FINAL SCRIPT: APPROVED FOR FILMING

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Title: Stimulation of Vascular Endothelial Cells Using Neutrophil Extracellular Traps in the Presence of Low-Density Lipoprotein

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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: 16
Number of Shots: 45

Introduction

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

1.1. **Takashi Obama:** Neutrophil extracellular traps (NETs) are involved in a variety of diseases including cardiovascular diseases. However, the synergistic effects of NET formation and lipoproteins on vascular cells remain unclear.

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. **Takashi Obama:** Utilizing this protocol, mechanistic analyses are ongoing to elucidate how inflammatory responses are induced in human aortic endothelial cells treated with neutrophil extracellular traps and low-density lipoprotein.

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

What significant findings have you established in your field?

1.3. **Takashi Obama:** We have demonstrated that high-density lipoprotein acts as a suppressor of NET formation that is promoted by oxidized low-density lipoprotein, oxidized phospholipids and lysophospholipids.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

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

Ethics Title Card

This research has been approved by the Ethics Committee at the Showa Medical University School of Pharmacy

Protocol

2. Isolation of Low-Density Lipoprotein (LDL) from Human Plasma

Demonstrator: Takashi Obama

2.1. To begin, centrifuge 45 to 50 milliliters of human whole blood obtained in a heparin tube at 700 *g* for 15 minutes at 4 degrees Celsius **[1]**. Slowly reduce the centrifuge speed after the spin **[2]**, then collect the upper layer containing the plasma fraction **[3]**.

2.1.1. WIDE: Talent loading conical tubes with whole blood into the centrifuge and starting the spin.

2.1.2. Talent slowly adjusting the centrifuge settings to reduce speed.

2.1.3. Talent carefully collecting the plasma fraction from the top layer of the centrifuged sample.

2.2. Centrifuge the plasma fraction two times at 700 *g* for 15 minutes at 4 degrees Celsius to remove blood cells completely, maintaining the same deceleration setting **[1]**. Then, add 1 microliter of 250 millimolar EDTA solution per 1 milliliter of plasma to prevent divalent metal ion-mediated oxidation of lipoproteins **[2]**.

2.2.1. Talent placing the plasma tube in the centrifuge and initiating the spin.

2.2.2. Talent adding the EDTA solution to the plasma using a micropipette.

2.3. Place 2.7 milliliters of the plasma into the ultracentrifuge tube **[1]**. Overlay with 900 microliters of PBS containing 250 micromolar EDTA **[2]**, then ultracentrifuge the tube at 600,000 *g* for 7 minutes at 4 degrees Celsius **[3]**.

2.3.1. Talent pipetting 2.7 milliliters of plasma into the ultracentrifuge tube.

2.3.2. Talent adding 900 microliters of PBS with EDTA onto the plasma layer.

2.3.3. Talent placing the tube into the ultracentrifuge and starting the run.

2.4. Next, discard 900 microliters from the top layer to eliminate the chylomicron fraction **[1]**. Add 900 microliters of PBS containing EDTA on top of the remaining plasma **[2]**, and ultracentrifuge at 600,000 *g* for 2.5 hours at 4 degrees Celsius **[3]**. Discard 900 microliters of the top layer to eliminate the very low-density lipoprotein fraction **[4]**.

2.4.1. Talent aspirating the top layer from the ultracentrifuge tube to remove chylomicrons.

2.4.2. Talent gently overlaying 900 microliters of PBS with EDTA onto the plasma.

2.4.3. Talent placing the tube in the ultracentrifuge and starting the long-duration spin.

2.4.4. Talent aspirating and discarding the upper layer containing very low-density lipoprotein.

2.5. Then, add 540 microliters of 0.5 grams per milliliter potassium bromide solution to the plasma to adjust the density to 1.063 **[1]**. Mix the contents gently using a pipette **[2]** and ultracentrifuge at 600,000 *g* for 2.5 hours at 4 degrees Celsius **[3]**. Collect 540 microliters of the top layer containing low-density lipoprotein **[4]**. Transfer the collected fraction into a dialysis membrane **[5]**, and dialyze against 2 liters of PBS containing EDTA at 4 degrees Celsius in the dark to eliminate potassium bromide **[6]**.

2.5.1. Talent adding potassium bromide solution to the sample tube.

2.5.2. Talent mixing the sample gently using a pipette.

2.5.3. Talent placing the sample in the ultracentrifuge and starting the spin.

2.5.4. Talent aspirating/collecting the top layer containing low-density lipoprotein into a new tube.

2.5.5. Talent transferring the low-density lipoprotein to a dialysis membrane.

2.5.6. Talent placing the dialysis membrane into a container with PBS containing EDTA.

3. Preparation of Poly-L-Lysine- and Gelatin-Coated Plates

3.1. Add 152 microliters of 0.01% poly-L-lysine or 0.1% gelatin solution to each well of a 12-well plate **[1]** and incubate at room temperature for at least 5 minutes **[2]**.

3.1.1. Talent pipetting 152 microliters of poly-L-lysine solution into a well of a 12-well plate.

3.1.2. Talent keeping the plate aside for incubation.

3.2. Then, remove the solution from the wells **[1]**, wash once with 200 microliters of sterile water **[2]**, and allow the wells to dry completely at room temperature **[3]**.

3.2.1. Talent aspirating the poly-L-lysine solution from each well.

3.2.2. Talent adding the wells with 200 microliters of sterile water, then aspirating the water.

3.2.3. Talent leaving the plate open on the work surface.

3.3. Store the prepared plate at room temperature until further use **[1]**. Prepare the gelatin-coated plates following the same procedure as demonstrated for Poly-L-lysine **[2]**.

3.3.1. Talent sealing and placing the plate in a cupboard.

3.3.2. Talent labeling another 12-well plate as “gelatin coated”.

4. Preparation of Neutrophil Extracellular Traps (NETs) with LDL

4.1. Culture 2 × 10^6 HL-60 cells per 10 milliliters in RPMI-1640 medium supplemented with 2 micromolar all-trans retinoic acid **[1]**. After 4 days of incubation with all-trans retinoic acid, collect the HL-60 cells **[2-TXT]**.

4.1.1. Talent mixing HL-60 cells with RPMI-1640 medium containing all-trans retinoic acid.

4.1.2. Talent removing the culture dishes from the incubator. **TXT: Incubation: 37 ^{0}C**

4.2. Centrifuge the collected cells at 220 *g* for 4 minutes at 18 to 22 degrees Celsius **[1]**. Aspirate the supernatant **[2]**, then wash the cells with an equal volume of serum-free RPMI-1640 medium **[3]**.

4.2.1. Talent placing the cell suspension into the centrifuge and starting the spin.

4.2.2. Talent carefully removing the supernatant from the cell pellet.

4.2.3. Talent adding serum-free RPMI-1640 to the cells.

4.3. Centrifuge the washed cells again at 220 *g* for 4 minutes at 18 to 22 degrees Celsius **[1]**, and resuspend the cells in serum-free RPMI-1640 medium **[2]**.

4.3.1. Talent placing the sample in the centrifuge.

4.3.2. Talent gently resuspending the cells in serum-free RPMI-1640.

4.4. After adjusting the cell concentration to 2 × 10^6 cells per milliliter **[1],** seed 0.5 milliliter of the suspension into each well of a 12-well plate pre-coated with poly-L-lysine **[2-TXT]**. Add 100 microliters of serum-free RPMI-1640 medium with or without 300 nanomolar phorbol 12-myristate 13-acetate or PMA **[3-TXT]**. Culture the cells for 30 minutes **[4]**.

4.4.1. Talent mixing the cells in a tube by inverting.

4.4.2. Talent pipetting the adjusted cell suspension into each well of the coated 12-

well plate. **TXT: Culture for at least 30 min**

4.4.3. Talent adding phorbol 12-myristate 13-acetate-supplemented or control medium to the wells. **TXT: Final concentration of PMA: 50 nM**

4.4.4. Talent placing the plate in an incubator to culture the cells.

4.5. Next, remove the medium from the wells **[1]**, wash the cells once with serum-free RPMI-1640 medium **[2]**, and replace it with serum-free RPMI-1640 containing either 0 or 20 micrograms per milliliter of low-density lipoprotein **[3]**. Continue culturing the cells for 2 hours **[4]**.

4.5.1. Talent aspirating the old medium from each well gently.

4.5.2. Talent adding serum-free RPMI-1640 and mixing gently .

4.5.3. Talent aspirating the medium gently and then adding fresh medium containing low-density lipoprotein or control.

4.5.4. Talent returning the plate to the incubator.

4.6. Collect the culture medium from each well into a tube **[1]**, and centrifuge at 700 *g* for 3 minutes at 18 to 22 degrees Celsius to remove cellular debris **[2]**.

4.6.1. Talent using a pipette to transfer culture medium from the wells to centrifuge tube.

4.6.2. Talent placing the tube in a centrifuge.

4.7. For stimulation, obtain the human aortic endothelial cell culture **[1]** and replace the spent medium with 0.5 milliliter of fresh culture medium and incubate the cells for 30 minutes **[2]**.

4.7.1. Talent placing the culture dish in the laminar hood.

4.7.2. Talent removing spent media and adding fresh media to the wells.

4.8. Finally, add 167 microliters of the culture medium collected from neutrophil-like cells containing neutrophil extracellular traps and LDL to human aortic endothelial cell dishes **[1]**.

4.8.1. Talent pipetting the collected conditioned medium into the human aortic endothelial cell culture dishes.

Results

5. Results

5.1. Stimulation of human aortic endothelial cells with neutrophil extracellular traps led to dose-dependent morphological changes from a cobblestone-like to an elongated shape **[1]**.

5.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight each row sequentially*

5.2. Morphological changes in human aortic endothelial cells were enhanced when stimulated with LDL-induced neutrophil extracellular traps **[1]**, with visible elongation apparent as early as 6 hours **[2]**.

5.2.1. LAB MEDIA: Figure 2B. *Video editor: Show all six panels sequentially*

5.2.2. LAB MEDIA: Figure 2B. *Video editor: Zoom in on the 6-hour panel*

1. Centrifuge

- **Pronunciation link (Merriam-Webster)**: Merriam-Webster provides a pronunciation entry with IPA YouTubeOxford Learner's Dictionaries+3Cambridge Dictionary+3YouTube+3Merriam-Webster+5Merriam-Webster+5teflpedia.com+5.
- **IPA**: /ˈsɛn-trə-fjuʤ/ Oxford Learner's Dictionaries+8Encyclopedia Britannica+8howtopronounce.com+8
- **Phonetic Spelling**: SEN-truh-fyo͞oj

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2. Plasma

- **Pronunciation link**: No confirmed link found (not essential for pronunciation).
- **IPA (American English)**: /ˈplæz·mə/
- **Phonetic Spelling**: PLAZ-muh

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3. Heparin

- **Pronunciation link**: No confirmed link found.
- **IPA (American English)**: /həˈpɛrɪn/
- **Phonetic Spelling**: huh-PEH-rin

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4. EDTA (Ethylenediaminetetraacetic acid)

- **Pronunciation link**: A pronunciation video is available (YouTube) YouTube+13YouTube+13TheFreeDictionary.com+13MyefeCambridge Dictionary.

- **IPA (American English)**: /ˌi·di·ˈti·eɪ/
- **Phonetic Spelling**: ee-dee-T-AY

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5. Ultracentrifuge

- **Pronunciation link**: No confirmed link found, but built from “ultra–” and “centrifuge”.
- **IPA (American English)**: /ˌʌl·trə-sɛn-trə-fjuʤ/
- **Phonetic Spelling**: UL-truh-SEN-truh-fyo͞oj

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6. Chylomicron

- **Pronunciation link**: No confirmed link found.
- **IPA (American English)**: /ˌkī·lō·mī·krɑn/
- **Phonetic Spelling**: KY-loh-MY-kron

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7. Lipoprotein

- **Pronunciation link**: A pronunciation video is available (YouTube) howtopronounce.com+3TheFreeDictionary.com+3Wiktionary+3TheFreeDictionary.com+3Wiktionary+3Cambridge Dictionary+3.
- **IPA (American English)**: /ˌlɪp·oʊ·ˈproʊ·ˌtēn/
- **Phonetic Spelling**: LIP-oh-PROH-tee-in

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8. Dialysis

- **Pronunciation link**: No confirmed link found.
- **IPA (American English)**: /dī-ˈæl·ə·səs/
- **Phonetic Spelling**: dye-AL-uh-sis