporation of Si-HAp increased the resolution of photolithographic hydrogels. The GelMA-Si-HAp composite hydrogel system can serve as an effective biomaterial in bone regeneration.

Keywords: gelatin methacrylate; hydroxyapatite; silanization; mesenchymal stem cells; digital light processing projection; 3D printing

1. Introduction

Bone tissue engineering aims to restore damaged bone by using a combination of cells, biomaterials, and growth factors [1,2]. Hydrogels are commonly used biomaterials in tissue engineering due to their ability to encapsulate a wide range of cell types and their high water retention [3]. Gelatin is a biocompatible and biodegradable collagen-derived hydrogel that provides the three-dimensional (3D) microenvironment to support cell proliferation and differentiation [4,5]. It exhibits greater solubility and less antigenicity than collagen [6]. However, the limitation of the gelatin hydrogel is its instability at body temperature [7,8]. To solve this problem, numerous methods have been adopted to enhance the stability of gelatin through chemical modification [9]. Functionalization of gelatin with methacrylate groups can generate a covalently crosslinked hydrogel, gelatin methacryloyl (GelMA), which is more stable than unmodified gelatin hydrogels are [10]. Recently, composite hydrogels have been constructed by incorporating various inorganic nanoparticles including graphene oxide, gold nanoparticles, and carbon nanotubes to improve the mechanical properties in tissue engineering [11,12]. To increase the potential of bone tissue engineering, an optimal hydrogel system should be able to regulate the material properties and encapsulate pro-osteogenic substances [13]. To mimic the in vivo microenvironment, selective

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02/27/2024 Cell Culture Troubleshooting

Nick Herbst - Feb 27, 2024, 12:25 PM CST

Title: Cell Culture Troubleshooting

Date: 02/27/2024

Content by: Nick Herbst

Goals: Look into troubleshooting tips for cell culture issues

Source: “Poor Cell Growth Troubleshooting.” Accessed: Feb. 27, 2024. [Online]. Available: https://www.sigmaaldrich.com/US/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/poor-cell-growth

Content:

- No or few viable cells after thawing
  - Stock was possibly thawed incorrectly
    - thaw rapidly, use pre-warmed media, remove cryoprotectant ASAP, don't centrifuge at high speeds
- Poor cell attachment
  - static electricity build up on TCP flask
    - increase room humidity (maybe room is dry since it is winter??), wipe outside of flask with damp towel
  - cell and media mixing is inadequate
    - mix better
- Slow cell growth
  - media is poor quality
    - use new media
  - too many passages
    - use stock of cells that have lower passage number
  - cells were too confluent at time of passage (contact inhibition)
    - passage at 80% confluency
  - CO_2 levels aren't right so pH is wrong
    - check incubator settings
  - fluorescent room lights cause media components to convert to free radicals
    - store cells and media in the dark
- Uneven cell growth
  - cell and media mixing is inadequate
    - mix better
- See attachment for PDF of webpage

Conclusions:

Since Will thawed the 3T3 fibroblasts for use in our project, we have been having multiple issues. The first thawing yielded no viable cells, and the second thawing yielded *very* few viable cells. When these very few cells were expanded, it took a very long time and the cells proliferated unevenly with some regions of the flask reaching confluency while other regions were sparse. In light of these struggles I looked into cell culture troubleshooting tips. It has been a while since I have thawed cells and maintained cell culture, so I will have to check with Will to know what exactly he did so I can help determine what the issue is.

Nick Herbst - Feb 27, 2024, 11:52 AM CST

Poor Cell Growth Troubleshooting

- Cell Growth: An Overview
- Assessing Cell Growth
- Common Causes and Solutions to Poor Cell Growth
- General Tips and Techniques for Preventing and Eliminating Cell Growth Problems
- Video Highlights
- Related Products

CELL GROWTH: AN OVERVIEW

Ensuring adequate cell growth is a critical part of collecting accurate data with cell cultures. Cells can be cultured in suspension, or as a monolayer that attaches to cultureware, such as a flask, dish or multiwell plate. The culture method is determined by the cells' endogenous phenotype and tissue of origin – so, cells derived from blood generally grow in suspension, while those derived from solid tissues typically grow in monolayers. Co-culturing a mixed population that contains both attached cells as well as phenotypes in suspension is also possible.

Download

Poor_Cell_Growth_Troubleshooting.pdf (1.54 MB)

03/08/2024 Identifying a Full LIVE/DEAD Staining Protocol

Nick Herbst - Mar 12, 2024, 12:18 PM CDT

Title: Identifying a Full LIVE/DEAD Staining Protocol

Date: 03/08/2024

Content by: Nick Herbst

Goals: Re-re-review literature to get a more fleshed-out LIVE/DEAD staining protocol for use in 3D hydrogels

Sources:

- Molecular Probes, “LIVE/DEAD Viability/Cytotoxicity Kit.” [Online]. Available: https://tools.thermofisher.com/content/sfs/manuals/mp03224.pdf
- S. Matsos, “Live/Dead Quantification Using Fiji - Step-by-Step Guide | Suuport,” Allevi. Accessed: Mar. 08, 2024. [Online]. Available: https://www.allevi3d.com/livedead-assay-quantification-fiji/

Content:

- Notes from Molecular Probes, Inc.'s LIVE/DEAD kit protocol
    - Calcein AM and Ethidium homodimer-1 Storage
        - Store in a -20 ºC freezer
        - Protected from light
            - Either kept in a box or wrap vials in foil
        - Before using the stock solutions, allow them to thaw to RT and centrifuge them briefly before opening them
            - Fluorophores can aggregate overtime
        - Calcein AM hydrolyzes when exposed to moisture, so be sure to keep it away from water (stock solution is in anhydrous DMSO)
            - Aqueous working solutions of calcein AM should be prepared immediately prior to use and must be used within one day
            - Ethidium homodimer-1 is not sensitive to moisture; Aqueous working solutions of it can be stored at -20 ºC for up to one year
        - Before refreezing stock solutions, seal all vials tightly
    - Optimal Concentration Determination
        - Note that the source says 2 µM calcein and 4 µM ethidium works for 3T3s
        - Prepare samples of live and dead cells
            - Can get the dead cells by killing cells with 70% methanol for 30 min
        - Use a sample of dead cells to optimize the EthD-1 concentration
            - Dilute the ethidium in PBS to varying concentrations and see the lowest conc that stains the dead cell nuclei *bright* red with *minimal* cytoplasmic staining
            - Try diluting to 0.1 - 10 µM
        - Use a sample of dead cells to start to optimize the calcein AM concentration
            - Dilute the calcein in PBS to varying concentrations and see the lowest conc which gives *minimal* cytoplasm staining
            - Try diluting to 0.1 - 10 µM
        - Use a sample of live cells to confirm the optimization of the calcein AM concentration
            - Dilute the calcein in PBS to the same conc used in the prior step and see if that gives a sufficient green stain in live cells
            - If not, try a higher concentration
    - **Important Notes**
        - Calcein AM is calcein acetoxymethylester, and it functions by permeating live cell plasma membranes and then getting hydrolyzed by intracellular esterases which releases the fluorescent calcein
            - Cells need to be washed with PBS prior to LIVE/DEAD staining because media can have esterases which hydrolyze the calcein AM and **cause extracellular fluorescence**
                - This could be the source of one of our issues! We may just need to wash the cells/gels more
        - Dim the lights of the work space and have cells covered in foil when not actively adding something or imaging
            - The fluorescent dyes are photosensitive
- Notes from Allevi's LIVE/DEAD ImageJ protocol
    - Need to split the images into respective channels
        - Image > Color > Split Channels
        - Gives 3 gray-scale windows labelled with their channel names
            - Close blue, keep red and green

- Change the images to 8-bit by Image > Type
- Clean up the images
    - Eliminate background noise with Image > Adjust > B/C and move minimum bar to the right until image is cleaner without losing important signals
        - Moveing the maximum bar to the left may also help to increase staining intensity
        - Hit "Apply" when done and then repeat for other channel
            - **Keep B/C settings constant between different images of the same channel** (all green should have same settings and all red should have same settings, but green and red don't have to have the same settings)
- Live/dead cell counting is done with image segmentation
    - convert grey-scale to binary by Image > Adjust > Threshold then drag top bar to highlight only the stained cells in red
        - Too high a threshold looses signal and too low threshold fuses cells for reduced count
        - Hit "Apply" when done to get cells as white w a black background and then repeat for other channel
    - Split fused cells with Process > Binary > Watershed
    - Analyze > Set Measurements > check Area
    - Analyze > Analyze Particles
        - If there is a lot of background noise still after processing, set a minimum particle size in pixel units
        - Check Add to Manager then Ok
        - Window > ROI Manager
            - Find a ROI of a cell that is small but still a stained cell then hit measure in the manager window to get the Area
        - Rerun Analyze Particles and use a number slightly smaller than the small cell area as the minimum particle size in area units
            - Dead cells are smaller than live cells so can't use same min measurement for both channels
        - Record how many ROIs there are for that channel
            - In ROI Manager click one ROI then do ctrl+A then Measure which gives data of area of all ROIs as well as ROI count
        - REPEAT above steps for the other channel
            - First delete all ROIs from previous measurement bc ROI manager is global
- Calculate % live cells by doing # green ROIs divided by sum of green *and* red ROIs then multiply by 100

Conclusions:

After preliminary LIVE/DEAD staining, we had background noise in the ethidium homodimer-1 stain and still had strange smearing in the calcein AM stain (as if the GelMA hydrogel is getting stained). Our advisor says that our current LIVE/DEAD staining protocol is "bare bones." We need to include information on troubleshooting and analysis, so I looked into the literature more. I found information on fluorescent dye concentration optimization and storage, background noise troubleshooting tips, and ImageJ analysis. I plan to add onto our current protocol to include this information as well as information on the RFP filter for ethidium homodimer-1 since the Nikon fluorescence microscope doesn't have a default RFP filter; Will needed to set that filter up. I will then make a more formal protocol for the ImageJ analysis.

04/09/2024 LIVE/DEAD Stained Cell Survival Length

Nick Herbst - Apr 09, 2024, 12:50 PM CDT

Title: LIVE/DEAD Stained Cell Survival Length

Date: 04/09/2024

Content by: Nick

Present: Nick

Goals: Find evidence in literature to support my belief that LIVE/DEAD staining is an endpoint and you can't put the stained cells back into culture to image again later

Source: F. L. Miles, J. E. Lynch, and R. A. Sikes, “Cell-based assays using calcein acetoxymethyl ester show variation in fluorescence with treatment conditions,” J Biol Methods, vol. 2, no. 3, p. e29, 2015, doi: 10.14440/jbm.2015.73.

Content:

- calcein AM is a transient label
    - cells don't retain fluorescence after 24 hours
- It is fluorophore so it is not stable in light, so the fluorescence decreases over time
- After extended exposure it is cytotoxic to cells

Conclusions:

The other team members have proposed that we LIVE/DEAD stain our cell-laden hydrogels, image them, then add media again and keep the stained cells in culture to image them again at later timepoints. I personally have *very* heavy doubts about this plan; I think that LIVE/DEAD staining is an end point and stained cells will not survive in culture to be imaged at later timepoints. Some of my team is under the assumption that live cells that were stained with calcein AM will lose their green fluorescence and stain with ethidium homodimer-1 once they die, and that since there is no fixing agent the cells will survive extended culture. I have expressed my concerns to my teammates and suggested we make more cell-laden gels than planned so we can culture them all and image subsets at different timepoints instead of imaging the same set of stained gels over and over. However, some of my teammates are very confident that you can keep LIVE/DEAD-stained cells in culture to image again at a later time, so I set out to look for literature to support my position.

The majority of what I found was not from peer-review journals because I couldn't find anything; this leads me to believe that this issue isn't normally found in literature because it is common practice use LIVE/DEAD staining *as an endpoint*. The majority of what I found was on discussion forums like ResearchGate. I was able to find some peer-reviewed literature and listed the key points above. Overall, it looks like my thinking was correct in that we can't put the stained cells back into culture and expect the staining to "update" when we image later.

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01/26/2024 Drying Hydrogels for Swelling Quantification

Nick Herbst - Jan 26, 2024, 5:26 PM CST

Title: Drying Hydrogels for Swelling Quantification

Date: 01/26/2024

Content by: Nick Herbst

Goals: Obtain evidence to support the idea that hydrogels can be air-dried for swelling quantification

Source: S. M. Bittner et al., “Swelling Behaviors of 3D Printed Hydrogel and Hydrogel-Microcarrier Composite Scaffolds,” *Tissue Eng Part A*, vol. 27, no. 11–12, pp. 665–678, Jun. 2021, doi: 10.1089/ten.tea.2020.0377.

Content:

- This paper goes into the swelling of 3D-printed hydrogel scaffolds
  - I only focused on the methods section that described how the authors quantified the swelling ratios of their hydrogels
- The authors swelled their hydrogels in PBS and then measured the wet weight, then dried the gels and measured the dry weight, then re-swelled the gels in PBS
  - They did multiple dry-swell cycles across different time points
  - To get the wet weight, the hydrogels were removed from the PBS, blotted to remove the surface water, and the weighed
  - To get the dry weight, the hydrogels were air-dried and then dried under a vacuum
- See attachment for the full paper

Conclusions:

During our first advisor meeting while we were discussing things to do this semester, Carley brought up quantifying the swelling of our GelMA hydrogels. Since the swelling ratio uses the dry weight and wet weight of a hydrogel, Carley suggested we lyophilize our hydrogels to dry them. However, I remembered that we calculated swelling ratios in BME 430 by letting hydrogels air-dry (for 30 days). Thus I looked for a paper to support the fact that we can air-dry our gels to get the dry weight. While it will take longer, this method requires no extra equipment.

Nick Herbst - Feb 02, 2024, 7:00 PM CST

ORIGINAL ARTICLE

Swelling Behaviors of 3D Printed Hydrogel and Hydrogel-Microcarrier Composite Scaffolds

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ten.tea.2020.0377.pdf (933 kB)

02/09/2024 LIVE/DEAD Staining Protocol

Nick Herbst - Feb 11, 2024, 3:06 PM CST

Title: LIVE/DEAD Staining Protocol

Date: 02/09/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Establish protocol for LIVE/DEAD staining

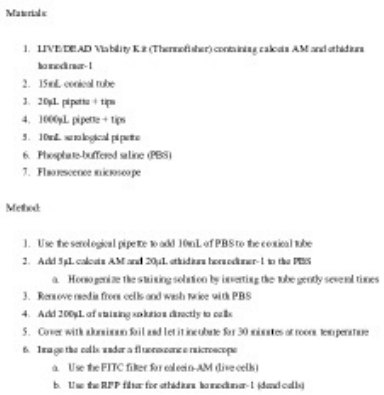
Content:

See attachment for protocol

Conclusions/action items:

Use this protocol to image the fibroblasts encapsulated in GelMA hydrogels. Cell viability can be determined by comparing the number of calcein AM positive cells to the number of ethidium homodimer-1 positive cells

Nick Herbst - Feb 11, 2024, 3:06 PM CST



Materials:

1. LIVE/DEAD Viability Kit (ThermoFisher) containing calcein AM and ethidium homodimer-1
2. 15mL conical tube
3. 20µL pipette + tips
4. 1000µL pipette + tips
5. 10mL serological pipette
6. Phosphate-buffered saline (PBS)
7. Fluorescence microscope

Method:

1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add 5µL calcein AM and 20µL ethidium homodimer-1 to the PBS
   a. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cells and wash twice with PBS
4. Add 200µL of staining solution directly to cells
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
   a. Use the FITC filter for calcein-AM (live cells)
   b. Use the RFP filter for ethidium homodimer-1 (dead cells)

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LIVE_DEAD_Staining_Protocol.pdf (35.8 kB)

03/12/2024 Improved LIVE/DEAD Staining Protocol

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Nick Herbst - Mar 12, 2024, 2:15 PM CDT

Title: Improved LIVE/DEAD Staining Protocol

Date: 03/12/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Rewrite protocol for LIVE/DEAD staining with more in-depth information

Content:

Materials:

- 15mL conical tube
- 20µL pipette + tips
- 1000µL pipette + tips
- 10mL serological pipette
- Phosphate-buffered saline (PBS)
- Fluorescence microscope
- LIVE/DEAD Viability Kit (Thermofisher) containing calcein AM and ethidium homodimer-1 (EthD-1)
    - Store in a -20 ºC freezer protected from light
        - Either keep the reagent vials in a box or wrap them in foil
    - Before using the stock solutions, allow them to thaw to RT and centrifuge them briefly before opening them
        - Fluorophores can aggregate overtime
    - Calcein AM hydrolyzes when exposed to moisture, so be sure to keep it away from water (stock solution is in anhydrous DMSO)
    - Aqueous working solutions of calcein AM should be prepared immediately prior to use and must be used within one day
    - Ethidium homodimer-1 is not sensitive to moisture; Aqueous working solutions of it can be stored at -20 ºC for up to one year
    - Before refreezing stock solutions, seal all vials tightly

Fluorescent Dye Concentration Optimization:

1. Prepare samples of live and dead cells
    1. Dead cells can be obtained by killing cells with 70% methanol for 30min
2. Use samples of dead cells to optimize the EthD-1 concentration
    1. Dilute the EthD-1 in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting EthD-1 to 0.1 - 10µM
    2. You want the lowest EthD-1 concentration which stains the dead cell nuclei bright red with minimal cytoplasmic staining
3. Use a sample of dead cells to start to optimize the calcein AM concentration
    1. Dilute the calcein AM in PBS to varying concentrations, add the dilutions to dead cells, incubate for 30min, then image
        1. Try diluting calcein AM to 0.1 - 10µM
    2. You want the lowest calcein AM concentration which gives minimal cytoplasm staining
4. Use a sample of live cells to confirm the optimization of the calcein AM concentration
    1. Dilute the calcein AM in PBS to the same concentration that used in the prior step, add it to live cells, incubate for 30min, then image
    2. See if the used concentration gives a sufficient green stain in live cells
        1. If not, try a higher concentration on another sample of live cells

LIVE/DEAD Staining:

1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add enough calcein AM and ethidium homodimer-1 to the PBS to achieve the concentrations determined in optimization process
    1. Anecdotally, 20µL of EthD-1 and 5µL of calcein AM in 10mL of PBS gives concentrations of 4µM and 2µM, respectively, and is suitable for 3T3 cells
    2. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cell-laden hydrogels and wash with PBS

Loading [MathJax]/extensions/Safe.js washes

4. Add 200µL of staining solution directly to the hydrogels
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
    1. Dim the lights in the working space and keep the gels covered whenever not imaging
    2. Use the FITC/GFP filter for calcein-AM (live cells)
    3. Use the TRITC/RFP filter for ethidium homodimer-1 (dead cells)

Tips:

- If you have extracellular fluorescence, **do additional and/or longer washes with PBS** prior to staining because media can have esterases which hydrolyze the calcein AM to cause fluorescence
    - Calcein AM is calcein acetoxymethyl ester, and it functions by permeating live cell plasma membranes and then getting hydrolyzed by intracellular esterases which releases the fluorescent calcein
- Be sure to dim the lights of the work space and have cells covered in foil when not actively adding something or imaging
    - The fluorescent dyes are photosensitive

Conclusions/action items:

Use this protocol to image the fibroblasts encapsulated in GelMA hydrogels. Use the Cell Viability Image Analysis protocol to analyze the captured fluorescent images

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03/20/2024 Cell Viability Image Analysis Protocol

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Nick Herbst - Apr 09, 2024, 11:37 AM CDT

Title: Cell Viability Image Analysis Protocol

Date: 03/20/2024

Content by: Nick Herbst

Present: Nick Herbst

Goals: Write step-by-step protocol for using ImageJ to analyze the LIVE/DEAD staining images to quantify cell viability

Content:

Materials:

- Computer
- ImageJ software with Bio-Formats plugin package
- Fluorescent LIVE/DEAD staining images

Methods:

1. Capture a fluorescent image and save the file with both the green (live) and the red (dead) channels
    1. If a Nikon fluorescence microscope was used, the file will save as a .nd2 \
2. Open the file in ImageJ
    1. File > Open > select the .nd2 file
    2. In the Bio-Formats Import Options window, set “View stack with:” to “Hyperstack” and “Color mode:” to “Default”
    3. Click “OK”
3. Split the image such that each channel is its own image
    1. Image > Color > Split Channels
    2. Close the blue channel since only the green and red channels will be used
4. Change the images’ type
    1. Image > Type > 8-bit
5. Eliminate some background noise by adjusting the brightness and contrast
    1. Image > Adjust > Brightness/Contrast
    2. A window will pop up with a pixel intensity histogram
        1. Move the “Minimum” slider to the right until the image looks cleaner without losing any important signal
        2. If necessary, move the “Maximum” slider to the left to increase the intensity of the staining
        3. When done, click “Apply”
    3. Repeat B/C adjustment for the other image
    4. **Keep B/C settings constant between different images of the same channel**
        1. All green images should have same settings and all red images should have same settings, but green and red images don't have to have the same settings
6. Convert the gray-scaled images into binary images via Thresholding
    1. Image > Adjust > Threshold
        1. Make sure dropdown menus say “Default” and “Red” and that “Dark background” is checked
    2. Move the top slider (the left bound of the the threshold) so that only the stained cells are highlighted in red
    3. Thresholding is a *critical* step
        1. Too high of a threshold results in losing a lot of signal
        2. Too low of a threshold will fuse cells close to each other and include background noise, making counting more difficult
    4. Click “Apply” to get a binary thresholded image
    5. Repeat thresholding for the other image
7. On both images, separate fused cells
    1. Process > Binary > Watershed
8. Set up the measurements window
    1. Analyze > Set Measurements
    2. Make sure “Area” is checked

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3. Click “OK”

9. Establish inclusion criteria for analysis
    1. Analyze > Analyze Particles
    2. Check “Add to Manager” and “Include holes”
    3. Set “Size” to “0-Infinity” and “Circularity” to “0.00-1.00”
    4. Click “OK”
    5. With the ROI Manager open, click on a ROI that is the smallest but is still clearly a stained cell, then click “Measure” in the window to get its area in μm^2
    6. Record this area, then click “Delete” to delete all ROIs from the manager
    7. Repeat this process for the other image
        1. Dead cells are smaller than live cells, so you cannot use the same minimum area for both images
10. Obtain stained cell counts for each image
    1. Once again, go to Analyze > Analyze Particles
    2. Check “Summarize” and “Include holes”
    3. Set “Circularity” to “0.00-1.00”
    4. Set “Size” such that the minimum is slightly smaller than the area that was recorded from the previous step and the maximum is “Infinity”
    5. Click “OK”
    6. In the Summary window that pops up, record the value in the “Count” column
        1. This is the number of stained cells for that particular channel
    7. Repeat this process for the other image
11. Calculate the percentage of live cells to obtain cell viability
    1. % Live = $(Count_{green} / (Count_{green} + Count_{red})) * 100$
        1. $Count_{green}$ = number of live cells stained with calcein AM in the green channel
        2. $Count_{red}$ = number of dead cells stained with ethidium homodimer-1 in the red channel

Conclusions/action items:

Use this protocol to analyze the captured fluorescent images

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01/26/2023 Prior Completed Trainings

Nick Herbst - Jan 26, 2024, 5:29 PM CST

Title: Prior Completed Trainings

Date: 01/26/2023

Content by: Nick Herbst

Present: Nick Herbst

Goals: Provide proof of training that was completed in prior semesters

Content:

WISCONSIN
UNIVERSITY OF WISCONSIN–MADISON

This certifies that Nicholas Herbst has completed training for the following course(s):

Course	Assignment	Completion	Expiration
Biosafety Required Training	Biosafety Required Training Quiz	8/21/2020	8/21/2025
Chemical Safety: The OSHA Lab Standard	Final Quiz	1/13/2022	
Responsible Conduct of Research	RCR Certification	9/7/2020	

Data Last Imported: 09/18/2022 08:50 PM

You have the following permits and upgrades:

Name	Date
Green Permit	01/29/2022
Lab Orientation	09/26/2020
Red Permit	01/26/2022
Laser 1	10/06/2020

Conclusions/action items:

- I have completed all necessary training for use of the TEAM Lab and the Teaching Lab

- I can get additional qualifications for the TEAM Lab/Makerspace if I want/need to

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2014/11/03-Entry guidelines

John Puccinelli - Sep 05, 2016, 1:18 PM CDT

Use this as a guide for every entry

- Every text entry of your notebook should have the **bold titles** below.
- Every page/entry should be **named starting with the date** of the entry's first creation/activity. subsequent material from future dates can be added later.

You can create a copy of the blank template by first opening the desired folder, clicking on "New", selecting "Copy Existing Page...", and then select "2014/11/03-Template")

Title: Descriptive title (i.e. Client Meeting)

Date: 9/5/2016

Content by: The one person who wrote the content

Present: Names of those present if more than just you (not necessary for individual work)

Goals: Establish clear goals for all text entries (meetings, individual work, etc.).

Content:

Contains clear and organized notes (also includes any references used)

Conclusions/action items:

Recap only the most significant findings and/or action items resulting from the entry.

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2014/11/03-Template

John Puccinelli - Nov 03, 2014, 3:20 PM CST

Title:

Date:

Content by:

Present:

Goals:

Content:

Conclusions/action items:

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