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Title: Functional Assessment of Mouse Gingival Epithelial Barrier Using Fluorescent Tracers

Authors and Affiliations:

Shuzheng Yuan^{1*}, Qianhui Ding^{1*}, Qihua He², Xin Cong¹

¹Department of Physiology and Pathophysiology, Peking University School of Basic Medical Sciences, State Key Laboratory of Vascular Homeostasis and Remodeling

²State Key Laboratory of Natural and Biomimetic Drugs, Peking University School

***These authors contributed equally**

Corresponding Authors:

Xin Cong (congxin@bjmu.edu.cn)

Email Addresses for All Authors:

Shuzheng Yuan (yuansz0419@163.com)

Qianhui Ding (dingqh@bjmu.edu.cn)

Qihua He (hqh@bjmu.edu.cn)

Xin Cong (congxin@bjmu.edu.cn)

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? **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. **No.**

Current Protocol Length

Number of Steps: 23

Number of Shots: 42

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**.

INTRODUCTION:

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

- 1.1. **Shuzheng Yuan:** Our research focuses on the development of a standardized protocol for quantitatively assessing murine gingival epithelial barrier integrity.
 - 1.1.1. 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.~~

- 1.1. **Shuzheng Yuan:** This protocol may be useful for studying barrier dysfunction diseases and testing barrier-restorative drugs.
 - 1.1.1. 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. **Qianhui Ding:** This protocol quantifies mouse gingival epithelial barrier permeability and integrity.
 - 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. **Qianhui Ding:** The protocol can be combined with micro-CT for multimodal imaging analysis.

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. **Qianhui Ding:** Future studies can explore barrier repair drugs and oral disease molecular mechanisms.

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.

Ethics Title Card

This research has been approved by the Ethics Committee of Animal Research, Peking

Protocol

2. Assessment of Gingival Epithelial Barrier Permeability in Mouse Periodontitis Models

Demonstrator: **NOTE: Not provided**

- 2.1. To begin, divide male wild-type C57BL/6J (*C-Fifty-Seven-B-L-bar-J*) mice into 3 groups consisting of an untreated control group, a ligation-induced periodontitis group, and a *Porphyromonas gingivalis*-induced periodontitis group [1].
 - 2.1.1. Talent placing mice into labeled cages according to their groups.
- 2.2. Select fluorescent tracers with non-overlapping spectra such as FITC (*Fit-C*)-labeled dextran and rhodamine B-labeled dextran [1].
 - 2.2.1. Shot of fluorescent tracer vials labeled FITC-dextran and rhodamine B-dextran.
- 2.3. To dilute the tracers, mix 1 gram per liter each of FD40 (*F-D-Forty*) and RD70 (*R-D-Seventy*) in 2 percent carboxymethylcellulose gel [1]. Store the prepared mixture on ice [2].
 - 2.3.1. Talent pipetting both tracers into a tube containing 2 percent carboxymethylcellulose gel and mixing thoroughly.
 - 2.3.2. Talent placing the prepared tracer gel on ice.
- 2.4. Next, use a mouth gag to hold the oral cavity of an anesthetized mouse, open [1-TXT]. Apply 50 microliters of the prepared tracer mixture topically to the labial gingival surface of the molar region [2]. Leave the mixture in place for 30 minutes and reapply if swallowed [3].
 - 2.4.1. Shot of mouth gag being used to keep the mouse's oral cavity open. **TXT: Anesthesia: 1.25% Tribromoethanol**
 - 2.4.2. Talent applying tracer mixture onto the gingival surface with a pipette.
 - 2.4.3. Shot showing tracer gel covering the gingival area.
- 2.5. After 30 minutes of tracer application, fix the mouse in a supine position on cardboard [1] and secure the limbs with tape to immobilize the animal [2].
 - 2.5.1. Talent positioning the mouse supine on cardboard.
 - 2.5.2. Shot of limbs being taped securely.
- 2.6. Now, open the oral cavity [1], and isolate the maxilla [2]. Gently clear away surrounding tissues [3] and rinse the specimen thoroughly with PBS [4].
 - 2.6.1. Talent opening the oral cavity and exposing the maxilla.
 - 2.6.2. Shot of maxilla being isolated.

- 2.6.3. Talent carefully removing surrounding tissue.
- 2.6.4. Talent rinsing the maxilla with phosphate-buffered saline.
- 2.7. Place the isolated maxilla on a glass slide [1]. Then, place modeling clay on both sides to stabilize and secure the coverslip over the sample [2].
 - 2.7.1. Shot of maxilla positioned on a glass slide.
 - 2.7.2. Talent placing modeling clay and positioning a coverslip over the specimen.
- 2.8. Now carefully position the dissected maxilla onto the custom-designed sample holder to ensure stable placement for imaging [1].
 - 2.8.1. Talent securing the mounted maxilla onto the microscope sample holder.
- 2.9. Using an upright single-photon microscope equipped with a 25X water-immersion objective, focus on the mounted support region beneath the maxilla and acquire images [1].
 - 2.9.1. Talent adjusting the upright single-photon microscope and focusing on the specimen.
- 2.10. Select the region of interest on the buccal surface of the second molar within the maxilla and begin imaging promptly [1].
 - 2.10.1. SCREEN: 2.10.1 (1).mp4: 00:02-00:04, 00:07-00:15
- 2.11. Acquire Z-stack images with a step size of 1 micrometer starting from the gingival surface to a depth of 100 to 200 micrometers for three-dimensional reconstruction [1].
 - 2.11.1. SCREEN: 2.11.1.mp4: 00:03-00:09, 00:19-00:36
Video editor: Please highlight the panel on the right hand side.
- 2.12. In the software, click **File** and select **New Folder** to create a new folder for the dataset and assign a descriptive name [1]. Under the **Acquisition** tab, set the XY format to 512 by 512 with a resolution of 0.99 micrometers per pixel [2]. Then, set the **Zoom factor** to 1 [3].
 - 2.12.1. SCREEN: 2.12.mp4: 00:25-00:44, 01:05-01:07 *Video editor: Please speed it up, and highlight the panel on the right hand side*
 - 2.12.2. SCREEN: 2.12.mp4: 01:10-01:17
 - 2.12.3. SCREEN: 2.12.mp4: 01:17 *Video editor: Please freeze the frame and highlight the zoom factor tab present on the right side just above the pixel size*
- 2.13. Now set the excitation wavelengths to 488 nanometers and 561 nanometers. Configure emission windows to 500 to 550 nanometers and 570 to 620 nanometers, respectively [1].
 - 2.13.1. SCREEN: 2.13.mp4: 00:04-00:30.

- 2.14. For Z-series and three-dimensional imaging, select **ND Acquisition** mode [1]. Set the **Z-step** to 1 micrometer and adjust if necessary [2].
- 2.14.1. SCREEN: 2.14.mp4: 00:03-00:11
- 2.14.2. SCREEN: 2.14.mp4: 00:14-00:18
- 2.15. After configuring parameters, select **Live** mode to visualize the gingival area in individual and merged fluorescence channels [1]. Then, click **Run Now** to start image acquisition [2].
- 2.15.1. SCREEN: 2.15.mp4: 00:07-00:14
- 2.15.2. SCREEN: 2.15.mp4: 00:21-00:24, 05:15-05:20
- 2.16. To measure permeability, separate the fluorescence intensity of FD40 and RD70 into different channels [1]. Merge the Z-axis slices without background subtraction [2]. Then, use ImageJ software to quantify fluorescence intensity to assess epithelial barrier permeability [3].
- 2.16.1. SCREEN: 2.16.mp4: 00:20-00:29
- 2.16.2. SCREEN: 2.16.mp4: 00:56-01:02
- 2.16.3. SCREEN: 2.16.mp4: 01:59-02:10
- 2.17. Click **File** and select **Open** to open the maxilla image in ImageJ [1]. Then press **Image** and change the image type to **8-bit** format for analysis [2].
- 2.17.1. SCREEN: 2.17.mp4: 00:12-00:15
- 2.17.2. SCREEN: 2.17.mp4: 00:17-00:20
- 2.18. Click **Analyze**, select **Set Measurements**, then check **Area**, **Mean**, and **IntDen** (*Integrated-Density*), and click **OK** to confirm [1].
- 2.18.1. SCREEN: 2.18.mp4: 00:06-end
- 2.19. Then, select the desired channel and adjust the threshold by clicking **Image**, choosing **Adjust**, and selecting **Threshold** [1]. Enable the **Dark Background** option to account for black backgrounds [2].
- 2.19.1. SCREEN: 2.19.mp4: 00:11-00:16
- 2.19.2. SCREEN: : 2.19.mp4: 00:16-00:18
- 2.20. Now click **Analyze** and select **Set Measurements**, then check **Mean gray value** and **Limit to threshold**, and confirm by clicking **OK** [1].
- 2.20.1. SCREEN: 2.20.mp4: 00:04-00:12
- 2.21. Using the **Freehand Selections** tool, manually trace the outline of the fluorescent signal region on the gingival tissue side [1]. Click **Analyze** and select **Measure** to obtain fluorescence intensity values where **IntDen** represents the fluorescence intensity [2].

2.21.1. SCREEN: 2.21.mp4: 00:06-00:27

2.21.2. SCREEN: 2.21.mp4: 00:28-end

2.22. Copy the measurement results into a spreadsheet for calculations and graphing [1].

2.22.1. SCREEN: 2.22.mp4: 00:03-00:12

2.23. Calculate the **mean fluorescence intensity** of the control group in the spreadsheet [1].
Divide each experimental group's fluorescence intensity value by the control group mean to obtain the relative fluorescence intensity [2].

2.23.1. SCREEN: 2.23.mp4: 00:06-00:09

2.23.2. SCREEN: 2.23.mp4: 00:11-00:20

Results

3. Results

- 3.1. In the control group, both fluorescent tracers were largely confined to the gingival surface [1]. In both ligation- and *Porphyromonas gingivalis*-treated groups, significant extravasation of both FD40 and RD70 into the gingival epithelium was observed [2].
 - 3.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the FD40 and the RD70 panels under "Control" showing green fluorescence limited to the surface.*
 - 3.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the FD40 and the RD70 panels under "Ligation" and "P. gingivalis"*
- 3.2. Semiquantitative analysis demonstrated significantly increased fluorescence intensity of both FD40 and RD70 in the ligation- and *Porphyromonas gingivalis*-treated groups [1].
 - 3.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the higher bars for "Ligation" and "P. gingivalis" in both FD40 and RD70 bar graphs*
- 3.3. 3D imaging revealed more extensive and diffuse tracer fluorescence distribution around the gingival vasculature in the ligation- and *Porphyromonas gingivalis*-treated groups compared to controls [1].
 - 3.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the "Ligation" and "P. gingivalis" images*

1. Ligation

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

IPA: /laɪˈɡeɪʃən/

Phonetic Spelling: ly·gay·shuhn

2. Periodontitis
Pronunciation link: <https://www.merriam-webster.com/dictionary/periodontitis>
IPA: /ˌpɛr.i.oo.dɑːnˈtɑɪ.tɪs/
Phonetic Spelling: peh·ree·oh·don·ty·tuhs
3. Porphyromonas gingivalis
Pronunciation link: <https://www.howtopronounce.com/porphyromonas-gingivalis>
IPA: /ˌpɔːr.fi.rooˈmoʊ.nəs ˌdʒɪn.dʒɪˈveɪ.lɪs/
Phonetic Spelling: por·fi·roh·moh·nuhs jin·ji·vay·luhs
4. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊˈres.ənt/
Phonetic Spelling: floo·reh·suhnt
5. Spectra
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectra>
IPA: /ˈspek.trə/
Phonetic Spelling: spek·truh
6. Dextran
Pronunciation link: <https://www.merriam-webster.com/dictionary/dextran>
IPA: /ˈdek.stræn/
Phonetic Spelling: dek·stran
7. Rhodamine
Pronunciation link: <https://www.merriam-webster.com/dictionary/rhodamine>
IPA: /ˈroʊ.də.miːn/
Phonetic Spelling: roh·duh·meen
8. Carboxymethylcellulose
Pronunciation link: <https://www.howtopronounce.com/carboxymethylcellulose>
IPA: /kɑːrˌbɑːk.siˌmɛθ.əlˈsɛl.jə.loʊs/
Phonetic Spelling: kar·bok·see·meth·uhl·sel·yuh·lohs
9. Anesthetized
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthetized>
IPA: /əˈnes.θə.taɪzd/
Phonetic Spelling: uh·nes·thuh·tyzed
10. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /ˈmaɪ.kroʊˌliː.t̬ər/
Phonetic Spelling: my·kroh·lee·terz
11. Gingival
Pronunciation link: <https://www.merriam-webster.com/dictionary/gingival>
IPA: /ˈdʒɪn.dʒə.vəl/
Phonetic Spelling: jin·juh·vuhl
12. Supine
Pronunciation link: <https://www.merriam-webster.com/dictionary/supine>
IPA: /ˈsuː.pam/
Phonetic Spelling: soo·pyne

13. Maxilla
 Pronunciation link: <https://www.merriam-webster.com/dictionary/maxilla>
 IPA: /mæk'sɪl.əl/
 Phonetic Spelling: mak·sil·uh
14. Phosphate-buffered saline
 Pronunciation link: <https://www.howtopronounce.com/phosphate-buffered-saline>
 IPA: /'fɑ:s.fert 'bʌf.əd sə'li:n/
 Phonetic Spelling: fos·fayt buf·erd suh·leen
15. Dissected
 Pronunciation link: <https://www.merriam-webster.com/dictionary/dissected>
 IPA: /dɪ'sektɪd/
 Phonetic Spelling: dih·sekt·id
16. Single-photon
 Pronunciation link: <https://www.howtopronounce.com/single-photon>
 IPA: /'sɪŋ.gəl 'foʊ.tɑ:n/
 Phonetic Spelling: sing·guhl foh·ton
17. Immersion
 Pronunciation link: <https://www.merriam-webster.com/dictionary/immersion>
 IPA: /ɪ'mɜ:.ʒən/
 Phonetic Spelling: ih·mur·zhuhn
18. Buccal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/buccal>
 IPA: /'bʌk.əl/
 Phonetic Spelling: buh·kuhl
19. Micrometer
 Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
 IPA: /maɪ'kra:.mə.t̬ər/
 Phonetic Spelling: my·krah·muh·ter
20. Excitation
 Pronunciation link: <https://www.merriam-webster.com/dictionary/excitation>
 IPA: /,ek.sə'teɪ.ʃən/
 Phonetic Spelling: ek·sy·tay·shuhn
21. Emission
 Pronunciation link: <https://www.merriam-webster.com/dictionary/emission>
 IPA: /ɪ'mɪʃ.ən/
 Phonetic Spelling: ih·mish·uhn
22. Nanometers
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
 IPA: /'næn.oʊ.mi.t̬ər/
 Phonetic Spelling: nan·oh·mee·terz
23. Fluorescence
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
 IPA: /flʊ'res.əns/
 Phonetic Spelling: floo·reh·suhns

24. Permeability
Pronunciation link: <https://www.merriam-webster.com/dictionary/permeability>
IPA: /ˌpɜː.mi.əˈbɪl.ə.t̬i/
Phonetic Spelling: pur·mee·uh·bil·uh·tee
25. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌep.ɪˈθiː.li.əl/
Phonetic Spelling: eh·puh·thee·lee·uhl
26. Threshold
Pronunciation link: <https://www.merriam-webster.com/dictionary/threshold>
IPA: /ˈθɹɛʃ.hoʊld/
Phonetic Spelling: thresh·hohld
27. Integrated-Density
Pronunciation link: <https://www.howtopronounce.com/integrated-density>
IPA: /ˈɪn.tɪɡreɪ.t̬ɪd ˈden.sə.t̬i/
Phonetic Spelling: in·tuh·gray·tuhd den·suh·tee
28. Extravasation
Pronunciation link: <https://www.merriam-webster.com/dictionary/extravasation>
IPA: /ˌɛk.strə.væˈseɪ.ʃən/
Phonetic Spelling: ek·struh·va·say·shuhn
29. Epithelium
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelium>
IPA: /ˌep.ɪˈθiː.li.əm/
Phonetic Spelling: eh·puh·thee·lee·uhm
30. Semiquantitative
Pronunciation link: <https://www.howtopronounce.com/semiquantitative>
IPA: /ˌsem.iˈkwɑːn.tə.teɪ.t̬ɪv/
Phonetic Spelling: sem·ee·kwon·tuh·tay·tiv
31. Vasculature
Pronunciation link: <https://www.merriam-webster.com/dictionary/vasculature>
IPA: /ˈvæs.kjə.lə.t̬ʃə/
Phonetic Spelling: vas·kyuh·luh·cher