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Title: 3D Flipwell Engineering for Developing Asynchronous Systems for Toxicologic and Immunomodulatory Therapies in Bacterial, Gut, and Immune Cells

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 34
Number of Shots: 60

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Furuta Saori:** We developed our 3D co-culture system that mimics the gut mucosal environment to study the cellular crosstalk and to evaluate drug responses, which could be utilized for drug screening and for understanding mucosal cellular interactions.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.4.2*

What are the most recent developments in your field of research?

1.2. **Furuta Lsaori:** Different groups have developed several different culture systems to model the gut mucosal environment by using complex bioengineering techniques. In contrast, we developed our co-culture system which is much easier to make using less expensive materials.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?
Videographer's Note: 1.3 mislabeled as "Maria Interview 1"

1.3. **Maria Beamer:** Our co-culture system revealed that a pro-immunogenic drug triggers coordinated responses among gut bacteria, epithelia, and immune cells, activating host immunity, likely via bacterial metabolites mediating cross-species communication.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:6.2*

What research questions will your laboratory focus on in the future?

1.4. **Maria Beamer :** We aim to study both aerobic and anaerobic bacterial strains and their metabolites to uncover immune-modulating effects, paving the way for novel immunotherapeutic strategies based on bacterial metabolites.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Assembly and Sterilization of the Flipwell Co-culture Insert Stack

Demonstrator: Maria Beamer

2.1. To begin, open a Petri dish cover and set it aside **[1]**. Place a scalpel blade on the edge of the Petri dish bottom **[2]**.

2.1.1. WIDE: Talent opening the Petri dish and setting lid aside.

2.1.2. Talent removing a scalpel blade from its casing and placing it on the edge of the dish.

2.2. Take a set of sterile tweezers and gently grab the insert bottom up, to remove it from the packaging **[1]**. With the other hand take the sterile scalpel blade and bring it up to the insert **[2]**.

2.2.1. Talent using tweezers to lift the insert out.

2.2.2. Talent positioning the blade near the insert.

2.3. With a C-shaped motion pierce the membrane at the edge and gently slide the scalpel blade around the insert bottom staying close to the plastic wall **[1]**.

2.3.1. Talent performing the C-shaped cut along the membrane edge.

2.4. Cut the PET *(P-E-T)* membrane out and use the second set of tweezers to remove it **[1]**. Then use the scalpel blade to scrape any white membrane shavings to clean the edge and rim of the insert **[2-TXT]**.

2.4.1. Talent removing the cut membrane with second tweezers.

2.4.2. Talent scraping shavings from the insert rim. **TXT: Carefully set the newly prepared insert back into its original sterile pack**

2.5. Next, gently spread a thin two to three millimeters bead of silicone all the way around the insert bottom being careful not to touch the membrane **[1]**.

2.5.1. Talent applying silicone bead around insert bottom.

2.6. Pick up the second set of sterile forceps and carefully lift the first insert without the membrane out of its protective pack **[1]**. Bring the two inserts together bottom-to-bottom so that the bottom rims align **[2-TXT]**.
Videographer's Note: 2.6.1 and 2.6.2 combined into 1 shot (2.6.1)

2.6.1. Shot of the first insert being lifted out of its pack.

2.6.2. Talent aligning and stacking the two inserts bottom to bottom. **TXT: Ensure final FCI stack has only 1 PET membrane in the middle**

2.7. When the Flipwell stack is glued place it inside the original sterile pack or a deep Petri dish to dry for 72 hours **[1]**. Use the lid from the specified Petri dishes to cover the stack assembly **[2]**.

2.7.1. Talent placing stack into pack or dish.

2.7.2. Talent covering with Petri dish lid.

2.8. To sterilize the stack assemblies, use sterile forceps to hang the Flipwell stack on the rim of the specified deep Petri dish rim **[1]**. Then close the glass sash of the biosafety cabinet and turn on the ultraviolet light **[2-TXT]**.
Videographer's Note: 2.8.1 and 2.8.2 combined into 1 shot (2.8.1)

2.8.1. Talent using forceps to hang the stack.

2.8.2. Talent closing cabinet sash and switching on UV light. **TXT: Step away and sterilize each membrane side for 30 min - 1 h**

3. Leakage Testing and Collagen Coating of the Flipwell Membrane

3.1. To test for leakage prior to collagen coating, first add 500 microliters of sterile PBS or sterile deionized water **[1-TXT]**. The next day, aspirate the liquid used for testing before drying the stacks for one to two hours inside the biosafety cabinet **[2]**.

3.1.1. Talent pipetting PBS or water into the Flipwell. **TXT: Cover dish and let it rest overnight**

3.1.2. Talent aspirating liquid.
Videographer's Note: do not use Take 1

3.2. To coat the membrane with collagen, open the Petri dish lid and let the Flipwell hang off the Petri dish rim **[1]**. Carefully add 200 microliters of collagen solution to one side of the insert stack **[2]**.

3.2.1. Talent opening lid and positioning Flipwell hanging off rim.
Videographer's Note: do not use Take 1

3.2.2. Talent pipetting collagen solution into one side. **TXT: Let sit for 1 h**
Videographer's Note: 3.2.1 and 3.2.2 combined into 1 shot (3.2.1)

3.3. Aspirate the collagen solution after 1 hour **[1]**. Then pipette 200 microliters of sterile PBS **[2]**.

3.3.1. Talent pipetting out the collagen solution.

3.3.2. Talent adding 200 µL of PBS.

3.4. After aspirating the PBS, cover the Petri dish with lid and let the membrane dry for 60

minutes inside the cabinet **[1]**. When the membrane is dry, use sterile forceps or tweezers and flip the Flipwell to the opposite side and let it hang off the Petri dish rim **[2]**.

3.4.1. Talent covering dish and letting dry in cabinet.

3.4.2. Talent flipping Flipwell with forceps and hanging it off rim.

3.5. To make the bacterial insert, place two sets of sterile forceps and the twenty-four-well inserts inside the biosafety cabinet **[1-TXT]**. With sterile tweezers lift the insert from its sterile pack **[2]**. With the second set of tweezers or forceps break off the small plastic feet at the bottom of the insert **[3]**.
Videographer's Note: 3.5.1-3.5.3 combined into 1 shot (3.5.1)

3.5.1. Talent arranging forceps and inserts in cabinet. **TXT: Making the bacterial insert is an optional step**

3.5.2. Talent lifting twenty-four-well insert.

3.5.3. Talent breaking off plastic feet.

3.6. Test the twenty-four-well insert by fitting it inside one of the sterile Flipwell co-culture insert stacks or the original twelve-well inserts **[1-TXT]**.

3.6.1. Talent inserting test insert into stack. **TXT: UV sterilize before use**

4. Epithelial and THP-1 Cell Seeding on the Flipwell Co-culture Insert

4.1. To begin seeding the Flipwells, seed 500 microliters of the epithelial cells lines on each side of the Flipwell's apical side **[1-TXT]**. Cover the assemblies with a Petri dish lid then incubate at 37 degrees Celsius with 5 percent carbon dioxide overnight until the cells attach **[2]**.

4.1.1. Talent pipetting cell suspension into apical side. **TXT: 7.5 x 10^4 cells per cm^2**

4.1.2. Talent covering assemblies with a Petri dish lid and placing them in an incubator.

4.2. Open the Petri dish lid and aspirate the DMEM media from the apical side of the Flipwell stack **[1]**. With sterile forceps, pick up the Flipwell carefully by the hook and rotate it by 180 degrees **[2]**.
Videographer's Note: 4.2.1-4.3.2 combined into 1 shot (4.2.1)

4.2.1. Talent aspirating apical DMEM.

4.2.2. Talent lifting Flipwell by hook and rotating it by 180°.

4.3. With the second set of sterile forceps, grab the Flipwell stack by the other hook now facing upward **[1]**. Then lower the assembly back into the Petri dish and hang the hook over the Petri dish rim **[2]**.

4.3.1. Talent re-grasping with second forceps after rotation.

4.3.2. Talent positioning assembly hanging on dish rim.

4.4. Add 500 microliters of THP-1 *(T-H-P-One)* cell suspension to the apical side of the co-culture insert stack **[1]**. Adjust the HT-29 *(H-T-Twenty-Nine)* media for the colon epithelial cells by adding media to the deep well Petri dish **[2]**.
Videographer's Note: 4.4.1-4.4.2 combined into 1 shot (4.4.1)

4.4.1. Talent adding THP-1 suspension to the apical side.

4.4.2. Talent topping up DMEM media for the gut epithelial cells in deep well dish.

4.5. To remove any air bubbles, hold the Flipwell stack by the arm with sterile forceps **[1]**. Carefully lower a gavage needle in and under the insert assembly and very gently place the soft tip up to the air bubble **[2]**.
Videographer's Note: 4.5.1-4.5.2 combined into 1 shot (4.5.1)

4.5.1. Talent grasping Flipwell arm with forceps.

4.5.2. Talent lowering needle and positioning near air bubble.

4.6. Carefully and very slowly pull the syringe plunger up **[1-TXT]** and watch the air bubble disappear slowly **[2]**. Then carefully remove the needle and syringe from the Flipwell **[3]**.
Videographer's Note: 4.6.1-4.6.3 combined into 1 shot (4.6.1)

4.6.1. Talent pulling plunger gradually. **TXT: Stop when bubble is 2 mm in diameter**

4.6.2. Talent pausing when bubble is two millimeters.

4.6.3. Talent removing needle and syringe.

4.7. After the air bubbles have been removed with a gavage needle, culture both cell types in the cell culture incubator ~~until THP-1 cells attach and proliferate for one to two days~~ **[1-TXT]**.

4.7.1. Talent placing in incubator and monitoring proliferation. **TXT: 37°C, 5% CO2, 100%, 1 - 2 days until cells attach and proliferate**

5. Bacterial Co-Culture, Fixation, and Immunostaining Preparation of the Flipwell System

5.1. With forceps, set the insert inside a sterile Petri dish and cover with the lid **[1]**. Transfer the Petri dish with the Flipwells ~~from the incubator~~ into the biosafety cabinet and set aside on the opposite side of the dish with bacterial inserts **[2]**.

5.1.1. Talent placing insert in dish and covering.

5.1.2. Talent placing dish opposite bacterial insert side.
Videographer's Note: Do not use Take 1

5.2. Pipette out 300 microliters of the DMEM media from the apical side of the Flipwell to allow for the bacterial insert **[1-TXT]**, then cover with the petri dish lid **[2].**

Videographer's Note: 5.2.1-5.2.2 combined into 1 shot (5.2.1)

5.2.1. Talent aspirating DMEM leaving two hundred microliters and covering assembly with a Petri dish lid. **TXT: Leave about 200 µL in the stack's top side**

5.2.2. Talent covering assemblies with lid.

5.3. Now add 50 to 100 microliters of *Bacillus subtilis* culture to the 24-well bacterial inserts and insert it into the Flipwell top side **[1]**.

5.3.1. Talent pipetting B. subtilis culture into bacterial inserts and transferring them into the flipwell.

5.4. Rotate the arm and hang it from the rim of the Flipwell co-culture insert stack **[1]**. Hold the stack with the second set of sterile forceps **[2]**. After a 3 hour-incubation, use sterile forceps to remove the bacterial insert from the Flipwell **[3-TXT]**.
Videographer's Note: 5.4.1-5.4.2 combined into 1 shot (5.4.1). Do not use first 2 takes

5.4.1. Shot of the arm being rotated and hung from the Flipwell rim.

5.4.2. Talent stabilizing stack with second forceps.

5.4.3. Talent removing bacterial insert carefully. **TXT: Label with a solvent-proof marker**

5.5. Place the Flipwell inside a 50-milliliter conical tube without disassembly **[1-TXT]**.

5.5.1. Talent placing assembly into conical tube. **TXT: Submerge in 4% PFA overnight or for longer storage, use 0.4% PFA**

5.6. To disassemble the Flipwell for electron microscopy, hold it with both hands and twist off each glued part of the stack **[1]**. Rotate the insert with the membrane and set it down with the membrane facing up **[2]**.
Videographer's Note: 5.6.1-5.6.2 combined into 1 shot (5.6.1)

5.6.1. Talent twisting apart the glued stack.

5.6.2. Talent placing the membrane insert membrane-side up.

5.7. Hold the insert, carefully cut out the membrane with a scalpel blade then place it inside a 1.7 milliliter tube containing paraformaldehyde solution **[1-TXT]**.

5.7.1. Talent cutting membrane of insert and placing the cut membrane into fixation vessel. **TXT: 500 µL - 1 mL of 0.4 - 4% PFA**

5.8. For confocal microscopy, add 300 microliters of sterile PBS to the apical side ~~after aspirating the DMEM~~ **[1]**. Carefully remove the stack from the Petri dish after pipetting out the PBS **[2]**.
Videographer's Note: 5.8.1-5.9.1 combined into 1 shot (5.8.1). Do not use first take

5.8.1. Talent adding three hundred microliters PBS, then aspirating PBS

5.8.2. Talent removing stack from dish.

5.9. Now flip the Flipwell and add 300 microliters of PBS to the now facing upward RPMI *(R-P-M-I)* side of the Flipwell co-culture insert stack then aspirate the phosphate-buffered saline **[1]**.

5.9.1. Talent flipping stack and adding and removing three hundred microliters PBS to RPMI side.

5.10. Using a 200-microliter pipette, create a large droplet of PBS **[1]**. Twist off the insert assembly to separate into the inserts **[2]**.
Videographer's Note: 5.10.1-5.10.2 combined into 1 shot (5.10.1)

5.10.1. Talent pipetting a two-hundred microliter large drop of PBS.

5.10.2. Talent twisting assembly to separate inserts.

5.11. Then carefully position the Flipwell stack with THP-1 cells on top of the 200-microliter drop of PBS **[1]**. Add 200 microliters of permeabilization buffer to the top side with colon epithelial cells and leave for ten minutes **[2-TXT]**.
Videographer's Note: 5.11.1-5.11.2 combined into 1 shot (5.11.2)

5.11.1. Talent positioning THP-1 insert over PBS drop.

5.11.2. Talent adding two hundred microliters permeabilization buffer. **TXT: Aspirate and add 200 µL PBS; Repeat wash 2 x**

5.12. For immunofluorescent staining, pipette 300 microliters of primary antibody to the top of the insert **[1]**. Add 300 microliters of primary antibody to the well of a twelve-well plate then place the insert inside the well on top of the droplet **[2-TXT]**. Cover the antibody well **[3].**

5.12.1. Talent pipetting antibody onto insert top.

5.12.2. Talent pipetting 300 µL primary antibody being pipetted to the well on 12-well plate then placing the insert inside the well, on top of the droplet. **TXT: Cover and incubate at 4 °C overnight**

Added shot 5.12.3: Talent covering the antibody well

Results

6. Results

6.1. Staining showed dramatic increases of mucous secretion in the gut epithelial compartment after Sepiapterin treatment, as indicated by the increases of the epithelial marker protein CK20 and mucosal protein MUC2 **[1].**

6.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight the SEP row in the left image and the CK20-SEP and MUC2 SEP columns in the right graph*

6.2. In THP-1 monocultures, Sepiapterin treatment significantly increased the expression of the M1 macrophage marker CD80 **[1]**, while downmodulating M2 macrophage marker CD163, indicating M1 polarization **[2].**

6.2.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the green CD80 panel in the left image and the CD80 SEP column in the graph on the right*

6.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the red CD163 panel in the left image and the CD163 SEP column in the graph on the right*

6.3. Scanning electron microscopy revealed increased mucus secretion from epithelial cells in SEP-treated co-cultures **[1]**. Sepiapterin treatment induced macrophage morphology resembling M1 phenotype in both mono-cultures and co-cultures **[2]**.

6.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the mucus-covered regions in panels O and P*

6.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight panel E,S and T*

6.4. Bacterial cells in Sepiapterin-treated mono-cultures displayed wrinkled surfaces characteristic of biofilm formation **[1]**.

6.4.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the Panel L*

Pronunciation Guide:

Asynchronous
Pronunciation link: https://www.merriam-webster.com/dictionary/asynchronous
IPA: /eɪˈsɪŋ.krə.nəs/
Phonetic: ay-SING-kruh-nuhs
Immunomodulatory
Pronunciation link: https://www.merriam-webster.com/dictionary/immunomodulatory
IPA: /ɪˌmjun.oʊˈmɒd.jəˌleɪ.tɔːr.i/
Phonetic: ih-MYOO-noh-MOD-yuh-lay-toh-ree
Co-culture
Pronunciation link: https://www.merriam-webster.com/dictionary/coculture
IPA: /ˈkoʊˌkʌl.tʃər/
Phonetic: KOH-kul-chur
Mucosal
Pronunciation link: https://www.merriam-webster.com/dictionary/mucosal
IPA: /ˈmjuːkə.səl/
Phonetic: MYOO-kuh-suhl
Epithelia
Pronunciation link: https://www.merriam-webster.com/dictionary/epithelia
IPA: /ˌɛpɪˈθiːliə/
Phonetic: eh-pi-THEE-lee-uh
Sepiapterin
Pronunciation link: No confirmed link found
IPA: /ˌsɛpiˈæp.tə.rɪn/
Phonetic: sep-ee-AP-tuh-rin
Polarization
Pronunciation link: https://www.merriam-webster.com/dictionary/polarization
IPA: /ˌpoʊ.lə.rəˈzeɪ.ʃən/
Phonetic: poh-luh-ruh-ZAY-shun
Biofilm
Pronunciation link: https://www.merriam-webster.com/dictionary/biofilm
IPA: /ˈbaɪ.oʊˌfɪlm/
Phonetic: BY-oh-film
Anaerobic
Pronunciation link: https://www.merriam-webster.com/dictionary/anaerobic
IPA: /ˌænəˈroʊbɪk/
Phonetic: an-uh-ROH-bik
Immunotherapeutic
Pronunciation link: No confirmed link found
IPA: /ɪˌmjun.oʊˌθɛrəˈpjuːtɪk/
Phonetic: ih-MYOO-noh-THER-uh-pyoo-tik