

Submission ID #: 70382

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Title: Single Nuclei Isolation from Coronary Endarterectomy Tissue of Coronary Artery Bypass Graft Patients

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? **Yes**

SCOPE SHOTS: 3.3.3, 5.3.2

Videographer: Please film the above shots using the scope kit

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? **Yes**

If **Yes**, how far apart are the locations? Same building but different floors

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: 25

Number of Shots: 56 (11 SC)

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.**

Answers are slightly edited in accordance with the journal guidelines

INTRODUCTION:

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

- 1.1. **Surya Ramachandran:** Our major research focus is to map cellular diversity in atherosclerotic plaques and identify cell to cell interactions which can be used to develop RNA based prognostic markers.
 - 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. **Khushi Jani:** Membrane fragility hinders single-cell isolation from tissue obtained during endarterectomy; therefore, this protocol isolates preserved nuclei, enabling transcriptomic profiling of high-risk plaques.
 - 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. **Khushi Jani:** The most significant challenge when performing this protocol is high calcification in the tissue, which critically impairs nuclei isolation quality and yield.
 - 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. **Rohini Nair:** Spatial Transcriptomics can be carried out to extract deeper biological insights. It will help to interpret spatial gene expression, cellular interactions, and tissue organization.

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.

Ethics Title Card

This research has been approved by the Human Ethical Committee of U. N. Mehta Institute of Cardiology and Research Centre and the Gujarat Biotechnology University

Protocol

2. Isolation of Nuclei from the Tissue Obtained During Coronary Endarterectomy

Demonstrator: Khushi Jani

- 2.1. To begin, gather all the reagents and ice box to perform the procedure [1]. Wash the tissue once or twice with serum-free RPMI medium to remove blood stains and fragments [2]. Using sterile forceps, transfer the tissue onto a sterile Petri dish [3] and record the weight [4].
 - 2.1.1. Talent arranging all reagents and ice box on the workbench.
 - 2.1.2. Talent washing the tissue in a container with serum-free Roswell Park Memorial Institute medium.
 - 2.1.3. Talent transferring the tissue onto a sterile Petri dish using sterile forceps.
 - 2.1.4. Talent recording the weight of the tissue on a weighing device.
- 2.2. Using a surgical blade number 10, mince the tissue into small pieces of approximately 1 cubic millimeter in size [1]. Now, add 1 milliliter chilled lysis buffer and the tissue to a 5-milliliter microcentrifuge tube [2].
 - 2.2.1. Talent mincing the tissue into small pieces on the sterile Petri dish using a surgical blade number 10.
 - 2.2.2. Talent adding chilled lysis buffer and tissue into a 5 milliliter microcentrifuge tube.
- 2.3. Lyse the tissue on ice using a micro pestle by applying a gentle, consistent force [1] and swirling the micro pestle clockwise and anticlockwise alternately [2]. Continue lysis for approximately 30 minutes while keeping the sample on ice [3].
 - 2.3.1. Talent inserting the micro pestle into the tissue tube and applying gentle force to lyse the tissue.
 - 2.3.2. Close shot of the micro pestle swirling clockwise and anticlockwise inside the tube.
 - 2.3.3. Talent continuing the lysis process with the tube kept on ice.
- 2.4. Filter the solution through a 100-micrometer pore-size strainer into a 15-milliliter centrifuge tube to remove debris and tissue chunks [1]. Centrifuge the nuclei at 500 g

- for 5 minutes at 4 degrees Celsius using a swinging bucket rotor [2].
- 2.4.1. Talent filtering the lysate through a 100 micrometer pore-size strainer into a 15 milliliter centrifuge tube.
 - 2.4.2. Talent placing the centrifuge tube containing the sample into the centrifuge with swinging bucket rotor.
- 2.5. Then, remove the supernatant using a regular-bore pipette tip without disturbing the pellet [1].
- 2.5.1. Talent carefully aspirating the supernatant from the tube using a regular-bore pipette tip while leaving the pellet undisturbed. **NOTE: After centrifugation, the pellet formation is shown and labeled as 2.5.1 and 2.9.1 (1st shot).**
- 2.6. Chill the nuclei wash and resuspension buffer on ice before use [1]. Resuspend the pellet in the chilled nuclei wash and resuspension buffer [2].
- 2.6.1. Talent placing the nuclei wash and resuspension buffer on ice to chill.
 - 2.6.2. Talent resuspending the pellet in the chilled nuclei wash and resuspension buffer by pipetting up and down.
- 2.7. Quickly assess lysis efficiency and cell viability by adding 5 microliters trypan blue to an aliquot of the sample [1] and counting the cells using a hemocytometer [2-TXT].
- 2.7.1. Talent adding 5 microliters trypan blue to the nuclei suspension in the tube.
 - 2.7.2. Talent loading the stained sample into a hemocytometer and counting the cells. **TXT: If viable cells are present, repeat the lysis, filtration and centrifugation steps; if no viable cells are observed, proceed further**
- 2.8. Then, filter the solution through a 30-micrometer pore-size strainer into a 15 milliliter centrifuge tube to remove debris and clumps [1]. Centrifuge the nuclei at 500 g for 5 minutes at 4 degrees Celsius using a swinging bucket rotor [2].
- 2.8.1. Talent filtering the nuclei suspension through a 30 micrometer pore-size strainer into a 15 milliliter centrifuge tube.
 - 2.8.2. Talent placing the centrifuge tube containing the filtered nuclei suspension into the centrifuge with a swinging bucket rotor.
- 2.9. After centrifugation, remove the supernatant using a regular-bore pipette tip without

disturbing the pellet [1] and resuspend the pellet in 1 milliliter nuclei wash and resuspension buffer [2].

2.9.1. Talent carefully aspirating the supernatant from the centrifuge tube using a regular-bore pipette tip while leaving the pellet undisturbed.

2.9.2. Talent resuspending the pellet in 1 milliliter nuclei wash and resuspension buffer by pipetting up and down.

3. Trypan Blue Staining for Assessing the Integrity of Nuclei

3.1. Take 15 microliters of the isolated nuclei suspension in a 1.5-milliliter microcentrifuge tube [1].

3.1.1. Talent pipetting 15 microliters of the isolated nuclei suspension into a 1.5 milliliter microcentrifuge tube.

3.2. Add 5 microliters trypan blue dye at 0.4 percent weight per volume to the nuclei suspension [1] and mix gently by pipetting [2].

3.2.1. Talent adding 5 microliters trypan blue dye into the tube containing the nuclei suspension.

3.2.2. Talent pipetting the mixture up and down to mix the dye and nuclei.

3.3. After a 5-minute incubation, load 10 microliters of the stained mixture onto a hemocytometer [1]. Observe the sample under a compound inverted microscope [2] to examine the morphology and count the number of nuclei [3].

3.3.1. Talent loading 10 microliters of the stained nuclei mixture onto a hemocytometer.

3.3.2. Talent placing the hemocytometer on the stage of a compound inverted microscope and viewing the sample.

3.3.3. SCOPE OR LAB MEDIA (Image): Show an image containing nucleic with good morphology. **Authors:** Please provide an image for this IF POSSIBLE (upload to your project page)

Videographer: Please film the above shot using the scope kit

4. Flow Cytometry Analysis of Single Nuclei Suspension

Demonstrators: Khushi Jani and Hemang Patel

- 4.1. Take 1 milliliter of the isolated nuclei suspension [1] and centrifuge at 500 *g* for 5 minutes at 4 degrees Celsius using a swinging bucket rotor [2].
 - 4.1.1. ~~Talent pipetting 1 milliliter of isolated nuclei suspension into a centrifuge tube.~~
NOTE: Not filmed, VO merged
 - 4.1.2. Talent placing the centrifuge tube into the centrifuge. N
- 4.2. Remove the supernatant using a regular-bore pipette tip without disturbing the pellet [1]. Resuspend the pellet in 200 microliters fluorescence-activated cell sorting buffer composed of PBS and 1 percent bovine serum albumin [2].
 - 4.2.1. Talent aspirating the supernatant from the centrifuge tube using a regular-bore pipette tip while leaving the pellet undisturbed.
 - 4.2.2. Talent resuspending the pellet in 200 microliters fluorescence-activated cell sorting buffer.
- 4.3. Divide the 200 microliters of resuspended nuclei into two aliquots [1]. Keep 100 microliters as the unstained control [2] and use the remaining 100 microliters as the stained sample [3].
 - 4.3.1. Talent splitting the resuspended nuclei suspension into two tubes.
 - 4.3.2. Talent labeling one tube as the unstained control containing 100 microliters.
 - 4.3.3. Talent labeling the second tube as the stained sample containing 100 microliters.
- 4.4. Add 1 microliter DAPI from a 1 milligram per milliliter stock solution to the stained sample to achieve a final concentration of 10 micrograms per milliliter [1]. Mix gently by pipetting [2] and incubate at room temperature in the dark for 5 minutes [3].
 - 4.4.1. Talent adding 1 microliter DAPI to the tube containing the stained sample.
 - 4.4.2. Talent gently pipetting the sample up and down to mix.
 - 4.4.3. Talent placing the tube in a dark location for incubation.
- 4.5. Set the flow cytometer to use the violet laser at 405 nanometers and detect using 448 by 45 nanometers [1]. Run the unstained control sample first [2] and acquire a minimum of 10,000 events of the total population [3].
 - 4.5.1. SCREEN: Flow cytometer software panel showing laser settings with the violet

laser set to 405 nanometers and detection set to 448 slash 45 nanometers.
Videographer: Please record the computer screen for the shots labeled as SCREEN

- 4.5.2. Talent loading the unstained control sample into the flow cytometer.
- 4.5.3. SCREEN: Acquisition window showing the event counter reaching at least 10,000 events.

- 4.6. Generate a plot of forward scatter area versus forward scatter height to distinguish singlets from doublets and aggregates [1]. Draw a gate around events displaying a linear relationship to retain only singlet nuclei [2].
 - 4.6.1. SCREEN: Flow cytometry software displaying a plot of forward scatter area versus forward scatter height.
 - 4.6.2. SCREEN: Cursor drawing a gate around the linear population representing singlet nuclei.

- 4.7. Next, generate a bivariate plot of forward scatter area versus side scatter area [1] and apply a broad gate encompassing the entire scatter population [2].
 - 4.7.1. SCREEN: Flow cytometry software displaying a plot of forward scatter area versus side scatter area.
 - 4.7.2. SCREEN: Cursor drawing a broad gate around the entire scatter population.

- 4.8. Now, create a 2D density pseudo color plot of side scatter area versus violet 450 nanometer area [1]. Use the unstained control to define background fluorescence and guide gate placement [2]. Identify and gate the distinct DAPI-positive population corresponding to intact nuclei [3].
 - 4.8.1. SCREEN: Flow cytometry software displaying a 2 dimensional density pseudo color plot of side scatter area versus violet 450 nanometer area. **NOTE: from video editor: 7509/7510) is a continues shot, which has the shot 4.8.1**
 - 4.8.3,. SCREEN: Display of the unstained control used to adjust background fluorescence and refine the gate. **NOTE: This shot should be before 4.8.2, VO is adjusted accordingly, the author says they labeled this 4.8.2 and the next shot 4.8.3**
 - 4.8.2,. SCREEN: Cursor identifying and drawing a gate around the distinct DAPI positive nuclei population. **This shot should be after 4.8.3**

5. Imaging the Isolated Nuclei using Confocal Microscopy

Demonstrator: Varsha Gupta

- 5.1. Take 50 microliters of nuclei suspension [1] and add 1 microliter DAPI from a 1 milligram per milliliter stock solution to achieve a final concentration of 10 micrograms per milliliter [2]. Mix gently by pipetting [3] and incubate at room temperature in the dark for 5 minutes before confocal imaging [4].
 - 5.1.1. Talent pipetting 50 microliters of nuclei suspension into a microcentrifuge tube.
 - 5.1.2. Talent adding 1 microliter DAPI to the nuclei suspension.
 - 5.1.3. Talent gently pipetting the sample up and down to mix.
 - 5.1.4. Talent placing the tube in a dark location for incubation.

 - 5.2. Mount 5 microliters of the stained nuclei suspension onto a clean glass slide [1] and cover with a coverslip [2].
 - 5.2.1. Talent pipetting 5 microliters of stained nuclei suspension onto a clean glass slide.
 - 5.2.2. Talent carefully placing a coverslip over the sample on the slide.

 - 5.3. Next, open the confocal microscope software [1] and observe the nuclei in 40x bright field [2].
 - 5.3.1. SCREEN: Confocal microscope software opening on the computer screen.
 - 5.3.2. ~~SCOPE or LAB MEDIA (IMAGE): Bright field view of nuclei at 40 times magnification.~~ **NOTE: Not filmed, cut the VO**
- Videographer: Please film the above shot using the scope kit***
-
- 5.4. Finally, acquire confocal images using the laser scanning confocal microscope [1]. Excite DAPI using a 405 nanometer laser and collect emission between 429 and 474 nanometers [2].
 - 5.4.1. Talent initiating image acquisition on the confocal microscope while observing the screen.
 - 5.4.2. SCREEN: Fluorescent nuclei visible under DAPI excitation with emission detected between 429 and 474 nanometers.

Results

6. Results

6.1. Following nuclear isolation and trypan blue staining, a distinct population of nuclei was observed without intact viable cells, confirming effective tissue lysis. Isolated nuclei appeared as dense, round, dark blue bodies under the microscope, indicating successful nuclear isolation [1].

6.1.1. LAB MEDIA: Figure 2D. *Video editor: Zoom in on the microscope view showing many small dark blue round dots.*

6.2. Calcified plaque tissue produced lower nuclear yield and reduced nuclear quality [1].

6.2.1. LAB MEDIA: Figure 2E.

6.3. Flow cytometry of the unstained control detected 77.88 percent singlets [1] and 0.19 percent DAPI-positive events reflecting baseline autofluorescence [2].

6.3.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the US-singlets plot and scatter 77.88 percent.*

6.3.2. LAB MEDIA: Figure 3A. *Video editor: Highlight US-scatter plot and box showing 0.19 %.*

6.4. The DAPI-stained sample showed 68.10 percent singlets [1] and 25.31 percent DAPI-positive nuclei indicating a clear fluorescence shift [2].

6.4.1. LAB MEDIA: Figure 3B. *Video editor: Highlight DAPI-singlets plot and box showing 68.1%.*

6.4.2. LAB MEDIA: Figure 3B. *Video editor: Highlight DAPI-Scatter plot and box showing 25.31%.*

6.5. Histogram analysis showed a rightward fluorescence shift of the DAPI-stained nuclei [1] relative to the unstained nuclei population [2].

6.5.1. LAB MEDIA: Figure 3C. *Video editor: Highlight blue curves*

6.5.2. LAB MEDIA: Figure 3C. *Video editor: Highlight red curves.*

6.6. Confocal imaging of the stained sample showed bright blue fluorescent nuclei that

appeared intact and uniformly labeled [1].

6.6.1. LAB MEDIA: Figure 3D.

1. RPMI
Pronunciation link: <https://www.merriam-webster.com/dictionary/RPMI>
IPA: /ˌär-pē-,em-'ī/
Phonetic Spelling: ar·pee·em·ee
2. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /ˌmaɪkroʊ'sentrɪfjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
3. Lysis
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysis>
IPA: /'laɪsɪs/
Phonetic Spelling: ly·suhs
4. Pestle
Pronunciation link: <https://www.merriam-webster.com/dictionary/pestle>
IPA: /'pesəl/
Phonetic Spelling: peh·suhl
5. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrɪfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
6. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /suː'pɜːneɪtənt/
Phonetic Spelling: soo·per·nay·tuht
7. Pellet
Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
IPA: /'pelɪt/
Phonetic Spelling: peh·luht
8. Hemocytometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/hemocytometer>
IPA: /ˌhiːmoʊ'saɪ'tɑːmɪtər/
Phonetic Spelling: hee·moh·sy·taa·muh·ter
9. Trypan
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypan>
IPA: /'traɪpæn/
Phonetic Spelling: try·pan
10. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

- IPA: /'ælikwa:t/
Phonetic Spelling: al·ih·kwaat
11. Microliter
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪkroʊˌli:tər/
Phonetic Spelling: my·kroh·lee·ter
 12. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊ'reɪsəns/
Phonetic Spelling: floor·eh·suhns
 13. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /saɪ'tɑ:mətri/
Phonetic Spelling: sy·taa·muh·tree
 14. Bovine
Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>
IPA: /'boʊvɪn/
Phonetic Spelling: boh·vyne
 15. Albumin
Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>
IPA: /æl'bjʊ:mɪn/
Phonetic Spelling: al·byoo·min
 16. Bivariate
Pronunciation link: <https://www.merriam-webster.com/dictionary/bivariate>
IPA: /baɪ'veəriət/
Phonetic Spelling: by·vair·ee·uht
 17. Autofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/autofluorescence>
IPA: /ˌɔ:təʊflʊ'reɪsəns/
Phonetic Spelling: aw·toh·floor·eh·suhns
 18. DAPI
Pronunciation link: <https://www.howtopronounce.com/dapi>
IPA: /'dæpi/
Phonetic Spelling: da·pee
 19. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kən'fəʊkəl/
Phonetic Spelling: kon·foh·kuhl
 20. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪ'kra:skəpi/
Phonetic Spelling: my·krah·skuh·pee