

Submission ID #: 69547

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Title: Preparation of Neovascular Tissues from Human Glioma Tissues for Quantitative Proteomics Analysis of Tumor Angiogenesis

Authors and Affiliations:

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**

Authors: Can these steps in step 3.3 be visualized on screen or under the microscope directly on the filming day? Our videographer will film them on the spot. If not, please let me know and we can use the images you have

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

Authors: There are only 2 shots with screen usage, our videographer will film them

3. Filming location: Will the filming need to take place in multiple locations? **YES**

If **Yes**, how far apart are the locations? The two laboratories are about 2 kilometers apart.

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: 21

Number of Shots: 56

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 will be slightly edited in accordance with the journal guidelines

INTRODUCTION:

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

- 1.1. **Xiaoxia Feng:** We perform proteomic profiling of glioma vasculature across tumor grades to identify biomarkers and therapeutic targets, revealing mechanisms driving angiogenesis and tumor progression.
 - 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. **Xiaoxia Feng:** Existing methods face protein degradation, incomplete lysis, scarce Grade I samples, and non-vascular contamination during LCM. This protocol optimizes handling and improves vascular protein profiling accuracy.
 - 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. **Xiaoxia Feng:** This protocol enables analysis of differential protein expression in glioma vasculature, identifying grade-specific angiogenesis-related proteins and quantifying proteomic changes using iTRAQ technology.
 - 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. **Xiaoxia Feng:** An important challenge is to locate tumor neovascularization under an optical microscope and collect the dissected vessels accurately.

- 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. **Xiaoxia Feng:** Our future studies will analyze grade-specific angiogenesis proteomes, PTMomics, pathway hubs, and multiomics-integrated biomarkers and drug targets.
 - 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**.

NOTE: Authors have written the answers for testimonial questions after the shoot, these were not scripted before.

What problem in your field of research does your article address?

- 1.6. **Xianquan Zhan, Professor at Shandong First Medical University** (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

Xianquan Zhan: Tumor angiogenesis is a very important pathophysiological phenomenon, it is a hallmark in cancer. Previous most of studies just focus on the angiogenesis inhibitor and activator research. Thus has many limitations. This video article used laser capture microdissection to purify and isolate neovascular tissues from glioma for direct proteomics and PTMomics analysis to find key protein and post-translational modification changes related to tumor angiogenesis, which has important scientific merits in clarification of molecular mechanism, discovery new biomarkers and drug targets, and even new anti-angiogeneiss drugs.

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

- 1.7. **Xianquan Zhan, Professor at Shandong First Medical University** (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

Xianquan Zhan: For the key takeaway after you watching the is video article, I think four items should be careful.
First, quality of control (QC) is very important.

Second, how to prevent the protein degradation

Third, how to accumulate the sufficient amounts of proteins for proteomic analysis, especially PTMomics analysis.

Forth, how to extract protein from the LCM-isolated neovascular tissues, and quantify the protein content, which must avoid any lose of proteins.

All of these items are very import for the successful experiments.

Ethics Title Card

This research has been approved by the Medical Ethics Review Committee of Shandong First Medical University

Protocol

2. Mounting Human Glioma Tissue on Laser-Capture Microdissection (LCM) Slide

Demonstrator: Xiaoxia Feng

- 2.1. To begin, obtain polyethylene naphthalate membrane and reusable slides [1]. Manually coat polyethylene naphthalate membrane onto the reusable slide [2] and store the coated slide at minus 80 degrees Celsius [3].
 - 2.1.1. WIDE: Talent placing the membrane and slides on the work desk.
 - 2.1.2. Talent manually coating polyethylene naphthalate membrane onto reusable slide. **Author's NOTE:** When photographer took photo/shot, there was no metal slide available, therefore, we used glass slide to replace it, simulate it. VO is modified accordingly
 - 2.1.3. Talent placing the coated slide into a minus 80 degrees Celsius freezer.
- 2.2. Maintain the cryostat at minus 25 degrees Celsius for both the chamber and specimen head [1]. Retrieve glioma tissue from minus 80 degrees Celsius storage and transfer it on ice [2]. Remove residual blood with filter paper [3].
 - 2.2.1. Talent adjusting the cryostat to minus 25 degrees Celsius for the chamber and specimen head.
 - 2.2.2. Talent removing glioma tissue from a minus 80 degrees Celsius freezer and placing it on ice.
 - 2.2.3. Talent gently blotting the tissue with filter paper to remove blood.
- 2.3. Apply embedding medium to the pre-cooled disc [1] and rapidly embed the tissue block [2]. Freeze the entire block at minus 25 degrees Celsius for 10 minutes to harden [3].
 - 2.3.1. Talent applying embedding medium onto the specimen disc.
 - 2.3.2. Talent positioning and embedding the tissue block in the medium.
 - 2.3.3. Talent placing the embedded tissue block in the cryostat set to minus 25 degrees Celsius.
- 2.4. After sectioning the tissue, mount the tissue sections onto a standard slide [1]. Stain the sections with the 0.5 percent toluidine blue solution [2] and screen neovascular vessels under an optical microscope [3].

2.4.1. ~~Talent sectioning the tissue at 8 micrometer thickness using the cryostat.~~
Author's NOTE: When photographer took photo/shot, the cryostat is broken, therefore, just took the picture of this instrument. We cannot have only a picture of the instrument without action, deleted and adjusted VO

2.4.2. Talent mounting tissue sections onto a standard slide.

2.4.3. Talent applying toluidine blue staining solution onto the tissue sections.

2.4.4. Talent examining stained sections under an optical microscope.

2.5. Then, transfer vessel-positive tissue section onto polyethylene naphthalate-coated laser capture microdissection slide [1]. Seal the slide, label it [2], and store at minus 80 degrees Celsius [3].

2.5.1. Talent transferring vessel-positive tissue section onto polyethylene naphthalate-coated laser capture microdissection slide.

2.5.2. Talent sealing the slide and sticking a label on the slide.

2.5.3. Talent placing the slide into a minus 80 degrees Celsius freezer.

2.6. Fix the slide-mounted tissue, stain it and cryopreserve the processed sample at minus 80 degrees Celsius [1 and 2].

2.6.1. *Reuse 2.4.3 and 2.5.3*

2.6.2. TEXT ON 2.6.1's BACKGROUND:

- **Fix:** Pre-chilled 70% ethanol (4 °C, 1 - 2 min)
- **Stain:** 0.5% Toluidine blue (4 °C, 30 s)
- **Rinse 2x:** ddH₂O at 4 °C using two separate wash baths
- **Add:** Protease inhibitor solution for 30 s

3. Isolating Neovascular Tissues from Mounted Glioma Sample Using Laser-Capture Microdissection (LCM)

3.1. Start the laser capture microdissection instrument in advance and preheat for 30 minutes [1]. Use the solid-state infrared laser to preheat for another 30 minutes [2] and install a 500-microliter microcentrifuge tube collection device [3].

3.1.1. Talent turning on the laser capture microdissection instrument for preheating.
Videographer's NOTE: 3.1.1 and 3.1.2 were merged and filmed as 3.1.1

- 3.1.2. Talent activating the solid-state infrared laser.
- 3.1.3. Talent installing a 500 microliter microcentrifuge tube collection device onto the instrument.
- 3.2. Next, load the tissue sample-mounted laser capture microdissection slide onto the prepared instrument [1] and adjust the focus on neovascular vessels in glioma tissues using the compatible software [2].
 - 3.2.1. Talent placing the laser capture microdissection slide onto the instrument stage.
 - 3.2.2. LAB MEDIA: LCM_20150515III-10_staining-to-find-neovascular-vessel.bmp.
Video editor: Highlight the dark purple snake kind of vessel at the center
Author's NOTE: This experiment was done 11 years ago in 2015 with a very old computer and we can only provide these images. We cannot provide a better-quality image as this is all we have for 3.2.2, 3.3.1 and 3.3.2
- 3.3. Outline the laser-marking line precisely along the outer edge of the neovascular vessel while avoiding the vascular wall [1]. Use the laser to burn and separate the neovascular vessel [2-TXT].
 - 3.3.1. LAB MEDIA: LCM_20150515III-11_drawing a line around neovascular vessel.bmp. *Video editor: Highlight the red outlined region at the center*
 - 3.3.2. LAB MEDIA: LCM_20150515III-14_cutting the neovascular vessel by laser.bmp.
Video editor: Highlight the white empty part at the center from where snake shaped vessel is removed
TXT: Collect the isolated vessel into the microcentrifuge tube cap
- 3.4. Invert and seal the collected neovascular vessel sample tube [1] and store the tube in a minus 80 degrees Celsius freezer [2].
 - 3.4.1. Talent inverting and sealing the microcentrifuge tube containing the collected neovascular vessel sample.
 - 3.4.2. Talent placing the sealed tube into a minus 80 degrees Celsius freezer.

4. Extraction of Proteins from LCM-Isolated Glioma Neovascular Tissues

- 4.1. Resuspend laser capture microdissection-collected glioma neovascular vessel tissue in

the protein extraction buffer [1]. Sonicate the suspension in an ice-water bath for 1 minute to achieve complete lysis [2].

4.1.1. Talent adding extraction buffer to the laser capture microdissection-collected tissue sample and resuspending it.

4.1.2. Talent placing the tube into an ice-water bath and sonication device for 1 minute.

4.2. Centrifuge the lysate at 16000 *g* for 10 minutes [1] and transfer the supernatant to a new microcentrifuge tube [2].

4.2.1. Talent placing the tube into a centrifuge set to 16000 *g* for 10 minutes. **Author's NOTE: Just took the picture of the machine.**

4.2.2. Talent transferring the clear supernatant into new microcentrifuge tube.

4.3. Now, aliquot a minimal volume of the supernatant for protein quantification [1-TXT]. Add dithiothreitol to the remaining supernatant to a final concentration of 1 millimolar and mix gently by pipetting to avoid bubble formation [2]. After aliquoting the treated supernatant, store it at minus 80 degrees Celsius [3].

4.3.1. Talent pipetting a small aliquot of supernatant into a separate tube for protein quantification. **TXT: Perform bicinchoninic acid (BCA) protein assay for quantification**

4.3.2. Talent adding dithiothreitol to the remaining supernatant and mixing by pipetting up and down.

4.3.3. Talent placing the aliquoted tubes into a minus 80 degrees Celsius freezer.

5. Preparing Tryptic Peptide Samples of the Extracted Neovascular Proteins

5.1. Add ice-cold acetone to each extracted protein sample at a sample to acetone ratio of 1 to 5 volume per volume [1]. After mixing, incubate the sample at minus 20 degrees Celsius for 1 hour [2] and then, centrifuge at 12000 *g* at 4 degrees Celsius for 15 minutes [3]. Discard the supernatant to remove sodium dodecyl sulfate, dithiothreitol, and Tris hydrochloride [4]. Resuspend the pellet in 20 microliters of dissolution buffer containing 1 microliter of 1 percent sodium dodecyl sulfate [5].

5.1.1. Talent adding ice-cold acetone to each extracted protein sample tube.

- 5.1.2. Talent placing the tubes into a minus 20 degrees Celsius freezer.
- 5.1.3. Talent placing the tube into a centrifuge set to 12000 g at 4 degrees Celsius.
Author's NOTE: Just took the picture of the machine.
- 5.1.4. Talent carefully discarding the supernatant without disturbing the pellet.
- 5.1.5. Talent adding dissolution buffer to the pellet and resuspending it.

5.2. Next, add 2 microliters of dithiothreitol reducing reagent to the sample and mix thoroughly [1]. Incubate at 60 degrees Celsius for 1 hour [3]. Then, add 1 microliter of iodoacetamide cysteine-blocking reagent and incubate at room temperature for 10 minutes [3].

- 5.2.1. Talent adding dithiothreitol to each sample tube and mixing the samples thoroughly.
- 5.2.2. Talent placing the tubes into a heating device set to 60 degrees Celsius for 1 hour.
- 5.2.3. Talent adding iodoacetamide to the sample and keeping the tube aside.

5.3. Add trypsin to each sample at a protein-to-trypsin ratio of 20 to 1 volume per volume [1]. Vortex-mix the sample [2] and incubate it at 37 degrees Celsius overnight [3].

- 5.3.1. Talent adding trypsin to each sample tube.
- 5.3.2. Talent vortex-mixing the sample.
- 5.3.3. Talent placing the tube into an incubator set to 37 degrees Celsius.

6. Preparation of iTRAQ-Labeled Tryptic Peptide Sample for Strong Cation Exchange (SCX) Fractionation

6.1. Reconstitute each iTRAQ (*eye-track*) sample with 50 microliters of isopropanol [1-TXT]. Add the corresponding tryptic peptide sample to the reagent tube and incubate at room temperature for 1 hour [2].

- 6.1.1. Talent adding 50 microliters of isopropanol to reagent tube and mixing to dissolve. **TXT: iTRAQ: Isobaric Tag for Relative and Absolute Quantification**
- 6.1.2. Talent transferring tryptic peptide sample into the corresponding reagent tube and placing the tube aside.

- 6.2. After incubation, add three times the reaction volume of ultrapure water to terminate the reaction [1] and incubate at room temperature for 30 minutes [2-TXT].
- 6.2.1. Talent adding ultrapure water to each reaction tube.
- 6.2.2. Talent keeping the tube aside. **TXT: Pool samples from 6 such tubes; Lyophilize the pooled sample**
- 6.2.3. ~~Talent placing the pooled sample into a lyophilizer.~~ **NOTE: Delete the shot as a picture for this won't look good, VO moved as on screen text**
- 6.3. Reconstitute the lyophilized peptides in 2 milliliters of Solution A containing 10 millimolar potassium dihydrogen phosphate, 25 percent acetonitrile, at pH 2.7 [1]. ~~After running the sample through a sulfoethyl functionalized cation exchange column, collect fractions every minute into separate tubes [2].~~
- 6.3.1. Talent adding Solution A to the lyophilized peptide sample and mixing to dissolve.
- 6.3.2. ~~Talent loading the reconstituted sample onto the sulfoethyl functionalized cation exchange column. **TXT: Column: 4.6 × 100 mm; 5 μm; 200 Å**~~
- Author's NOTE: Did not film as the column was not available**
- 6.4. After running the sample through a sulfoethyl functionalized cation exchange column, collect fractions using suitable buffer gradients into separate tubes [1] ~~[1-TXT]. Monitor absorbance at 214 nanometers throughout the process and collect fractions every minute into separate tubes for a total of 30 fractions [2].~~
- 6.4.1. ~~Talent operating the chromatography system to run the specified gradient at 1 milliliter per minute. **TXT: 0% → 10% (2 min) ; 10% → 20% (25 min) ; 20% → 45% (5 min) ; 50% → 100% (5 min)**~~
- 6.4.2. ~~Shot of Shot of fraction tubes with solution being collected sequentially during monitoring at 214 nanometers.~~ **Author's NOTE: Did not film this step as the column was not available, we took a picture of the fractionation result instead**
- 6.5. Finally, concentrate each fraction by vacuum centrifugation [1]. Reconstitute each fraction with 40 microliters of 0.1 percent volume per volume trifluoroacetic acid [2] and store all fractions at minus 80 degrees Celsius [3]. Perform LC-MS-MS to analyze each fractionated peptide sample [4].
- 6.5.1. Talent placing collected fraction tubes into a vacuum centrifuge.
- 6.5.2. Talent adding 0.1 percent trifluoroacetic acid to each dried fraction and mixing.

6.5.3. Talent placing the reconstituted fractions into a minus 80 degrees Celsius freezer.

Added shot: Shot of the LC MS instrument

Results

7. Results

7.1. The distribution of mass deviation of identified peptides exhibited a normal distribution centered around zero [1].

7.1.1. LAB MEDIA: Figure 1A

7.2. The peptide length distribution showed that the vast majority of peptides had lengths of 8 to 20 amino acid residues [1].

7.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the 8 bars from the left FROM [5,7] to [19,21].*

7.3. The distribution of relative mass of all identified proteins shows that almost 100% of the protein molecular weights are distributed between zero to 200 kilodaltons [1], and most of the protein isoelectric points are distributed between 4 to 10 [2].

7.3.1. LAB MEDIA: Figure 1C.

7.3.2. LAB MEDIA: Figure 1D. *Video editor: Highlight the bars.*

7.4. Quantitative analysis identified 371 differentially expressed proteins in glioma neovascular tissues compared to controls [1], including 170 upregulated proteins [2] and 201 downregulated proteins [3].

7.4.1. LAB MEDIA: Figure 2.

7.4.2. LAB MEDIA: Figure 2. *Video editor: Highlight the label "Upregulated DEPs (n=170)".*

7.4.3. LAB MEDIA: Figure 2. *Video editor: Highlight the label "Downregulated DEPs (n=201)".*

7.5. Gene ontology analysis identified 76 statistically significant cellular components [1], 75 statistically significant biological processes [2], and 46 statistically significant molecular functions [3] among the 371 differentially expressed proteins [4].

7.5.1. LAB MEDIA: Figure 3. *Video editor: Highlight the cellular component panel labeled "CC".*

7.5.2. LAB MEDIA: Figure 3. *Video editor: Highlight the biological process panel labeled "BP".*

7.5.3. LAB MEDIA: Figure 3. *Video editor: Highlight the molecular function panel labeled “MF”.*

7.5.4. LAB MEDIA: Figure 3

7.6. Kyoto Encyclopedia of Genes and Genomes pathway analysis identified 23 statistically significant signaling pathway changes among the 371 differentially expressed proteins [1].

7.6.1. LAB MEDIA: Figure 4.

7.7. Protein–protein interaction network analysis identified 25 hub differentially expressed proteins using 4 algorithms [1].

7.7.1. LAB MEDIA: Figure 5. *Video editor: Highlight the panels labeled Degree, MNC, MCC, and DMNC in the network analysis figure.*

1. Glioma
Pronunciation link: <https://www.merriam-webster.com/dictionary/glioma>
IPA: /gli'oomə/
Phonetic Spelling: glee·oh·muh
2. Polyethylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyethylene>
IPA: /,pɑ:li'εθəli:n/
Phonetic Spelling: pah·lee·eth·uh·leen
3. Naphthalate
Pronunciation link: <https://www.merriam-webster.com/dictionary/naphthalate>
IPA: /'næfθə,leit/
Phonetic Spelling: naf·thuh·layt
4. Cryostat
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryostat>
IPA: /'kraɪə,stæt/
Phonetic Spelling: kry·uh·stat
5. Microdissection
Pronunciation link: <https://www.merriam-webster.com/dictionary/microdissection>
IPA: /,maɪkroudi'sɛkʃən/
Phonetic Spelling: my·kroh·duh·sek·shuhn
6. Neovascular
Pronunciation link: <https://www.merriam-webster.com/dictionary/neovascular>
IPA: /,ni:oo'væskjələr/
Phonetic Spelling: nee·oh·vas·kyuh·ler

7. Toluidine
Pronunciation link: <https://www.merriam-webster.com/dictionary/toluidine>
IPA: /təˈluːəˌdiːn/
Phonetic Spelling: tuh·loo·uh·deen
8. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
9. Lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /ˈlaɪˌseɪt/
Phonetic Spelling: ly·sayt
10. Dithiothreitol
Pronunciation link: <https://www.merriam-webster.com/dictionary/dithiothreitol>
IPA: /daɪˌθaɪoʊˈθriːəl/
Phonetic Spelling: dye·thy·oh·three·awl
11. Iodoacetamide
Pronunciation link: <https://www.merriam-webster.com/dictionary/iodoacetamide>
IPA: /ˌaɪoʊdoʊəˈsiːtəˌmaɪd/
Phonetic Spelling: eye·oh·doh·uh·see·tuh·myd
12. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrɪpsɪn/
Phonetic Spelling: trip·sin
13. Isopropanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/isopropanol>
IPA: /ˌaɪsoʊˈproʊpəˌnɔːl/
Phonetic Spelling: eye·soh·proh·puh·nawl
14. Lyophilize
Pronunciation link: <https://www.merriam-webster.com/dictionary/lyophilize>
IPA: /laɪˈɑːfəˌlaɪz/
Phonetic Spelling: ly·ah·fuh·lyz
15. Acetonitrile
Pronunciation link: <https://www.merriam-webster.com/dictionary/acetonitrile>
IPA: /əˌsiːtoʊˈnætraɪl/
Phonetic Spelling: uh·see·toh·ny·tryl
16. Trifluoroacetic
Pronunciation link: <https://www.merriam-webster.com/dictionary/trifluoroacetic>
IPA: /ˌtraɪˌfluəroʊəˈsiːtɪk/
Phonetic Spelling: try·floor·oh·uh·see·tik
17. Chromatography
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatography>
IPA: /ˌkroʊməˈtɑːɡrəfi/
Phonetic Spelling: kroh·muh·taa·gruh·fee

18. Protease

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

IPA: /'prouti, eɪs/

Phonetic Spelling: 360h·tee·ays

19. Bicinchoninic

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

IPA: /ˌbaɪsɪnkoo'niːnɪk/

Phonetic Spelling: by·sin·koh·nin·ik

20. Isoelectric

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

IPA: /ˌaɪsoʊi'lektrɪk/

Phonetic Spelling: eye·soh·ih·lek·trik