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Title: Exploring the Application of Surface-Enhanced Raman Scattering-Based Biosensing of Individual sEVs in Disease Diagnosis and Therapeutics

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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**

Videographer: Please record the computer screen for the shots labeled as SCREEN

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: 10
Number of Shots: 26 (23 SC)

Introduction

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

INTRODUCTION:

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

1.1. **Jun Liu:** We use surface-enhanced Raman scattering combined with machine learning to tackle cancer diagnostics and deliver biomedical applications.

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

~~What are the current experimental challenges?~~

1.2. **Siddharth Srivastava:** The current experimental challenges include high inherent heterogeneity of sEVs and complexity in analyzing high-dimensional spectral data, compounded by low signal-noise-ratio.

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. **Jun Liu:** We have established single-vesicle biochemical fingerprinting to distinguish disease sources, showing potential for early diagnosis.

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

~~What advantage does your protocol offer compared to other techniques?~~

1.4. **Siddharth Srivastava:** Our spectroscopic technique is non-invasive, does not require lysing of particles, and is highly sensitive.

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

~~What questions will future research focus on?~~

1.5. **Jun Liu:** In the future, we will correlate the spectral profile to the proteomic profile, which in turn would help correlate exosome content with specific functions, such as diagnosis**.**

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.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Jun Liu, Graduate Student, UCLA:** (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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Siddharth Srivastava, Graduate Student, UCLA**: (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

Ethics Title Card

This research was approved by the Institutional Review Board (IRB) at University of California Los Angeles

Protocol

2. Sample Preparation and Spectra Acquisition

NOTE: The authors have nor provided demonstrator's name

2.1. To begin, pipette approximately 5 microliters of the small extracellular vesicle sample onto the designated plasmonic substrate **[1]**. Place the substrate in a desiccator to let the droplet dry completely for approximately 15 minutes **[2]**.

2.1.1. WIDE: Talent pipetting 5 microliters of the small extracellular vesicle sample onto a plasmonic substrate.

2.1.2. Talent placing the substrate inside a desiccator and sealing it.

2.2. Then, transfer the dried substrate to a confocal Raman microscope **[1]**. Using the instrument software WiRE *(wire)* version 4.4, initiate data collection **[2]**. Set the excitation laser to 785 nanometers at 5 milliwatts and calibrate the system **[3]**.

2.2.1. Talent placing the prepared substrate into the stage of a confocal Raman microscope.

2.2.2. SCREEN: Launch WiRE version 4.4 software to initiate data collection. ***Videographer: Please record the computer screen for the shots labeled as SCREEN*** **Videographer's NOTE:** don't show "start acquisition" being selected yet

2.2.3. SCREEN: Adjust the settings to set the laser excitation wavelength to 785 nanometers and power to 5 milliwatts.**Videographer's NOTE:** take4 is only setting power

2.2.4. ~~SCREEN: Calibrate the Raman system by aligning the silicon reference peak to 520 inverse centimeters~~. . **NOTE**: Not filmed

2.3. Perform a scouting scan over a 300 by 300-micrometer area to locate single vesicles using static mode with 50% laser power, 0.1-second exposure, and 10-micrometer step size **[1]**. Once the vesicles are located, conduct high-resolution mapping in static mode within a 5-by-5 grid using a 1-micrometer step size **[2-TXT]**.

2.3.1. SCREEN: Set up a scouting scan in static mode over a 300 by 300 micrometer region with 50% laser power, 0.1s exposure time, and 10 micrometer step size. **Videographer's NOTE:** did not shoot, use 2.2.3

2.3.2. SCREEN: Configure a 5 by 5 grid for high-resolution mapping in static mode with 1 micrometer step size, 50 percent power, and 0.2 second exposure per point. **TXT: 50% laser power; 0.2 s exposure/point; Next, perform a scouting scan to locate individual vesicles and high-resolution mapping**

~~2.4. Next, perform a scouting scan over a 300 by 300-micrometer area on the graphene substrate to locate individual vesicles **[1 TXT]** and then perform high-resolution mapping in extended mode within a 2 by 2 grid **[2 TXT].**~~

~~2.4.1. SCREEN: Configure a scouting scan over a 300 by 300 micrometer region on the graphene substrate in static mode. **TXT: 50% laser power; 0.1 s exposure; 10 µm step size**~~

2.4.2. ~~SCREEN: Set up high resolution mapping in extended mode over a 2 by 2 grid. **TXT: 1% laser power; 10 s exposure; 1 µm step size**~~ **Videographer's NOTE:** did not shoot

3. Quality Control and Data Normalization

3.1. Screen the initial raw spectra and exclude any that display excessive noise or abnormal spectral shapes **[1]**. ~~Remove isolated cosmic ray spikes using threshold-based detection or local deviation filtering **[2]**~~.

3.1.1. SCREEN: Display raw Raman spectra and highlight examples of highly noisy or distorted shapes being deselected.

3.1.2. ~~SCREEN: Apply a spike removal tool and show cosmic ray spikes being detected and eliminated from the spectra using a local deviation filter~~. . **NOTE**: Not required

3.2. Apply a Savitzky-Golay filter to each spectrum to reduce fluorescence background and smooth the spectral data **[1]**. Calculate the signal-to-noise ratio for each spectrum using either the peak-to-baseline method or the standard-deviation method **[2]**. For each spectrum, calculate the signal-to-noise ratio as the ratio of the peak intensity after baseline subtraction at a representative Raman band to the standard deviation of the noise **[3]**.

3.2.1. SCREEN: Apply Savitzky-Golay filtering to a raw spectrum and show reduction in fluorescence and smoothened data.

3.2.2. SCREEN: Show a menu with signal-to-noise ratio calculation options and initiate the computation using the peak-to-baseline method.

3.2.3. SCREEN: Demonstrate how signal-to-noise ratio is calculated by measuring peak intensity at a selected Raman band and dividing by noise level.

3.3. Estimate noise by subtracting a Savitzky-Golay-smoothed version of the spectrum, using a window size of 11 and a polynomial order of 3, from the original spectrum **[1]**. Rank all spectra in ascending order of signal-to-noise ratio and evaluate the persistence of diagnostic peaks across the ranked spectra to determine the threshold **[2]**. Then, set the threshold at the point where any of these peaks disappear into baseline noise, corresponding to a signal-to-noise ratio of 28 **[6]**.

3.3.1. SCREEN: Apply Savitzky-Golay smoothing with specified parameters and display the residual spectrum used to estimate noise. **Videographer's NOTE**: did not shoot, use 3.2.3

3.3.2. SCREEN: Display a ranked list of spectra by signal-to-noise ratio and highlight presence or absence of diagnostic peaks across the list.

3.3.3. SCREEN: Annotate the point where the diagnostic peaks vanish into the noise and mark the corresponding signal-to-noise ratio threshold of 28.

3.4. Exclude spectra that fall below the signal-to-noise ratio threshold of 28 to ensure only high-quality spectra are retained for downstream analysis **[1]** and normalize each remaining spectrum to either its maximum peak intensity or the total area under the curve **[2]**.

3.4.1. SCREEN: Filter out spectra with a signal-to-noise ratio below 28 from the dataset. **Videographer's NOTE**: do not use take1

3.4.2. SCREEN: Apply normalization to the accepted spectra and show both maximum peak and area-under-curve options being implemented.

4. Spectral Analysis with Machine Learning

4.1. Assign a label to each spectral measurement based on its origin, such as a gastric cancer patient or a healthy control **[1]**. Input the labeled spectral data into a Support Vector Classifier with a linear kernel **[2]** and apply a stratified shuffle split for five-fold cross-validation to ensure balanced representation in each fold **[3]**. Now, average the accuracy metrics across all folds to assess overall model performance **[4]**.

4.1.1. SCREEN: Display a dataset where each spectral measurement is tagged with a label like "gastric cancer" or "healthy control."

4.1.2. SCREEN: Import the dataset into a Support Vector Classifier interface and select the linear kernel option.

4.1.3. SCREEN: Apply a stratified shuffle split method for five-fold cross-validation and initiate the process.

4.1.4. SCREEN: Show a table or plot displaying the accuracy scores from each fold and the averaged performance metric.

4.2. Finally, use Linear Discriminant Analysis or LDA to reduce the dimensionality of the surface-enhanced Raman scattering dataset for visualization **[1]**. Employ the trained Linear Discriminant Analysis model to perform direct classification between the sample groups **[2-TXT]**.

4.2.1. SCREEN: Apply Linear Discriminant Analysis to the high-dimensional dataset and display a 2D or 3D plot showing reduced dimensions.

4.2.2. SCREEN: Show classification output from the Linear Discriminant Analysis model, highlighting separation between sample groups. **TXT: Evaluate the trained models on a holdout set of blinded patient samples** **Videographer's NOTE**: do not use take1

Results

5. Results

5.1. Raman spectra with the corresponding average spectra revealed distinct molecular signatures across each sample type acquired from single small extracellular vesicles isolated from tissue, blood, and saliva samples **[1]**.

5.1.1. LAB MEDIA: Figure 2. *Video editor: Sequentially Highlight panels A , B and C*.

5.2. Linear Discriminant Analysis revealed clear clustering of extracellular vesicle data according to their source—tissue, blood, or saliva **[1]**.

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight panel (D), pointing to the three clustered color-coded groups labeled tissue (purple), blood (turquoise), and saliva (orange)*.

5.3. Binary classification using a Support Vector Machine classifier achieved the highest accuracy for tissue samples at 90.1% **[1]**, followed by blood at 70.9% **[2]**, and saliva at 60.7% **[3]**.

5.3.1. LAB MEDIA: Figure 2E. *Video editor: Highlight BLUE BAR*.

5.3.2. LAB MEDIA: Figure 2E. *Video editor: Highlight CYAN BAR*

5.3.3. LAB MEDIA: Figure 2E. *Video editor: Highlight ORANGE BAR*

5.4. LDA-based classification splits clearly separated healthy and gastric cancer samples in tissue-derived extracellular vesicle data **[1]**.

5.4.1. LAB MEDIA: Figure 2 F G H. *Video editor: Highlight panel (F), showing the green and red split plots*.

5.5. Similar LDA-based classification splits for blood- and saliva-derived extracellular vesicle data showed less optimal separation between healthy and cancer samples **[1]**.

5.5.1. LAB MEDIA: Figure 2 F G H. *Video editor: Highlight panel (G) and (H)*

5.6. Raman spectra of doxorubicin-incubated vesicles without graphene showed consistent peaks at approximately 1081, 1206, and 1440 centimeters inverse **[1]**.

5.6.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the blue-labeled DOX peaks*.

5.7. With graphene, an additional D peak at approximately 1350 centimeters inverse and a G peak at approximately 1580 centimeters inverse were observed, serving as internal standards **[1]**.

5.7.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the purple-labeled D and G peaks*.

5.8. The ratio of the doxorubicin peak at 442 centimeters inverse to the graphene G peak increased with higher drug concentrations **[1]** and longer incubation times **[2]**.

5.8.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the 3 yellow bars* .

5.8.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the tallest yellow bar in the first group corresponding to "0.5DOX Conc" . and "2" incubation time*

1. Microliters
   Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
   IPA: /ˈmaɪ.kroʊˌliː.tər/
   Phonetic Spelling: my·kroh·lee·ter
2. Extracellular
   Pronunciation link: https://www.merriam-webster.com/dictionary/extracellular
   IPA: /ˌɛk.strəˈsɛl.jə.lɚ/
   Phonetic Spelling: ek·struh·sel·yuh·ler
3. Vesicle
   Pronunciation link: https://www.merriam-webster.com/dictionary/vesicle
   IPA: /ˈvɛsɪkəl/
   Phonetic Spelling: ves·ih·kuhl
4. Plasmonic
   Pronunciation link: https://www.merriam-webster.com/dictionary/plasmonic
   IPA: /plæzˈmɑːnɪk/
   Phonetic Spelling: plaz·mon·ik
5. Desiccator
   Pronunciation link: https://www.merriam-webster.com/dictionary/desiccator
   IPA: /ˈdɛsɪˌkeɪtər/
   Phonetic Spelling: dess·ih·kay·ter
6. Confocal
   Pronunciation link: https://www.merriam-webster.com/dictionary/confocal
   IPA: /kɑnˈfoʊkəl/
   Phonetic Spelling: kon·foh·kuhl
7. Raman
   Pronunciation link: https://www.merriam-webster.com/dictionary/Raman
   IPA: /ˈrɑːmən/
   Phonetic Spelling: rah·muhn

8. Nanometers
   Pronunciation link: https://www.merriam-webster.com/dictionary/nanometer
   IPA: /ˈnænəˌmiːtər/
   Phonetic Spelling: nan·uh·mee·ter
9. Milliwatts
   Pronunciation link: https://www.merriam-webster.com/dictionary/milliwatt
   IPA: /ˈmɪlɪˌwɑːt/
   Phonetic Spelling: mil·ih·wot
10. Silicon
    Pronunciation link: https://www.merriam-webster.com/dictionary/silicon
    IPA: /ˈsɪlɪkən/
    Phonetic Spelling: sil·ih·kuhn
11. Micrometer
    Pronunciation link: https://www.merriam-webster.com/dictionary/micrometer
    IPA: /maɪˈkrɑːmɪtər/
    Phonetic Spelling: my·krah·mih·ter
12. Graphene
    Pronunciation link: https://www.merriam-webster.com/dictionary/graphene
    IPA: /ˈgræfˌiːn/
    Phonetic Spelling: graf·een
13. Spectra
    Pronunciation link: https://www.merriam-webster.com/dictionary/spectra
    IPA: /ˈspɛktrə/
    Phonetic Spelling: spek·truh
14. Savitzky-Golay
    Pronunciation link: https://www.howtopronounce.com/savitzky-golay
    IPA: /səˈvɪtski ɡoʊˈleɪ/
    Phonetic Spelling: suh·vits·kee goh·lay
15. Fluorescence
    Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
    IPA: /ˌflʊrˈɛsəns/
    Phonetic Spelling: floor·ess·uhns
16. Signal-to-noise
    Pronunciation link: https://www.howtopronounce.com/signal-to-noise
    IPA: /ˈsɪgnəl tuː nɔɪz/
    Phonetic Spelling: sig·nuhl too noyze
17. Polynomial
    Pronunciation link: https://www.merriam-webster.com/dictionary/polynomial
    IPA: /ˌpɑːlɪˈnoʊmiəl/
    Phonetic Spelling: pah·luh·noh·mee·uhl
18. Baseline
    Pronunciation link: https://www.merriam-webster.com/dictionary/baseline
    IPA: /ˈbeɪsˌlaɪn/
    Phonetic Spelling: bays·line

19. Normalization
    Pronunciation link: https://www.merriam-webster.com/dictionary/normalization
    IPA: /ˌnɔːrmələˈzeɪʃən/
    Phonetic Spelling: nor·muh·luh·zay·shuhn
20. Support Vector Machine
    Pronunciation link: https://www.howtopronounce.com/support-vector-machine
    IPA: /səˈpɔːrt ˈvɛktər məˈʃiːn/
    Phonetic Spelling: suh·port vek·ter muh·sheen
21. Classifier
    Pronunciation link: https://www.merriam-webster.com/dictionary/classifier
    IPA: /ˈklæsɪˌfaɪər/
    Phonetic Spelling: klas·uh·fyer
22. Stratified
    Pronunciation link: https://www.merriam-webster.com/dictionary/stratified
    IPA: /ˈstrætəˌfaɪd/
    Phonetic Spelling: strat·uh·fyed
23. Cross-validation
    Pronunciation link: https://www.merriam-webster.com/dictionary/cross-validation
    IPA: /ˌkrɔːsˌvæləˈdeɪʃən/
    Phonetic Spelling: kross·val·uh·day·shuhn
24. Linear Discriminant Analysis
    Pronunciation link: https://www.howtopronounce.com/linear-discriminant-analysis
    IPA: /ˈlɪniər dɪˈskrɪmɪnənt əˈnæləsɪs/
    Phonetic Spelling: lin·ee·er dih·skrimh·uh·nuhnt uh·nal·uh·siss
25. Dimensionality
    Pronunciation link: https://www.merriam-webster.com/dictionary/dimensionality
    IPA: /dɪˌmɛnʃəˈnælɪti/
    Phonetic Spelling: dih·men·shuh·nal·ih·tee
26. Gastric
    Pronunciation link: https://www.merriam-webster.com/dictionary/gastric
    IPA: /ˈgæstrɪk/
    Phonetic Spelling: gas·trik
27. Doxorubicin
    Pronunciation link: https://www.merriam-webster.com/dictionary/doxorubicin
    IPA: /ˌdɑːksoʊˈruːbɪsɪn/
    Phonetic Spelling: dok·soh·roo·buh·sin
28. Incubation
    Pronunciation link: https://www.merriam-webster.com/dictionary/incubation
    IPA: /ˌɪnkjəˈbeɪʃən/
    Phonetic Spelling: in·kyuh·bay·shuhn
29. Inverse centimeters
    Pronunciation link: https://www.howtopronounce.com/inverse-centimeters
    IPA: /ˈɪnvɜːrs ˈsɛntɪˌmiːtərz/
    Phonetic Spelling: in·vers sen·tuh·mee·terz

30. Bimodal
    Pronunciation link: https://www.merriam-webster.com/dictionary/bimodal
    IPA: /ˌbaɪˈmoʊdəl/
    Phonetic Spelling: bye·moh·duhl