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Project Page Link: <https://review.jove.com/account/file-uploader?src=21446428>

Title: Development of Mesenchymal Stem Cell Membrane-Enveloped Nanovesicles for Enhanced Gene Delivery

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.**

Videographer: Please film the computer screen for shots 5.5.1 to 5.7.2

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. **Yes.**

Current Protocol Length

Number of Steps: 28

Number of Shots: 55

Introduction

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

INTRODUCTION:

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

- 1.1. **Yaxuan Liang:** In this protocol, we introduce a method to prepare mesenchymal stem cell derived nanovesicles, which resemble extracellular vesicles and encapsulate AAVs for improved gene delivery.
 - 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. **Yaxuan Liang:** We call this AAV-containing-nanovesicles "CME-AAV". Compared with the emerging gene delivery vector EV-AAV, CME-AAV offers greater flexibility in cell sources and higher yield.
 - 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?~~

- 1.4. **Yaxuan Liang:** This protocol describes steps to obtain purified cell membrane-enveloped AAVs, or CME-AAVs, via extrusion and density gradient ultracentrifugation.
 - 1.4.1. 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.~~

- 1.5. **Yaxuan Liang**: Two key factors require careful consideration: preprocessing cell fragments for extrusion and optimizing the membrane fragment-AAV mixing ratio to control CME-AAV particle-to-genome ratio.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.6. **Yaxuan Liang**: Future studies can further explore cell membrane enclosure to enhance immune protection and functional surface properties, optimizing CME-AAV as a versatile platform for AAV delivery.

1.6.1. 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 (OPTIONAL):

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.

What problem in your field of research does your JoVE article address?

- 1.3. **Yaxuan Liang, Associate Professor, Beijing Normal University**: (authors will present their testimonial statements live)
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What value do you expect this video article to have for other researchers working in your field?

- 1.4. **Yaxuan Liang, Associate Professor, Beijing Normal University**: (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.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the Beijing Normal University at Zhuhai

Protocol

2. Isolation and Preparation of Human Mesenchymal Stem Cell (hMSC) Membrane Fractions

Demonstrator: Yue Su

- 2.1. To begin, remove the culture medium from a culture dish containing human mesenchymal stem cells at 70 to 90 percent confluency [1]. Using a cell scraper, detach the cells from the culture dish [2] and transfer them into a conical tube containing PBS [3].
 - 2.1.1. WIDE: Talent aspirating medium from a culture dish.
 - 2.1.2. Talent scraping mesenchymal stem cells with a cell scraper.
 - 2.1.3. Talent transferring detached cells into a conical tube containing PBS
- 2.2. Centrifuge the collected cells at 300 *g* for 5 minutes at 4 degrees Celsius to pellet the cells [1]. Then wash the cell pellet twice with PBS and centrifuge again [2].
 - 2.2.1. Talent placing the conical tube into a refrigerated centrifuge.
 - 2.2.2. Talent resuspending the pellet in PBS and placing the tube into the centrifuge.
- 2.3. Resuspend the final cell pellet in PBS supplemented with a protease inhibitor cocktail [1]. Then subject the cell suspension to 3 freeze-thaw (*Freeze-Thaw*) cycles to disrupt the plasma membrane and generate a crude lysate [2].
 - 2.3.1. Shot of the pellet being resuspended in PBS with protease inhibitor cocktail.
 - 2.3.2. Talent transferring the tube between a freezing source and room temperature to perform freeze and thaw cycles.
- 2.4. Using a pipette, homogenize the lysate thoroughly [1]. Then centrifuge at 1000 *g* for 20 minutes at 4 degrees Celsius to remove cellular debris [2].
 - 2.4.1. Talent pipetting the lysate repeatedly.
 - 2.4.2. Talent placing the tube into a refrigerated centrifuge.
- 2.5. Transfer the supernatant to a new tube [1]. Centrifuge at 18000 *g* for 1 hour at 4 degrees Celsius to pellet the membrane fraction [2].
 - 2.5.1. Talent carefully transferring supernatant into a clean tube.
 - 2.5.2. Talent setting the centrifugation parameters.
- 2.6. Discard the supernatant [1] and resuspend the membrane pellet in PBS to obtain the crude mesenchymal stem cell membrane fraction [2].
 - 2.6.1. Talent removing supernatant.

2.6.2. Talent resuspending the pellet in PBS.

2.7. Now homogenize the membrane suspension by sequentially passing it through syringes fitted with 20 to 27-gauge needles [1].

2.7.1. Talent passing the suspension through syringes with progressively smaller gauge needles.

2.8. Determine the protein concentration of the homogenized membrane suspension using a bicinchoninic acid assay [1]. Aliquot the suspension [2] and store at minus 80 degrees Celsius until needed [3]. Re-homogenize the suspension using a 27-gauge syringe after each thaw cycle [4].

2.8.1. Talent performing a bicinchoninic acid protein assay.

2.8.2. Talent aliquoting the membrane suspension into labeled tubes.

2.8.3. Talent placing the aliquots into a minus 80 degrees Celsius freezer.

2.8.4. Talent passing the thawed suspension through a 27 G syringe.

3. Encapsulation of Adeno-Associated Virus (AAVs) within Mesenchymal Stem Cell Membrane Vesicles by Extrusion

3.1. Mix the prepared membrane suspension with Adeno-Associated Virus or AAVs at a ratio of 150 micrograms protein per 1×10^{10} genome copies [1-TXT]. Incubate the mixture at 37 degrees Celsius for 30 minutes with gentle shaking [2].

3.1.1. Talent combining membrane suspension and viral solution in a tube.

3.1.2. Talent placing the tube in a 37 degrees Celsius shaker.

3.2. Load the membrane and virus mixture into a mini extruder fitted with a 400-nanometer polycarbonate membrane [1]. Pass the mixture through the extruder 20 times to promote encapsulation [2].

3.2.1. Talent loading the sample into a mini extruder with a 400 nanometer membrane.

3.2.2. Talent repeatedly passing the mixture through the extruder.

3.3. Replace the 400-nanometer membrane with a 200-nanometer membrane [1] and repeat extrusion [2].

3.3.1. Talent replacing the membrane with a 200 nanometer polycarbonate membrane.

3.3.2. Talent performing 20 extrusion passes with the new membrane.

3.4. Collect the processed suspension containing encapsulated virus, along with unencapsulated virus and empty vesicles [1]. Pool multiple batches if required and proceed immediately to purification [2].

- 3.4.1. Talent collecting the extruded suspension into a clean tube.
- 3.4.2. Shot of pooled tubes being combined.

4. Purification of Membrane-Encapsulated Adeno-Associated Virus by Iodixanol Density Gradient Ultracentrifugation

- 4.1. Dilute the stock iodixanol solution with 10x PBS and sterile deionized water to prepare 40 and 25% solutions [1].
 - 4.1.1. Talent preparing iodixanol dilutions in sterile tubes.
- 4.2. Using a pipette, underlay 1 milliliter of 40 percent iodixanol at the bottom of a 13.2 milliliter open-top thin-wall Ultra-Clear tube [1]. Underlay 3 milliliters of 25 percent iodixanol above the 40 percent layer to form a discontinuous gradient [2].
 - 4.2.1. Talent pipetting 1 mL of 40% iodixanol into the bottom of a 13.2 mL open-top, thin-wall ultra-clear tube.
 - 4.2.2. Talent pipetting 3 mL of 25% iodixanol 25 percent layer above the denser solution.
- 4.3. Gently overlay 7 milliliters of the processed suspension onto the top of the gradient [1]. Balance the tubes and load them into a swing bucket ultracentrifuge rotor [2].
 - 4.3.1. Talent layering 7 mL sample onto the gradient. **NOTE: Shot an additional close-up between 4.3.1 and 4.3.2**
 - 4.3.2. Talent balancing tubes and placing them into the ultracentrifuge rotor.
- 4.4. Centrifuge at 150,000 *g* for 3 hours at 4 degrees Celsius using fast acceleration and slow deceleration [1].
 - 4.4.1. Talent placing the rotor into the ultracentrifuge and starting the run with specified settings.
- 4.5. Then carefully remove the tubes without disturbing the gradient [1]. Collect 1 milliliter fractions from top to bottom into labeled 1.5-milliliter microcentrifuge tubes [2]. Keep all fractions on ice [3].
 - 4.5.1. Talent removing tubes from the rotor.
 - 4.5.2. Talent fractionating the gradient into labeled microcentrifuge tubes.
 - 4.5.3. Shot of the tubes being placed on ice.
- 4.6. Now identify and pool fractions 7 and 8 containing purified CME-AAVs (*C-M-E-A-A-Vees*) into a new open-top ultracentrifuge tube [1]. Make up the volume to 11 milliliters with PBS [2]. Then gently mix with a 1-milliliter pipette [2].
 - 4.6.1. Close up of combining fractions 7 and 8 into a single tube.

- 4.6.2. Talent adding phosphate buffered saline to final volume of 11 mL.
- 4.6.3. Talent gently mixing the suspension with 1 mL pipette.
- 4.7. Using fast acceleration and deceleration, centrifuge the pooled fractions at 150,000 *g* for 3 hours at 4 degrees Celsius [1]. Discard the supernatant carefully [2]. Resuspend the pellet in 50 to 200 microliters of sterile PBS, depending on the desired concentration [3].
 - 4.7.1. Talent loading the tube into the ultracentrifuge for the second spin.
 - 4.7.2. Talent removing supernatant with a pipette.
 - 4.7.3. Talent resuspending the pellet in a small volume of PBS.
- 4.8. Aliquot the purified preparation [1]. Then store at minus 80 degrees Celsius until further use [2].
 - 4.8.1. Talent aliquoting the purified preparation into labeled tubes.
 - 4.8.2. Shot of the tubes in a minus 80 degrees Celsius freezer.

5. In Vitro Transduction and Fluorescence Imaging of Cells with Encapsulated and Control Adeno-Associated Virus

- 5.1. Prepare working solutions of encapsulated virus and control virus in sterile PBS [1-TXT].
 - 5.1.1. Shot of labeled 1.5 ml Eppendorf tube containing the vectors. **TXT: Vectors: CME-AAV9.EGFP and AAV9.EGFP**
- 5.2. Add equal genome copies of encapsulated virus or control virus directly to the culture medium in each well [1]. Include negative control wells with an equivalent volume of PBS or empty virus particles [2].
 - 5.2.1. Talent pipetting viral solution into wells of a 48-well plate.
 - 5.2.2. Talent adding control solutions to designated wells.
- 5.3. Gently swirl the plate to ensure uniform distribution [1] and incubate for 24 to 72 hours, depending on experimental design [2].
 - 5.3.1. Talent gently swirling the culture plate.
 - 5.3.2. Talent placing the plate into a cell culture incubator.
- 5.4. Next, gently aspirate the culture medium [1]. Wash the cells twice with PBS, without dislodging them [2]. Stain nuclei with Hoechst 33342 according to the manufacturer's instructions [3].
 - 5.4.1. Talent gently aspirating medium.
 - 5.4.2. Shot of PBS being pipetted over the cell suspension.

- 5.4.3. Talent adding Hoechst staining solution to the wells.
- 5.5. Perform live cell fluorescence imaging using an inverted fluorescence microscope with appropriate filter sets for enhanced green fluorescent protein and Hoechst [1].
 - 5.5.1. SCREEN: Live fluorescence image showing enhanced green fluorescent protein and Hoechst stained nuclei. *Videographer: Please film the computer screen*
- 5.6. Select 10 or 20X objectives for quantification and 63X objective for high resolution imaging if needed [1].
 - 5.6.1. SCREEN: Images at 10 – 20 X are seen. *Videographer: Please film the computer screen*
- 5.7. Acquire at least 5 fields of view per well using identical imaging parameters [1]. Save all images in raw and grayscale formats for quantitative analysis [2].
 - 5.7.1. SCREEN: Capture multiple fields of view using consistent exposure and gain settings. *Videographer: Please film the computer screen*
 - 5.7.2. SCREEN: Save image files in raw and grayscale formats in designated folders. *Videographer: Please film the computer screen*

Results

6. Results

6.1. Transmission electron microscopy revealed that CME-AAVs exhibited size dimensions and membrane-bound structures similar to extracellular vesicles [1],

6.1.1. LAB MEDIA: Figure 1A.

6.2. The mesenchymal stem cell membrane markers CD73 (*C-D-Seventy-three*) and CD90 (*C-D-Ninety*) were detected in CME-AAV by Western blot analysis [1]. The 3 major adeno-associated virus capsid proteins VP1 (*V-P-One*), VP2, and VP3 were identified in CME-AAV by SDS-PAGE [2].

6.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the bands labelled "CD73" and "CD90" in the lane labelled "CME-AAV9.EGFP".*

6.2.2. LAB MEDIA: Figure 1C. *Video editor: Highlight the bands labelled "VP1", "VP2", and "VP3" in the lane labelled "CME-AAV9.EGFP".*

6.3. NanoSight (*Nano-sight*) analysis showed that CME-AAV9.EGFP (*C-M-E-A-A-V-Nine-E-G-F-P*) had a mean diameter of 128.6 nanometers and a total particle concentration of 1.32×10^{10} particles per milliliter [1].

6.3.1. LAB MEDIA: Figure 1D. *Video editor: Highlight the main peak around 120 nanometer*

6.4. Fluorescence microscopy demonstrated that CME-AAV9.EGFP produced a markedly higher level of enhanced green fluorescent protein expression in HEK293T (*H-E-K-Two-Ninety-Three-T*) cells [1]. The membrane-enveloped virus achieved a markedly higher level of gene expression [2].

6.4.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the green fluorescence images of the rows labelled "CME-AAV9.EGFP" and "AAV9.EGFP" and emphasize the stronger green signal in the CME-AAV9.EGFP row.*

6.4.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the bar labelled "CME-AAV9.EGFP"*

6.5. In vivo bioluminescence imaging showed that the liver exhibited the strongest luminescence signal following CME-AAV administration [1]. Quantitative analysis of liver regions revealed that CME-AAV administration resulted in significantly higher gene expression levels [2].

6.5.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the bright signal in the liver region of the mouse images in the CME-AAV9.Luc group.*

6.5.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar labelled "CME-AAV9.Luc"*

1. Mesenchymal
Pronunciation link: <https://www.merriam-webster.com/dictionary/mesenchymal>
IPA: /ˌmez.ənˈkaɪ.məl/
Phonetic Spelling: mez·uhn·kai·muhl
2. Confluency
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluency>
IPA: /kənˈfluː.ən.si/
Phonetic Spelling: kuhn·floo·uhn·see
3. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsen.trəˌfjuː.dʒ/
Phonetic Spelling: sen·truh·fyooj
4. Protease
Pronunciation link: <https://www.merriam-webster.com/dictionary/protease>
IPA: /ˈproʊ.tiˌeɪs/
Phonetic Spelling: proh·tee·ays
5. Lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /ˈlaɪ.seɪt/
Phonetic Spelling: lie·sayt
6. Homogenize
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenize>
IPA: /həˈmɑː.dʒəˌnaɪz/
Phonetic Spelling: huh·mah·juh·nyze
7. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuː.pərˈneɪ.tənt/
Phonetic Spelling: soo·per·nay·tuhnt
8. Bicinchoninic
Pronunciation link: <https://www.merriam-webster.com/dictionary/bicinchoninic>
IPA: /ˌbaɪ.sɪn.kəˈnɪn.ɪk/
Phonetic Spelling: bye·sin·kuh·nin·ik
9. Adeno-Associated
Pronunciation link: <https://www.merriam-webster.com/dictionary/adeno>
IPA: /əˈdiː.nou/ ; /əˈsoʊ.si.eɪ.tɪd/
Phonetic Spelling: uh·dee·noh ; uh·soh·see·ay·tuhd
10. Encapsulation
Pronunciation link: <https://www.merriam-webster.com/dictionary/encapsulation>
IPA: /ɪnˌkæp.sjəˈleɪ.ʃən/
Phonetic Spelling: in·kap·syuh·lay·shuhn
11. Extrusion
Pronunciation link: <https://www.merriam-webster.com/dictionary/extrusion>
IPA: /ɪkˈstruː.ʒən/
Phonetic Spelling: ik·stroo·zhuhn

- 12. Polycarbonate**
Pronunciation link: <https://www.merriam-webster.com/dictionary/polycarbonate>
IPA: /ˌpɑːliˈkɑːr.bə.nert/
Phonetic Spelling: pah·lee·kar·buh·nayt
- 13. Iodixanol**
Pronunciation link: <https://www.howtopronounce.com/iodixanol>
IPA: /ˌaɪ.ooˈdɪk.sə.nɔːl/
Phonetic Spelling: eye·oh·dik·suh·nawl
- 14. Ultracentrifugation**
Pronunciation link: <https://www.howtopronounce.com/ultracentrifugation>
IPA: /ˌʌl.trəˌsen.trəˌfjuːˈgeɪ.jən/
Phonetic Spelling: ul·truh·sen·truh·fyoo·gay·shuhn
- 15. Bioluminescence**
Pronunciation link: <https://www.merriam-webster.com/dictionary/bioluminescence>
IPA: /ˌbaɪ.ooˌluː.məˈnes.əns/
Phonetic Spelling: bye·oh·loo·muh·ness·uhns
- 16. Fluorescence**
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flɔːˈres.əns/
Phonetic Spelling: flaw·ress·uhns
- 17. Extracellular**
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>
IPA: /ˌek.strəˈsel.jə.lə/
Phonetic Spelling: ek·struh·sel·yuh·ler
- 18. Transduction**
Pronunciation link: <https://www.merriam-webster.com/dictionary/transduction>
IPA: /trænzˈdʌk.jən/
Phonetic Spelling: tranz·duk·shuhn
- 19. Hoechst**
Pronunciation link: <https://www.howtopronounce.com/hoechst>
IPA: /hɜːkst/
Phonetic Spelling: hurkst
- 20. NanoSight**
Pronunciation link: <https://www.howtopronounce.com/nanosight>
IPA: /ˈnæn.oo.sait/
Phonetic Spelling: nan·oh·site
- 21. HEK293T**
Pronunciation link: No confirmed link found
IPA: /eɪtʃ iː keɪ tuː ˈnɑːn.ti θriː tiː/
Phonetic Spelling: aitch·ee·kay·two·ninety·three·tee
- 22. Bioluminescence**
Pronunciation link: <https://www.merriam-webster.com/dictionary/bioluminescence>
IPA: /ˌbaɪ.ooˌluː.məˈnes.əns/
Phonetic Spelling: bye·oh·loo·muh·ness·uhns

23. Luminescence

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

IPA: /ˌluː.məˈnes.əns/

Phonetic Spelling: loo·muh·ness·uhns

24. Capsid

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

IPA: /ˈkæp.sɪd/

Phonetic Spelling: kap·sid

25. Vesicles

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

IPA: /ˈves.ɪ.kəlz/

Phonetic Spelling: vess·ih·kuhlz