FINAL SCRIPT: APPROVED FOR FILMING

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Title: Quantification of Endothelial Fatty Acid Uptake Using Fluorescent Fatty Acid Analogs

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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? **YES**

   My office where the interviewing portions of the filming will take place is about 100 feet from the tissue culture room and main research lab room, where the majority of the filming will take place. Also, the plate reader is located in a nearby, adjoined building that is 200 feet away, and requires going up and down a flight of stairs (or use of an elevator, which would increase the distance by about 500 feet).

Current Protocol Length
Number of Steps: 13
Number of Shots: 29

Introduction

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

1.1. **Ayon Ibrahim:** Blood-borne nutrients like fatty acids must cross the capillary endothelial barrier to enter metabolic tissue, via poorly understood mechanisms. Clarifying these processes could reveal new therapeutic targets for metabolic diseases such as type 2 diabetes.

1.1.1. 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?

1.2. **Ayon Ibrahim**: We found that skeletal muscle and adipose tissue regulate endothelial lipid transport via paracrine metabolites, partly controlled by the endocrine system, and that mitochondrial ATP unexpectedly contributes to endothelial fat uptake despite their reliance on glycolytic ATP.

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

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

1.3. **Ayon Ibrahim:** Using the assay outlined in this protocol, we plan to probe deeper into the molecular mechanisms of endothelial lipid uptake and transport, including how it might be regulated by other tissue.

1.3.1. 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

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.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.4. **Ayon Ibrahim, Assistant Professor, Department of Biological Sciences, Union College:**
(authors will present their testimonial statements live)

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

Protocol

2. Gelatin Coating of 96-Well Plate and Cell Seeding

Demonstrator: Ayon Ibrahim

2.1. To begin, prepare a 0.1 percent gelatin solution by adding 25 milliliters of 2 percent gelatin stock to 475 milliliters of PBS **[1]**. Mix the gelatin solution thoroughly **[2]** and filter-sterilize it using a 0.2-micrometer vacuum filter or autoclave **[3]**.

2.1.1. WIDE: Talent pouring 25 milliliters of gelatin to a bottle containing 475 milliliters of PBS.

2.1.2. Talent swirling and inverting the bottle by hand to thoroughly mix the diluted gelatin solution. **Videographer's NOTE**: Forgot to slate

2.1.3. Talent attaching the vacuum filter unit and pouring the solution into the reservoir for sterilization.

2.2. Using a pipette, add 100 microliters of the 0.1 percent gelatin solution into each well of a black, clear-bottom 96-well plate **[1]**. Place the plate in a 37-degree Celsius incubator for at least 30 minutes or overnight **[2]**.

2.2.1. Talent pipetting the gelatin solution out of a reagent reservoir into each well of the 96-well plate.

2.2.2. Talent placing the plate inside a 37 degree Celsius incubator.

2.3. After incubation, remove extra gelatin solution from the pre-coated 96-well plate **[1].** Wash the plate with PBS once **[2].** Then, pipette 100 microliters of the cell suspension into each well, using a seeding density that allows the cells to reach confluence by the next day **[3]**.

2.3.1. Talent removing extra remaining gelatin solution from the pre-coated 96-well plate by flicking the whole plate.

2.3.2. Talent washing the plate once with PBS, and removing that solution as well.

2.3.3. Talent pipetting the cell suspension out of a reagent reservoir into each well of the pre-coated 96-well plate.

3. Treatment Reagent Preparation and Application

3.1. Prepare 20 millimolar solutions of 3-hydroxyisobutyrate and lactate as positive controls. For the negative control, use 1 micromolar niclosamide **[1-TXT]**. Then, pipette the solutions into a separate 96-well plate **[2].**

3.1.1. Talent pipetting and mixing solutions to prepare 20 millimolar 3-hydroxyisobutyrate and lactate, and a 1 micromolar solution of niclosamide. **TXT: Solutions are preincubated in PBS with Ca^{2+} and Mg^{2+}**

3.1.2. Talent pipettes the prepared solutions into a separate 96-well plate.

3.2. Wash the endothelial cells once with pre-warmed divalent PBS, pipetting at a steep angle to avoid disrupting the plated cells **[1]**. Add 50 microliters per well of the appropriate treatment reagent **[2]** and incubate the plate at 37 degrees Celsius for 30 minutes or 60 minutes **[3]**.

3.2.1. Talent discarding the media and washing the wells with pre-warmed divalent phosphate-buffered saline.

3.2.2. Talent pipetting 50 microliters of treatment reagent into each well of the plate.

3.2.3. Talent placing the plate inside a 37-degree Celsius incubator.

3.3. To prepare the BODIPY-fatty acid:BSA *(Bo-di-Pee-Fatty-Acid-B-S-A)* complex, mix a 2 micromolar BODIPY-fatty acid solution with 1 micromolar fatty acid-free BSA in PBS **[1]**. Incubate the mixture at room temperature in the dark for 10 minutes before use **[2]**.

3.3.1. Talent mixing BODIPY-fatty acid with fatty acid-free bovine serum albumin in a 15 milliliter centrifuge tube.

3.3.2. Talent placing the tube in a room temperature drawer to protect solution from light.

3.4. Next, add 50 microliters of the prepared BODIPY-fatty acid:BSA complex to each treated well **[1]** and incubate the plate at 37 degrees Celsius for 5 minutes **[2-TXT]**.

3.4.1. Talent pipetting BODIPY-fatty acid:BSA complex into the treated wells. **TXT: Avoid adding to the outer columns to use as controls** **Videographer's NOTE:** Do not use the first couple of seconds, use footage after the talent removes the end tube

3.4.2. Talent placing the plate in a 37 degree Celsius incubator. **Videographer's NOTE:** This scene was shot several times earlier, use any of them

4. Fluorescence Quenching and Intracellular Signal Detection

4.1. Prepare a 1 micromolar solution of fatty acid-free BSA in PBS as a wash buffer **[1]** and pre-warm it to 37 degrees Celsius **[2]**. Remove the BODIPY-fatty acid:BSA complex from

the wells **[3]** and wash the entire plate twice with 50 microliters of the pre-warmed wash buffer, performing each wash for 1.5 minutes **[4]**.

4.1.1. Talent preparing the 1 micromolar bovine serum albumin wash buffer.

4.1.2. Talent placing the buffer in a 37 degree Celsius water bath.

4.1.3. Talent discarding the BODIPY-fatty acid:BSA solution.

4.1.4. Talent performing two washes with the pre-warmed buffer using a multichannel pipette.

4.2. Then, add 50 microliters of 0.08 percent Trypan Blue to each well to quench extracellular fluorescence **[1]**. Using a microplate reader, immediately measure intracellular fluorescence **[2-TXT]**.

4.2.1. Talent pipetting 50 microliters of 0.08 percent Trypan Blue into each well of the plate.

4.2.2. Talent places the plate in the microplate reader with the screen showing the setting visible. **TXT: Excitation: 488 nm**, **Emission: 515 nm**, **Cutoff: 495 nm**, **Mode**: **Bottom-read**

5. Hoechst Nuclear Staining for Cell Normalization

5.1. After removing the Trypan Blue solution from the wells, gently wash the cells with PBS **[1]**.

5.1.1. Talent washing the wells with phosphate-buffered saline using a multichannel pipette.

5.2. Add 4 micrograms per milliliter of Hoechst dye diluted in 10 percent media to each well **[1-TXT]**. Incubate the plate at 37 degrees Celsius for 30 minutes **[2]**.

5.2.1. Talent pipetting Hoechst staining solution into each well. **TXT: Do not add Hoechst to the outer columns**

5.2.2. Talent placing the plate inside a 37 degree Celsius incubator.

5.3. Then, wash the plate once with PBS to remove excess dye **[1]**.

5.3.1. Talent performing a single wash of all wells with phosphate-buffered saline using a multichannel pipette.

5.4. After that add fresh PBS to each well **[1]** and measure Hoechst fluorescence using a microplate reader **[2-TXT]**.

5.4.1. Talent pipetting fresh phosphate-buffered saline into each well.

5.4.2. Talent places the plate in the reader and starts it. **TXT: Excitation: 350 nm**, **Emission: 461 nm**, **Cutoff: 455 nm Mode**; **Bottom-read**

Results

6. Results

6.1. In both HUVEC *(HUE-Vek)* and EA.hy926 *(E-A-H-Y-Nine-Two-Six)* cells, intracellular BODIPY-C_{12} *(BODI-PEE-C-Twelve)* signal increased significantly with higher BODIPY-Fatty acid concentrations at 1 minute **[1],** 5 minutes **[2],** and 10 minutes of incubation **[3].**

6.1.1. LAB MEDIA: Figure 2A,B. *Video editor: Highlight the increasing bars for 0.5 µM, 1 µM, and 2 µM BODIPY-FA at 1-minute timepoint under HUVEC and EA.hy926*

6.1.2. LAB MEDIA: Figure 2A,B. *Video editor: Highlight the increasing bars for 0.5 µM, 1 µM, and 2 µM BODIPY-FA at 5-minute timepoint under HUVEC and EA.hy926*

6.1.3. LAB MEDIA: Figure 2A,B. *Video editor: Highlight the increasing bars for 0.5 µM, 1 µM, and 2 µM BODIPY-FA at 10-minute timepoint under HUVEC and EA.hy926*

6.2. Lactate treatment for 1 hour increased BODIPY-C_{12} uptake in a dose-dependent manner at 5 millimolar and 20 millimolar concentrations **[1].** Treatment with 3-hydroxyisobutyrate also significantly enhanced BODIPY-C_{12} uptake in a dose-dependent fashion, with the highest uptake observed at 20 millimolar **[2].**

6.2.1. LAB MEDIA: Figure 3A*. Video editor: Highlight the three bars labeled 0, 5, and 20 millimolar lactate, showing progressively higher heights*

6.2.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the increasing bar heights at 0, 5, and 20 millimolar 3-HIB*

6.3. Treatment with 1 micromolar niclosamide for 30 minutes led to a significant reduction in BODIPY-C_{12} uptake compared to untreated DMSO *(D-M-S-O)* control **[1].**

6.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the bar at 1 micromolar niclosamide, which is visibly shorter than the bar at 0 micromolar*

6.4. Following 5-minute incubation with BODIPY-C_{16} in HUVECs, lactate treatment at 10 millimolar and 20 millimolar significantly increased uptake in a dose-dependent manner **[1].** Treatment with 1 micromolar niclosamide significantly decreased BODIPY-C_{16} uptake compared to the untreated DMSO control **[2].**

6.4.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the upward trend in bar heights across the 0, 10, and 20 millimolar lactate groups*

6.4.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the bar labeled 1 micromolar niclosamide.*

- **gelatin**
Pronunciation link: https://www.merriam-webster.com/dictionary/gelatin
IPA: /ˈdʒɛl·ə·tɪn/
Phonetic Spelling: JEL-uh-tin

- **phosphate** *(as in "phosphate-buffered saline / PBS")*
Pronunciation link: https://www.merriam-webster.com/dictionary/phosphate
IPA: /ˈfɑːs·feɪt/ or /ˈfɒs·feɪt/ (American often /ˈfɑsˌfeɪt/)
Phonetic Spelling: FAHS-fayt

- **incubator**
Pronunciation link: https://www.merriam-webster.com/dictionary/incubator
IPA: /ˈɪŋ·kjə·ˌbeɪ·tɚ/
Phonetic Spelling: ING-kyoo-BAY-ter

- **microliter** *(µL)*
Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
IPA: /ˈmaɪ·kroʊˌli·tər/
Phonetic Spelling: MY-kroh-ly-ter

- **centrifuge**
Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛn·trəˌfjuːdʒ/ or /ˈsɛn·trəˌfjuʒ/
Phonetic Spelling: SEN-truh-fyoohj

- **albumin** *(as in "bovine serum albumin" / BSA)*
Pronunciation link: https://www.merriam-webster.com/dictionary/albumin
IPA: /ælˈbju·mɪn/ or /ælˈbju·mən/
Phonetic Spelling: al-BYOO-min

- **fluorescence**
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
IPA: /flʊˈɔr·ə·səns/ or /fləˈrɛs·əns/ (Am. often /flʊˈrɔːr·ə·səns/)
Phonetic Spelling: floh-OR-uh-sens

- **quench / quenching**
Pronunciation link: https://www.merriam-webster.com/dictionary/quench
IPA: /kwɛntʃ/
Phonetic Spelling: KWENCH

- **Trypan** *(as in "Trypan Blue" — note this is a trade / common name; pronunciation based on typical usage)*
Pronunciation link: No confirmed link found on Merriam-Webster.

IPA (approximate): /ˈtraɪ·pæn/
Phonetic Spelling: TRY-pan

- **Hoechst** *(name of a dye — German-derived; approximate American rendering)*

Pronunciation link: No confirmed link found on Merriam-Webster.
IPA (approximate): /ˈhoʊst/ or /ˈhoʊkʃt/
Phonetic Spelling: HOHST (or HOHKSHT)

- **niclosamide**

Pronunciation link: https://www.merriam-webster.com/dictionary/niclosamide
IPA: /ˌnɪk·ləˈsæm·aɪd/
Phonetic Spelling: nik-luh-SAM-ide

- **divalent** *(as in "divalent PBS")*

Pronunciation link: https://www.merriam-webster.com/dictionary/divalent
IPA: /daɪˈveɪ·lənt/
Phonetic Spelling: dy-VAY-luhnt

- **multichannel** *(as in "multichannel pipette")*

Pronunciation link: No confirmed link found on Merriam-Webster (compound word).
IPA (approximate): /ˌmʌl·tiˈtʃæn·əl/
Phonetic Spelling: MUL-tee-CHAN-uhl

- **complex** *(as in "BODIPY-fatty acid:BSA complex")*

Pronunciation link: https://www.merriam-webster.com/dictionary/complex
IPA: /ˈkɑm·plɛks/
Phonetic Spelling: KOM-pleks

- **confluence** *(as in "cells reach confluence")*

Pronunciation link: https://www.merriam-webster.com/dictionary/confluence
IPA: /ˈkɑn·flə·wəns/ or /ˈkɒn·flʊ·əns/ (Am. often /ˈkɑn·flu·əns/)
Phonetic Spelling: KON-floo-ens