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Title: Elastic Fiber Profiling in Lung Tissue Using Quantitative Imaging Analyses

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? **YES.**

- 3. Filming location:** Will the filming need to take place in multiple locations? **YES**
within 50 meter on the same floor but separate room

- 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: 38

Number of Shots: 54

Interviews

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

INTRODUCTION:

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

- 1.1. **Veronika Lukacs-Kornek:** We study how fibroblasts and the extracellular matrix shape tissue function in health and disease.
 - 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. **Veronika Lukacs-Kornek:** Existing methods lack standardized microscopy-based approaches to quantitatively assess elastic fiber architecture in tissues.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.3. **Pablo S. Bravo:** This protocol can be applied to microscopy images across organs, disease models, and aging studies involving elastic fibers.
 - 1.3.1. 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?~~

- 1.4. **Xue Qian:** This protocol quantifies elastic fiber architecture, topology, and remodeling directly from tissue microscopy images.
 - 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. **Qianying Chen:** Future studies can link elastic fiber remodeling to cellular states, disease mechanisms, and treatment responses.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **NOTE: Added Shot: 5.2**

Added statement

~~Describe the most important consideration or challenge when performing this protocol.~~

"Added Shot" 4.2 Xue Qian: Consistent image quality and accurate fiber segmentation are essential for robust and reproducible measurements

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

Ethics Title Card

This research has been approved by the Animal Ethics Committee of the state of North Rhine-Westphalia, Germany

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Elastic Fiber Staining

Demonstrator: NOTE: Not provided

2.1. To begin, take paraffin-embedded lung tissue sections [1], immerse the sections in xylene mixed isomers two times for 5 minutes each to deparaffinate the tissue sections [2].

2.1.1. Establishing shot of the talent with tissue slides placed in front of him.

***Videographer:** Please obtain CU shot as well for this shot.*

2.1.2. Talent placing the slides into a staining chamber containing xylene mixed isomers.

2.2. Rehydrate the slides using a descending ethanol series of 100% [1], 95% [2], and 80% ethanol for 3 minutes each at room temperature. Repeat this process 3 times. [3].

2.2.1. Talent transferring the slides into the staining chamber containing 100% ethanol.

Added Shot" 2.2.2 Talent transferring the slides into the staining chamber containing 100% ethanol.

Added Shot" 2.2.3 Talent transferring the slides into the staining chamber containing 95% ethanol.

Added Shot" 2.2.4 Talent transferring the slides into the staining chamber containing 80% ethanol.

NOTE: It's the same thing the authors have shown it separately for every solution

2.3. Then, transfer the slide into a new staining chamber containing the Resorcin-Fuchsin solution and incubate for 20 to 30 minutes at room temperature [1].

2.3.1. Talent placing the slide into the staining chamber containing the Resorcin-Fuchsin solution and keeping it aside.

2.4. Place the slide in a staining chamber under running distilled water and wash for 1 minute [1].

2.4.1. Talent placing the slide in a staining chamber under running distilled water.

2.5. Transfer the slide to 95 to 96 percent ethanol and differentiate for 15 to 60 seconds at room temperature [1]. Monitor the differentiation under the microscope [2].

- 2.5.1. Talent placing the slide into a staining chamber containing 95 to 96 percent ethanol.
- 2.5.2. SCOPE: Elastic fibers becoming distinctly visible during differentiation **NOTE: 2.5.2. filmed with the phone. Filename is: IMG_1940**
- 2.6. Immediately transfer the slide into bidistilled water to stop the differentiation and rinse for 30 seconds **[1]**.
 - 2.6.1. Talent transfers the slide into a staining chamber containing bidistilled water.
- 2.7. Then, dehydrate the slides sequentially in 95 percent ethanol for 1 to 2 minutes **[1]**, followed by 100 percent ethanol two times for 1 to 2 minutes each to remove the residual water **[2] [3]**. Clear the tissue sections in xylene two times for 3 minutes until the tissue appears optically transparent **[4]**.
 - ~~2.7.1. Talent transferring the slides through the ethanol staining chambers in sequence. **NOTE: Do not use this**~~
 - “Added Shot” 2.7.1.1** Talent transferring the slides into the staining chamber containing 95% ethanol.
 - “Added Shot” 2.7.1.2** Talent transferring the slides into the staining chamber containing 100% ethanol.
 - “Added Shot” 2.7.1.3** Talent transferring the slides into the staining chamber containing 100% ethanol.
 - 2.7.2. Talent placing the slides into a staining chamber containing xylene.
 - Added Shot” 2.7.2.1**Talent transferring the slides into the staining chamber containing xylol
 - Added Shot” 2.7.2.2** Talent transferring the slides into the staining chamber containing xylol
- NOTE: It's the same thing the authors have shown it two times**
- 2.8. Using a xylene-compatible permanent synthetic resin, mount the coverslips and allow the slides to dry flat at room temperature **[1]**.
 - 2.8.1. Talent mounting the coverslips using a xylene-compatible permanent synthetic resin and placing it aside to dry.

3. Image Deconvolution: Stain Vector Estimation in QuPath

Demonstrator: Pablo S. Bravo

- ~~3.1. After acquiring high-resolution brightfield images of the lung tissue sections using a slide scanner with a 40× **(Forty X)** objective **[1]**, draw a region of interest or ROI **(R-O-I)** encompassing the entire lung tissue section to ensure complete coverage during scanning **[2]**. **NOTE: Not filmed**~~

~~3.1.1. SCREEN: 00:04-00:18~~

~~3.1.2. SCREEN: 00:18-00:28~~

~~3.2. Define a background reference area without tissue to measure and subtract the background intensity during the image analysis [1].~~

~~3.2.1. SCREEN: Background reference area without tissue being selected.~~

~~3.3. Use the scanner's automatic focus detection to assign an optimal number of focal points across the tissue [1]. Acquire the image using uniform illumination and exposure settings [2].~~

~~3.3.1. SCREEN: Focal points are being assigned across the tissue.~~

~~3.3.2. SCREEN: Final image being acquired using uniform illumination and exposure settings.~~

3.4. After obtaining high-resolution images of lung tissue sections, open the image in QuPath (*Qu-Path*) and select the Brightfield image type [1]. Draw an ROI containing predominantly elastic fibers to define the first stain component [2].

3.4.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:05-00:07, 00:13-00:18

3.4.2. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:18-00:28

3.5. In the Image tab, navigate to the Stain Vectors section [1]. Click Stain 1 and select Set stain vector from ROI to define the color vector for the elastic fibers [2].

3.5.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:28-00:31

3.5.2. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:31-00:36

3.6. Select a region of interest containing non-elastic tissue. Click Stain 2 and select Set stain vector from ROI to define the secondary color component [1].

3.6.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:36-00:49

3.7. Allow QuPath to automatically calculate Stain 3, corresponding to the background [1].

3.7.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:49-00:54

3.8. Save the stain vectors as custom stain vectors and apply them for consistent color deconvolution across all samples [1].

3.8.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 00:58-01:00,01:32-01:38

4. Image Color Deconvolution in Fiji

4.1. Open Fiji and load the brightfield image of the stained tissue section [1].

- 4.1.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 01:43-01:47, 01:52-01:56, 02:11-02:12
- 4.2. If the image is not already in the RGB (*R-G-B*) format, select **Image**, **Type**, and **RGB Color** to convert the image [1]. Then, save the new image to preserve the original raw data [2].
 - 4.2.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 02:12-02:23
 - 4.2.2. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 02:26-02:30, 02:38-02:41
- 4.3. Select **Image**, **Color**, **Color Deconvolution**, and **User Values** to perform the color deconvolution [1].
 - 4.3.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 02:41-02:50
- 4.4. Then, enter the custom stain vector obtained from QuPath [1].
 - 4.4.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 03:02-03:05, 03:38-03:41
- 4.5. Run the plugin to generate 3 separate grayscale images corresponding to the elastic fibers, the tissue excluding the elastic fibers, and the background [1].
 - 4.5.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 03:42-03:45. 06:56-07:06
- 4.6. Use the generated images directly for TWOMBLI (*Twombli*) without additional preprocessing [1]. Save the elastic fiber channel as a TIFF file for the downstream quantification and analysis [2].
 - 4.6.1. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 07:13-07:21.
 - 4.6.2. SCREEN: Screen-Recording-2026-07-20-at-11.21.25.MOV: 07:21-07:27, 07:34-07:36

5. ROI Selection for Fiber Analysis

- 5.1. Select 5 unbiased square ROI in the alveolar region and peribronchiolar region, each measuring 100 by 100 micrometers [1].
 - 5.1.1. SCREEN: Screen-Recording-2026-07-20-at-12.40.30.MOV: 00:19-00:33, 00:37-00:41, 01:23-01:27, 05:19-05:22, 06:27-06:29
- 5.2. Select **Image** and **Duplicate** to duplicate each region of interest on the raw image and preserve the original data [1]. Then, duplicate the same regions of interest on the deconvoluted elastic fiber image [1].
 - 5.2.1. SCREEN: Screen-Recording-2026-07-20-at-12.40.30.MOV: 01:35-01:40, 02:25-02:33
 - 5.2.2. SCREEN: Screen-Recording-2026-07-20-at-12.40.30.MOV: 03:35-03:44, 04:18-

04:23

5.3. Save all duplicated ROI as individual TIFF files for the downstream analysis [1].

5.3.1. SCREEN: Screen-Recording-2026-07-20-at-12.40.30.MOV: 04:35-04:40

5.4. Next, open the image in QuPath and set the image type to **Brightfield** [1].

5.4.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.Mov: 00:04-00:11

5.5. From the Tools menu, select the desired annotation tool to create the initial annotations [1].

5.5.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 00:11-00:13

5.6. Draw square annotations in the lung tissue, isolating either an alveolar region or a peribronchial area for analysis [1].

5.6.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 00:13-00:18

5.7. Navigate to Annotations and select Lock Annotations to prevent accidental movement or modification [1]. Click the + (*Plus*) button next to Class List to add the classes, alveoli, and the peribronchial area [1]. Select each annotation. Choose the corresponding class and click **Set Selected** to assign the annotation [2].

5.7.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 00:33-00:37

5.7.2. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 00:37-00:52

5.7.3. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 00:53-00:57, 01:03-01:08

5.8. Open the corresponding script and execute it [1]. Confirm that the ROIs have been generated successfully [2].

5.8.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 03:20-03:30

5.8.2. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 03:30-03:33

5.9. Then, select the generated ROI. Navigate to File, Export Images, and Original Pixels to export the ROI [1] and save the exported ROI as TIFF files [2].

5.9.1. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 03:34-03:38

5.9.2. SCREEN: Screen-Recording-2026-07-20-at-12.27.25.MOV: 03:58-04:03, 04:10-04:12

6. TWOMBLI Analysis

6.1. Save all ROI images of the elastic fibers in a dedicated folder for analysis [1].

6.1.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 00:04-00:19

6.2. Create a "Mask" folder for TWOMBLI binary masks [1] and an "HDM" folder for High-

Density Matrix images [2].

6.2.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 00:24-00:29

6.2.2. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 00:19-00:24

6.3. Open Fiji and select Plugins, Macros, and Run to launch TWOMBLI [1].

6.3.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 00:30-00:41

6.4. Select the appropriate analysis parameter file [1] and choose the Dark fibers over the light background option [2].

6.4.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 00:54-01:03

6.4.2. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 01:03-01:05

6.5. Generate the binary masks and save them in the Mask folder [1]. Then, generate the binary images and save them in the HDM folder [2].

6.5.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 01:20-01:34

6.5.2. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 01:36-01:43

6.6. Finally, extract the quantitative network parameters, including the fiber length, branch points, curvature, density, and alignment, followed by appropriate statistical analysis[1].

6.6.1. SCREEN: Screen-Recording-2026-07-20-at-12.58.33.MOV: 01:45-01:55

Results

7. Results

7.1. Reproducibility testing of the workflow demonstrated similar quantitative outputs when datasets were independently analyzed by two researchers using the same optimized parameters [1]. Similar results were also obtained across samples from independent animals, demonstrating consistency across operators and biological replicates [2].

7.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the dots for A and B groups in all the graphs*

7.1.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the dots for M1, M2, and M3 groups in all the graphs*

1. Paraffin
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraffin>
IPA: /'perəfɪn/
Phonetic Spelling: PAIR·uh·fin
2. Xylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/xylene>
IPA: /'zaɪ,li:n/
Phonetic Spelling: ZYE·leen
3. Deparaffinate
Pronunciation link: No confirmed link found
IPA: /di:pə'ræfə,neɪt/
Phonetic Spelling: dee·puh·RAF·uh·nayt
4. Rehydrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/rehydrate>
IPA: /,ri:'haɪ,dreɪt/
Phonetic Spelling: ree·HYE·drayt
5. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /'εθə,nɒl/
Phonetic Spelling: ETH·uh·nawl

6. Resorcin-Fuchsin
Pronunciation link: No confirmed link found
IPA: /rɪˈzɔrsɪn ˈfjuːksɪn/
Phonetic Spelling: rih·ZOR·sin FYOOK·sin
7. Bidistilled
Pronunciation link: No confirmed link found
IPA: /ˌbaɪdɪˈstɪld/
Phonetic Spelling: bye·dih·STILD
8. Dehydrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/dehydrate>
IPA: /diːˈhaɪˌdreɪt/
Phonetic Spelling: dee·HYE·drayt
9. Deconvolution
Pronunciation link: No confirmed link found
IPA: /ˌdiːkɒnvəˈluːʃən/
Phonetic Spelling: dee·kon·vuh·LOO·shuhn
10. QuPath
Pronunciation link: No confirmed link found
IPA: /ˈkjuːˌpæθ/
Phonetic Spelling: KYOO·path
11. Brightfield
Pronunciation link: No confirmed link found
IPA: /ˈbraɪtˌfiːld/
Phonetic Spelling: BRYTE·feeld
12. Elastic
Pronunciation link: <https://www.merriam-webster.com/dictionary/elastic>
IPA: /ɪˈlæstɪk/
Phonetic Spelling: ih·LAS·tik
13. Fiji
Pronunciation link: <https://www.merriam-webster.com/dictionary/Fiji>
IPA: /ˈfiːdʒi/
Phonetic Spelling: FEE·jee
14. Grayscale
Pronunciation link: <https://www.merriam-webster.com/dictionary/grayscale>
IPA: /ˈɡreɪˌskeɪl/
Phonetic Spelling: GRAY·skayl
15. TWOMBLI
Pronunciation link: No confirmed link found
IPA: /ˈtwɒmbli/
Phonetic Spelling: TWOM·blee
16. Quantification
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>
IPA: /ˌkwɒntəfəˈkeɪʃən/
Phonetic Spelling: kwon·tuh·fuh·KAY·shuhn

17. Alveolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/alveolar>
IPA: /æɪˈviːələr/
Phonetic Spelling: al·VEE·uh·ler
18. Peribronchiolar
Pronunciation link: No confirmed link found
IPA: /ˌpɛrɪˌbrʌŋkiˈoʊlər/
Phonetic Spelling: pair·ih·brong·kee·OH·ler
19. Peribronchial
Pronunciation link: <https://www.merriam-webster.com/medical/peribronchial>
IPA: /ˌpɛrəˈbrʌŋkiəl/
Phonetic Spelling: pair·uh·BRONG·kee·uhl
20. Annotations
Pronunciation link: <https://www.merriam-webster.com/dictionary/annotation>
IPA: /ˌænəˈteɪʃənz/
Phonetic Spelling: an·uh·TAY·shuhnz
21. Binary
Pronunciation link: <https://www.merriam-webster.com/dictionary/binary>
IPA: /ˈbɑːnəri/
Phonetic Spelling: BYE·nuh·ree
22. Curvature
Pronunciation link: <https://www.merriam-webster.com/dictionary/curvature>
IPA: /ˈkɜːvətʃər/
Phonetic Spelling: KER·vuh·cher
23. Reproducibility
Pronunciation link: <https://www.merriam-webster.com/dictionary/reproducibility>
IPA: /rɪˌprɑːdəsəˈbɪləti/
Phonetic Spelling: rih·prah·duh·suh·BIL·uh·tee
24. Quantitative
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantitative>
IPA: /ˈkwʌntəˌteɪtɪv/
Phonetic Spelling: KWON·tuh·tay·tiv
25. Biological
Pronunciation link: <https://www.merriam-webster.com/dictionary/biological>
IPA: /ˌbaɪəˈlɒdʒɪkəl/
Phonetic Spelling: bye·uh·LOJ·ih·kuhl
26. Replicates
Pronunciation link: <https://www.merriam-webster.com/dictionary/replicate>
IPA: /ˈrɛplɪkəts/
Phonetic Spelling: REP·lih·kuhts