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Title: Exogenous Mitochondrial Transfer in Differentiating Brown Adipocytes and AGPAT2-Deficient Preadipocytes

Authors and Affiliations:

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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**

- 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: 20

Number of Shots: 36

Interviews

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Antonio Moreno:** This study examines if mitochondrial mass drives adipogenesis and whether Exogenous Mitochondrial transfer can rescue impaired differentiation caused by AGPAT2 gene deficiency.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the research gap or limitation in existing methods and how this protocol addresses them.**~~

- 1.2. **Antonio Moreno:** Genetic methods alter signaling alongside mitochondrial mass; this protocol uses exogenous transfer to isolate mass effects during adipogenesis.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Antonio Moreno:** This protocol measures the specific impact of mitochondrial mass on cell differentiation, network integration, metabolic rescue, and cellular bioenergetic function.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Antonio Moreno:** A key challenge is maintaining the structural integrity and bioenergetic viability of isolated mitochondria to ensure their functional integration into the host cell network.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Antonio Moreno:** Following this procedure, Seahorse assays for bioenergetics can be performed, and mitochondrial fusion-fission dynamics can be measured.
 - 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 at Pontificia Universidad Católica de Chile

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Donor Cells for Mitochondrial Isolation

Demonstrator: Antonio Moreno

- 2.1. To begin, place 100-millimeter culture dishes in the biosafety cabinet [1].
 - 2.1.1. WIDE: Talent placing the dishes in the biosafety cabinet. ***Videographer:** Please obtain CU shot as well for this shot.*
- 2.2. Seed 2×10^6 donor HepG2 cells or preadipocytes in the dish [1]. Grow the HepG2 cells to 80% confluence and preadipocytes to 100% confluence [2].
 - 2.2.1. Talent seeding the donor cells into the 100 millimeter culture dishes in the biosafety cabinet.
 - 2.2.2. Shot of the donor cells at the appropriate confluence in the culture dish. **NOTE:** Videographer provided an image for this.
- 2.3. Stain the donor cells with 100-nanomolar MitoTracker Deep Red FM in the FBS-free medium [1]. Incubate the cells for 30 minutes at 37 degrees Celsius in a 5% carbon dioxide incubator [2].
 - 2.3.1. Talent adding the MitoTracker Deep Red FM staining solution to the donor cells.
 - 2.3.2. Talent placing the culture dish into the incubator.
- 2.4. Wash the cells 2 times with the growth medium to remove the excess dye [1].
 - 2.4.1. Talent washing the cells by adding the growth medium and removing it.
- 2.5. Next, detach the cells using 0.25% trypsin A [1]. Collect the cell suspension [2] and centrifuge it at 300 g for 5 minutes at 4 degrees Celsius [3].
 - 2.5.1. Talent adding trypsin to detach the cells.
 - 2.5.2. Talent collecting the cell suspension.
 - 2.5.3. Talent placing the cell suspension into the centrifuge.

NOTE: Treat this as a separate section

Isolation of Donor Mitochondria

- 2.6. Resuspend the pellet in 1 milliliter of the mitochondrial isolation buffer supplemented with BSA, protease inhibitors, and phosphatase inhibitors [1]. Disrupt the plasma membrane by passing the suspension through a tuberculin syringe 5 times [2]. Then homogenize the suspension by passing it through an insulin syringe at least 10 times, until the solution appears light brown with no visible clumps [3].
 - 2.6.1. Talent resuspending the pellet in the supplemented mitochondrial isolation buffer.
 - 2.6.2. Talent passing the suspension through the tuberculin syringe multiple times.
 - 2.6.3. Talent passing the suspension through the insulin syringe until a homogeneous light-brown solution is obtained.
- 2.7. Centrifuge the homogenate at 900 *g* for 11 minutes at 4 degrees Celsius [1].
 - 2.7.1. Talent placing the homogenate into the centrifuge.
- 2.8. Collect the supernatant [1] and centrifuge it at 10,000 *g* for 10 minutes at 4 degrees Celsius to pellet the mitochondria [2]. Wash the pellet 3 times with the mitochondrial isolation buffer supplemented with BSA [3].
 - 2.8.1. Talent collecting the supernatant.
 - 2.8.2. Talent placing the supernatant into the centrifuge.
 - 2.8.3. Talent washing the mitochondrial pellet with the mitochondrial isolation buffer supplemented with bovine serum albumin.
- 2.9. Resuspend the mitochondrial pellet in 100 microliters of BSA-free mitochondrial isolation buffer [1]. Quantify the protein concentration using the bicinchoninic acid protein assay kit [2].
 - 2.9.1. Talent resuspending the mitochondrial pellet in bovine serum albumin-free mitochondrial isolation buffer.
 - 2.9.2. Talent adding the bicinchoninic acid protein assay reagent to a sample tube.

NOTE: Treat this as a separate section

Mitochondrial Transfer to Recipient Cells

2.10. Seed the recipient primary preadipocytes derived from P-0.5 neonatal mice at 5×10^5 cells per well in a 12-well plate containing 18-millimeter coverslip [1]. Incubate for 24 hours before the assay [1].

2.10.1. WIDE: Talent seeding the recipient preadipocytes into the 12-well plates containing 18 millimeter coverslips.

2.10.2. Reuse 2.11.3

2.11. Add the isolated donor mitochondria directly to the recipient medium at 10 micrograms of mitochondrial protein per 10^5 cells [1]. Rock the plates gently to distribute the mitochondria [2]. Incubate the cells for 24 to 120 hours at 37 degrees Celsius in a humidified incubator with 5% carbon dioxide [3].

2.11.1. Talent adding the isolated donor mitochondria to the recipient medium.

2.11.2. Talent gently rocking the plate to distribute the mitochondria.

2.11.3. Talent placing the plate into the incubator.

2.12. At the desired time point, counterstain the recipient cells with MitoTracker Green and image them by confocal microscopy [1].

2.12.1. Talent adding stain to the cells.

Results

3. Results

- 3.1. Flow cytometry showed that approximately 60% of preadipocytes were positive for the MitoTracker Deep Red signal, indicating efficient uptake of exogenous mitochondria [1].
 - 3.1.1. LAB MEDIA: Figure 1E. *Video editor: Highlight the 2 curves labelled "MitoTracker Deep Red".*
- 3.2. Live-cell confocal microscopy revealed colocalization of exogenous MitoTracker Deep Red-labeled mitochondria with the endogenous mitochondrial network labeled with MitoTracker Green [1].
 - 3.2.1. LAB MEDIA: Figure 1F. *Video editor: Highlight the merged images in the 3rd column for row 2 and 3 (Mito rows).*
- 3.3. Because approximately 50% of cells remained negative for the MitoTracker Deep Red signal, the overall mitochondrial incorporation efficiency was about 50% [1].
 - 3.3.1. LAB MEDIA: Figure 1G. *Video editor: Highlight the 2 Mito bars*
- 3.4. Human mitochondrial DNA remained detectable throughout 1 [1], 3 [2], and 5 days of differentiation [3].
 - 3.4.1. LAB MEDIA: Figure 1H. *Video editor: Highlight the data point at day 1 for the differentiated + hMito group.*
 - 3.4.2. LAB MEDIA: Figure 1H. *Video editor: Highlight the data point at day 3 for the differentiated + hMito group.*
 - 3.4.3. LAB MEDIA: Figure 1H. *Video editor: Highlight the data point at day 5 for the differentiated + hMito group.*
- 3.5. In contrast, human mitochondrial DNA levels rapidly declined in cells that were not induced to differentiate [1], becoming nearly undetectable by day 3 [2].
 - 3.5.1. LAB MEDIA: Figure 1H. *Video editor: Highlight the line for the non-differentiated + hMito group.*
 - 3.5.2. LAB MEDIA: Figure 1H. *Video editor: Highlight the data point at day 3 for the non-differentiated + hMito group.*

1. Biosafety cabinet
Pronunciation link: <https://www.merriam-webster.com/dictionary/biosafety>
IPA: /ˌbaɪoʊˈseɪfti ˈkæbəneɪt/
Phonetic Spelling: bye·oh·sayf·tee kab·uh·nuht
2. HepG2
Pronunciation link: No confirmed link found
IPA: /hep dʒiː tuː/
Phonetic Spelling: hep gee two
3. Preadipocytes
Pronunciation link: <https://www.howtopronounce.com/preadipocyte>
IPA: /ˌpriːəˈdɪpəˌsaɪts/
Phonetic Spelling: pree·uh·dip·uh·sytes
4. Confluence
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>
IPA: /ˈkɒnfluəns/
Phonetic Spelling: kon·floo·uhns
5. MitoTracker
Pronunciation link: No confirmed link found
IPA: /ˈmaɪtoʊˌtrækər/
Phonetic Spelling: my·toh·trak·er
6. Nanomolar
Pronunciation link: No confirmed link found
IPA: /ˌnænoʊˈmoʊlər/
Phonetic Spelling: nan·oh·moh·ler
7. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪŋkjəˌbeɪt/
Phonetic Spelling: ing·kyuh·bayt
8. Carbon dioxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/carbon%20dioxide>
IPA: /ˌkɑːrbən daɪˈɑːksaɪd/
Phonetic Spelling: kar·buhn dye·ok·side
9. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: trip·sin
10. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
11. Resuspend
Pronunciation link: No confirmed link found

- IPA: /ˈriːsəˈspend/
Phonetic Spelling: ree·suh·spend
12. Mitochondrial
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondrion>
IPA: /ˈmaɪtoʊˈkɑːndriəl/
Phonetic Spelling: my·toh·kon·dree·uhl
13. Protease
Pronunciation link: <https://www.merriam-webster.com/dictionary/protease>
IPA: /ˈproʊtiˌeɪs/
Phonetic Spelling: proh·tee·ays
14. Phosphatase
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphatase>
IPA: /ˈfɑːsfəˌteɪs/
Phonetic Spelling: fos·fuh·tays
15. Plasma membrane
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasma%20membrane>
IPA: /ˈplæzməˈmemˌbreɪn/
Phonetic Spelling: plaz·muh mem·brayn
16. Tuberculin
Pronunciation link: <https://www.merriam-webster.com/dictionary/tuberculin>
IPA: /tuːˈbɜːkjəlɪn/
Phonetic Spelling: too·bur·kyuh·lin
17. Homogenize
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenize>
IPA: /həˈmɑːdʒəˌnaɪz/
Phonetic Spelling: huh·mah·juh·nyze
18. Homogenate
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenate>
IPA: /həˈmɑːdʒəˌneɪt/
Phonetic Spelling: huh·mah·juh·nayt
19. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·nay·tuhnt
20. Bovine serum albumin
Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>
IPA: /ˈboʊvənˈsɪrəm ælˈbjuːmɪn/
Phonetic Spelling: boh·vine seer·uhm al·byoo·min
21. Bicinchoninic
Pronunciation link: <https://www.howtopronounce.com/bicinchoninic>
IPA: /ˌbaɪsɪnˌkoʊˈnɪnɪk/
Phonetic Spelling: bye·sin·koh·nin·ik
22. Coverslip
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>

- IPA: /'kʌvərˌslɪp/
Phonetic Spelling: kuv·er·slip
23. Micrograms
Pronunciation link: <https://www.merriam-webster.com/dictionary/microgram>
IPA: /'maɪkroʊˌɡræm/
Phonetic Spelling: my·kroh·gram
24. Humidified
Pronunciation link: <https://www.merriam-webster.com/dictionary/humidify>
IPA: /hjuː'mɪdəˌfaɪd/
Phonetic Spelling: hyoo·mid·uh·fyd
25. Counterstain
Pronunciation link: No confirmed link found
IPA: /'kaʊntərˌsteɪn/
Phonetic Spelling: kown·ter·stayn
26. Confocal microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kən'foʊkəl mə'kraːskəpi/
Phonetic Spelling: kuhn·foh·kuhl my·kros·kuh·pee
27. Flow cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /floʊ saɪ'tɑːmətri/
Phonetic Spelling: floh sy·tom·uh·tree
28. Colocalization
Pronunciation link: No confirmed link found
IPA: /koʊˌloʊkələˈzeɪʃən/
Phonetic Spelling: koh·loh·kuh·luh·zay·shuhn
29. Exogenous
Pronunciation link: <https://www.merriam-webster.com/dictionary/exogenous>
IPA: /ɛk'sɑːdʒənəs/
Phonetic Spelling: ek·sah·juh·nuhs
30. Incorporation
Pronunciation link: <https://www.merriam-webster.com/dictionary/incorporation>
IPA: /ɪnˌkɔːrpeɪʃən/
Phonetic Spelling: in·kor·puh·ray·shuhn
31. Mitochondrial DNA
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondrion>
IPA: /ˌmaɪtoʊ'kɑːndriəl ˌdiːən'eɪ/
Phonetic Spelling: my·toh·kon·dree·uhl dee·en·ay
32. Detectable
Pronunciation link: <https://www.merriam-webster.com/dictionary/detectable>
IPA: /dɪ'tektəbəl/
Phonetic Spelling: dih·tek·tuh·buhl
33. Differentiate
Pronunciation link: <https://www.merriam-webster.com/dictionary/differentiate>

IPA: /ˌdɪfəˈrɛnʃiˌeɪt/

Phonetic Spelling: dif·uh·ren·shee·ayt

34. Undetectable

Pronunciation link: <https://www.merriam-webster.com/dictionary/undetectable>

IPA: /ˌʌndɪˈtɛktəbəl/

Phonetic Spelling: un·dih·tek·tuh·buhl