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Title: Intranasal Delivery of mRNA Polyplexes via Rayleigh Breakup Aerosols: An In Vitro Method for Nasal Deposition and Functional Testing

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: 21

Number of Shots: 35

Introduction

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 statements with sufficient context.

The answers are slightly edited according to journal guidelines

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Mazen Kafienah:** We worked on and validated a non-destructive and gentle method to deliver nasal mRNA vaccines via Rayleigh breakup aerosolisation.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What research gap are you addressing with your protocol?~~

- 1.2. **Ziheng Zheng:** Current nebulizers damage fragile mRNA vectors. Our protocol demonstrates a low-shear aerosolization approach that preserves mRNA integrity.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What significant findings have you established in your field?~~

- 1.3. **Mazen Kafienah:** We showed that Rayleigh breakup preserves mRNA nanoparticle integrity while enabling targeted turbinate deposition and functional gene expression.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How will your findings advance research in your field?~~

- 1.4. **Ziheng Zheng:** Our findings advance the field by establishing a fully validated, non-destructive intranasal mRNA aerosolization framework, enabling safer and more effective respiratory delivery research.

1.4.1. 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.

Videographer's NOTE: The first room the Authors gave me to do the headshots was quite dark with just white walls. It had no natural light coming in to support the light I had with me.

Protocol

2. Setting Up the Atomizer and Alberta Idealized Nasal Inlet (AINI)

Demonstrator: Mazen Kafienah

- 2.1. To begin the device setup [1], assemble the Rayleigh breakup nasal atomizer device [2] with the Alberta Idealized Nasal Inlet or AINI (A-I-N-I) model [3].

2.1.1. **WIDE:** Talent assembling the Rayleigh breakup nasal atomizer device

Added shot 2.0.0 Show the orientation of the pieces. **Videographer's NOTE:** Shot 2.0.0 is an extra shot showing the pieces on the table

Added shot 2.1.2: Assembling the pieces in close-up **Videographer's NOTE:** Shot 2.1.2 is an extra shot. The wide shot of 2.1.1 doesn't show as much as the Authors wanted to show. They asked me to create this extra shot of the pieces being assembled

- 2.2. After placing the required components in the next generation impactor [1], attach the calibrated flow meter to the Next Generation Impactor [2]

Added shot 2.1.3: Talent placing the plates in the next generation impactor. **Videographer's NOTE:** Shot 2.1.3 is an extra shot of the plates being put in Next Generation Impactor.

2.2.1. Talent attaching the calibrated flow meter to the Next Generation Impactor.

- 2.3. Next, use Parafilm to seal the device properly to the Next Generation Impactor [1].

2.3.1. Talent wrapping Parafilm around the interface to seal the device to the AINI.

- 2.4. Connect the outlet of the impactor to a vacuum pump to draw airflow through the nasal passages [2].

2.4.1. Talent connecting the outlet of the Next Generation Impactor to the vacuum pump tubing.

- 2.5. ~~Set the airflow rate to 7.5 liters per minute using a calibrated flow meter attached to the induction port of the Next Generation Impactor [1].~~ Adjust the pump controls until the flow meter reads 7.5 liters per minute [2].

2.5.1. ~~Talent attaching the calibrated flow meter to the induction port of the Next~~

~~Generation Impactor.~~ **Videographer's NOTE:** Shot 2.5.1 was not filmed. The Authors told me it is no longer needed

2.5.2. Talent adjusting the vacuum pump settings. **Videographer's NOTE:** Shot 2.5.2 Take 3 is the best shot to use because the air pump is connected and my reflection in the glass is not seen in the shot

2.6. Check if the airflow of the air pump is delivering 7.5 liters per minute through the AINI (A-I-N-I) and turn off the air pump [1].

2.6.1. Close-up of the air pump display showing 7.5 liters per minute. **Videographer's NOTE:** Shot 2.6.1 needs a caption saying to turn off air pump from mains (press switch).

2.7. Remove the calibrated flow meter and attach the AINI component in its place

2.7.1. Talent attaching the AINI to the induction port of the Next Generation Impactor.

2.8. Ensure that all connections, model sections, and tubing to the pump are airtight [2].

2.8.1. Talent checking and tightening all connections to confirm an airtight system.

2.9. Then, gently mix the prepared polyethyleneimine mRNA suspension to ensure homogeneity while avoiding bubble formation [1].

2.9.1. Talent gently mixing the polyethyleneimine messenger ribonucleic acid suspension in its container.

2.10. Draw the prepared sample into the atomizer reservoir [1] and load it into the atomizer device [2].

2.10.1. Talent aspirating the sample into the atomizer reservoir. **Videographer's NOTE:** Shots 2.10.1 & 2.10.2 are merged into one shot. Take 2 of this shot is the one to use

2.10.2. Talent inserting the filled reservoir into the atomizer device to complete sample loading.

3. Aerosol Generation and Sample Collection from AINI

Demonstrator: Ziheng Zheng

- 3.1. After turning on the air pump and attaching the syringe pump to the atomizer [1], insert the Rayleigh breakup atomizer nozzle into the entrance of the AINI nostril at an angle of 45 degrees [2]. Activate the Rayleigh breakup atomizer to begin aerosolization [3].

Added shot: 3.0.0 is an extra shot. Showing the attachment of the syringe to the spring aid

- 3.1.1. Talent positioning and inserting the atomizer nozzle into the vestibule section at a 45-degree angle. **Videographer's NOTE: Shots 3.1.1, 3.1.2 & 3.2.1 were merged together.**
 - 3.1.2. Talent pressing the control or trigger to activate the Rayleigh breakup atomizer.
- 3.2. Turn off the air pump promptly after spraying to prevent deposited droplets from being drawn further downstream or drying unevenly [1].
 - 3.2.1. Talent switching off the air pump immediately after completion of the spray.
- 3.3. Then, carefully disassemble the AINI model into its individual anatomical segments to collect deposited nanoparticles from each region [1].
 - 3.3.1. Talent separating the AINI model into individual sections on the bench.
- 3.4. Next, identify and prepare to collect deposits from six compartments, including the atomizer device [1], the nasal vestibule section, the turbinate section [2], the olfactory region, the nasopharynx section [3], and any downstream capture such as attached Next Generation Impactor stages or a final filter [4].
 - 3.4.1. Talent pointing to or arranging the atomizer device as a collection compartment.
 - 3.4.2. Talent showing or pointing to the nasal vestibule section and turbinate section.
 - 3.4.3. Talent pointing to the olfactory region section and the nasopharynx section.
 - 3.4.4. Talent showing the downstream capture components.
- 3.5. For each compartment, use a measured volume of cell culture medium to wash out the deposited material and recover the samples into a separate container [1]. Repeat the sample collection step for all parts of the device [2].
 - 3.5.1. Talent adding an individual compartment with a measured volume of cell culture

medium and collecting sample into a separate container. **Videographer's NOTE:** Shots 3.5.1 & 3.5.2 were merged together. There are 5 components that needed to be filmed for this so I split it up into letters. A, B, C, D, E to represent each component being filmed. **Show clip A where they clean and add the medium/sample from one part here for 3.5.1**

3.5.2. Talent repeating the adding and collection steps from other parts of the device. **Videographer's NOTE:** Show clips B, C, D, E using split screens to show sample retrieval from all parts

3.6. Then, retrieve the liquid from the Next Generation Impactor stages, if used, by rinsing the stages with DMEM to trap fine particles [1-TXT].

3.6.1. Talent rinsing the Next Generation Impactor stages by adding Dulbecco's Modified Eagle Medium. **Videographer's NOTE:** Shot 3.6.1 was split into two shots - A & B. **TXT: Clean the Rayleigh breakup atomizer and the AINI model after each run**

3.6.2. ~~Talent placing a cap on the collection tube and sealing it.~~

~~3.7. Finally, place the collected samples in an incubator set to 37 degrees Celsius for a short period of time until use [1].~~

3.7.1. ~~Talent placing the capped sample tubes into an incubator set to 37 degrees~~
Videographer's NOTE: Shot 3.7.1 was not filmed. The Authors told me it is not needed

Results

4. Results

- 4.1. Z-average diameters of polyethyleneimine mRNA polyplexes remained within the expected range of 136 to 160 nanometers before aerosolization [1], and 145 to 162 nanometers after aerosolization, indicating that Rayleigh breakup did not cause significant size changes [2].
 - 4.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight the 3 bars labeled "Pre-Aerosolized"*.
 - 4.1.2. LAB MEDIA: Figure 1. *Video editor: Highlight the 3 bars labeled "Aerosolized"*.
- 4.2. The zeta potential and Peak 1 mean diameters by intensity remained largely unchanged before and after aerosolization [1].
 - 4.2.1. LAB MEDIA: Figure 1. *Video editor: Show only the graph on the top left with "Zeta Potential (mV)" written on Y axis and the bottom right graph with "Peak 1 Mean by Intensity (nm)" written on Y axis.*
- 4.3. Numerous blue-stained nuclei were observed in cells from the turbinate region, indicating successful DAPI staining of A549 cells [1].
 - 4.3.1. LAB MEDIA: Figure 6. *Video editor: Highlight the dense blue-stained nuclei distributed across the entire image.*
- 4.4. Green-fluorescing cells were observed in A549 wells after exposure to aerosolized messenger RNA, indicating successful transfection [1].
 - 4.4.1. LAB MEDIA: Figure 3. *Video editor: Highlight the scattered green dots across the image, representing transfected cells.*
- 4.5. Merged DAPI and green fluorescent protein signal in A549 cells confirmed transfection feasibility and validated cellular uptake of the mRNAs [1].
 - 4.5.1. LAB MEDIA: Figure 4.
- 4.6. The green fluorescent protein signal was observed in HEK293T cells exposed to the aerosolized mRNA, confirming that the method produced detectable reporter expression in a second epithelial cell line that served as a positive control [1].
 - 4.6.1. LAB MEDIA: Figure 5. *Video editor: Highlight the bright green-fluorescing signals.*

5. Rayleigh
Pronunciation link: <https://www.merriam-webster.com/dictionary/Rayleigh>
IPA: /'reɪli/
Phonetic Spelling: ray·lee
6. Atomizer
Pronunciation link: <https://www.merriam-webster.com/dictionary/atomizer>
IPA: /'ætə,maɪzər/
Phonetic Spelling: a·tuh·mai·zer
7. Nasal
Pronunciation link: <https://www.merriam-webster.com/dictionary/nasal>
IPA: /'neɪzəl/
Phonetic Spelling: nay·zuhl
8. Alberta
Pronunciation link: <https://www.merriam-webster.com/dictionary/Alberta>
IPA: /æl'bɜ:tə/
Phonetic Spelling: al·bur·tuh
9. Idealized
Pronunciation link: <https://www.merriam-webster.com/dictionary/idealized>
IPA: /aɪ'di:əlaɪzd/
Phonetic Spelling: eye·dee·uh·lized
10. Impactor
Pronunciation link: <https://www.merriam-webster.com/dictionary/impactor>
IPA: /ɪm'pæktər/
Phonetic Spelling: im·pak·ter
11. Calibrated
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibrated>
IPA: /'kælɪ,breɪtɪd/
Phonetic Spelling: ka·luh·bray·tid
12. Parafilm
Pronunciation link: <https://www.merriam-webster.com/dictionary/Parafilm>
IPA: /'pærə,fɪlm/
Phonetic Spelling: pair·uh·film
13. Polyethyleneimine
Pronunciation link: <https://www.howtopronounce.com/polyethyleneimine>
IPA: /,pɑ:li,εθɪl'i:n,aɪ,mɪ:n/
Phonetic Spelling: pol·ee·eth·uh·leen·eye·meen
14. Messenger
Pronunciation link: <https://www.merriam-webster.com/dictionary/messenger>
IPA: /'mɛsɪndʒər/
Phonetic Spelling: meh·sin·jer
15. Ribonucleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleic>
IPA: /,raɪboonu'kli:ɪk/
Phonetic Spelling: rye·boh·noo·klee·ik

16. Aerosolization
 Pronunciation link: <https://www.merriam-webster.com/dictionary/aerosolization>
 IPA: /,erə,sa:lə'zeɪʃən/
 Phonetic Spelling: air·uh·saw·luh·zay·shuhn
17. Vestibule
 Pronunciation link: <https://www.merriam-webster.com/dictionary/vestibule>
 IPA: /'vestə,bju:l/
 Phonetic Spelling: ves·tuh·byool
18. Turbinate
 Pronunciation link: <https://www.merriam-webster.com/dictionary/turbinate>
 IPA: /'tɜ:bənət/
 Phonetic Spelling: tur·buh·nit
19. Olfactory
 Pronunciation link: <https://www.merriam-webster.com/dictionary/olfactory>
 IPA: /ɑ:l'fæktəri/
 Phonetic Spelling: all·fak·tuh·ree
20. Nasopharynx
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nasopharynx>
 IPA: /,neɪzou'færɪŋks/
 Phonetic Spelling: nay·zoh·fair·inks
21. Nanoparticles
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanoparticle>
 IPA: /,nænou'pɑ:rtɪkəl/
 Phonetic Spelling: nan·oh·par·ti·kuhl
22. Dulbecco's
 Pronunciation link: <https://www.merriam-webster.com/dictionary/Dulbecco>
 IPA: /du:l'bəkoo/
 Phonetic Spelling: dool·bek·oh
23. Zeta
 Pronunciation link: <https://www.merriam-webster.com/dictionary/zeta>
 IPA: /'zertə/
 Phonetic Spelling: zay·tuh
24. Polyplexes
 Pronunciation link: <https://www.howtopronounce.com/polyplexes>
 IPA: /'pɑ:li,pleksɪz/
 Phonetic Spelling: pol·ee·pleks·iz
25. DAPI
 Pronunciation link: <https://www.howtopronounce.com/dapi>
 IPA: /'deɪpi/
 Phonetic Spelling: day·pee
26. Transfection
 Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
 IPA: /træn'sfɛkʃən/
 Phonetic Spelling: tran·sfek·shuhn

27. Fluorescent

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

IPA: /flʊ'resənt/

Phonetic Spelling: floor·eh·suhnt

28. Epithelial

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

IPA: /,ɛpɪ'θi:liəl/

Phonetic Spelling: eh·puh·thee·lee·uhl

29. Incubator

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

IPA: /'ɪŋkjə,betər/

Phonetic Spelling: ing·kyuh·bay·ter