

Submission ID #: 70517

Scriptwriter Name: Pallavi Sharma

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Title: Evaluation of 3D Spheroids for AAV Transduction Studies

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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'**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**
3 minutes away from each other

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: 26

Number of Shots: 45

Interviews

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. **Ronit Mazor**: We developed a sensitive, physiologically relevant 3D spheroid platform to evaluate AAV transduction activity and improve the prediction of vector performance for gene therapy.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Ronit Mazor**: This protocol is applicable for evaluating AAV activity in vector potency testing, serotype screening, transgene optimization, neutralization studies, and preclinical gene therapy.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

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

- 1.3. **Susana Nagera**: The key challenge when performing this protocol is maintaining high cell viability, producing uniform compact spheroids, and optimizing AAV dose for each cell type and serotype.
 - 1.3.1. 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?~~

- 1.4. **Susana Nagera**: This protocol can assess transgene expression and functionality using RT-qPCR, western blotting, ELISA, enzymatic activity assays, and onset-time kinetics analyses.

- 1.4.1. 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.~~

- 1.5. **Susana Nagera**: Future studies can incorporate co-cultures, organoids, microfluidics, immune cells, or vasculature to better recapitulate in vivo AAV transduction environments.
- 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.

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 is the key takeaway you hope viewers remember after watching your video article?

- 1.6. **Susana S Najera, FDA/ORISE Fellow**: (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Susana S Najera, FDA/ORISE Fellow**: (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

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

2. Seeding and Size Optimization of Spheroid

Demonstrator: Susana S Najera

- 2.1. To start, grow the cells in a T75 (*T-Seventy-Five*) cell culture flask until they reach 70% confluency under standard conditions [1-TXT]. After trypsinizing the cells, prepare a 1 to 10 dilution for routine cell passaging [2]. Incubate the cells again until they reach 70% confluency, then harvest them. [3].
 - 2.1.1. Talent placing the cells in the incubator. **TXT: Cells used: HEK293T, Huh7, HEP3B, A375, and HEPG2** ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent adding cells to cells to the culture flask
 - 2.1.3. Talent placing the cells in the incubator.
- 2.2. After assessing cell viability, thoroughly pipette the cell suspension to ensure it is well mixed [1].
 - 2.2.1. Talent pipetting the cell suspension up and down.
- 2.3. Seed 1×10^4 cells in 200 microliters in an ultra-low attachment 96-well plate, using varying cell densities for different cell lines [1].
 - 2.3.1. Talent seeding the cells into the ultra-low attachment 96-well round-bottom plate.
- 2.4. Add 1 volume of complete medium to all rows, except the top row [1]. Then, add 2 volumes of 1×10^4 cells per well in the top row [1]. Serially dilute row 1A by adding 1 volume of row 1A into row 1B and continue in the same manner [1].
 - 2.4.1. Talent adding complete medium to rows 1B through 1H.
 - 2.4.2. Talent adding cells to row 1A.
 - 2.4.3. Talent transferring 1 volume from row 1A into row 1B to continue the serial dilution.
- 2.5. Centrifuge the ultra-low attachment plate at 350 g for 5 minutes to pull the cells to the center and promote cell-to-cell contact [1]. Incubate at 37 degrees Celsius undisturbed for 24 to 48 hours for spheroid formation. Allow 3 days for the diameter to reach about 400 micrometers. [2].

2.5.1. Talent placing the ultra-low attachment plate in the centrifuge.

2.5.2. Talent places the plates at 37 °C.

2.6. Check spheroid size uniformity, ensuring cells are resuspended evenly and plates are flat [1].

2.6.1. SCREEN: 3-plate.mp4: 00:01-00:05.

2.7. Monitor spheroid morphology. Select mature spheroids that exhibit tight cell-to-cell adhesion rather than loose cell aggregates [1].

2.7.1. SCREEN: 3-plate.mp4: 00:08-00:13

3. Live-Cell Real-Time Spheroid Monitoring

3.1. Once the cell suspensions are spun down, transfer the plate to a 37 degrees Celsius incubator equipped with a live-cell imaging instrument [1].

3.1.1. WIDE: Talent transferring the plate into the incubator containing the live-cell imaging instrument.

3.2. Log into the acquisition software [1]. Select **Schedule To Acquire** and **Scan On Schedule**. Then, select Create Vessel to New [3].

3.2.1. SCREEN: 4.mp4: 00:04-00:08

3.2.2. SCREEN: 4.mp4: 00:11-00:20

3.3. Then, choose **Scan Type** and select **Spheroid** [1]. Pick the channels of interest, select Phase + Brightfield (*Phase-with-Brightfield*), then choose **Green** or **Red**. Set the desired magnification to 10× (*Ten-X*) [2].

3.3.1. SCREEN: 4.mp4: 00:21-00:23

3.3.2. SCREEN: 4.mp4: 00:24-00:28

3.4. Pick the plate model and its position in the drawer [1]. Select the wells to image, specify the number of images to acquire per well [2], and enter the experiment name [3].

3.4.1. SCREEN: 4.mp4: 00:29-00:32

3.4.2. SCREEN: 4.mp4: 00:33-00:36

3.4.3. SCREEN: 4.mp4: 00:38-00:47

3.5. For the analysis setup, select **Defer Analysis Until Later** [1].

3.5.1. SCREEN: 4.mp4: 00:47-00:48

~~3.6. Right-click on the timeline and click Set Selected Scan Group Interval [1].~~

~~3.6.1. SCREEN: A right-click is being performed on the timeline, and set Selected Scan Group Interval is being selected.~~ **NOTE: Not filmed**

3.7. Set scans to be added every 6 hours and scan indefinitely. Check daily for spheroid

growth by logging into the imaging software [1]. ~~Set the desired starting time at least 1 hour after the start of incubation in the automated imaging apparatus [2].~~

3.7.1. SCREEN: 4.mp4: 01:07-01:10

3.7.2. ~~SCREEN: A start time at least 1 hour after incubation is being set in the automated imaging apparatus.~~ NOTE: Not filmed

3.8. ~~Check daily for spheroid growth by logging into the imaging software [1].~~ NOTE: Merged with the previous shot

3.8.1. SCREEN: The experiment dashboard displaying spheroid growth is being shown.

3.9. Select View Recent Scans and double-click on the desired experiment [1]. After selecting **Brightfield** in the image channels panel, use the **Measure Image** feature to measure the diameters of the spheroids [2].

3.9.1. SCREEN: 4.mp4: 00:00-00:06

3.9.2. SCREEN: 4.mp4: 00:06-00:21

3.10. Remove the plate on the day of optimized spheroid formation and use it for Adeno-associated virus or AAV (A-A-V) transduction [1].

3.10.1. Talent removing the plate from the imaging apparatus.

4. AAV Transduction and Live-Cell Imaging in 3D Spheroids

4.1. Prepare a 96-well round plate containing 2% FBS supplemented DMEM without antibiotics [1]. Mix 33 microliters of AAV stock with 297 microliters of medium to obtain a starting multiplicity of infection or MOI (M-O-I) of 1×10^6 [2].

4.1.1. Talent adding a 96-well round plate containing 2% fetal bovine serum supplemented DMEM without antibiotics.

4.1.2. Talent mixing 33 microliters of AAV stock with 297 microliters of medium.

4.2. Perform 10-fold serial dilutions, starting with a MOI of 1×10^6 , by adding 33 microliters to 297 microliters of 2% FBS-supplemented DMEM [1].

4.2.1. Talent transferring 33 microliters of the previous dilution into 297 microliters of fresh medium.

4.3. Aspirate all medium from the previously prepared spheroid plate using a multichannel pipette [1].

4.3.1. WIDE: Talent aspirating medium from the spheroid plate with a multichannel pipette.

4.4. Transfer 100 microliters of the desired MOI of AAV from the dilution plate to the treatment plate using a multichannel pipette. [1].

4.4.1. Talent transferring 100 microliters from the dilution plate to the treatment plate

with a multichannel pipette.

- 4.5. Place the plates back into the imaging instrument [1]. Start monitoring transduction by tracking green fluorescent intensity over 72 hours [2].
 - 4.5.1. Talent placing the plates into the imaging instrument.
 - 4.5.2. SCREEN: : 5.5-and-5.7.mp4 00:02-00:12
- 4.6. Add 100 microliters of complete DMEM per well every 3 days to replenish nutrients [1].
 - 4.6.1. Talent adding complete DMEM to each well using a multichannel pipette.
- 4.7. Place the plate back in the imaging instrument [1] and start recording transduction efficiency every 6 hours
 - 4.7.1. Talent placing the treatment plate into the imaging instrument.
 - 4.7.2. SCREEN: 5.5-and-5.7.mp4: 00:20-00:25
- 4.8. Analyze the data by creating an analysis definition specific to the cells and reporter genes of interest [1]. Perform endpoint analysis using fluorescence readouts [1]
 - 4.8.1. SCREEN: 5.8.1.mp4: 00:12-00:28, 00:47-00:52
 - 4.8.2. Talent in front of the computer with selected wells showing fluorescence.
- 4.9. Similarly, perform AAV transduction in 3D spheroids with luminescent reporters [1], followed by transduction analysis using bioluminescent imaging [2].
 - 4.9.1. Talent adding cells to the 96-well plate.
 - 4.9.2. Talent placing the plate in the live cell imaging system.

Results

5. Results

5.1. Each cell line exhibited distinct morphological features and varying levels of compaction after 3 days of seeding [1].

5.1.1. LAB MEDIA: Figure 2.

5.2. Hepatic cell lines formed more compact, denser spheroids than other cell lines [1]. HEK293T (*H-E-K-Two-Nine-Three-T*) formed a very round spherical appearance with a distinct necrotic core [2]. A375 (*A-Three-seventy-Five*) formed an irregularly shaped cell aggregate [3].

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the spheroids labeled Huh7, HEPG2, and HEP3B.*

5.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the spheroid labeled HEK293T*

5.2.3. LAB MEDIA: Figure 2. *Video editor: Highlight spheroid labeled A375*

5.3. Spheroids were highly susceptible to AAV transduction, with strong transduction detected at very low AAV titers across different cell lines for self-complementary GFP [1].

5.3.1. LAB MEDIA: Figure 3. *Video editor: Show the blue curves in panels A–D and highlight the point on the x-axis at which the curve shows an upward trend*

5.4. Spheroid transduction of single-stranded GFP was observed at lower levels than self-complementary GFP [1].

5.4.1. LAB MEDIA: Figure 3. *Video editor: Compare the red and blue curves in panels A–D and emphasize that the red curves remain lower.*

5.5. A 100-fold difference in relative fluorescence was observed between the self-complementary and single-stranded transgenes at a MOI of 10^6 in HEK293T cells [1].

5.5.1. LAB MEDIA: Figure 3A. *Video editor: Highlight HEK293T panel.*

5.6. A 3-fold difference in relative fluorescence was observed between the self-complementary and single-stranded transgenes in HEP3B (*HEP-Three-B*) cells [1].

5.6.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the HEP3B*

5.7. All 4 AAV serotypes were successfully detected using both the fluorescent plate reader and the live-cell imaging system [1]. AAV2 showed the highest transduction efficacy, followed by AAV1, in both cell lines [2].

- 5.7.1. LAB MEDIA: Figure 4.
- 5.7.2. LAB MEDIA: Figure 4A–D. *Video editor: Highlight the red and blue curve .*
- 5.8. All AAV serotypes showed efficient transduction using the luminescence system, enabling precise differentiation of transduction efficacy [1].
- 5.8.1. LAB MEDIA: Figure 5.
- 5.9. AAV2 was the most effective serotype in HeLa spheroids, while AAV9 had the lowest transduction efficacy [1].
- 5.9.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the red (AAV2) and yellow (AAV9) curve .*
- 5.10. AAV2 self-complementary GFP transduction was detectable at infection rates as low as 10^1 , with a noticeable increase in signal after about 34 hours compared to untreated cells [1].
- 5.10.1. LAB MEDIA: Figure 6C. *Video editor: Highlight the blue curve*
- 5.11. The AAV2 single-stranded GFP signal was weaker and slower than the self-complementary GFP signal [1].
- 5.11.1. LAB MEDIA: Figure 6D–G. *Video editor: Highlight the red curves.*

1. Confluency
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluency>
IPA: /kənˈfluː.ən.si/
Phonetic Spelling: kuhn·floo·uhn·see
2. Trypsinizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsinize>
IPA: /ˈtrip.səˌnaɪz/
Phonetic Spelling: trip·suh·nyze
3. Passaging
Pronunciation link: <https://www.merriam-webster.com/dictionary/passage>
IPA: /ˈpæs.ɪ.dʒɪŋ/
Phonetic Spelling: pass·ih·jɪŋ
4. Viability
Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>
IPA: /ˌvaɪ.əˈbɪl.ə.ti/
Phonetic Spelling: vy·uh·bil·uh·tee
5. Ultra-low attachment
Pronunciation link: No confirmed link found

- IPA: /'ʌl.trə loʊ ə'tætʃ.mənt/
Phonetic Spelling: uhl·truh loh uh·tach·muhnt
6. Serial dilution
Pronunciation link: <https://www.merriam-webster.com/dictionary/serial>
IPA: /'sɪr.i.əl daɪ'luː.ʃən/
Phonetic Spelling: seer·ee·uhl dye·loo·shuhn
7. Spheroid
Pronunciation link: <https://www.merriam-webster.com/dictionary/spheroid>
IPA: /'sfɪr.ɔɪd/
Phonetic Spelling: sfeer·oyd
8. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔːr'fɑː.lə.dʒi/
Phonetic Spelling: mor·fol·uh·jee
9. Automated
Pronunciation link: <https://www.merriam-webster.com/dictionary/automated>
IPA: /'ɔː.tə.meɪ.tɪd/
Phonetic Spelling: aw·tuh·may·tid
10. Brightfield
Pronunciation link: No confirmed link found
IPA: /'brɑːt fiːld/
Phonetic Spelling: bryte·feeld
11. Magnification
Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
IPA: /,mæɡ.nə.fə'keɪ.ʃən/
Phonetic Spelling: mag·nuh·fuh·kay·shuhn
12. Adeno-associated virus
Pronunciation link: <https://www.merriam-webster.com/dictionary/adeno-associated>
IPA: /ə'diː.noʊ ə'soʊ.si.eɪ.tɪd 'vaɪ.rəs/
Phonetic Spelling: uh·dee·noh uh·soh·see·ay·tid vy·ruhs
13. Transduction
Pronunciation link: <https://www.merriam-webster.com/dictionary/transduction>
IPA: /trænz'dʌk.ʃən/
Phonetic Spelling: tranz·duk·shuhn
14. Multiplicity of infection
Pronunciation link: No confirmed link found
IPA: /,mʌl.tə'plɪs.ə.ti əv ɪn'fek.ʃən/
Phonetic Spelling: mul·tuh·pliss·uh·tee uhv ɪn·fek·shuhn
15. Fetal bovine serum
Pronunciation link: No confirmed link found
IPA: /'fiː.təl 'boʊ.vɑɪn 'sɪr.əm/
Phonetic Spelling: fee·tuhl boh·vyne seer·uhm
16. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>

- IPA: /flɒːˈres.ənt/
Phonetic Spelling: flor·ess·uhnt
17. Bioluminescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/bioluminescence>
IPA: /ˌbaɪ.lʊː.luː.məˈnes.ənt/
Phonetic Spelling: bye·oh·loo·muh·ness·uhnt
18. Compaction
Pronunciation link: <https://www.merriam-webster.com/dictionary/compaction>
IPA: /kəmˈpæk.ʃən/
Phonetic Spelling: kuhm·pak·shuhn
19. Hepatic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hepatic>
IPA: /həˈpæt.ɪk/
Phonetic Spelling: huh·pat·ik
20. Necrotic
Pronunciation link: <https://www.merriam-webster.com/dictionary/necrotic>
IPA: /nəˈkraː.tɪk/
Phonetic Spelling: nuh·krot·ik
21. Aggregate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aggregate>
IPA: /ˈæg.rə.gət/ (noun), /ˈæg.rə.geɪt/ (verb)
Phonetic Spelling: ag·ruh·guht; ag·ruh·gayt
22. Susceptible
Pronunciation link: <https://www.merriam-webster.com/dictionary/susceptible>
IPA: /səˈsep.tə.bəl/
Phonetic Spelling: suh·sep·tuh·buhl
23. Serotype
Pronunciation link: <https://www.merriam-webster.com/dictionary/serotype>
IPA: /ˈsɪr.oʊ.taɪp/
Phonetic Spelling: seer·oh·type
24. Efficacy
Pronunciation link: <https://www.merriam-webster.com/dictionary/efficacy>
IPA: /ˈef.ə.kə.si/
Phonetic Spelling: ef·uh·kuh·see
25. HeLa
Pronunciation link: <https://www.merriam-webster.com/dictionary/HeLa>
IPA: /ˈhiː.lɑː/
Phonetic Spelling: hee·lah
26. Luminescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/luminescence>
IPA: /ˌluː.məˈnes.əns/
Phonetic Spelling: loo·muh·ness·uhns
27. HEK293T
Pronunciation link: No confirmed link found

- IPA: No verified IPA available
Phonetic Spelling: H-E-K two·nine·three·T
28. Huh7
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: huh·seven
29. HEP3B
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: hep·three·B
30. HEPG2
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: H-E-P-G two
31. A375
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: A three·seventy·five