FINAL SCRIPT: APPROVED FOR FILMING

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Title: Characterization of Adipocyte-Derived Extracellular Vesicle Secretion Using a CD63-GFP Reporter Mouse Model In Vivo and In Vitro

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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, done**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, 100 ft apart**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 34
Number of Shots: 56

Introduction

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

Videographer's NOTE: Authors made various text changes to the interview statement sections

INTRODUCTION:

1.1. **Sofia Krylova:** We study adipocyte-derived EVs to define their roles in adipose tissue, particularly how they regulate the crosstalk between adipocytes and their progenitor cells.

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

1.2. **Sofia Krylova:** There are currently no in vivo systems to study AdEV secretions, and the existing purification protocols are not yet fully optimized.

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

CONCLUSION:

1.3. **Sofia Krylova:** The protocol provides a robust and reproducible system to study AdEV secretion in vivo and in vitro, as well as an optimized adipocyte EV purification method.

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

1.4. **Sofia Krylova:** The protocol for the first time describes and validates the use of serial low speed centrifugations combined with size exclusion chromatography to purify EVs derived from mature adipocytes.

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

1.5. **Sofia Krylova:** Our findings allow us to ask new questions about how adipocytes use EVs to communicate with other cell types under physiological and pathological conditions.

1.5.1. 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.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the Albert Einstein College of Medicine

Protocol

2. Isolation of Adipocyte Progenitor Cells from Inguinal Adipose Tissue

Demonstrator: Sofia Krylova

2.1. To begin, use sterile technique to excise inguinal adipose tissue from euthanized mice **[1].** Place 1 gram of tissue into a 2-milliliter tube containing 0.5 milliliters of digestion buffer **[2-TXT]**. Using sterile scissors, mince the tissue into pieces no larger than 1 millimeter **[3]**.

2.1.1. WIDE: Talent placing dissected adipose tissue on a sterile dish. **Videographer's NOTE**: 2.1.1-2.1.2 filmed as 2.1.1 - author performed both steps.

2.1.2. Talent adding tissue into a 2 mL eppendorf tube with 0.5 milliliters of digestion buffer. **TXT: 0.5 mL 1 M HEPES, 0.5 g BSA, 50 mL DMEM**

Talent using sterile scissors to finely mince the tissue into small fragments. **Videographer's NOTE**: 2.1.3 was mislabeled on the slate as 2.3

2.2. Suspend the minced tissue in 10 milliliters of digestion buffer containing 0.5 milligrams per milliliter Liberase and 50 units per milliliter DNase I *(D-N-Ase)* **[1]**. Incubate the sample at 37 degrees Celsius on an orbital shaker **[2-TXT].**

2.2.1. Talent adding the minced tissue to labeled tube with 10 milliliters of digestion buffer containing Liberase and DNase I.

2.2.2. Talent placing the tube in an orbital shaker. **TXT: Incubation: 37 °C, 100 rpm, 30 - 60 mins**

2.3. During incubation, gently invert the tube several times **[1]**. When the solution appears cloudy and no visible tissue fragments remain, the digestion is complete **[2]**.

2.3.1. Talent removing the tube and gently inverting it.

2.3.2. Shot of the cloudy solution with no visible fragments.

2.4. To isolate stromal vascular fraction or SVF, dilute the digested suspension with two volumes of digestion buffer **[1-TXT].** Gently invert the tube three to four times to mix well **[2]**.

2.4.1. Talent adding digestion buffer.

2.4.2. Talent gently inverting the tube multiple times.

2.5. Pass the suspension through a sterile 100 micrometer filter to remove undigested tissue **[1]**. Then centrifuge the filtrate at 300 *g* for 5 minutes at 4 degrees Celsius **[2]**. Label the pellet as SVF **[3]**.

2.5.1. Talent pouring the suspension through a filter into a clean tube.

2.5.2. Talent placing the tube in a centrifuge, setting to 300 g for 5 minutes at 4 degrees Celsius, and starting the spin.

2.5.3. Talent pipetting out the upper adipocyte layer.

~~2.5.4. Talent writing "SVF" on the tube containing the pellet.~~ **Videographer's NOTE**: REMOVE 2.5.4

2.6. Incubate the stromal vascular fraction in freshly prepared red blood cell lysis buffer on ice for 5 minutes, protected from light **[1]**.

2.6.1. Talent adding 1×red blood cell (RBC) lysis buffer to the tube on ice and covering it with foil. **Videographer's NOTE**: 2.6.1 added a shot B

2.7. Add two volumes of wash buffer **[1]** and centrifuge at 400 *g* for 5 minutes at 4 degrees Celsius **[2]**.

2.7.1. Talent adding wash buffer.

2.7.2. Talent placing the tube in a centrifuge. **Videographer's NOTE**: 2.7.2 Please use Clip B112_B163_11247W_001 Clip had to be re-filmed due to wrong color of buffer in tube.

2.8. Resuspend the stromal vascular fraction in 80 microliters of buffer per gram of tissue **[1-TXT]** and transfer the contents to a new tube **[2]**. Add 20 microliters of non-adipocyte progenitor depletion cocktail per gram of tissue **[3]**. Incubate the sample for 15 minutes at 2 to 8 degrees Celsius in the dark **[4]**. **NOTE**: VO added for the extra shot

2.8.1. Talent pipetting the appropriate volume of buffer into the SVF tube. **TXT: Buffer: PBS, pH 7.2, 0.5 % BSA, 2 mM EDTA**

Added shot 2.8.2. : Talent transfer SVF from 50 mL tube to 1.5 mL Eppendorf tube. **Videographer's NOTE**: 2.8.2 ADD Step Transfer SVF from tube to 1.5ml eppendorf tube.

2.8.3, Talent pipetting depletion cocktail into the SVF suspension and gently mixing.

2.8.4, Talent placing the tube on a cold block and wrapping it in foil to shield from light. **Videographer's NOTE**: RENAMED scripted 2.8.2 to 2.8.3 to 2.8.3 to 2.8.4

2.9. Pass the sample through an LS *(L-S)* column and collect the flow-through containing the Lineage-negative cell fraction **[1]**. Centrifuge the flow-through at 300 *g* for 5 minutes at 4 degrees Celsius to obtain the adipocyte progenitor cells **[2]**.

2.9.1. Shot of the flow-through being collected into a labeled tube.

2.9.2. Talent placing the tube in a centrifuge.

3. Confocal and Flow Cytometry-Based Detection of Secreted GFP-Tagged Adipocyte Extracellular Vesicles

Demonstrator: Sofia Krylova

3.1. Incubate 250 microliters of isolated mouse adipocyte progenitor cells mice onto collagen-coated 8-well chamber slides for 6 hours **[1-TXT].**

3.1.1. WIDE: Talent pipetting 250 microliters of APC suspension into a collagen-coated 8-well chamber slide. **TXT: APC concentration: 1 x 10^4 cells/mL; Source: AdipCD63-GFP or CD63-GFP mice**

3.2. Fix the cells with 4 percent paraformaldehyde for 10 minutes to preserve cellular and extracellular vesicle structures for imaging **[1]**. Next, wash the slides three times with PBS **[2].** Then mount with antifade medium **[3-TXT]**.

3.2.1. Talent gently adding 4 percent paraformaldehyde to the wells.

3.2.2. Talent pipetting PBS over the slides.

3.2.3. Talent mounting the slide with antifade medium. **TXT: Capture GFP signals using a confocal microscope**

~~3.3. Capture GFP signals using a confocal microscope equipped with a 63 X oil immersion objective **[1]**. Acquire images as z-series stacks with a 0.5 micrometer step size **[2-TXT].** Authors: Please create videos of the shots labeled as SCOPE/SCREEN and upload the files to your project page as soon as possible: **https://review.jove.com/account/file-uploader?src=21249398**~~

~~3.3.1. SCOPE/SCREEN: Shot of the tissue under confocal microscope.~~

~~3.3.2. SCOPE/SCREEN: Images are being captured under z-series. **TXT: Excitation: 488 nm; Emission: 500 - 550 nm**~~

3.4. Next, after counting the adipocyte progenitor cells, suspend them in dilute viability dye **[1-TXT]**. Incubate on ice for 20 minutes, protected from light **[2]**.

3.4.1. Talent suspending cells in the viability dye mix. **TXT: Cell concentration: 1 - 10 × 10^6/ 100 µL**

3.4.2. Talent placing the tube on ice under dark conditions.

3.5. Wash the cells once with wash buffer and centrifuge **[1-TXT].** Suspend the cells in brilliant staining buffer so that the final concentration is 1 million cells per 100 microliters **[2]**.

3.5.1. Talent adding wash buffer to the stained cells. **TXT: Centrifugation: 300 x *g*, 5 min, 4 °C**

3.5.2. Talent placing the suspension in a centrifuge.

3.6. Block nonspecific binding by adding Fc receptor blocking reagent at a 1 to 100 ratio and incubate **[1-TXT]**.

3.6.1. Talent adding Fc blocking reagent to the cell suspension. **TXT: Incubation: On ice, 10 min**

3.7. Pre-spin the Sca-1 *(S-C-A-One)* antibody at 6,000 *g* for 10 minutes at 4 degrees Celsius **[1]**. Add the Sca-1 antibody to the APC tube at a 1 to 100 dilution, then place the tube on ice protected from light **[2]**. **NOTE**: VO added for the extra shot

3.7.1. Talent spinning down the Sca-1 antibody in a microcentrifuge.

Added shot 3.7.2. : Talent then transferring 1 microliter Sca-1 antibody into the APC tube on an ice rack, keeping it covered under foil.

3.8. Wash the cells twice with 1 milliliter of wash buffer **[1]**, then centrifuge at 300 *g* for 5 minutes at 4 degrees Celsius **[2]**.

3.8.1. Talent pipetting 1 mL wash buffer into the tube with the cells ~~twice~~.

3.8.2. Shot of the tube being placed in a centrifuge.

3.9. Suspend the cells in wash buffer for flow cytometry sorting **[1-TXT]**. Then acquire the data for Sca-1 positive and GFP positive cells on a spectral flow cytometer equipped with a violet and blue laser **[2]**.

3.9.1. Talent resuspending the final cell pellet in 500 microliters of wash buffer. **TXT: Final concentration: 1 x 10^6 cells per 500 µL**

3.9.2. TEXT ON PLAIN BACKGROUND:
405 nm violet laser: 450/50 nm filter for Pacific Blue
488 nm blue laser: 530/30 nm filter for GFP

4. Detection of Secreted Adipocyte-Derived Extracellular Vesicles In Vitro

Demonstrator: Sofia Krylova, Daniel Zamith-Miranda

4.1. To begin differentiation, coat dishes with 0.2 percent gelatin for 30 minutes at room temperature **[1]**. Seed freshly isolated APCs in ~~antibiotic~~ supplemented DMEM/F12 *(D-M-E-M-F-Twelve)* medium and incubate until confluency **[2-TXT]**.

4.1.1. WIDE: Talent pipetting 0.2 percent gelatin into dishes.

4.1.2. Talent pipetting supplemented DMEM/F12 medium into the dishes. **TXT: Medium: DMEM/F12, 15% FBS, 1% Penicillin/Streptomycin (P/S), 0.1% Primocin**

4.2. Replace the medium from Day 1 to Day 8 with MesenCult adipogenic differentiation medium **[1]**.

4.2.1. TEXT ON PLAIN BACKGROUND:
MesenCult adipogenic differentiation medium: 50 mL
45 mL of base
5 mL of supplement
0.5 mL of GlutaMAX
50 µL of Primocin

Replace with fresh differentiation medium on Day 4
Video Editor: please play both shots side by side in a split screen

4.3. On Day 9, wash the adipocytes twice with PBS **[1].** Then replace the medium with mature adipocyte culture medium containing DMEM, 1 % BSA, and 0.2% Primocin **[2-TXT]**.

4.3.1. Talent sucking off the differentiation medium, then pipetting PBS into the dishes.

4.3.2. Talent sucking off PBS, then adding mature adipocyte culture medium. **TXT: Incubate for 16 h**

4.4. To isolate adipocyte secreted extracellular vesicles, first pass 15 milliliters of culture medium through a 40-micrometer filter followed by a 30-micrometer filter **[1]**. Centrifuge the filtrate at 500 *g* for 5 minutes, then centrifuge the supernatant at 2,000 *g* for 10 minutes to remove any residual cells and debris **[2]**.

4.4.1. Talent filtering culture medium through the 40 micrometer filter into a collection tube.

4.4.2. Talent placing the sample in a centrifuge and setting the parameters.
AUTHORS: Please perform any 1 centrifugation step since this is only for demonstration

4.5. After passing the resulting supernatant ~~filtrate~~ through a 0.8 micrometer filter, centrifuge at 4,000 g for 15 minutes ~~centrifuge at 4,000 g for 15 minutes~~ to concentrate the medium **[1-TXT]**.

4.5.1. Talent centrifuges the ultrafiltration unit containing the sample at 4,000 g. **TXT: Final volume: 0.5 mL**

4.6. Load the concentrated sample onto a size exclusion chromatography column connected to an automatic fraction collector and collect fractions F1 through F8 **[1]**.

4.6.1. Talent loading sample into the chromatography column.

4.7. To perform western blot analysis, concentrate each fraction from 400 microliters to 25 microliters **[1]**.

4.7.1. Talent transferring samples to 2 mL ultrafiltration unit, and centrifuges 10,000g, 10 minutes at 4 degree.**TXT: Verify with Western blot, NTA and TEM analysis**

4.8. Pipette 50 microliters of radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors to the concentrate **[2]**. Add 75 microliters of 2 X SDS loading buffer to denature the sample **[3]** and resolve proteins on a 4 to 12 percent tris-glycine gel **[4]**. **NOTE**: VO added for the extra shot

4.8.1. Shot of labeled tube with concentrated sample.

4.8.2. Talent pipetting 50 µL of radioimmunoprecipitation assay buffer into the tube with concentrated sample.

4.8.3. Talent adding sodium dodecyl sulfate buffer

Added shot: Talent then loading the sample onto a 12% tris-glycine gel. **Videographer's NOTE:** SPLIT 4.8.3 into 2 steps. 4.8.3 Talent adding sodium dodecyl sulfate buffer. ADD 4.8.3 - Loading the sample onto a 12% tris-glycine gel.

4.9. For nanoparticle tracking analysis, dilute extracellular vesicle samples with 0.1 micrometer filtered sterile PBS and mix gently **[1-TXT]**.

4.9.1. Talent diluting extracellular vesicle sample with PBS .
TXT: Sample to PBS: 1: 1000; Final particle concentration: 10^7–10^9 particles/mL;

4.10. Load the diluted sample into the NTA instrument equipped with a high-sensitivity scientific complementary metal–oxide–semiconductor camera and a 532 nanometer green laser **[1]**. Set detection for particles between 10 nanometers and 1000 nanometers and within a concentration of 10^6 to 10^9 particles per milliliter **[2]**.

4.10.1. Talent loading the sample into the NTA instrument.

4.10.2. SCREEN: 69670_screenshot_1.mp4 00:17-00:21 and 00:35-00:40.

4.11. Use the NS Xplorer software suite to perform imaging and quantification using a dynamic observation volume-based concentration algorithm **[1]**.

4.11.1. SCREEN: 69670_screenshot_1.mp4 06:35-06:40.

Results

5. Results

5.1. Adipose progenitor cells from Stop$^{fl/fl}$/CD63-GFP *(Stop-flox-flox-C-D-Sixty-Three-G-F-P)* mice showed no detectable green fluorescent protein puncta under confocal microscopy **[1]**, while cells from AdipCD63 *(A-dip-C-D-Sixty-Three)* -GFP mice displayed numerous distinct green fluorescent protein puncta throughout the cytoplasm **[2]**.

5.1.1. LAB MEDIA: Figure 1 *Video Editor: Please highlight image A*

5.1.2. LAB MEDIA: Figure 1 *Video Editor: Please highlight image B*

5.2. Flow cytometry analysis showed that 1.2% of Sca1*(Ska-One)*-positive cells in the reporter mice expressed green fluorescent protein **[1]**, compared to 12.6% in AdipCD63-GFP mice **[2]**.

5.2.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the gated region labeled "P6"*

5.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the gated region labeled "P6"*

5.3. Western blotting of size-exclusion chromatography fractions showed that extracellular vesicle markers CD63 and HSP70 *(H-S-P-Seventy)* were enriched in fractions F1 and F2 **[1]**, while markers of endoplasmic reticulum and mitochondria were absent, confirming vesicle purity **[2]**.

5.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the dark bands for CD63 and HSP70 in lanes labeled F1 and F2.*

5.3.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the absence of bands for Calnexin and COX IV in fractions F1 and F2.*

5.4. Nanoparticle tracking analysis revealed that adipocyte-derived extracellular vesicles ranged in size from 50 nanometers to 500 nanometers **[1]**, and transmission electron microscopy confirmed the vesicles exhibited intact double membrane structures **[2]**.

5.4.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the main peak in the red line graph between 50 and 500 nanometers on the x-axis.*

5.4.2. LAB MEDIA: Figure 4B.

5.5. Western blot confirmed knockout of STX4 *(S-T-X-Four)* in adipocytes **[1]**.

5.5.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the absence of the STX4 band in the KO lane*

5.6. Confocal microscopy showed that CD63-GFP-positive vesicles were dispersed

throughout the cytosol in wild-type adipocytes **[1]**, but accumulated near the plasma membrane in STX4 knockout adipocytes **[2]**.

5.6.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the WT image*

5.6.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the KO image.*

5.7. The concentration of secreted adipocyte-derived extracellular vesicles was significantly reduced in STX4 knockout adipocytes compared to wild-type **[1]**.

5.7.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the small red bar labeled KO in the bar graph.*

5.8. Western blot of equal protein amounts of intracellular vesicles showed reduced expression of CD63 and CD81 in STX4 knockout adipocytes compared to wild-type **[1]**.

5.8.1. LAB MEDIA: Figure 5D. *Video editor: Highlight the reduced intensity of CD63 and CD81 bands in the KO lane*

- **nguinal**

Pronunciation link: https://www.merriam-webster.com/dictionary/inguinal
IPA: /ˈɪŋgwɪnəl/
Phonetic Spelling: ING-gwi-nuhl

- **adipose**

Pronunciation link: https://www.merriam-webster.com/dictionary/adipose
IPA: /ˈædɪˌpoʊs/
Phonetic Spelling: AD-ih-pohs

- **euthanized**

Pronunciation link: https://www.merriam-webster.com/dictionary/euthanize
IPA: /ˈjuːθəˌnaɪzd/
Phonetic Spelling: yoo-THUH-nized

- **milliliter**

Pronunciation link: https://www.merriam-webster.com/dictionary/milliliter
IPA: /ˈmɪlɪˌliːtər/
Phonetic Spelling: MIL-ih-lee-ter

- **digestion**

Pronunciation link: https://www.merriam-webster.com/dictionary/digestion
IPA: /dɪˈdʒɛstʃən/
Phonetic Spelling: dih-JES-chuhn

- **buffer**
Pronunciation link: https://www.merriam-webster.com/dictionary/buffer
IPA: /ˈbʌfər/
Phonetic Spelling: BUH-fuhr

- **centrifuge**
Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyooj

- **pellet**
Pronunciation link: https://www.merriam-webster.com/dictionary/pellet
IPA: /ˈpɛlɪt/
Phonetic Spelling: PEL-it

- **lysis**
Pronunciation link: https://www.merriam-webster.com/dictionary/lysis
IPA: /ˈlaɪsɪs/
Phonetic Spelling: LY-sis

- **stromal**
Pronunciation link: No confirmed link found
IPA: /ˈstroʊməl/
Phonetic Spelling: STROH-muhl

- **vascular**
Pronunciation link: https://www.merriam-webster.com/dictionary/vascular
IPA: /ˈvæskjələr/
Phonetic Spelling: VAS-kyuh-luhr

- **fraction**
Pronunciation link: https://www.merriam-webster.com/dictionary/fraction
IPA: /ˈfrækʃən/
Phonetic Spelling: FRAK-shuhn

- **adipocyte**
Pronunciation link: No confirmed link found
IPA: /ˈædɪˌpoʊˌsaɪt/
Phonetic Spelling: AD-ih-poh-syt

- **progenitor**
Pronunciation link: https://www.merriam-webster.com/dictionary/progenitor
IPA: /prəˈdʒɛnɪtər/
Phonetic Spelling: proh-JEN-ih-tuhr

- **confocal**

Pronunciation link: https://www.merriam-webster.com/dictionary/confocal
IPA: /kənˈfoʊkəl/
Phonetic Spelling: kuhn-FOH-kuhl

- **microscopy**

Pronunciation link: https://www.merriam-webster.com/dictionary/microscopy
IPA: /maɪˈkrɒskəpi/ or /maɪˈkrɑːskəpi/
Phonetic Spelling: my-KROS-kuh-pee

- **paraformaldehyde**

Pronunciation link: No confirmed link found
IPA: /ˌpærəˌfɔːrməlˈdɛhaɪd/
Phonetic Spelling: par-uh-FOR-mal-DYE-hyde

- **immersion**

Pronunciation link: https://www.merriam-webster.com/dictionary/immersion
IPA: /ɪˈmɜːrʒən/ (AmE: /ɪˈmɝʒən/)
Phonetic Spelling: ih-MUR-zhuhn

- **cytoplasm**

Pronunciation link: https://www.merriam-webster.com/dictionary/cytoplasm
IPA: /ˈsaɪtəˌplæzəm/ or /ˈsaɪtəˌplæzm/
Phonetic Spelling: SY-tuh-plaz-um

- **viability**

Pronunciation link: https://www.merriam-webster.com/dictionary/viability
IPA: /ˌvaɪəˈbɪlɪti/
Phonetic Spelling: vy-uh-BIL-ih-tee