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Title: Updated Protocol for the Assembly and Use of the Minibioreactor Arrays (MBRAs)

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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: 26
Number of Shots: 59

Introduction

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

1.1. **Jason Pizzini:** Our research examines how the gut microbiome can be engineered to protect against harmful pathogens while revealing the ecological rules and mechanisms behind colonization resistance.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

1.2. **Jason Pizzini:** Next-generation sequencing, advanced bioinformatics, germ-free mice, and in vitro gut models are all technologies that are transforming how we study microbial communities and their role in health and disease.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What are the current experimental challenges?

1.3. **Jason Pizzini:** Many in vitro gut models are challenging to operate at high throughput. Models that allow the functional interrogation of microbial communities at scale are needed.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Jason Pizzini:** The Minibioreactor array is a continuous flow culture system that aims to overcome the lack of throughput capacity in other systems, letting us scale up experiments while still capturing complex and reproducible microbial community behavior.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

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

Protocol

2. Preparation for Minibioreactor Array (MBRA) Assembly

Demonstrator: Jason Pizzini

2.1. To begin, ensure that the Minibioreactor array strips are 3D printed and contain 6 independent bioreactor chambers **[1-TXT]**. Arrange all the components required for the assembly **[2]**. Using a one-quarter inch-28NF fraction tap with a T-handle tap wrench, thread the three one-quarter inch ports in each chamber to insert fittings **[3]**.

2.1.1. WIDE: Talent showing a completed 3D-printed Minibioreactor array strip with 6 chambers. **TXT: MBRA: Minibioreactor Array**

2.1.2. LAB MEDIA: Table 1 *Video editor: Please show the 3 pages of the table sequentially so that all the components are shown*

2.1.3. Talent threading the ports using a one-quarter inch-28NF fraction tap with a T-handle tap wrench. ***Videographer: Please shoot the assembly steps in close-up so that the pieces being assembled are clear visible***

2.2. After washing the chamber with water, place a 10 by 3-millimeter magnetic stir bar into each chamber and add 1 milliliter of distilled water **[1]**. Then, position a rubber washer on top of each port of the bioreactor **[2]**.

2.2.1. Talent placing a magnetic stir bar and pouring water into a chamber.

2.2.2. Talent placing a rubber washer onto a port.

2.3. For each chamber, screw in one waste straw-threaded male luer, one media straw-threaded male luer, and one empty threaded male luer into the ports **[1]**.

2.3.1. Talent screwing the specified luers into the ports.

2.4. Now, insert 6 rubber septa onto three thirty-second-inch female luer barbs **[1]** and fold the upper sleeve of each septum down to cover the neck **[2]**. Attach these to the designated ports of each chamber **[3]**.

2.4.1. Talent placing rubber septa onto female luer barbs. **NOTE**: 2.4.1, 2.4.2 and 2.4.3 are combined into 1 shot

2.4.2. Talent folding the septum sleeve over the neck.

2.4.3. Talent attaching the septa to the designated ports.

2.5. Cut C-flex tubing strips of the desired length and number **[1-TXT]**. Attach a one-eighth inch female luer barb to one end and a male luer lock connector to the opposite end of each length of tubing **[2]**.

2.5.1. Talent cutting a C-flex tubing. **TXT: Cut two of each of the following lengths: 2 3/8”; 3 11/16”; 5; 6 1/2” , 7 13/16”; 9”**

2.5.2. Talent attaching a female luer barb and a male luer lock connector to each tubing length. **TXT: Cut C-flex tubing strips of desired lengths**

2.6. Then, insert a one-sixteenth-inch female luer barb into each end of the red two-stop E-lab tubing with a 1.14-millimeter inner diameter **[1-TXT]** and the orange two-stop E-lab tubing with a 0.89-millimeter inner diameter **[2-TXT]**.

2.6.1. Talent inserting one-sixteenth inch female luer barb into end of the red two-stop E-lab tubing. **TXT: Briefly submerge the E-lab tubing’s ends in near-boiling water to soften them**

2.6.2. Talent inserting into orange two-stop E-lab tubing with 0.89 millimeter inner diameter. **TXT: Repeat this process 6x for each MBRA strip**

2.7. Connect the prepared E-lab tubing to the C-flex tubing, and ensure each of the 6 C-flex tubing lengths is connected to one red and one orange E-lab line via female luers **[1]**.

2.7.1. Talent connecting orange and red E-lab tubing the C-flex tubing.

2.8. Next, cut the C-flex tubing to different lengths as required **[1-TXT]**. Attach one-eighth inch female luer barb and a male luer lock connector to both ends of one 3-inch piece and the 12-inch piece of C-flex tubing **[2]**. Attach male luer lock connectors to both ends of the remaining pieces **[3]**.

2.8.1. Talent cutting the C-flex tubing. **TXT: 1” – 21 pieces; 2” – 1 piece; 3” – 3 pieces; 12” – 1 piece**

2.8.2. Talent attaching a female luer barb and a male luer lock connector to the 3 inch and 12 inch tubing.

2.8.3. Talent attaching male luer lock connectors to both ends of the remaining tubing.

2.9. Assemble the waste line tree according to the 3D diagram **[1]**.

2.9.1. LAB MEDIA: Figure 3B.

2.10. Attach the exposed ends of the red two-stop E-lab tubing to the terminal male luer locks on the waste line tree in ascending order based on C-flex tubing length **[1]**. Then, connect the 3-inch C-flex tubing with the one-eighth-inch female luer barb and male luer lock connector to the top of the waste line tree **[2]**.

2.10.1. Talent attaching red E-lab tubing to the waste line tree. **NOTE**: 2.10.1, 2.10.2 are combined into 1 shot

2.10.2. Talent securing the 3 inch C-flex tubing to the top of the assembly.

2.11. Assemble the feed line tree according to the 3D diagram **[1]**.

2.11.1. LAB MEDIA: Figure 3B.

2.12. Bridge the exposed ends of the orange two-stop E-lab tubing to the terminal male luer locks on the feed line tree in ascending order based on C-flex tubing length **[1]** and attach the 12-inch C-flex tubing to the top of the feed line tree **[2]**.

2.12.1. Talent attaching orange E-lab tubing to the feed line tree. **NOTE**: 2.12.1, 2.12.2 are combined into 1 shot

2.12.2. Talent securing the 12 inch C-flex tubing to the top of the assembly.

3. MBRA Assembly Using the Prepared Components

3.1. Attach the variable-length C-flex tubing at the end of the feed line tree to the bioreactor **[1]**, arranging them in ascending order from the shortest line on the left to the longest on the right **[2]**.

3.1.1. Talent attaching the C-flex feed lines to the bioreactor ports.

3.1.2. Close-up of the feed lines arranged from shortest on the left to longest on the right.

3.2. Attach the variable-length C-flex tubing at the end of the waste line tree to the bioreactor strip in descending order **[1]**, with the longest line on the left and the shortest on the right to accommodate pump placement **[2]**. Bundle all C-flex feed lines together on the left side of the strip and secure them with a twist tie **[3-TXT]**.

3.2.1. Talent connecting waste lines to the bioreactor.

3.2.2. Close-up showing waste lines arranged from longest on the left to shortest on the right.

3.2.3. Talent bundling and tying the feed lines. **TXT: Repeat the process for the waste lines on the right side**

3.3. Form a loop with the orange two-stop E-lab tubing between the C-flex lines and secure the loop using autoclave tape **[1]** and repeat the process for the red two-stop E-lab tubing on the waste side of the bioreactor strip **[2]**.

3.3.1. Talent looping and securing the orange tubing with autoclave tape.

3.3.2. Talent looping and securing the red tubing with autoclave tape.

3.4. Cover the female luer at the end of the waste and feed line trees with foil to prevent contamination **[1]**. Loosen the male threaded luers with septa on each bioreactor chamber to allow steam to escape during autoclaving **[2]**.

3.4.1. Talent wrapping foil over the female luers.

3.4.2. Talent loosening threaded luers on each chamber.

3.5. After placing the assembly into an autoclave bin, stretch out the feed and waste line trees into separate bins adjacent to the one containing the MBRA strips **[1-TXT]**.

3.5.1. Talent stretching feed and waste line trees into separate bins. **TXT: Autoclave: 121 °C, ≥ 15 psi for 25 min; Once cooled, retighten the threaded male luers with the septa**

4. MBRA Connection and Operation

4.1. To attach the system to the pumps, remove the autoclave tape securing the E-lab tubing for both waste and feed lines **[1]** and untie the bundles of C-flex tubing **[2]**. Position the MBRA between the two pumps on top of the stir plate **[3]**. Clamp it down using the 3D-printed holders and align it with the marked stirring positions on the plate **[4]**.

4.1.1. Talent peeling off autoclave tape from the E-lab tubing. **NOTE**: 4.1.1, 4.1.2, 4.1.3 are combined into 1 shot

4.1.2. Talent untying bundled C-flex tubing.

4.1.3. Talent placing the MBRA between two pumps.

4.1.4. Talent clamping the MBRA into position on the stir plate.

4.2. Now, attach the feed line E-lab tubing to the peristaltic pump cartridges **[1]** and position the tubing stops into the cartridge slots **[2]**. Repeat the process for the waste line E-lab

tubing on the pump located to the right of the stir plate **[3]**. Then, lock the peristaltic pump cartridges into the pump **[4-TXT]**.

4.2.1. Talent attaching feed line E-lab tubing to pump cartridges. **NOTE**: 4.2.1, 4.2.2 are combined into 1 shot

4.2.2. Close-up of tubing stops seated in the cartridge slots.

4.2.3. Talent attaching waste line E-lab tubing to the right-hand pump.

4.2.4. Talent locking cartridges into the pump. **TXT: Ensure the cartridges are seated fully**

4.3. Arrange the C-flex tubing neatly using the 3D-printed tube holders **[1]**. Connect the end of the waste line tree to the tubing connected to the waste bottles **[2]**. Next, attach the female luer on the feed line entry tubing to the male connector on the 12 inch tube from the media bottle cap **[3]**.

4.3.1. Talent placing C-flex tubing into the 3D-printed tube holders.

4.3.2. Talent connecting waste line tree tubing to waste bottle tubing.

4.3.3. Talent connecting feed line entry tubing to media bottle cap tubing.

4.4. Turn on both pumps to start media flow **[1]** and ensure both pumps are set to clockwise rotation when waste is positioned to the right of the pumps **[2]**.

4.4.1. Talent powering on both pumps. **NOTE**: 4.4.1, 4.4.2 are combined into 1 shot

4.4.2. Close-up of pump settings showing clockwise rotation.

4.5. Observe the droplet size and cadence in each bioreactor chamber. If any variability **[1]** or abnormality is noticed **[2]** replace the orange two-stop E-lab tubing connected to the affected chamber to reduce flow rate variation **[3]**. Once the chambers are full, shut off both pumps and allow the bioreactors to sit for 24 to 48 hours to check for contamination before starting the experiment **[4]**.

4.5.1. Close-up of left most reactor where no droplet is falling. **NOTE**: Please draw a circle around the left most reactor to show that drops are not falling

Added shot: pointing to the offending reactor. **NOTE**: This is slated as 4.5.2 and shot along with the next shot of replacing the tube

4.5.2. Talent following the line to the orange stop tubing and replacing the orange E-lab tubing line.

4.5.3. Talent switching off the pumps.

5. MBRA Disassembly and Refurbishment

5.1. Switch the media input to a 1 liter container of 10 percent bleach in deionized water **[1]** and increase the flow rate on both pumps to maximum to displace the contents of the bioreactor chambers with bleach **[2]**.

5.1.1. Talent replacing the media input line with a container of bleach solution.

5.1.2. Shot of Pump control panel showing the flow rate being increased to maximum.

5.2. Once the chambers are clear of media, invert the MBRA to disinfect above the fill line for 5 minutes **[1]**. After 5 minutes, right the system and wait an additional 5 minutes for sterilization **[2]**.

5.2.1. Talent inverting the MBRA assembly.

5.2.2. Talent placing the MBRA upright on the bench.

5.3. After the chambers have been cleared of media and have been sterilized for 10 minutes **[1]**, replace the bleach with 1 liter of deionized water and flush the system until the water has passed through **[2]**. Then, disconnect the bioreactor E-lab tubing from the pumps and remove the MBRAs **[3]**.

5.3.1. Shot of the clean system.

ADDED SHOT: Talent switching from 10% bleach to DI water

5.3.2. Talent detaching the E-lab tubing from the pumps and removing the MBRA assembly.

5.4. Remove the used septa from the bioreactor **[1]** and drain each chamber until only 1 milliliter of water remains **[2].** Replace the septa and the orange 2-stop E-lab tubing before autoclaving the assembled strip **[3]**. Finally, refurbish and repair the MBRA as required **[4]**.

5.4.1. Talent removing septa from the chamber ports.

5.4.2. Talent tilting the MBRA to drain water, leaving a small volume behind.

5.4.3. Talent replacing the septa and the orange 2-stop E-lab tubing.

5.4.4. TEXT ON PLAIN BACKGROUND:

- Autoclave up to 3 reuse cycles

- After 3rd reuse: Fully disassemble MBRA

  Replace C-flex tubing and sterilize parts

  Replace if damaged

- Reapply epoxy to waste and feed PTFE connections if brittle

Results

6. Results

6.1. A human fecal sample was prepared and grown in the MBRA system. After four days of continuous flow **[1]**, the microbial community in all nine bioreactors was dominated by 18 bacterial genera, each comprising at least 2% of relative abundance in any replicate **[2]**.

6.1.1. LAB MEDIA: Figure 5A.

6.1.2. LAB MEDIA: Figure 5A. *Video editor: Sequentially Highlight the colored stacked bars in the chart*.

6.2. Twenty-two out of the 65 detected genera were present in all nine bioreactor replicates, demonstrating high reproducibility **[1]**.

6.2.1. LAB MEDIA: Figure 5A.

6.3. Alpha diversity analysis showed minimal variation between replicates **[1]** in both the observed operational taxonomic units **[2]** and Shannon diversity index **[3]**.

6.3.1. LAB MEDIA: Figure 5B.

6.3.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the top panel showing "Observed OTUs"* .

6.3.3. LAB MEDIA: Figure 5B. *Video editor: Highlight the bottom panel showing "Shannon Diversity"* .

1. Bioreactor

Pronunciation link:
https://www.howtopronounce.com/bioreactor
youtube.com+4oxfordlearnersdictionaries.com+4howjsay.com+4howtopronounce.com+9howtopronounce.com+9justpronounce.com+9

IPA (American English): /baɪˈɒr.iˌæk.tər/

Phonetic Spelling: bye-OR-ee-ak-ter

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2. Luer

Pronunciation link:
https://www.merriam-webster.com/medical/Luer%20syringe youglish.com+11merriam-webster.com+11youtube.com+11

IPA (American English): /ˈluː.ər/

Phonetic Spelling: LOO-er

2. Septum

Pronunciation link:
https://dictionary.cambridge.org/pronunciation/english/septum youtube.com+15dictionary.cambridge.org+15dictionary.cambridge.org+15

IPA (American English): /ˈsɛp.təm/ dictionary.cambridge.org+1

Phonetic Spelling: SEP-tuhm