FINAL SCRIPT: APPROVED FOR FILMING

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Title: Real-Time Metabolic Detection in Living Cells Using Hyperpolarized ^{13}C NMR

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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: 21
Number of Shots: 53 (10 SC)

Introduction

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

1.1. **Tomoto Ura:** We develop non-invasive methods to measure real-time cellular metabolism using advanced hyperpolarized NMR techniques. Our goal is creating accessible tools for various biological research applications.

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 research gap are you addressing with your protocol?

1.2. **Tomoto Ura:** Several hyperpolarized NMR systems for cellular metabolism have been reported. However, standardized and reproducible protocols for broader research implementation have not been available until now.

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 advantage does your protocol offer compared to other techniques?

1.3. **Natsuko Miura:** Our protocol enhances accessibility using commercially available components. We also provide comprehensive validation including in-situ mixing advantages and repeated measurement capabilities for broader adoption.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.2*

What new scientific questions have your results paved the way for?

1.4. **Natsuko Miura:** Our results enable real-time tracking of dynamic metabolic changes in living cells without destruction. This opens new possibilities for long-term drug studies and diverse metabolic probe applications.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.1.2*

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

1.5. **Yoichi Takakusagi:** Our approach has limitations including probe options and automation requirements. Solving these challenges will establish cellular metabolic measurement techniques and bridge our laboratory's DNP-MRI research efforts in the future.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.8.1*

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

Protocol

2. Alginate Gel Preparation

Demonstrator: Tomoto Ura

2.1. To begin, add 2 milliliters of 0.25 percent volume-to-volume trypsin-EDTA solution to a 10-centimeter dish **[1]**. Tilt the dish gently to ensure the entire surface is covered **[2]**, then aspirate the excess trypsin-ethylenediaminetetraacetic acid solution **[3]**.

2.1.1. WIDE: Talent adding 2 milliliters of trypsin-ethylenediaminetetraacetic acid to the 10 centimeter dish using a pipette.

2.1.2. Talent gently tilting the dish to evenly coat the surface with the solution.

2.1.3. Talent aspirating the excess trypsin-ethylenediaminetetraacetic acid using a pipette or vacuum aspirator.

2.2. Place the dish in an incubator set at 37 degrees Celsius and incubate for 2 minutes **[1]**.

2.2.1. Talent placing the dish inside a 37 degrees Celsius incubator and closing the door.

2.3. Then, add 10 milliliters of culture medium to the dish **[1]**. Using a pipette, gently pipette up and down to detach the cells from the surface **[2]** and collect the entire cell suspension into a 15-milliliter conical tube **[3]**.

2.3.1. Talent adding 10 milliliters of culture medium to the dish. **NOTE**: The shots 2.3.1, 2.3.2 and 2.3.3 may have been shot together

2.3.2. Talent pipetting up and down gently to detach the cells.

2.3.3. Talent transferring the cell suspension into a 15 milliliter conical tube using a pipette.

2.4. Now, take approximately 10 microliters of the cell suspension and load it into a cell counter to determine the number of cells **[1]**. Ensure that the measured count confirms a yield of approximately 10 million cells, or adjust as needed **[2]**.

2.4.1. Talent pipetting 10 microliters of cell suspension into the cell counter.

2.4.2. Display of cell count results on the cell counter monitor.

2.5. Centrifuge the remaining cell suspension at 120 *g* for 5 minutes **[1]**. After centrifugation, aspirate and discard the supernatant **[2]**. Resuspend the resulting cell pellet in 4 milliliters of 2 percent weight-to-volume alginate solution **[3]** and pipette slowly to minimize bubble formation **[4]**.

2.5.1. Talent placing the conical tube into the centrifuge.

2.5.2. Talent removing the supernatant from the centrifuged tube using a pipette.

2.5.3. Talent adding 4 milliliters of 2 percent alginate solution to the pellet.

2.5.4. Talent pipetting the mixture slowly and carefully to avoid bubbles.

2.6. Now, add 10 milliliters of 50 millimolar calcium chloride solution to a 50-milliliter centrifuge tube **[1]**.

2.6.1. Talent pipetting 10 milliliters of calcium chloride solution into a labeled 50 milliliter centrifuge tube.

2.7. Secure the syringe to the cap of the centrifuge tube containing calcium chloride solution **[1].** Draw 300 microliters of the cell-alginate mixture into a 0.5-milliliter syringe fitted with a 3- gauge, 10 millimeter-needle **[2]**.

2.7.2 Talent attaching and securing the syringe to the cap of the centrifuge tube

2.7.1. Talent filling a 0.5 milliliter syringe with 300 microliters of the cell-alginate mixture. **NOTE**: The shots and VO are inverted. Show 2.7.2 first

2.8. Then, close the centrifuge tube tightly with the syringe fixed to the cap **[1]** and place the assembly into the centrifuge to spin gently at 200 *g* for 5 minutes **[2]**. Remove the supernatant after centrifugation **[3]** and resuspend the resulting gel in culture medium **[4]**.

2.8.1. Talent sealing the centrifuge tube with the syringe in place. **NOTE**: The shots 2.8.1, 2.8.2 were shot together

2.8.2. Talent placing the assembly into the centrifuge and initiating the spin at 200 g.

2.8.3. Talent removing the supernatant.

2.8.4. Talent gently resuspending the gel in culture medium by pipetting up and down.

2.9. Next, using a Pasteur pipette, transfer the cell-encapsulated gel into an NMR tube **[1]** and insert the sponge fixture to ensure a tight seal **[2]**.

2.9.1. Talent transferring the gel from the tube to the NMR tube using a Pasteur pipette.

2.9.2. Talent inserting the sponge fixture into the NMR tube and securing the seal.

2.10. Pre-warm the medium reservoir containing 5 percent volume-to-volume deuterium oxide in a 37 degrees Celsius water bath **[1]**. Begin circulation at a rate of approximately 500 microliters per hour to stabilize the conditions **[2]**.

2.10.1. Talent placing the medium reservoir in the water bath set at 37 degrees Celsius.

2.10.2. Talent activating the circulation system.

2.11. Inspect the setup to ensure there are no leaks before proceeding **[1]**.

2.11.1. Talent examining all connections in the setup.

3. Preparation of Hyperpolarized Probe using a DNP Polarizer

Demonstrator: Keita Saito

3.1. Add 18 microliters of Carbon 13 labeled pyruvic acid at a concentration of 14.2 molar, doped with 25 millimolar OX063 *(ox-63)*, to a sample vial **[1]**. Connect the vial to the fluid path of the dynamic nuclear polarization polarizer system **[2]**.

3.1.1. Talent pipetting 18 microliters of [1-13C] pyruvic acid doped with OX063 into a labeled sample vial.

3.1.2. Talent connecting the sample vial to the tubing of the dynamic nuclear polarization polarizer system.

3.2. Next, insert the connected vial into the bore unit of the dynamic nuclear polarizer **[1-TXT]**. Apply microwave irradiation at approximately 188 gigahertz with a power of 22 milliwatts for a duration of 60 to 80 minutes **[2]**.

3.2.1. Talent placing the vial assembly into the bore unit of the polarizer system. **TXT: Polarizer settings: 6.7 T; 1.25 K; 1.2 mbar** **NOTE**: 2 takes were shot

3.2.2. Talent adjusting settings for 188 gigahertz, 22 milliwatts. **NOTE**: 2 takes were shot

3.3. Use the solid-state nuclear magnetic resonance spectrometer integrated into the dynamic nuclear polarization system to monitor the carbon-13 signal intensity **[1]**. Record the signal data every 5 minutes using the system's control software **[2]**.

3.3.1. Talent switching on SPINit software and setting up the monitoring time for the polarization build-up. **NOTE**: 2 takes were shot

3.3.2. SCREEN: 3.3.2. 13:00-13:10 **NOTE**: Screen recordings are on the project page

3.4. Now, rapidly dissolve the polarized sample in 3.2 milliliters of dissolution buffer preheated to biological temperature **[1-TXT]**. Ensure the final solution reaches a temperature between 308 and 313 kelvin and a pH of approximately 7 before transferring for NMR measurement **[2]**.

3.4.1. Talent dissolving the sample in 3.2 milliliters of heated dissolution buffer quickly after polarization. **TXT: Dissolution buffer: 40 mM Tris; 50 mM NaCl; 80 mM NaOH; 100 mg/L EDTA Videographer's NOTE:** This shot covers all talent shots from 3.4.1 to 4.5.2. This is to show how quickly they need to inject the solution into the NMR tube. The shot includes a pan to follow Tomoto. We have closer shots of all of the steps that can also be used. This shot starts by adding the buffer then 3-4 minute wait for it to be ready. The main shot is at the end of the clip**.**

3.4.2. Talent measuring the pH of the solution using pH test paper.

4. Nuclear Magnetic Resonance (NMR) Setup and Measurement

Demonstrator: Tomoto Ura

4.1. Load the prepared sample into an NMR tube and insert the tube into the spectrometer **[1]**. On the NMR console, initiate the automated procedure to perform locking, tuning, matching, and shimming with the sample in place to ensure optimal spectral resolution and signal stability **[2]**.

4.1.1. ~~Talent adding the sample to the NMR tube~~. **NOTE**: Delete the shot. VO merged with the next shot

4.1.2. Talent inserting the sample-filled NMR tube into the spectrometer slot.

4.1.3. SCREEN: 4.1.3 .01:45-01:59

4.2. Load a carbon-13 pulse-acquire sequence, with proton decoupling enabled using a setting like 13-CPD **[1-TXT]**. Set the flip angle to 90 degrees with a 1 microsecond pulse labeled as P1 **[2]**.

4.2.1. SCREEN: 4.2.1**. TXT: ^{13}C pulse-acquire sequence example: zg2d**

4.2.2. SCREEN: 4.2.2.

4.3. Next, define the acquisition time as 1.376 seconds, spectral width as 23,663 hertz, and acquisition points or TD as 65,536 **[1]**. Set the relaxation delay D1 to zero seconds **[2]**. Assign 150 time increments to TD1 for acquiring 150 sequential spectra in pseudo two-dimensional mode **[3]** and adjust the receiver gain to a low value, such as approximately 1, to avoid signal saturation caused by the hyperpolarized samples **[4]**.

4.3.1. SCREEN: 4.3.1. 00:15-00:30.

4.3.2. SCREEN: 4.3.2.

4.3.3. SCREEN: 4.3.3.

4.3.4. SCREEN: 4.3.4.

4.4. Now, stop the peristaltic pump to temporarily halt the circulation of the medium before injecting the hyperpolarized solution through the inlet tubing **[1]**. Begin the spectral acquisition approximately 10 seconds before dissolving the hyperpolarized substrate to ensure signal capture upon arrival at the detection site **[2]**. When the solution exits the dynamic nuclear polarization polarizer, draw 1 milliliter of it into a syringe **[3]**.

4.4.1. Talent stopping the peristaltic pump.

4.4.2. ~~Talent injecting the hyperpolarized solution through the inlet tubing~~. **NOTE**: Delete the shot. VO merged with the next shot

4.4.3. SCREEN: 4.4.3-4.6.1. 00:00-00:15.

4.4.4. Talent drawing 1 milliliter of hyperpolarized solution into a syringe from the polarizer outlet.

4.5. Switch the three-way valve at the bioreactor inlet to Position B to direct the

hyperpolarized solution into the NMR tube **[1]**. After the injection is complete, switch the valve back to Position A to resume medium circulation and prevent any backflow **[2]**.

4.5.1. Talent switching the valve to Position B and injecting the solution into the NMR tube.

4.5.2. Talent switching the valve back to Position A to restore medium circulation.

4.6. Finally, confirm that the injection was successful after noting an increase in the Free Induction Decay signal on the acquisition software **[1]**.

4.6.1. SCREEN: 4.4.3-4.6.1. 00:30-00:35 and 05:04-05:08.

Results

5. Results

5.1. Hyperpolarized carbon-13 NMR spectra of the same SCCVII *(S-C-C-7)* cell-encapsulated sample were acquired at 1.5-hour intervals to evaluate the feasibility of repeated non-destructive measurements **[1]**. The first NMR measurement showed a markedly increased lactate signal, indicating active pyruvate-to-lactate metabolic conversion **[2]**.

5.1.1. LAB MEDIA: Figure 5.

5.1.2. LAB MEDIA: Figure 5. *Video editor: Highlight the data points for lactate in A*.

5.2. Subsequent measurements at 1.5-hour intervals revealed a progressive reduction in lactate signal intensity, indicating decreased metabolic activity over time **[1]**.

5.2.1. LAB MEDIA: Figure 5. *Video editor: Highlight the data points for lactate in B and C*.

5.3. In HeLa cells, initial measurements confirmed successful pyruvate-to-lactate conversion with clearly discernible signal peaks **[1]**.

5.3.1. LAB MEDIA: Figure 6. *Video editor: Highlight the data points for lactate between 5 to 50 seconds on X axis*

5.4. Although the signal decreased gradually due to T_1 relaxation, the signal-to-noise ratio remained adequate throughout the 60-second acquisition, suggesting that the current protocol is broadly applicable and can serve as a versatile tool for real-time metabolic profiling in various cell types **[1]**.

5.4.1. LAB MEDIA: Figure 6.

Pronunciation guides:

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1. trypsin

Pronunciation link: https://www.merriam-webster.com/dictionary/trypsin
IPA: /ˈtraɪpˌsɪn/
Phonetic spelling: TRYPE-sin

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2. ethylenediaminetetraacetic

Pronunciation link: https://www.merriam-webster.com/dictionary/ethylenediaminetetraacetic
IPA: /ˌɛθəˌliːn daɪˌæmɪnɛˌtɛtrəəˈsɛtɪk/
Phonetic spelling: ETH-uh-leen dye-am-in-uh-NEH-tet-ruh-uh-SET-ik

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3. aspirate

Pronunciation link: https://www.merriam-webster.com/dictionary/aspirate
IPA: /ˈæs.pəˌreɪt/
Phonetic spelling: AS-puh-rayt

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4. conical

Pronunciation link: https://www.merriam-webster.com/dictionary/conical
IPA: /ˈkɑ.nɪ.kəl/
Phonetic spelling: KAH-ni-kuhl

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5. alginate

Pronunciation link: https://www.merriam-webster.com/dictionary/alginate
IPA: /ˈæl.dʒɪˌneɪt/
Phonetic spelling: AL-jih-nayt

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6. Pasteur (as in Pasteur pipette)

Pronunciation link: https://www.merriam-webster.com/dictionary/Pasteur
IPA: /pɑːˈstɜr/
Phonetic spelling: pah-STUR

7. deuterium

Pronunciation link: https://www.merriam-webster.com/dictionary/deuterium
IPA: /duːˈtɪə.ri.əm/
Phonetic spelling: doo-TEER-ee-um

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8. hyperpolarized

Pronunciation link: https://www.merriam-webster.com/dictionary/hyperpolarize
IPA: /ˌhaɪ.pərˈpoʊ.ləˌraɪzd/
Phonetic spelling: HY-per-poh-luh-ryzd

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9. pyruvic

Pronunciation link: https://www.merriam-webster.com/dictionary/pyruvic
IPA: /ˌpaɪˈruː.vɪk/
Phonetic spelling: pie-ROO-vik

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10. polarization

Pronunciation link: https://www.merriam-webster.com/dictionary/polarization
IPA: /ˌpoʊ.lə.rɪˈzeɪ.ʃən/
Phonetic spelling: poh-luh-ruh-ZAY-shuhn

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11. peristaltic

Pronunciation link: https://www.merriam-webster.com/dictionary/peristaltic
IPA: /ˌpɛr.ɪˈstæl.tɪk/
Phonetic spelling: per-ih-STAHL-tik
