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Title: Isolation and Purification of Plant Extracellular Vesicles from Arabidopsis Leaves Using an Optimized Apoplastic Wash Collection Method

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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? **Yes**
If **Yes**, how far apart are the locations?
From the first floor to the third floor.
- 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: 55

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 full sentence with sufficient context**. Also, the statements have been edited for length and clarity.

INTRODUCTION:

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

- 1.1. **Baoye He:** Our study focuses on plant extracellular vesicles and how they transport molecules that mediate communication between plants and microbial pathogens.

- 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. **Baoye He:** Plant extracellular vesicle isolation methods vary across laboratories, causing inconsistent results; this protocol provides a standardized, reliable, and reproducible workflow.

- 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 **Baoye He:** This protocol enables researchers to isolate plant extracellular vesicles and analyze their RNA, protein, and other molecular cargo.

- 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 **Baoye He:** A key challenge in extracellular vesicle isolation is preserving sample purity while avoiding contamination from intracellular components.

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 **Baoye He:** This study enables future studies to investigate how extracellular vesicles shape plant immunity and plant-microbe communication.

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.

Protocol

NOTE: The step-and-shot numbering has been changed in the author-provided file. Based on the videographer's notes, it appears that the same updated numbering has been followed. Please confirm.

1. Leaf Preparation and Vacuum Infiltration

Demonstrator: Baoye He

- 1.1. To begin, cut the *Arabidopsis thaliana* plant shoots from the soil [1]. Place the freshly cut plant shoots on a clean surface [2], cut at the base of the leaf blade to remove the petiole and collect the distal blade zones [3].

- 1.1.1. WIDE: The talent cutting the *Arabidopsis thaliana* plant shoots from the soil.

Videographer's NOTE: 1.1.1 and 1.1.2 are filmed in single shots

- 1.1.2. Show the experimental set up with the freshly cut plant shoots on a clean surface.

- 1.1.3. Talent cutting the leaf blade at the base, removing the petiole and collecting the distal blade sections.

- 1.2. Wash the leaves three times with RO (R-O) water [1], place the leaves on paper towels, and gently pat dry [2]. **NOTE: The VO is adjusted for the additional shot**

Added shot: 1.2.0: Washing leaves

- 1.2.1. Talent placing the washed leaves on paper towels and patting them dry.

- 1.3. Then, load the leaves into a 120-milliliter syringe and tap gently to settle them till the 50-milliliter mark [1].

- 1.3.1. Talent transferring leaves into a syringe and gently tapping it..

Videographer's NOTE: 1.3.1 and 1.4.1 are filmed in the same shot

- 1.4. Add approximately 60 milliliters of vesicle isolation buffer or VIB (V-I-B) into the syringe until leaves are fully immersed [1]. Gently press the plunger to expel trapped air [2]. Seal the syringe tip with a piece of parafilm folded four times to prevent leakage [3].

- 1.4.1. Talent adding the buffer into the syringe until leaves are fully immersed.

Videographer's NOTE: 1.4.2, 1.4.3, 1.5.1, 1.5.2 are filmed in the same Shot

- 1.4.2. Talent pressing the plunger to release air bubbles.
- 1.4.3. Talent securing the syringe tip using the folded parafilm.
- 1.5. To infiltrate leaves, pull the plunger outward to create a vacuum [1]. Now, hold the plunger in the extended position for 5 seconds, then slowly release [2-TXT]. Repeat the process until leaves appear darker and translucent, indicating successful infiltration [3].
 - 1.5.1. Talent pulling the plunger to create a vacuum.
 - 1.5.2. Talent holding the position for 5 sec and then releasing it. **TXT: Repeat infiltration cycle 2-3 times**
 - 1.5.3. Shot of the leaves that have darkened and turned translucent after successful infiltration.
- 1.6. Now, pull out the plunger and drain the excess buffer into the sink [1].
 - 1.6.1. Talent pulling out the plunger and pouring the excess buffer into the sink.

2. Collection of Apoplastic Washing Fluid (AWF)

Demonstrators: Baoye He, Brisa Davila, Faris Alsaïd, Pankaj Kumar Yadav

- 2.1. To collect the apoplastic washing fluid or AWF (A-W-F) [1], on a clean surface, cut a 12-inch strip of Scotch tape and place it sticky side up [2]. Wrap one end of the tape around an empty 1-milliliter needleless syringe, just below the wide end [3]. Lay 50 to 70 infiltrated leaves on the tape overlapping each other [4], with bases toward the syringe and tips upward [5].
 - 2.1.1. WIDE: Briefly show the lab setup, including the clean surface with the infiltrated leaves, tape, and syringe neatly arranged and ready for use.
 - 2.1.2. Talent cutting a 12-inch strip of Scotch tape and placing it sticky side up. **NOTE: 2.1.2 and 2.1.4 are filmed in the same shot**
 - 2.1.3. Talent wrapping one end of the tape around an empty 1 mL needleless syringe.
 - 2.1.4. Talent laying infiltrated leaves on the tape in overlapping layers with base toward the syringe and tips upward.
Authors: Please show it for a few leaves only.

2.1.5. CU: Shot of the correct leaf placement and orientation on the adhesive surface.

2.2. Wrap the tape-bound infiltrated leaves tightly around the syringe [1]. Secure the top with two strips of tape [2]. Place the wrapped syringe into a 50-milliliter conical tube [3] with the cut leaf ends and syringe base facing down [4].

2.2.1. Talent wrapping the tape-bound infiltrated leaves around the syringe.

2.2.2. Talent securing the top with two strips of tape.

2.2.3. Talent placing the wrapped syringe into the conical tube with the cut leaf ends and syringe base facing down. NOTE: 2.2.3 and 2.2.4 are filmed in the same shot

2.2.4. CU: Shot of the final setup with the wrapped syringe fitted into the conical tube.

2.3. Now, spin the conical tube containing the wrapped syringe in a refrigerated centrifuge equipped with a fixed-angle rotor [1-TXT]. Then, collect the supernatant or the AWF in a new 15-milliliter conical tube and place the sample on ice [2-TXT].

2.3.1. Talent fitting the wrapped syringe and a balance tube into the centrifuge, closing the lid and initiating the spin. TXT: $900 \times g$, 10 min, 4 °C

2.3.2. Talent collecting the AWF in a new 15 milliliter conical tube and placing the sample on ice. TXT: Collect AWF from all infiltrated leaves

3. Extracellular Vesicles (EV) Isolation via Differential Centrifugation

Demonstrator: Baoye He

3.1. Centrifuge the AWF using a swing-bucket rotor with standard acceleration and deceleration settings [1-TXT]. Collect the supernatant and filter it through a 0.45-micron nylon cell filter to remove large cellular debris [2]. Transfer the filtered supernatant to clean, labelled centrifuge tubes [3].

3.1.1. Talent fitting the tubes symmetrically in the rotor, closing the lid of the centrifuge and initiating spin. TXT: $2,000 \times g$, 20 min, 4 °C

3.1.2. Talent filtering the supernatant using a 45µm nylon cell filter.

3.1.3. Talent transferring the filtered supernatant into a clean labelled centrifuge tube.

3.2. Centrifuge the filtered supernatant using a refrigerated centrifuge equipped with a fixed-angle rotor, applying standard acceleration and deceleration settings [1-TXT]. Collect the supernatant for further processing [2].

3.2.1. Talent fitting the tubes symmetrically in the rotor, closing the lid of the centrifuge and initiating spin. **TEXT: 10,000 × g, 30 min, 4 °C**

3.2.2. Talent collecting the supernatant after centrifugation.

3.3. Transfer the supernatant into 17-milliliter ultracentrifuge tubes and spin the samples in an ultracentrifuge equipped with a swing-bucket rotor [1-TXT]. After centrifugation, discard the supernatant and wash the pellet in approximately 16 milliliters of VIB [2]. Repeat the ultracentrifugation - washing cycle as demonstrated to collect the extracellular vesicles or EVs (*E-Vees*) [3]. Use a pipette tip to gently scrape the bottom of the tube to recover the EV pellet [4].

3.3.1. Talent loading 17 mL ultracentrifuge tubes and initiating the spin. **TEXT: Centrifuge at 100,000 × g for 1 hr at 4 °C**

3.3.2. Talent adding 16 ml of VIB to pellet and washing it.

3.3.3. Representative shot of talent placing the tube in ultracentrifuge to repeat the ultracentrifugation - washing cycle. **NOTE: Reuse 3.3.1**

3.3.4. Talent using a pipette tip to gently scrape the bottom of the tube to recover the EV pellet after centrifugation.

4. Top-Loading and Bottom-Loading Sucrose Density Gradient Separation of EVs

4.1. Keep 10 to 90 percent sucrose gradient stocks in fixed concentrations, freshly prepared and ready to use [1]. To prepare the top-loading discontinuous sucrose gradient, begin by taking a clean 17-milliliter ultracentrifuge tube [2]. Using a pipette, carefully add 1 milliliter of the most concentrated sucrose solution to the bottom of the tube [3]. Next, gently layer 1 milliliter of the next lower concentration sucrose solution on top of the first layer [4-TXT].

Authors: Please label the tubes with % gradient details.

4.1.1. **TEXT ON A PLAIN BACKGROUND:**

Sucrose gradient stock concentrations prepared using VIB:

10%, 16%, 22%, 28%, 34%, 40%, 46%, 52%, 58%, 64%, 70%, and 90%

4.1.2. Shot of the talent holding a clean 17 mL ultracentrifuge tube.

- 4.1.3. Talent transferring 1 mL of the most concentrated sucrose solution from labeled vial to the bottom of the tube using a pipette. **NOTE: 4.1.3 and 4.1.4 are filmed in same shot**
- 4.1.4. Talent gently layering 1 mL of the next lower concentration sucrose solution on top of the first layer. **TXT: Continue layering using descending concentration; Omit the 10% solution**
- 4.2. Now, mix 100 microliters of the resuspended EV pellet with 1 milliliter of the previously prepared 10 percent sucrose solution [1]. Add 1.1 milliliter of this mixture as the final layer in the ultracentrifuge tube to yield a total volume of around 12.1 milliliters [2]. Spin the top-loaded sucrose gradient mixture in an ultracentrifuge [3].
 - 4.2.1. Talent mixing 100 μ L of the resuspended EV pellet with the 1 mL of 10% sucrose solution.
 - 4.2.2. Talent adding 1.1 mL of this mixture as the final layer to yield a total volume of around 12.1 milliliters.
 - 4.2.3. Talent putting the tube in the ultracentrifuge. **TXT: Centrifuge at 100,000 x g, 16 hr at 4 °C** **NOTE: Reuse 3.3.1**
- 4.3. After ultracentrifugation, collect 2-milliliter fractions per sample by pipetting from the top [1] and transfer each fraction into a new 17-milliliter ultracentrifuge tube [2]. Once the fractions are centrifuged at 100,000 g for 1 hr at 4 degrees Celsius, resuspend the pellet in 50 microliters of VIB [3]. **NOTE: The VO has been edited by the authors**
 - 4.3.1. Talent pipetting out 2-mL fractions per sample from the top. **NOTE: 4.3.1 and 4.3.2 are filmed in the same shot**
 - 4.3.2. Talent transferring the fraction into a new 17 mL ultracentrifuge tube.
 - 4.3.3. Representative shot of talent resuspending the pellet in 50 μ L VIB.
- 4.4. For bottom loading, mix 100 microliters of the resuspended EV pellet with 1 milliliter of 90 percent sucrose [1]. Using a pipette, carefully load 1.1 milliliter of this sample to the bottom of the 17-milliliter ultracentrifuge tube to form the first layer of the discontinuous sucrose gradient [2]. Next, gently layer 1 milliliter of the next lower concentration sucrose solution on top of the first layer [3-TXT]. After preparing the bottom-loaded gradient, carry out the centrifugation and fraction-collection steps as demonstrated for the top-loaded method [4-TXT].
 - 4.4.1. Talent mixing 100 μ L of the resuspended EV pellet with 1 mL of 90% sucrose. **NOTE: 4.4.1-4.4.3 are filmed in a single shot**

- 4.4.2. Talent carefully loading 1.1 mL of the sample to the bottom of the ultracentrifuge tube to form the first layer.
- 4.4.3. Talent gently layering 1 mL of the next lower concentration sucrose solution on top of the first layer. **TXT: Continue layering using descending concentration**
- 4.4.4. Representative shot of the talent putting the tube in the ultracentrifuge. **TXT: Centrifuge at 100,000 x g, 72 hr at 4 °C** **NOTE: Add the onscreen text. Reuse 3.3.1**

5. Size Exclusion Chromatography Column Setup and EV Fractionation

- 5.1. Prepare VIB and all additional buffers fresh and filter the buffers through a 0.22-micron membrane [1]. De-gas the buffers under vacuum before use [2].
 - 5.1.1. Talent filtering a buffer through the 0.22 µm membrane.
 - 5.1.2. Talent de-gassing a buffer solution.
- 5.2. Set up the size-exclusion chromatography column with a 35-nanometer pore size at room temperature and open the top cap [1]. Then remove the bottom cap and allow the buffer to drain from the column [2].
 - 5.2.1. Show the column set up for 1-2 seconds and then show the talent opening the top cap of the column.
 - 5.2.2. Show the talent opening the bottom cap and the buffer draining out of the column.
- 5.3. To equilibrate the column, flush the column with three column volumes of VIB buffer [1-TXT].
 - 5.3.1. Talent flushing the column with de-gassed filtered buffer.
- 5.4. Now, adjust the EV samples to a final volume of 500 microliters using VIB [1]. Then, apply this EV suspension directly to the loading frit at the top of the column [2]. Wait for it to fully enter the resin bed [3]. Now, add 2 milliliters of VIB and let it elute completely [4]. After discarding the eluate, add 400 microliters of VIB and collect 400 microliters of the eluate, as fraction 1, in 1.5-milliliter microcentrifuge tubes [5-TXT].
 - 5.4.1. Talent using VIB to adjust the final volume of EV samples to 500 µL. **NOTE: 5.4.1 and 5.4.2 are filmed in the same shot**
 - 5.4.2. Talent adding the EV suspension directly to the loading frit at the top of the column.
 - 5.4.3. CU: Shot of the column once the sample has fully entered the resin bed.
 - 5.4.4. Talent adding 2 mL of VIB and letting it fully drain out of the column.

5.4.5. Talent adding 400 μ L of VIB and collecting 400 μ L of the eluate as fraction 1 in 1.5 mL microcentrifuge tubes.

TXT: Repeat this process to collect fractions 2 - 6

Results

6. Results

- 6.1. The preparation of leaves using the optimized protocol described in this article showed less cell damage than the existing method [1]. Consistently, the AWF produced using the optimized protocol contained less chlorophyll levels [2] and a weaker chloroplast marker protein signal, indicating low tissue damage and cytoplasmic contamination [3].
 - 6.1.1. LAB MEDIA: Figure 3A
Video editor: Please show the full image and then emphasize Method 1 image.
 - 6.1.2. LAB MEDIA: Figure 3B
Video editor: Please show the full image and then emphasize the shorter bar.
 - 6.1.3. LAB MEDIA: Figure 3C
Video editor: Please show the full image and then encircle the band for Method 1.
- 6.2. Sucrose density gradient centrifugation effectively enriched and purified EVs [1]. The EVs were primarily detected in fractions 4 and 5 [2], with fraction 4 showing the highest particle concentration [3]. Transmission electron microscope analysis of fraction 4 confirmed vesicles of the expected size and typical cup-shaped morphology [4].
 - 6.2.1. LAB MEDIA: Figure 5
 - 6.2.2. LAB MEDIA: Figure 5A
Video editor: Please show the full image and then emphasize the bars for fractions 4 and 5.
 - 6.2.3. LAB MEDIA: Figure 5 B and 5C
Video editor: Please display both images side by side and then in Figure 5B, highlight the dark band in the bottom panel under F4 and 1.14. Also, at the same time highlight the set of the 3 overlapping tall peaks between 100 and 200 in Figure 5C.
 - 6.2.4. LAB MEDIA: Figure 5D
Video editor: Please add arrows pointing to two prominent circular structures with dark borders. Also, ensure that the scale bar is clearly visible.
- 6.3. Size-exclusion chromatography effectively purified plant EVs, and nanoparticle tracking analysis and transmission electron microscope analysis of the resulting fraction confirmed vesicles of expected size with typical cup-shaped morphology [1].

6.3.1. LAB MEDIA: Figure 7

Video editor: Please show the full image.

In Figure 7A, emphasize the bar for fraction 2; in Figure 7B, emphasize the band for fraction 2; in Figure 7C, highlight the set of the tallest overlapping peaks between 100 and 200 and, in Figure 7D, please add an arrow pointing to the disc-shaped structure with a dark border located near the center of the image.

1. Arabidopsis
Pronunciation link: <https://www.merriam-webster.com/dictionary/Arabidopsis>
IPA: /ˌærəbəˈdapsəs/
Phonetic Spelling: air·uh·buh·dop·suhs ([merriam-webster.com](https://www.merriam-webster.com))
2. petiole
Pronunciation link: <https://www.merriam-webster.com/dictionary/petiole>
IPA: /ˈpeti,ool/
Phonetic Spelling: pet·ee·ohl ([merriam-webster.com](https://www.merriam-webster.com))
3. distal
Pronunciation link: <https://www.merriam-webster.com/dictionary/distal>
IPA: /ˈdistəl/
Phonetic Spelling: dis·tuhl ([merriam-webster.com](https://www.merriam-webster.com))
4. vesicle
Pronunciation link: <https://www.merriam-webster.com/dictionary/vesicle>
IPA: /ˈvesɪkəl/
Phonetic Spelling: ves·ih·kuhl ([merriam-webster.com](https://www.merriam-webster.com))
5. syringe
Pronunciation link: <https://www.merriam-webster.com/dictionary/syringe>
IPA: /səˈrɪndʒ/, /ˈsɪrɪndʒ/
Phonetic Spelling: suh·rinj, sir·inj ([merriam-webster.com](https://www.merriam-webster.com))
6. parafilm
Pronunciation link: <https://www.howtopronounce.com/parafilm>
IPA: /ˈpærəfilm/
Phonetic Spelling: pair·uh·film ([howtopronounce.com](https://www.howtopronounce.com))
7. translucent
Pronunciation link: <https://www.merriam-webster.com/dictionary/translucent>
IPA: /træn(t)sˈlu:sənt/, /trænzˈlu:sənt/
Phonetic Spelling: trans·LOO·suhnt, tranz·LOO·suhnt ([merriam-webster.com](https://www.merriam-webster.com))
8. apoplastic
Pronunciation link: <https://www.howtopronounce.com/apoplastic>

- IPA: /ˌæpəˈplæstɪk/
Phonetic Spelling: ap·uh·plas·tik (howtopronounce.com)
9. centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj (merriam-webster.com)
10. supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·NAY·tuht (merriam-webster.com)
11. chromatography
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatography>
IPA: /ˌkroʊməˈtɑɡrəfi/
Phonetic Spelling: kroh·muh·TOG·ruh·fee (merriam-webster.com)
12. sucrose
Pronunciation link: <https://www.merriam-webster.com/dictionary/sucrose>
IPA: /ˈsuːkroʊs/, /ˈsuːkroʊz/
Phonetic Spelling: soo·krohs, soo·krohz (merriam-webster.com)
13. extracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>
IPA: /ˌɛkstrəˈsɛljələr/
Phonetic Spelling: ek·struh·SEL·yuh·luhr (merriam-webster.com)
14. equilibrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibrate>
IPA: /ɪˈkwɪləˌbreɪt/
Phonetic Spelling: ih·KWIL·uh·brayt (merriam-webster.com)
15. eluate
Pronunciation link: <https://www.merriam-webster.com/dictionary/eluate>
IPA: /ˈɛljəwət/, /-ˌwət/
Phonetic Spelling: el·yuh·wuht, el·yuh·waht (merriam-webster.com)