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Title: Exploring Adipose Tissue Structure by Methylsalicylate Clearing and 3D Imaging

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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 similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **32**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Jérôme Gilleron**: This protocol allows the analysis of the three-dimensional structures of very large human and mouse adipose tissues by light microscopy, facilitating the linkage of pathological dysfunctions with adipose tissue structural changes **[1]**.

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

REQUIRED:

1.2. **Jérôme Gilleron**: This clearing procedure is simple, very well adapted to clearing human and whole mice adipose tissues, and uses less-toxic solvents, such as ethanol and methylsalicylate, compared to other clearing protocols **[1]**.

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

Protocol

2. Mouse and Human White Adipose Tissue Fixation, Permeabilization, and Saturation

2.1. Begin by immersing the harvested mouse or human white adipose tissue in at least 10 milliliters of 4% paraformaldehyde in PBS *(P-B-S)* in a 15-milliliter plastic tube **[1]**.

2.1.1. WIDE: Talent adding tissue to tube, with PFA container visible in frame
Videographer: Important step

2.2. Shake the tissue on a rolling plate at room temperature for 1 hour **[1]** before transferring the tube to a rolling plate at 4 degrees Celsius overnight **[2]**.

2.2.1. Talent placing tube onto plate at room temperature

2.2.2. Talent placing tube onto the rolling plate in the 4 °C room and shot of tube shaking on rolling plate at 4 °C

2.3. The next morning, rinse the fixed white adipose tissue in 10 milliliters of PBS for 5 minutes at room temperature to remove all traces of fixative **[1]** and transfer the tissue to a 15-milliliter tube containing 10 milliliters of 0.3% glycine in PBS **[2]** for a 1-hour incubation at room temperature on an orbital shaker at 100 revolutions per minute **[3]**.

2.3.1. Talent adding tissue to tube with PBS container visible in frame

2.3.2. Talent adding tissue to tube, with glycine container visible in frame

2.3.3. Talent placing tube into the orbital shaker and shot of tube shaking on shaker

2.4. When the remaining free aldehyde groups have been quenched, immerse the tissue in a 15-milliliter tube containing 10 milliliters of 0.2% Triton X-100 in PBS for a 2-hour incubation with shaking at 37 degrees Celsius **[1]**.

2.4.1. Talent placing tissue into tube, with Triton X-100 container visible in frame

2.5. At the end of the incubation, treat the tissue with 10 milliliters of 0.2% Triton X-100 and 20% DMSO (D-M-S-O) with shaking at 37 degrees Celsius overnight **[1-TXT]**.

2.5.1. Talent placing tube into the orbital shaker and shot of the tube shaking on shaker, with Triton X-100 and DMSO containers visible in frame **TEXT: DMSO: dimethyl sulfoxide**

2.6. The next morning, immerse the tissue in a 15-milliliter plastic tube containing 10 milliliters of 0.1% Tween-20, 0.1% Triton X-100, 0.1% deoxycholate, and 20% DMSO in PBS for an at least 24-hour incubation at 37 degrees Celsius with shaking **[1]**.

2.6.1. Talent transferring tissue to tube, with Tween-20, Trion X-100, deoxycholate, and DMSO containers visible in frame

2.7. At the end of the incubation, rinse the tissue with 10 milliliters of 0.2% Triton X-100 in PBS on an orbital shaker at 100 revolutions per minute for 1 hour **[1]** followed by immersion in 15 milliliters of 0.2% Triton X-100, 10% DMSO, and 3% BSA *(B-S-A)* in PBS for 12 hours at 37 degrees Celsius with shaking **[2-TXT]**.

2.7.1. Talent placing tube into the orbital shaker and shot of the tube shaking on shaker, with Triton X-100 container visible in frame

2.7.2. Talent adding tissue to tube, with Triton X-100, DMSO, and BSA solution container visible in frame **TEXT: BSA: bovine serum albumin**

3. Mouse and Human White Adipose Tissue Staining

3.1. For staining of the white adipose tissue, transfer the tissue into 300 microliters of 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS supplemented with the primary antibodies of interest in a 1.5-milliliter microtube protected from with aluminium foil, and place the tube into a 50-milliliter Falcon tube **[1].** Place the tube into the orbital shaker for a 2-day incubation at 37 degrees Celsius with shaking **[2]**.

3.1.1. WIDE: Talent adding tissue to a tube containing antibody cocktail, with Triton X-100, DMSO, and BSA solution and antibody containers visible in frame. Talent wrapping the tube into aluminium foil and place the light protected tube into a 50ml falcon tube. *Videographer: Important step*

3.1.2. Talent placing tube into the orbital shaker and shot of the tube shaking on shaker *Videographer: Important step*

3.2. At the end of the incubation, rinse the tissue two times in 10 milliliters of 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS for 5 hours at 37 degrees Celsius with shaking and protection from light **[1]**.

3.2.1. Talent removing the eppendrof tube from the 50ml falcon tube, removing the aluminium foil, and adding tissue to tube, with Triton X-100, DMSO, and BSA solution container visible in frame. Talent wrapping the tube into aluminium foil.

3.3. After the second rinse, wash the tissue in 10 milliliters of fresh 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS for 16 to 48 hours at 37 degrees Celsius with shaking **[1]**. Then, transfer the tissue to a 1.5-milliliter tube containing 300 microliters of 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS supplemented with the appropriate secondary antibodies and protect the tube from light using aluminium foil **[2]**.

3.3.1. Talent placing tube into the orbital shaker and shot of the tube shaking on shaker

3.3.2. Talent removing the aluminium foil and adding tissue to an ependrof tube containing antibody, antibody secondary visible in frame. Talent wrapping the tube into aluminium foil.

3.4. After a 2-day incubation at 37 degrees Celsius on a shaker, wash the tissue two times in 10 milliliters of 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS for 5 hours at 37 degrees with shaking protected from light **[1]**.

3.4.1. Talent placing tube protected from light into the orbital shaker and shot of the tube shaking on shaker

3.5. After the second wash, rinse the tissue into a tube containing 10 milliliters of fresh 0.2% Triton X-100, 10% DMSO, and 3% BSA in PBS for 16-48 hours at 37 degrees Celsius with shaking and light protection **[1]** followed by a 2-hour wash in 10 milliliters of PBS alone at 37 degrees Celsius with shaking and light protection **[2]**.

3.5.1. Talent removing the aluminium foil and adding tissue to tube, with Triton X-100, DMSO, and BSA solution container visible in frame. Talent wrapping the tube into an aluminium foil.

3.5.2. Talent placing tube protected from light into the orbital shaker ~~and shot of the tube shaking on shaker~~

4. Mouse and Human White Adipose Tissue Clearing

4.1. For white adipose tissue clearing, at the end of the PBS wash, immerse the tissue in 10 milliliters of an ascending ethanol concentration series for a 2-hour incubation per concentration at room temperature with shaking protected from light as indicated **[1-TXT]**.

4.1.1. WIDE: Talent adding tissue to tube, with 15-milliliter tubes of ethanol concentrations visible in frame and protect the tubes from light by wrapping them into aluminium foil **TEXT: 50% -> 70% -> 95% -> 100% -> 100% EtOH O/N**

4.2. The next morning, immerse the tissue in a 20-milliliter glass bottle with a plastic cap containing 5 milliliters of methyl salicylate in a fume hood **[1]**. The white adipose tissue is still clearly visible at this stage **[1bis].** Shake the container protected from light at room temperature for at least 2 hours. The white adipose tissue becomes completely transparent **[2]**.

4.2.1. Talent filling up the 20-milliliter glass bottle with methyl salicylate from the stock bottle visible in frame. Talent removing the aluminium foil from the tube and adding tissue to the bottle. Talent wrapping the bottle into aluminium foil. *Videographer: Important step*

4.2.1.bis ADDED SHOT : Close view shot of the glass bottle at the beginning of the clearing when adipose tissue is still visible

4.2.2. *Close view s*hot of an almost invisible tissue at the end of the clearing *Videographer: Important step*

5. 3D Confocal Imaging

5.1. For 3D confocal imaging of the stained and cleared white adipose tissue, mount a metallic imaging chamber equipped with a glass bottom and, in a fume hood, transfer the tissue to the chamber **[1]. F**ill the chamber with fresh methyl salicylate **[2]**. NOTE: 5.1.1 – 5.2.1 shot together

5.1.1. WIDE: Talent at fume hood, mount the metallic chamber by adding the large glass cover slip to the bottom metallic part, and screw the top metallic part to form the chamber. Talent removing the aluminium foil and transferring tissue to the chamber *Videographer: Important step*

5.1.2. Talent filling chamber with methyl salicylate *Videographer: Important step*

5.2. Finalize the mountaging of the chamber by adding a small coverslip to stabilize the tissue and a large coverslip to close the chamber **[1]. P**lace the chamber onto an inverted confocal microscope stage **[2]** for tissue imaging at a low magnification to generate a few cubic-centimeter-3D maps of the whole adipose tissue sample **[3]**.

5.2.1. Talent adding small coverslips to chamber to stabilize the tissue. Talent adding a large coverslip to close the chamber. Talent removing the excess of methyl salicylate. Talent showing the chamber fully mounted with the almost invisible tissue inside

5.2.2. Talent placing chamber onto stage

5.2.3. Talent at microscope, looking into the eye pieces to place the tissue at the correct position, setting up imaging parameters and imaging sample, with monitor visible in frame

5.3. After 3D-map generation, use a 20x long distance air objective that provides a good ratio between the resolution and depth to select several areas for tissue sampling at a higher magnification **[1]**.

5.3.1. SCREEN: 5.3.1.: 01:44-02:13 *Video Editor: please speed up*

6. 3D Adipose Tissue Image Quantitative Result Extraction

6.1. For cell segmentation, convert 3D stacks onto the software format to free-up memory space **[1]** before opening the **Cell** module of the software **[2]**.

6.1.1. WIDE: Talent converting stacks, with monitor visible in frame

6.1.2. SCREEN: 6.1.2. - 6.2.1. – 6.3.1.: 00:28-00:32

6.2. Change the **Cell Detection** setting to plasma membrane staining and select the fluorescent channel of the marker used to delineate the cell periphery **[1]**.

6.2.1. SCREEN: 6.1.2. - 6.2.1. – 6.3.1.: 00:34-00:52 *Video Editor: can speed up*

6.3. Then set up the thresholds, provide a range of the expected cell sizes, and run the segmentation **[1]**.

6.3.1. SCREEN: 6.1.2. - 6.2.1. – 6.3.1.: 00:53-01:08 *Video Editor: can speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.1., 3.1., 4.2., 5.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
5.1.

Results

7. Results: Representative Adipose Tissue Labeling and Analysis

7.1. The effects of clearing on human **[1]** and mouse white adipose tissue can be observed with the naked eye **[2]**.

7.1.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize left column of images*
7.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize right column of images*

7.2. Clearing also drastically improves the image depth of the tissue that is able to be acquired **[1]** and facilitates whole tissue 3D imaging and 3D-map generation at a 4x magnification **[2]**.

7.2.1. LAB MEDIA: Figures 3B and 3C *Video Editor: please sequentially emphasize 150, 300, and 450 micrometer image columns*
7.2.2. LAB MEDIA: Suppl video 1-1

7.3. 3D mapping further enables the selection of different areas of interest to be acquired at a 20x magnification **[1]** to a depth of 2 millimeters **[2]**.

7.3.1. LAB MEDIA: Figures 3D and 3E *Video Editor: please emphasize right column images in Figure 3D*
7.3.2. LAB MEDIA: Figures 3D and 3E *Video Editor: please emphasize Figure 3E*

7.4. Specific staining of lipid droplets and adipocytes **[1]** can be achieved using perilipin **[1]** and anti-Glut4 (glute-four) antibodies, respectively **[3]**.

7.4.1. LAB MEDIA: Figures 4A and 4B
7.4.2. LAB MEDIA: Figures 4A and 4B *Video Editor: please emphasize green signal in Figure 4A*
7.4.3. LAB MEDIA: Figures 4A and 4B *Video Editor: please emphasize red signal in Figure 4B*

7.5. Blood vessels can be detected using either CD31 (C-D-thirty-one) antibody **[1]** or intravenous injection of lectin-DyLight649 (dye-light-six forty-nine) shortly before sacrifice **[2]**.

7.5.1. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize white signal in Figure 4C*

7.5.2. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize red signal in Figure 4D*

7.6. Macrophages and T cells can be visualized using anti-CD301 (C-D-three-oh-one)-PhycoErythrin antibody **[1]** and anti-TCR (T-C-R)-beta-Pacific Bleu antibody **[2]**.

7.6.1. LAB MEDIA: Figures 4D and 4E *Video Editor: please emphasize green signal in Figure 4D*
7.6.2. LAB MEDIA: Figures 4D and 4E *Video Editor: please emphasize green signal in Figure 4E*

7.7. The peripheral nerve network can be detected using anti-Tyrosine Hydroxylase antibody **[1]**.

7.7.1. LAB MEDIA: Figures 4F and 4G *Video Editor: please emphasize yellow signal in Figure 4G*

7.8. Using these labelling data, the mean size and size distribution **[1]** and blood vessel network density of the adipocytes within the adipose tissue can then be determined **[2]**.

7.8.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize graphs in Figure 5B*
7.8.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize Figure 5C*

Conclusion

8. Conclusion Interview Statements

8.1. **Jérôme Gilleron**: It is crucial to follow the incubation times as demonstrated, as a poor sample preparation will not result in a good image quality **[1]**.

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

8.2. **Jérôme Gilleron**: This protocol can be combined with advanced imaging systems or can be coupled to post-processing image analysis freeware **[1]**.

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

NOTE to Video Editor: Please add the image LastSlide.pdf at the very end of the video. It's a group photo to acknowledge all the people from the lab.