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Title: A Continuous Culture Millifluidic Device for the Study of *Escherichia Coli* Under Low and Fluctuating Nutrient Conditions

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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? **Yes, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Juanita Lara-Gutierrez, Postdoc at ETH Zürich

Current Protocol Length

Number of Steps: 12

Number of Shots: 35

Interviews

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Juanita Lara-Gutierrez:** My research focuses on examining how bacteria adapt physiologically to the low and fluctuating nutrient conditions typical of natural environments.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Juanita Lara-Gutierrez:** Conventional cultivation methods poorly replicate low, dynamic nutrient conditions; whereas our system enables cultivation under these environmentally relevant conditions.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Juanita Lara Gutierrez:** The main challenge during low-nutrient cultivation is maintaining adequate cell densities to prevent clogging, pressure buildup, and nutrient depletion.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Juanita Lara-Gutierrez:** Following this procedure, cells can be harvested for downstream analyses, including proteomics, metabolomics, and other omics approaches.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Juanita Lara-Gutierrez:** Future studies can investigate microbial adaptation to nutrient limitation and fluctuations using integrated quantitative omics analyses.
 - 1.5.1. 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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

~~What value do you expect this video article to have for other researchers working in your field?~~

- 1.6. **Juanita Lara-Gutierrez, Postdoc at ETH Zürich:** (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How does the video format of your article help you to share your work with the broader scientific community?~~

- 1.7. **Juanita Lara-Gutierrez, Postdoc at ETH Zürich:** (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: SCREEN timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sri Lakshmi Priya) for queries regarding lab media.

2. Preparation of Bacterial Pre-culture and Medium

Demonstrator: Juanita Lara-Gutierrez

- 2.1. Prepare the overnight or ON (*ON*) culture the day before the experiment [1-TXT]. Prepare a pre-culture by adding 6 microliters of ON culture to a tube containing 6 milliliters of full rich-defined media or RDM (*R-D-M*) [2]. Divide the mixture equally into two 15-milliliter culture tubes and label them as A and B [3]. Incubate both tubes at 37 degrees Celsius with shaking at 200 rpm [4].
 - 2.1.1. WIDE: Talent sitting next to the prepared ON culture tube kept on the workbench. **TXT: Strain: *E. coli* NCM3722** *Videographer: Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent adding ON culture into a tube containing full RDM.
 - 2.1.3. Talent dividing the culture into two tubes and labeling them A and B.
 - 2.1.4. Talent placing the tubes into an incubator shaker.
- 2.2. Monitor the optical density at 600 nanometers using tube B and do not proceed until the optical density reaches approximately 0.5 [1].
 - 2.2.1. SCREEN: 70652_2.2_t2.mp4: 00:00-00:10
- 2.3. During incubation, prepare the low RDM medium [1] by mixing the required reagents and allocating the total volume across bottles for different time points [2]. Then, seal each bottle with caps fitted with three tubes [3].
 - 2.3.1. Talent arranging the reagents on the workbench.
 - 2.3.2. TEXT ON A PLAIN BACKGROUND:
Low RDM Medium:
10x MOPS modified rich buffer - 620 mL
0.132 M potassium phosphate dibasic solution - 62 mL
Autoclaved milliQ water - 5518 mL
5x EZ supplement - 0.124 mL
10x ACGU solution - 0.248 mL

Medium allocation:

- ~900 milliliters: 1 liter bottle (time points 1 and 2)
- ~2100 milliliters: 2.5 liter bottle (time points 3 and 4)
- ~3200 milliliters: 4 liter bottle (time points 5 and 6)
- 2.3.3. Talent sealing bottles with 3 tubed caps.

3. Setting up the Millifluidic Continuous Culture Device System

- 3.1. Connect the third-widest silicone tube to a 0.2 micrometer vent filter for sterile air exchange [1]. Attach one end of each peristaltic tube with free Male Luer lock fittings to the smaller diameter silicone tubes [2] while keeping the aluminum foil cover on the free fitting [3].
 - 3.1.1. Talent connecting a vent filter to the third silicone tube.
 - 3.1.2. Talent attaching peristaltic tubing to the smaller diameter silicone tubes.
 - 3.1.3. Shot of aluminum foil cover on the free fitting.
- 3.2. Place the bottles and peristaltic pump into an incubator set to 37 degrees Celsius [1]. Secure all peristaltic tubing into the channels of the same pump head, ensuring identical flow rates across all filters [2].
 - 3.2.1. Talent placing bottles and pump into the incubator.
 - 3.2.2. Talent fixing tubing into the pump head channels.
- 3.3. Dilute the pre-culture at a ratio of 1 to 10 by adding 600 microliters of culture into a tube containing 5.4 milliliters of low RDM [1]. Using a 1-milliliter pipette, introduce 1-milliliter of diluted culture into each of six 0.45-micrometer polyvinylidene fluoride or PVDF (*P-V-D-F*) filters [2].
 - 3.3.1. Talent pipetting pre-culture into a tube containing low RDM.
 - 3.3.2. Talent loading diluted culture into PVDF filters.
- 3.4. After rinsing one-way check valves with autoclaved ultrapure water, attach them to each end of the filters [1]. Remove the aluminum foil covers from tubing fittings [2]. Connect each filter to the peristaltic tubing fittings [3].
 - 3.4.1. Talent rinsing check valves and attaching them to filters.
 - 3.4.2. Talent removing foil covers from tubing.
 - 3.4.3. Talent connecting filters to tubing fittings.
- 3.5. Set the flow rate to 2 milliliters per minute on the peristaltic pump and start the flow [1-TXT]. Place a waste receptacle below the filters to collect outflow and start the flow [2]. NOTE: VO is edited as per the revised shot
 - 3.5.1. SCREEN: 70652_3.5.1_t3.mp4: 00:02-00:10 TXT: Time 0
 - 3.5.2. Talent positioning a waste container beneath the filters and starting the pump.

4. Sample Collection and Analysis

- 4.1. After 2 hours, unplug one of the filters connected to the 1-liter bottle [1]. Unscrew the one-way check valves [2] and place the filter into a 50-milliliter centrifuge tube with the inlet facing downward [3].
 - 4.1.1. Talent disconnecting a filter from the setup.
 - 4.1.2. Talent unscrewing the check valve.
 - 4.1.3. Talent placing the filter into a centrifuge tube with the inlet facing downward.
- 4.2. Centrifuge at 2000 g for 2 minutes [1]. Using a sterilized pipe cutter, apply gentle pressure at the outlet end of the plastic casing to open the filter [2]. Release the inner cylinder and discard the membrane-containing section [3].
 - 4.2.1. Talent placing the tube into a centrifuge.
 - 4.2.2. Talent pressing the outlet end of the plastic casing with pipe cutter and opening the filter.
 - 4.2.3. Talent removing and discarding the membrane section.
- 4.3. Rinse the inner walls of the filter casing with the collected culture medium [1]. Measure the total collected volume using a 5-milliliter serological pipette [2].
 - 4.3.1. Talent rinsing the inner casing walls with collected medium.
 - 4.3.2. Talent measuring liquid volume with a pipette.
- 4.4. Take three 100 microliter aliquots from the sample [1] and fix them with glutaraldehyde to a final concentration of 1.5 percent [2].
 - 4.4.1. Talent pipetting aliquot into a tube.
 - 4.4.2. Talent adding glutaraldehyde into the tube.
- 4.5. Unplug the corresponding filters [1] at subsequent time points and repeat the collection procedure [2]. Label the collected samples according to their respective time points [3]. Label the samples with the fluorescent DNA dye [4] and count the cells using flow cytometry [5].
 - 4.5.1. Talent unplugging the filters.
 - 4.5.2. TEXT ON A PLAIN BACKGROUND:
4 hours: 1-liter bottle
6 hours and 8 hours: 2.5-liter bottle
10 hours and 12 hours: 4-liter bottle
 - 4.5.3. Close-up shot of labeled centrifuge tubes arranged in order of collection time points while the talent writes or applies labels to each sample tube.
 - 4.5.4. Close-up shot of the talent adding the fluorescent DNA dye into the collected sample tube using a pipette.
 - 4.5.5. Shot of the labeled sample tube being loaded into the flow cytometer sample holder for cell counting.

Results

5. Results

- 5.1. Over time, the log₁₀-transformed (*log-ten-transformed*) cell counts followed linear trajectories [1], indicating exponential growth in all conditions [2].
 - 5.1.1. LAB MEDIA: Figure 4A–C. *Video editor: Show the straight fitted lines across the data points.*
 - 5.1.2. LAB MEDIA: Figure 4A–C. *Video editor: Highlight the data points aligning closely along the straight lines.*
- 5.2. The growth rates were 0.88 plus or minus 0.03 per hour for the 0.5 percent RDM condition [1], 0.64 plus or minus 0.06 per hour for the 0.25 percent condition [2], and 0.18 plus or minus 0.002 per hour for the 0.01 percent condition [3].
 - 5.2.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the label “ $\mu = 0.88$ ” in the 0.5% RDM panel.*
 - 5.2.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the label “ $\mu = 0.64$ ” in the 0.25% RDM panel.*
 - 5.2.3. LAB MEDIA: Figure 4C. *Video editor: Highlight the label “ $\mu = 0.18$ ” in the 0.01% RDM panel.*
- 5.3. The cell counts reached up to 1 billion cells per sample [1], demonstrating that the millifluidic continuous culture device or MCCD (*M-C-C-D*) supports both exponential growth and high biomass yield at nanomolar to micromolar nutrient concentrations [2].
 - 5.3.1. LAB MEDIA: Figure 4A–C. *Video editor: Highlight the dots just below the “9” mark on the y-axis at the right end of each graph.*
 - 5.3.2. LAB MEDIA: Figure 4A–C.
- 5.4. Cells grown in 0.5 percent RDM had a mean length of 1.8 plus or minus 0.3 micrometers [1], whereas cells grown in 0.01 percent rich-defined medium had a mean length of 1.1 plus or minus 0.28 micrometers [2].
 - 5.4.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the inset image showing a single larger rod-shaped cell.*
 - 5.4.2. LAB MEDIA: Figure 4E. *Video editor: Highlight the inset image showing a single smaller rod-shaped cell.*

Pronunciation guides:

1. Millifluidic
Pronunciation link: No confirmed link found
IPA: /ˌmɪlɪfluˈɪdɪk/
Phonetic Spelling: mil·ee·floo·id·ik
2. Silicone
Pronunciation link: <https://www.merriam-webster.com/dictionary/silicone>
IPA: /ˈsɪlɪˌkoʊn/
Phonetic Spelling: sil·ih·kohn
3. Peristaltic
Pronunciation link: <https://www.merriam-webster.com/dictionary/peristaltic>
IPA: /ˌperəˈstɔːltɪk/
Phonetic Spelling: peh·ruh·stawl·tik
4. Luer
Pronunciation link: <https://www.merriam-webster.com/dictionary/Luer>
IPA: /ˈluːər/
Phonetic Spelling: loo·er
5. Aluminum
Pronunciation link: <https://www.merriam-webster.com/dictionary/aluminum>
IPA: /əˈluːmənəm/
Phonetic Spelling: uh·loo·muh·nuhm
6. Polyvinylidene
Pronunciation link: <https://www.merriam-webster.com/medical/polyvinylidene>
IPA: /ˌpɑːliˌvaɪnəˈlɪdɪn/
Phonetic Spelling: pol·ee·vy·nuh·lid·een
7. Fluoride
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluoride>
IPA: /ˈflʊr.aɪd/
Phonetic Spelling: floor·ide
8. Receptacle
Pronunciation link: <https://www.merriam-webster.com/dictionary/receptacle>
IPA: /rɪˈseptɪkəl/
Phonetic Spelling: rih·sep·ti·kuhl

9. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
10. Sterilized
Pronunciation link: <https://www.merriam-webster.com/dictionary/sterilize>
IPA: /ˈsterəˌlaɪz/
Phonetic Spelling: stair·uh·lyze
11. Serological
Pronunciation link: <https://www.merriam-webster.com/dictionary/serological>
IPA: /ˌsɪrəˈlɑːdʒɪkəl/
Phonetic Spelling: seer·uh·laa·ji·kuhl
12. Aliquots
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
IPA: /ˈælɪˌkwɑːt/
Phonetic Spelling: al·ih·kwaat
13. Glutaraldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/glutaraldehyde>
IPA: /ˌgluːtərˈældəˌhaɪd/
Phonetic Spelling: gloo·ter·al·duh·hyde
14. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊˈresənt/
Phonetic Spelling: floo·res·uhnt
15. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /saɪˈtɑːmətri/
Phonetic Spelling: sy·taa·muh·tree
16. Trajectories
Pronunciation link: <https://www.merriam-webster.com/dictionary/trajectory>
IPA: /trəˈdʒektəri/
Phonetic Spelling: truh·jek·tuh·ree
17. Exponential
Pronunciation link: <https://www.merriam-webster.com/dictionary/exponential>
IPA: /ˌɛkspəˈnenʃəl/
Phonetic Spelling: ek·spuh·nen·shuhl
18. Biomass
Pronunciation link: <https://www.merriam-webster.com/dictionary/biomass>

IPA: /'baɪoʊˌmæs/

Phonetic Spelling: by·oh·mas

19. Nanomolar

Pronunciation link: No confirmed link found

IPA: /ˌneɪnoʊˈmoʊlər/

Phonetic Spelling: nay·noh·moh·ler

20. Micromolar

Pronunciation link: No confirmed link found

IPA: /ˌmaɪkroʊˈmoʊlər/

Phonetic Spelling: my·kroh·moh·ler

21. Rod-shaped

Pronunciation link: <https://www.merriam-webster.com/dictionary/rod-shaped>

IPA: /'rɔːdˌʃeɪpt/

Phonetic Spelling: raad·shaypt