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Title: Creating Rapid Oxygen Oscillations in Microbial Single-Cell Growth Analysis Using a Microfluidic Double-Layer Device

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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**

Current Protocol Length
Number of Steps: 25
Number of Shots: 55

Introduction

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

1.1. **Keitaro Kasahara:** We have developed a double-layer microfluidic chip that creates rapid oxygen oscillations, allowing us to observe microbial growth behavior under rapidly oscillating oxygen conditions mimicking natural and industrial environments.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.3*

What are the current experimental challenges?

1.2. **Keitaro Kasahara:** Traditional experimental setups lack the precision and speed needed to mimic rapid oxygen fluctuations and do not support continuous, high-resolution monitoring of microbes.

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

What advantage does your protocol offer compared to other techniques?

1.3. **Keitaro Kasahara:** The double-layer PDMS microfluidic chip enables microbial single-cell growth analysis under controlled oxygen conditions, including rapidly oscillating oxygen conditions.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.4*

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

Protocol

2. Fabrication and Bonding of a Multilayer PDMS Microfluidic Device

Demonstrator: Keitaro Kasahara

2.1. To begin, thoroughly mix the PDMS *(P-D-M-S)* base and curing agent in a 10 to 1 ratio to prepare the pre-cure solution **[1]**. Place the mixture in a desiccator and degas for 1 hour **[2]**.

2.1.1. WIDE: Talent adding PDMS base and curing agent into a beaker and mixing thoroughly with a spatula.

2.1.2. Talent placing the beaker with PDMS mixture into a desiccator and closing the lid.

2.2. Clean the top layer mold with isopropanol **[1]** and dry it using compressed air **[2]**.

2.2.1. Talent rinsing the top layer mold with isopropanol.

2.2.2. Talent using compressed air to dry the mold completely.

2.3. Now, pour the PDMS mixture into the cleaned top layer mold **[1]**. Then thermally cure the mold at 80 degrees Celsius for 20 minutes to partially cure the PDMS **[2]**. Check the PDMS stickiness by gently pressing it with tweezers or by hand **[3-TXT]**.

2.3.1. Talent pouring the PDMS mixture into the top layer mold.

2.3.2. Talent placing the filled mold into an oven set at 80 degrees Celsius.

2.3.3. Talent checking PDMS surface stickiness using tweezers. **TXT: If the PDMS is still sticky, adjust heating time**

2.4. Once cured, peel the PDMS out of the mold **[1]** and cut it into 20 by 18-millimeter single pieces **[2]**.

2.4.1. Talent peeling the cured PDMS from the mold.

2.4.2. Talent using a blade to cut the PDMS into rectangular pieces. **AUTHOR'S NOTE:** Please don't use the last seconds

2.5. Punch 0.75-millimeter diameter holes for gas inlets into each piece **[1]**. Then rinse the bottom surface with isopropanol **[2]**. ~~After drying with compressed air, attach adhesive tape **[3].**~~

2.5.1. Talent punching holes into each PDMS piece using a hole punch.

2.5.2. Talent rinsing the bottom surface of the PDMS with isopropanol.

~~2.5.3. Talent applying adhesive tape to the bottom surface of each piece.~~
Note: 2.5.3 removed by the authors

2.6. Next, clean the bottom layer mold with isopropanol and dry it **[1]**. Fix the mold on a spin coater **[2]** then pour the PDMS mixture at the center, close to the wafer, to minimize bubble formation **[3-TXT]**.

2.6.1. Talent cleaning the bottom mold with isopropanol.

2.6.2. Shot of the mold being fixed on a spin coater.

2.6.3. Talent pouring PDMS onto the center of the mold. **TXT: Use tweezers/needles to move visible air bubbles to the outer edge**

2.7. Spin coat the PDMS at 1000 revolutions per minute for 60 seconds **[1]**. Thermally cure the coated PDMS at 80 degrees Celsius for 10 minutes **[2-TXT]**.

2.7.1. Talent setting the spin coater and starting the spin coating process.

2.7.2. Talent placing the mold in the oven for 10 minutes at 80 degrees Celsius. **TXT: Check stickiness and adjust heating time, if necessary**

2.8. Now place the top PDMS pieces onto the bottom PDMS layer on the mold **[1]** and press firmly to ensure surface attachment **[2]**.

2.8.1. Talent placing the rectangular top PDMS pieces onto the bottom layer.

2.8.2. Talent using gloved fingers or a roller to press and attach both layers.

2.9. Thermally cure the assembled layers at 80 degrees Celsius for at least 1 hour to complete the bonding **[1]**.

2.9.1. Talent placing the assembled chip back into the oven and setting the timer for 1 hour.

2.10. Then gently cut the bottom PDMS layer into individual pieces **[1]** and carefully peel off the entire PDMS chip from the mold **[2]**.

2.10.1. Talent using a scalpel to cut the bottom layer into chip-sized pieces.

2.10.2. Talent gently peeling the PDMS chip from the mold by hand. **AUTHOR's NOTE:** 2.10.1 and 2.10.2 are in one clip

2.11. Punch 0.5-millimeter diameter holes for fluid inlets and outlets at both ends of the fluid channels **[1]**. Rinse the bottom surface of the chip with isopropanol **[2].** ~~and attach scotch tape to the dried chip **[3].**~~

2.11.1. Talent punching small holes at both ends of the PDMS fluid channels.

2.11.2. Talent rinsing the chip bottom surface with isopropanol.

~~2.11.3. Talent applying scotch tape to the bottom surface of the chip.~~
Note: 2.11.3 removed by the authors

2.12. Activate the PDMS surface by plasma oxidation for 25 seconds **[1]** and bond it to a 0.175-millimeter-thick cover glass **[2]**.

2.12.1. Talent placing the chip and a cover glass in a plasma cleaner.

2.12.2. Talent aligning and pressing the PDMS chip onto the glass substrate.

2.13. After placing the chip on the glass, flush the top layer with pressurized air to ensure that the PDMS membrane is properly attached to the glass **[1]**. Heat the bonded chip at 80 degrees Celsius for 10 seconds to 1 minute to increase bonding stability **[2]**.

2.13.1. Talent flushing the top layer with air.
AUTHOR's NOTE: 2.12.2 and 2.13.1 are in one clip

2.13.2. Talent placing the bonded chip into the oven and setting a short heating cycle.

3. Microscopy Setup, Dye Perfusion, and Gas Control for FLIM-Based Oxygen Sensing

3.1. Select the appropriate objective lens on the microscope, such as 20X for oxygen sensing or 100X for microbial observation **[1-TXT]**. Then fix the double-layer chip on the chip holder using adhesives **[2]**.

3.1.1. Talent rotating the microscope turret to select the objective lens. **TXT: Add immersion oil if necessary**

3.1.2. Talent fixing the double layer chip onto the chip holder with adhesives

3.2. Next prepare an oxygen-sensitive dye solution at the appropriate concentration **[1-TXT]**.

3.2.1. Shot of prepared dye solution in a labeled microtube. **TXT: 3 mM tris(2,2'-bipyridyl)dichlororuthenium(II)hexahydrate, RTDP is being used here**

3.3. Calibrate the FLIM *(Flim)* camera using a reference slide with a known lifetime, such as 3.75 nanoseconds **[1]**. Measure the signal intensity with the FLIM camera **[2-TXT].**

3.3.1. Talent placing a calibration slide under the FLIM camera.

3.3.2. SCREEN: 3.3.2.mp4 00:06-00:15
TXT: Adjust exposure time to reach signal intensity between 0.68 - 0.72

3.4. Fix the chip holder on the microscope stage and connect the appropriate tubing to the fluid inlet and outlet **[1]**. Then start perfusing the oxygen-sensitive dye solution at a constant flow rate of 100 nanoliters per minute using a syringe pump **[2]**.

3.4.1. Talent securing the chip holder onto the microscope stage and attaching inlet and outlet tubing to the chip. AUTHOR's NOTE: 3.4.1 is in two clips. Second clip is from " connecting the tubing"

3.4.2. Talent programming the syringe pump to initiate dye perfusion.

3.5. Next, connect the gas inlet and mass flow controllers with suitable tubing **[1]**. Begin flushing gas with controlled oxygen concentrations **[2-TXT]**.

3.5.1. Talent connecting gas tubing to the chip and mass flow controllers.

3.5.2. Talent setting the flow rates on the gas controller and starting the gas flow. **TXT: 0% and 21% oxygen, total mass flow rate 600 mL/min**

3.6. Measure the phase lifetime in the absence of oxygen and at another known oxygen concentration **[1]**. Calculate the quenching constant using the Stern-Volmer equation **[2]**.

3.6.1. SCREEN: 3.6.1.mp4 00:00-00:17

3.6.2. SCREEN: 3.6.2.mp4 00:00-00:11
AND
TEXT ON PLAIN BACKGROUND:

$$[O_2] = \frac{1}{K_q}\left(\frac{\tau_0}{\tau} - 1\right) = \frac{1}{K_q}\left(\frac{I_0}{I} - 1\right)$$

Video editor: Please play both shots side by side

4. Cell Seeding, Cultivation, and Time-Lapse Imaging under Controlled Oxygen Conditions

4.1. Prepare a seeding solution by diluting the *Escherichia coli* culture to the desired optical density **[1]**. Transfer the solution into a 1-milliliter syringe for loading **[2]**.

4.1.1. Talent measuring the E. *coli* culture's optical density and diluting it in a tube.

4.1.2. Talent drawing the seeding solution into a 1 milliliter syringe.

4.2. Fix the chip holder on the microscope stage **[1].** ~~Allow the chip and holder to warm up in the incubator at 37 degrees Celsius for several hours to reduce defocusing during imaging **[2].**~~

4.2.1. Shot of the chip holder being fixed on a microscope stage.

~~4.2.2. Talent placing the chip holder with the mounted chip onto the microscope stage inside an incubator.~~
Note: 4.2.2 removed by the authors

4.3. Before starting cultivation, begin gassing with the desired initial oxygen concentration by adjusting the oxygen and nitrogen mixture while maintaining the total and carbon dioxide flow rates constant **[1]**. Then connect the gas inlet tubing to the chip and mass flow controllers **[2]**.

4.3.1. Talent adjusting the gas controller settings to set the oxygen, nitrogen, and carbon dioxide mixture.

4.3.2. Talent connecting the gas inlet tubing securely between the chip and the flow controller ports.

4.4. Connect the seeding syringe to the fluid outlet and manually push the syringe to begin seeding the bacteria onto the chip **[1]**. Observe under the microscope to verify

successful trapping of cells in the cultivation chambers **[2]**.

4.4.1. Talent connecting the syringe with seeding solution to the fluid outlet.

4.4.2. Talent observing cells in the cultivation chambers on the screen.

4.5. After cell inoculation, disconnect the seeding syringe and attach a syringe filled with fresh medium **[1]**. Manually push the syringe to flush out any remaining cells from the medium supply channel **[2]**.

4.5.1. Talent removing the seeding syringe and replacing it with a fresh medium syringe.

4.5.2. Talent manually flushing the channel with medium using the syringe.

4.6. Begin perfusing the medium at a constant rate of 100 nanoliters per minute using the syringe pump **[1-TXT]**.

4.6.1. Talent starting the syringe pump and monitoring the medium flow. **TXT: Ensure that flow rate is enough to prevent chamber drying**

4.7. Start time-lapse image acquisition **[1]**. For cultivation under oscillating oxygen conditions, begin automatic control of mass flow controllers simultaneously with image capture **[2-TXT]**.

4.7.1. SCREEN: 4.7.1.mp4 00:00-00:08

4.7.2. Talent starting time-lapse image acquisition and automatic gas control. **TXT: If there is any delay, record the timing difference**

Results

5. Results

5.1. The oxygen concentration in the fluid channel of the microfluidic chip decreased from 21% to 0% within 10 seconds **[1]** and increased back to 21% within a similar time frame upon reoxygenation **[2]**.

5.1.1. LAB MEDIA: Figure 2B (left panel). *Video editor: Highlight the red box in 2 B left curve.*

5.1.2. LAB MEDIA: Figure 2B (right panel). *Video editor: Highlight the steep rise in the oxygen concentration curve*

5.2. After 3 hours of cultivation, *E. coli* colonies grown under 21% oxygen conditions appeared visibly larger than those under 0% oxygen conditions **[1]**.

5.2.1. LAB MEDIA: Figure 3A-B. *Video editor: Please highlight images of 3A*

5.3. The normalized colony area increased more rapidly at higher oxygen concentrations, with a clear trend shown from 0% to 21% oxygen **[1]**. The exponential growth rate μ *(mew)* increased sharply from 0% to 0.5% oxygen and plateaued beyond 5% **[2].**

5.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the curves corresponding to 10 and 21% O_2*

5.3.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the steep portion of the plot from 0% to 0.5% oxygen.*

5.4. Under oscillating oxygen with a 60-minute cycle, *E. coli* growth displayed three phases, a rapid response to deoxygenation **[1]**, a recovery phase with gradual increase **[2]**, and a final stabilization around the anaerobic growth rate **[3]**.

5.4.1. LAB MEDIA: Figure 4 (T'=60 min). *Video editor: Highlight the area pointed at by "response"*

5.4.2. LAB MEDIA: Figure 4 (T'=60 min). *Video editor: Highlight the area pointed at by "Recovery"*

5.4.3. LAB MEDIA: Figure 4 (T'=60 min). *Video editor: Highlight the area pointed at by "Stabolization"*

5.5. As the switching interval T' *(T-dash)* decreased from 60 to 1 minute, the growth rate oscillations became more synchronized with gas phases, and adaptation effects diminished **[1]**.

5.5.1. LAB MEDIA: Figure 4 (all panels). *Video editor: Sequentially show the graphs from T' = 60 min to T'=1 min*

5.6. Single-cell area measurements showed faster growth during aerobic phases and clear drops during anaerobic phases across all tested switching intervals **[1]**.

5.6.1. LAB MEDIA: Figure 5. *Video editor: Please highlight all blue dots in the white portions of each graph*

Pronunciation Guide:

1. Escherichia
Pronunciation link: https://www.merriam-webster.com/medical/Escherichia YouTube+8Merriam-Webster+8YouTube+8
IPA: /ˌɛʃəˈrɪkiə/
Phonetic Spelling: ESH-uh-RIK-ee-ə
2. Escherichia coli
Pronunciation link: https://www.collinsdictionary.com/us/dictionary/english/escherichia-coli YouGlish+1Merriam-Webster+1YouTube+4Collins Dictionary+4YouGlish+4
IPA: /ˌɛʃəˈrɪkiə ˈkoʊlaɪ/
Phonetic Spelling: ESH-uh-RIK-ee-ə COH-lye
3. E. coli
Pronunciation link: https://www.merriam-webster.com/dictionary/E.%20coli YouTube+4Collins Dictionary+4Definitions+4Merriam-Webster+9YouGlish+9YouTube+9Merriam-Webster+1YouTube+1
IPA: /ˌiː ˈkoʊ·laɪ/
Phonetic Spelling: ee COH-lye
4. microfluidic
Pronunciation link: https://www.howtopronounce.com/microfluidic YouTube+8How To Pronounce+8How To Pronounce+8 ***(via HowToPronounce domain, default resource)***
IPA: /ˌmaɪkroʊfluˈɪdɪk/
Phonetic Spelling: my-kroh-flu-ID-ik
5. PDMS (polydimethylsiloxane)
Pronunciation link: https://www.howtopronounce.com/pdms Definitions+15How To Pronounce+15How To Pronounce+15Merriam-Webster+13YouGlish+13pronounce.voanews.com+13
IPA: /ˌpiː.diː.emˈɛs/
Phonetic Spelling: P-D-M-S (pee-dee-em-ess)
6. Stern–Volmer
Pronunciation link: https://www.synonyms.com/pronounce/stern-volmer YouGlish+7synonyms.com+7pronouncekiwi.com+7
IPA: /stɜnˈvɔlmər/
Phonetic Spelling: STERN-VOL-mer
7. FLIM (fluorescence lifetime imaging microscopy)
Pronunciation link: No confirmed link found ***(acronym commonly pronounced "F-L-I-M", each letter spoken)***
IPA: /ɛf-ɛl-aɪ-ɛm/
Phonetic Spelling: F-L-I-M
8. tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate
Pronunciation link: No confirmed link found ***(too complex for standard entries; approximate)***
IPA: /traɪs tuːˌbaɪpɪˈrɪdəl daɪklɔːroʊˈruːθiːniəm/
Phonetic Spelling: trys too by-pi-RID-il dye-KLOR-oh-ROO-thee-nee-əm

9. spin coater
Pronunciation link: No specific entry—standard terms: “spin” and “coater”
IPA: /spɪn ˈkoʊtər/
Phonetic Spelling: spin COH-ter
10. nanoliters
Pronunciation link: Merriam-Webster (implied from “nanoliter”)
IPA: /ˈnænəˌliːtərz/
Phonetic Spelling: NAN-oh-LEE-terz