Journal of Visualized Experiments

Immunohistochemical staining of CD8 protein in tissue sections from patients with tumors

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57945R4
Full Title:	Immunohistochemical staining of CD8 protein in tissue sections from patients with tumors
Keywords:	immunohistochemistry, CD8+ TILs , tumor immunotherapy , protein, antigen, antibody
Corresponding Author:	Shiqiang Zhang Sun Yat-Sen University Shenzhen, Guangdong CHINA
Corresponding Author's Institution:	Sun Yat-Sen University
Corresponding Author E-Mail:	zhangshq9@mail2.sysu.edu.cn;shiqiangzhang@foxmail.com
Order of Authors:	Shiqiang Zhang
	Jun Wang
	Juan Fang
	Binyuan Yan
	Haiyun Xiong
	Fu Shi
	Guolong Liao
	Xiaofang Lu
	Jun Pang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	No. 628, Zhenyuan Road, Xinhu Street, Guangming New District, Shenzhen, P.R. China

Dear Editor,

Please find enclosed our manuscript entitled " *Immunohistochemical staining of CD8 protein in tissue sections from patients with tumors"* that we would like to be considered for publication in Journal of Visualized Experiments. This paper highlights a protocol for *Immunohistochemistry*. We consider of value publishing these data in Journal of Visualized Experiments, as they *can show the image of specific antigen located on tissue sections and can be used to determine the expression relative level of aimed protein or distribution of target cells.* The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working in the field of *life sciences, such as tumor*.

Shiqiang Zhang, Fang Juan Binyuan Yan, Haiyun Xiong, Fu Shi, Guolong Liao, Xiaofang Lu and Pang Jun designed the procedures described in the manuscript. *Shiqiang Zhang and Fang Juan* performed the experiments and analyzed the data. Finally, *Shiqiang Zhang , Fang Juan and Jun Wang* wrote the manuscript.

During the preparation and submission of this manuscript, we have been kindly assisted by *Rachel Service*.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

Shiqiang Zhang and Jun Pang

TITLE:

Immunohistochemical Staining of CD8 Protein in Tissue Sections from Patients with Tumors

AUTHORS & AFFILIATIONS:

Shiqiang Zhang[1]*, Jun Wang[2]*, Fang Juan[3]*, Binyuan Yan[1], Haiyun Xiong[1], Fu Shi[1], Guolong Liao[1], Xiaofang Lu[4], Jun Pang[1]

[1]Department of Urology, the Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China
[2]Zhongshan School of Medicine, Sun-Yat Sen University, Guangzhou, China
[3]Guanghua School of Stomatology, Sun Yat-Sen University, Guangzhou, China
[4]Department of Pathology, the Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China

*Shiqiang Zhang, Jun Wang and Fang Juan contributed equally to this work.

Corresponding author:

Jun Pang, M.D., Ph.D. (pangjun530@qq.com)

Email Addresses of Co-Authors:

Shiqiang Zhang (zhangshq9@mail2.sysu.edu.cn)
Jun Wang (wangj256@mail2.sysu.edu.cn)
Fang Juan (fangj6@mail2.sysu.edu.cn)
Binyuan Yan (yanby6@mail.sysu.edu.cn)
Haiyun Xiong (xionghy7@mail.sysu.edu.cn)
Fu Shi (shif8@mail.sysu.edu.cn)
Guolong Liao (liaoglong@mail.sysu.edu.cn)
Xiaofang Lu (zdluxiaofang2003@163.com)

KEYWORDS:

immunohistochemistry, CD8+ TILs, tumor immunotherapy, protein, antigen, antibody

SUMMARY:

The present study presents a protocol for immunohistochemistry (IHC) that can image specific antigens located on tissue sections and determine the relative expression level of a target protein or the distribution of target cells.

ABSTRACT:

Immunohistochemistry (IHC) is one of the most useful detection techniques in scientific research and clinical practice. IHC can give researchers a direct view of a target protein or target cells through bi-colored images of histological tissue sections from patients. Cell nuclei are stained with hematoxylin and target proteins are stained via the chromogenic reaction of 3,3',4,4'-Biphenyltetramine tetrahydrochloride (DAB) in classic IHC, which can show both the relative expression level of a target protein and its location within the tissue. The principle utilized in IHC is the specific binding between an antigen and an antibody, which partially guarantees the

accuracy of the results. IHC is also widely used to study cell subsets because it can show the exact location of target cell subsets in organs or tissues. This can help us understand their effects and functional mechanisms. Clinical data suggest that T-cell surface glycoprotein CD8 (CD8)+ tumor-infiltrating lymphocytes (TILs) could serve as an indication of the effectiveness of anti-programmed cell death 1 (PD-1) / programmed cell death ligand 1 (PD-L1) therapy in patients with tumors; therefore, IHC staining of CD8 protein to evaluate the CD8+ TILs in tissue sections becomes very important. IHC has several advantages. Samples are more accessible and last for a long time in storage. The reagents and equipment have been commercialized for years. However, there are also limitations. Lymphocyte infiltration into tumors is a dynamic process, and the results of IHC only reflect the infiltration at one specific time point, and not the dynamic changes over time. This disadvantage partly inhibits its clinical application in tumor immunotherapy, which mostly depends on T cell infiltration into the tumor microenvironment.

INTRODUCTION:

The presence of tumor-infiltrating lymphocytes (TILs) is considered to be associated with better clinical outcomes in different kinds of cancer[1–5]. T-cell surface glycoprotein CD8 (CD8)+ TILs are the most important effectors to prevent tumor development among all TILs[6–8]. The application of IHC can be used to help researchers and/or pathologists accurately observe the CD8+ TILs of individual patients. Assessment of CD8+ TILs can help to determine the prognosis for patients. In addition, CD8+ TIL evaluation can be one of the indicators for tumor immunotherapy[9].

IHC is the most commonly used detection method to evaluate specific proteins in patients' organs or tissues, especially human tumor tissues, both qualitatively and quantitatively[10,11]. IHC is widely used in pathological diagnosis. Specimen collection and storage for IHC are relatively easy. For example, there is little time limitation on the use of a sample as long as tissues are infiltrated with paraffin wax. The forms of antigen are well preserved. Thus, it is possible to handle many tissue samples at the same time. The experimental conditions can be controlled to avoid human interference. However, there are still some limitations of IHC in terms of sensitivity and background reduction.

PROTOCOL:

The Ethical Committee of the Seventh Affiliated Hospital, Sun Yat-sen University approved all experimental methods used in the study.

1. Preparation of the sample

1.1. Immerse the fresh human tumor tissue (within 30 min after resection; cut into 2 cm x 2 cm x 0.3 cm) in 10% formalin for 24 h (at least 2 h). The volume of formalin should be at least ten times greater than that of the tissue.

1.2. Set the program of the tissue processor as follows: 70% ethanol for 60 min, 80% ethanol for 60 min, 2x 95% ethanol for 60 min, 95% ethanol for 70 min, 2x 100% ethanol for 70 min, 2x xylene for 40 min, 5x paraffin wax for 40 min.

1.3. Immerse the paraffin-infiltrated sample with liquid wax in a mold. Cover with an embedding box. Cool the wax to immobilize the sample by putting it on an ice table (−2 °C to −8 °C). Make sure to locate the sample at the center of the mold.

1.4. Use a microtome to cut the wax block into 4 µm sections. Float the sections on the surface of a 55 °C water bath and mount sections on adhesive microscope slides with a positive charge. Bake the slides for at least 60 min at 65 °C to dry the slides, which can help the sections to better adhere to the slides.

1.5. Coat the tissue block with paraffin wax to protect the surface antigen if the samples are not to be handled immediately.

2. De-paraffinization and rehydration

2.1. Bake slides at 60 °C for 30 min. Wipe off the melted wax using a paper towel without touching the sections.

NOTE: The representative slides were selected upon review of hematoxylin and eosin-stained slides, which had tumor and tumor-adjacent stroma and more TILs.

2.2. Immerse slides in xylene for 30 min to remove as much extra wax as possible.

2.3. Transfer slides through liquid based on the order below to remove all the wax in the sections and rehydrate them: 2x xylene for 10 min, 2x 100% ethanol for 5 min, 90% ethanol for 5 min, 80% ethanol for 5 min, 70% ethanol for 5 min, and deionized water for 5 min.

3. Antigen retrieval

3.1. In a pressure cooker, boil the tissue slides in 10 mM citrate buffer (pH 6.0) for antigen retrieval at 120 °C for 5 min or at 100 °C for 30 min. Do not open the pressure cooker before the temperature drops to 70 °C by water rinsing, otherwise the tissue sections may fall off.

3.2. Cool the slides in a water bath at room temperature for 60 min, rinse slides with phosphate-buffered saline (PBS, pH 7.5) for 5 min, and then repeat the rinsing with PBS twice.

NOTE: The container should be placed on a shaking table when rinsing the slides.

4. Staining

4.1. Wipe off the liquid on the slides without touching the tissue sections. Draw a circle to surround the tissue using a hydrophobic pen to create a hydrophobic boundary.

4.2. Add 100 µL of 3% hydrogen peroxide onto the circle to fully cover the tissue section. Incubate

the slides at room temperature for 15 min, rinse slides with PBS for 5 min, and repeat twice.

4.3. Add 100 µL of blocking buffer onto the circle to fully cover the tissue section. Incubate the slides at room temperature for 15 min, rinse slides with PBS for 5 min, and then repeat twice.

4.4. Dilute the primary CD8 protein antibody with Antibody Diluent at a ratio of 1:100. Add 100 µL of the diluted antibody onto the circle to fully cover the tissue section. Incubate the slides in a moist chamber at 4 °C overnight (about 12 h).

4.5. Rewarm the slides at room temperature for 30 min, rinse slides with PBS for 5 min, and then repeat twice.

4.6. Dilute the secondary antibody with PBS at a ratio of 1:500. Place 100 µL of the diluted antibody onto the circle to fully cover the tissue section. Incubate the slides at room temperature for 60 min, rinse slides with PBS for 5 min, and then repeat twice.

4.7. Dissolve the DAB particles according to the manufacturer's instructions. Mix well before use. Add 100 µL of DAB solution onto the circle to fully cover the tissue section. Color development will take 1 min. The slides can be placed on white paper for better color observation.

4.8. Rinse the slides with ddH_2O immediately after color development. Accurate time control can reduce background staining.

4.9. Immerse the slides in hematoxylin for 60 s, rinse the slides with running water for 1–2 min, and then immerse the slides in 1% hydrochloric-alcohol solution for 1 s.

4.10. Rinse the slides with running water for 1–2 min, immerse the slides in 0.037 M ammonium hydroxide 6x–8x, and then rinse the slides with running water for 1–2 min.

NOTE: All incubation steps should be carried out in a moist chamber. Make sure that the liquid is well enclosed by the hydrophobic boundary, or the tissue section may dry out.

5. Dehydration and mounting sections

5.1. Wash slides in the following order to dehydrate the tissue sections: 80% ethanol for 5 min, 90% ethanol for 5 min, 2x 100% ethanol for 5 min, and 2x xylene for 10 min.

5.2. Add one drop of environmentally friendly resin to cover the section. Mount the section with a cover slip. Make sure no bubbles are trapped. Dry the slides in the air and then view them under a microscope.

REPRESENTATIVE RESULTS:

Successful immunohistochemical staining shows the CD8+ TILs in the tumor sections (bladder cancer). CD8 protein expression (the surface marker of CD8+ TILs) are defined and quantified

using the brown signal in the image (**Figure 1**); the blue signal represents the cell nucleus. Meanwhile, the location of CD8+ TILs can be determined by observing the distribution of both the brown and blue signals (**Figure 2**). **Figure 3** and **Figure 4** depict oral cancer tissues used as 'positive' and 'negative' controls, respectively, in our experiments

FIGURE LEGENDS:

Figure 1. CD8+ TILs are well infiltrated into the tumor. Normal tissue cannot be observed in this image; most CD8+ T cells are in the tumor area. An arrow indicates CD8+ T cells. 100x magnification.

Figure 2. CD8+ TILs are distributed mainly at the interface of the tumor and tumor-adjacent stroma. There are few CD8+ T cells in the tumor area. 200x magnification.

Figure 3. Negative control (Oral cancer tissue). There are no CD8+ T cells in this tissue.

Figure 4. Positive control (Oral cancer tissue). Many CD8+ T cells are infiltrated into the tumor.

DISCUSSION:

The presence of CD8+ TILs has been reported in different kind of tumors[6–8]. The retrieval of the antigen denatures the CD8 protein and exposes the antigen epitope. Binding of the anti-CD8 antibody and subsequent horseradish peroxidase–labeled secondary antibody are quite effective and the color development is well established. The results of CD8 IHC can help to provide a direct view of CD8+ TILs in the tumor microenvironment, as shown in **Figure 1** and **Figure 2**.

To better display the results of IHC in sections, the materials and steps could be more effective. The primary antibody is the key factor for the success of the whole experiment. The specificity of the primary antibody can determine the outcome. There will be a high background if the primary antibody binds to other proteins non-specifically. The species origin of the primary antibody is also important. The antibodies cannot be generated in the same species as the samples, or the secondary antibody will bind to both the primary antigen and non-targeted sample proteins, causing a high background. The primary antibody concentration and the primary antibody incubation time with tissues should be individualized for a specific protein. During the entire operation, the slices should not be dried; otherwise, there will be non-specific staining. Furthermore, negative and positive control samples are needed to validate the results.

IHC has several advantages compared to other detection methods. Samples are more accessible and last long in storage. The reagents and equipment have been commercialized for years, which lowers the cost. Information on the expression and location can be presented at the same time to help describe features of individual patients. However, there are also limitations. Lymphocyte infiltration into tumors is a dynamic process, because the infiltration, expansion, and apoptosis of immune cells can influence the equilibrium of the body. The results of IHC only reflect the infiltration at one specific time point, and not the dynamic changes over time. This disadvantage partly inhibits its clinical application in tumor immunotherapy, which mostly depends on T cell infiltration into tumor microenvironment.

ACKNOWLEDGMENTS:

This study was supported by Research Project of Shenzhen Health Family Planning System (grant#: SZBC2018001), the Natural Science Foundation of China (grant#: 81772754), and the Natural Science Foundation of Guangdong Province (grant#: 2017A030308009).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

[1] Galon, J. et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. **313** (5795), 1960-1964 (2006).

[2] Hwang, W.T., Adams, S.F., Tahirovic, E., Hagemann, I.S., Coukos, G. Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecologic Oncology*. **124** (2), 192-198 (2012).

[3] Laghi, L. et al. CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study, *Lancet Oncology*. **10** (9), 877-884 (2009).

[4] Pages, F. et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *New England Journal of Medicine*. **353** (25), 2654-2666 (2005).

[5] Zhang, L. et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *New England Journal of Medicine*. **348** (3), 203-213 (2003).

[6] Sharma, P. et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*. **104** (10), 3967-3972 (2007).

[7] Mahmoud, S.M. et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *Journal of Clinical Oncology*. **29** (15), 1949-1955 (2011).

[8] Savas, P. et al. Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nature Medicine*. **24** (7), 986-993 (2018).

[9] Tumeh, P.C. et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. **515** (7528), 568-571 (2014).

[10] Kourea, H., Kotoula, V. Towards tumor immunodiagnostics. *Annals of Translational Medicine*. **4** (14), 263 (2016).

[11] Smith, N.R., Womack, C. A matrix approach to guide IHC-based tissue biomarker development in oncology drug discovery. *Journal of Pathology*. **232** (2), 190-198 (2014).

Figure 1

Click here to access/download;Figure;Figure 1-344521 10X-1.tif

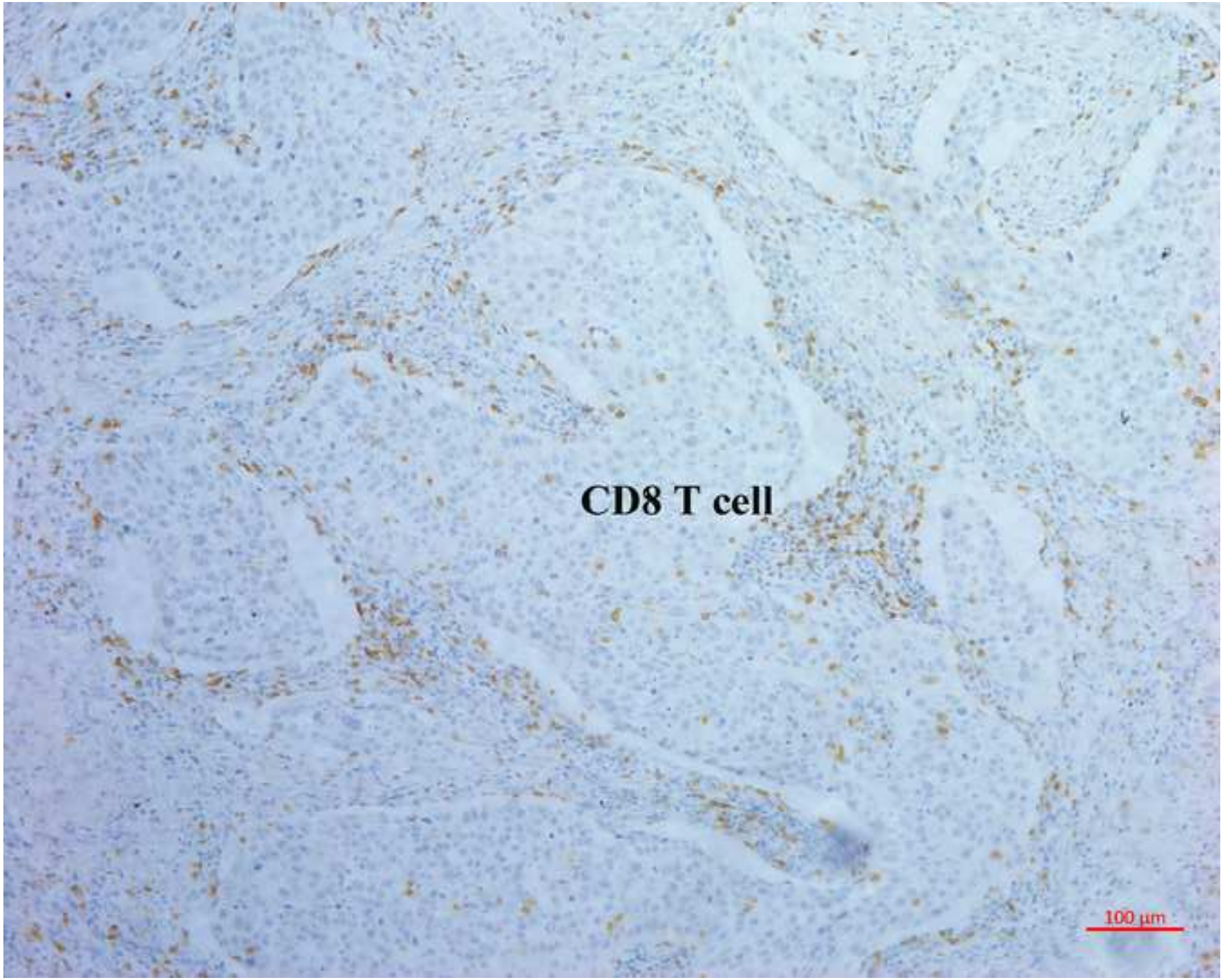

Figure 2

Click here to access/download;Figure;renamed_449b3.tif

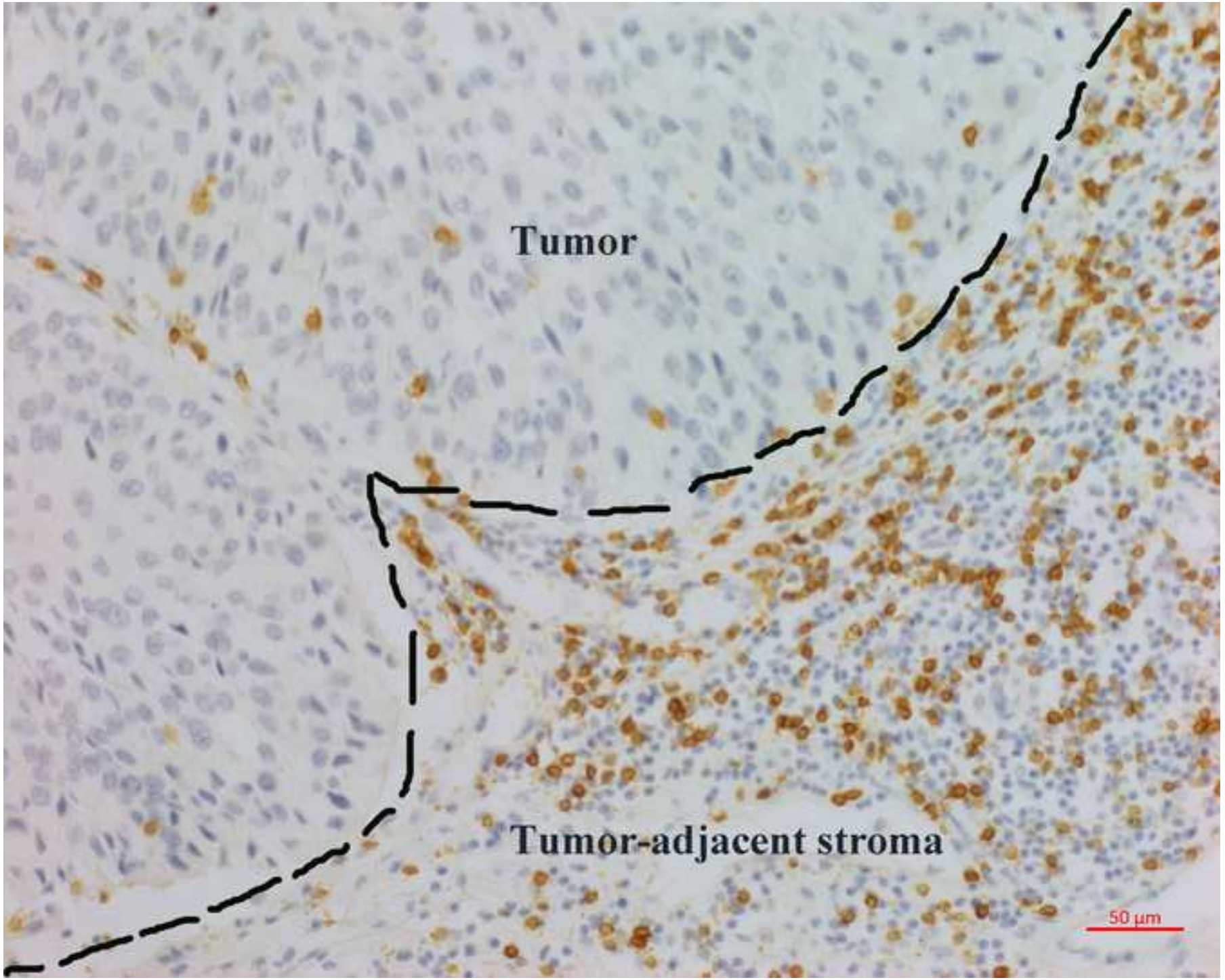

Figure 3

Click here to access/download;Figure;Figure 3-Negative control-CD8-20X2-1.tif

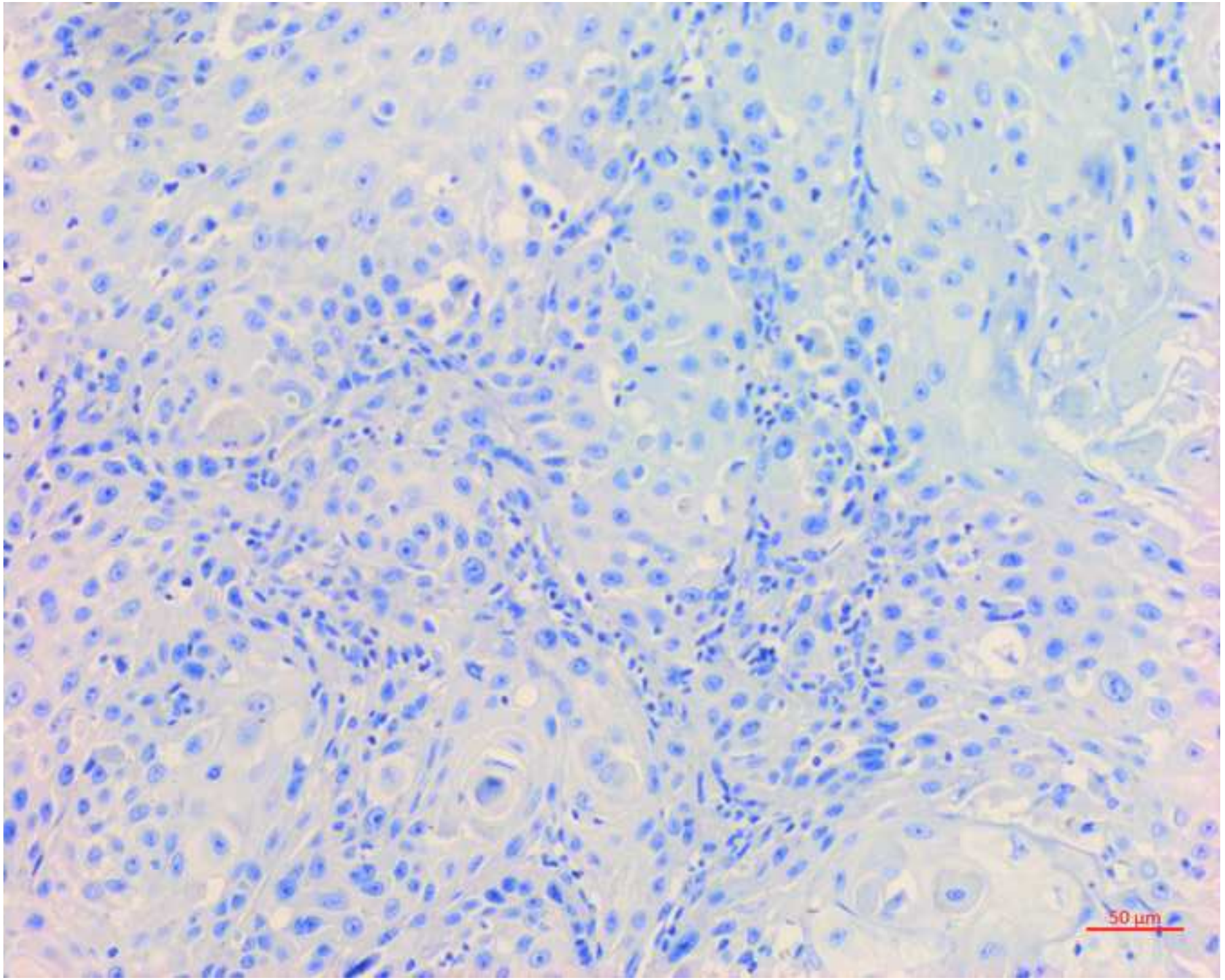

Figure 4

Click here to access/download;Figure;Figure 4-Positive control-CD8+ 20X1-1.tif

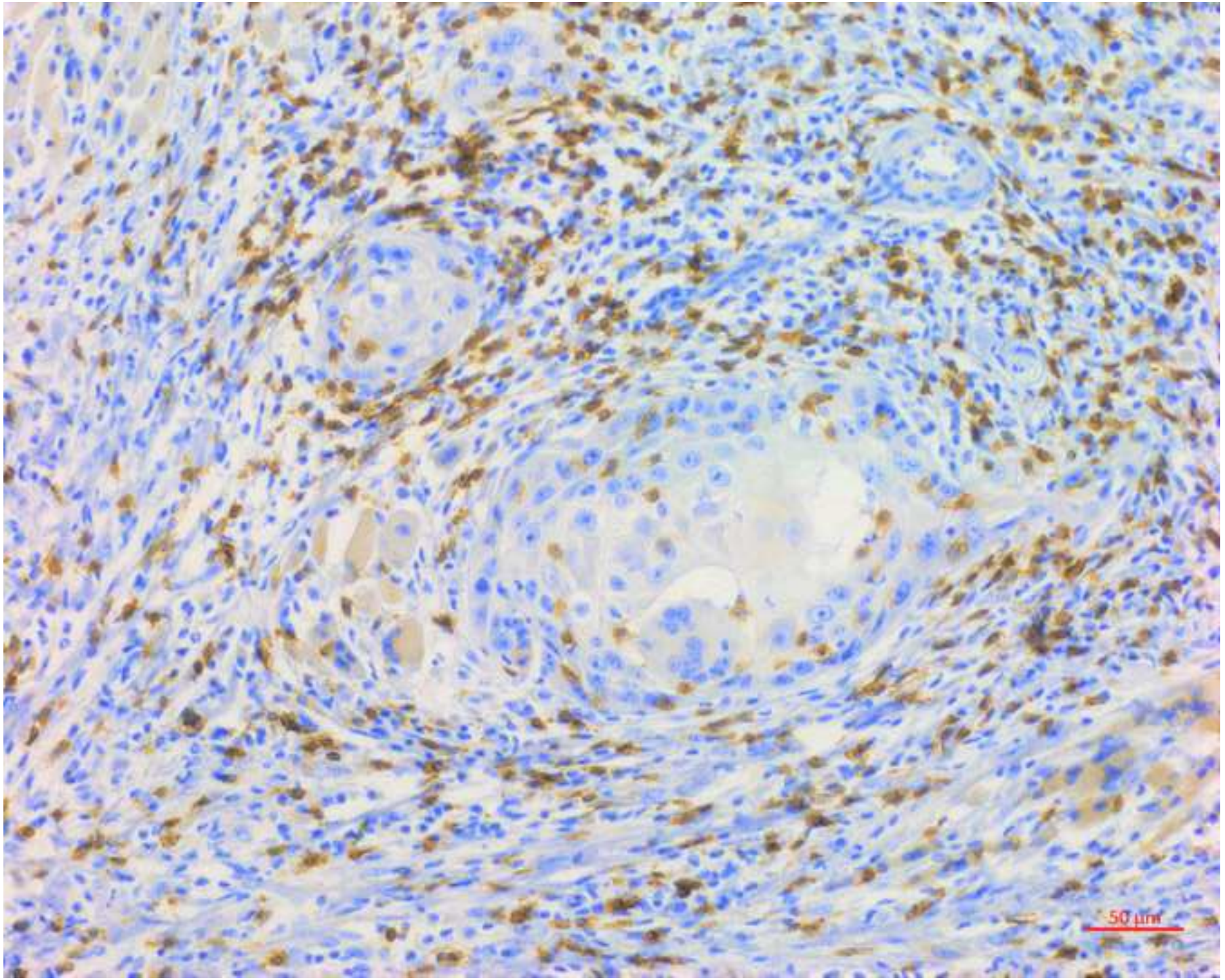

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Ammonium hydroxide	Guangzhou Chemical Reagent Factory	1336-21-6
Antibody Diluent, Background Reducing	DAKO	S302281
Blocking buffer	Serotec	BUF029
CD8, Rabbit Monoclonal Antibody	Thermo Scientific	RM-9116-S
Citrate buffer	MXB Biotechnologies	MVS-0066
DAB	MXB Biotechnologies	DAB-0031
Ethanol	Guangzhou Chemical Reagent Factory	
Formalin	Xiuwei Commerce	XW-RS-019
Goat anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, HRP conjugate	Thermo Scientific	31462
Hematoxylin	Xiuwei Commerce	XW-RS-001
Hydrochloric	Guangzhou Chemical Reagent Factory	7647-01-0
Hydrogen peroxide	Guangzhou Chemical Reagent Factory	7722-84-1
Paraffin	Leica	39601006
Phosphate-buffered saline(PBS)	MXB Biotechnologies	PBS-0060
Pressure cooker	Supor	
Resin	Xiuwei Commerce	XW-RS-005
Tissue processor	Leica	TP1020
Xylene	Guangzhou Chemical Reagent Factory	1330-20-7

Comments/Description

Antigen retrieval buffer, pH 6.0

pH 7.5

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Shiqiang Zhang

Department: Urology

Institution: The Seventh Affiliated Hospital of Sun Yat-Sen University

Article Title: Immunohistochemical staining of CD8 protein to evaluate the CD8+TILs in tissue sections from tumor patients

Signature: Shiqiang Zhang Date: Dec. 21, 2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Immunohistochemical staining of CD8 protein to evaluate CD8+ TILs in tissue sections from

Author(s): Shigang Zhang, Jun Wang, Juan Feng, Jun Pang tumor patients

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

Dear Phillip,

The following is the document that addresses each of the editorial comments individually with the revised manuscript. We haven't finished the professional proofreading service because of the limited time. If there are still contents that need to be revised and improved, please contact us immediately.

Best,

Shiqiang

Editorial comments:

1. The manuscript as a whole is still unclear in many places; please proofread further (and consider using a professional proofreading service).

This process probably needs another two weeks.

2. Please include an ethics statement before the numbered protocol steps indicating that the protocol follows the guidelines of your institution's human research ethics committee.

The Ethical Committee of the Seventh Affiliated Hospital, Sun Yat-sen University approved all experimental methods in the study.

3. Protocol: What are the 'Focus' sections? JoVE guidelines do not allow these; they should be 'NOTE's or numbered protocol steps.
Have revised.

4. 1.1: Where does the tumor tissue come from here? How fresh should it be? Around how large?
The tumor tissues come from patients who had tumor resection. The tumor is considered as fresh specimen fixed **in 10% formalin** within half an hour after resected from human body. The fixed tissue is 2*2*0.3cm.

5. 1.3: Please include the tissue processor in the Table of Materials

Leica TP1020 Semi-enclosed Benchtop Tissue Processor

6. 1.4: What is the fridge temperature?

The temperature is -2~-8℃.

7. 1.7: How exactly are slides coated? How much paraffin?
In this place we used the wrong word “slides”, it should be tissue wax block. It was done by putting the cutting side of the wax block in liquid paraffin twice.

8. 2.1: How exactly is melted wax wiped away?
Wipe off melted wax on the slides with a paper towel without touching the sections if there is melted wax on the slides which can be wiped off after baking.

9. 3.1: So are slides boiled in a pressure cooker? It’s not mentioned in the Table of Materials. How is the cooker cooled and for around how long?

Yes，slides are boiled in a pressure cooker. The cooker is cooled by water rinsing, which takes about 10 min.

10. 3.2: Repeat what twice?
Have revised.

11. Results: These are still vague, and important concerns raised by the reviewers have not been addressed. In particular:

a) Please explain what the blue samples are, and please explain the distribution of the signals more thoroughly.

The blue signal stands cell nucleus . We have annotated the image
b) Please explain more thoroughly how these results arise from the given protocol.

c) Please include and explain your positive and negative controls.
Have done.
d) Please include and explain additional types of tumors you have done this staining on.
We had done oral cancer tissues and used them as positive and negative controls..

12. Figures 1 and 2: Please explain what type of tumors these are. Please also more explicitly (e.g., with additional labeling and borders drawn in) point out important features of these figures (e.g., the exact boundaries of the tumors and tumor-adjacent stroma). Please do this for any new Figures included as well.
Have revised.

Dear Phillip,

The following is the document that addresses each of the editorial comments individually with the revised manuscript. We haven't finished the professional proofreading service because of the limited time. If there are still contents that need to be revised and improved, please contact us immediately.

Best,

Shiqiang

Editorial comments:

1. The manuscript as a whole is still unclear in many places; please proofread further (and consider using a professional proofreading service).

This process probably needs another two weeks.

2. Please include an ethics statement before the numbered protocol steps indicating that the protocol follows the guidelines of your institution's human research ethics committee.

The Ethical Committee of the Seventh Affiliated Hospital, Sun Yat-sen University approved all experimental methods in the study.

3. Protocol: What are the 'Focus' sections? JoVE guidelines do not allow these; they should be 'NOTE's or numbered protocol steps.
Have revised.

4. 1.1: Where does the tumor tissue come from here? How fresh should it be? Around how large?
The tumor tissues come from patients who had tumor resection. The tumor is considered as fresh specimen fixed **in 10% formalin** within half an hour after resected from human body. The fixed tissue is 2*2*0.3cm.

5. 1.3: Please include the tissue processor in the Table of Materials

Leica TP1020 Semi-enclosed Benchtop Tissue Processor

6. 1.4: What is the fridge temperature?

The temperature is -2~-8℃.

7. 1.7: How exactly are slides coated? How much paraffin?
In this place we used the wrong word “slides”, it should be tissue wax block. It was done by putting the cutting side of the wax block in liquid paraffin twice.

8. 2.1: How exactly is melted wax wiped away?
Wipe off melted wax on the slides with a paper towel without touching the sections if there is melted wax on the slides which can be wiped off after baking.

9. 3.1: So are slides boiled in a pressure cooker? It’s not mentioned in the Table of Materials. How is the cooker cooled and for around how long?

Yes，slides are boiled in a pressure cooker. The cooker is cooled by water rinsing, which takes about 10 min.

10. 3.2: Repeat what twice?
Have revised.

11. Results: These are still vague, and important concerns raised by the reviewers have not been addressed. In particular:

a) Please explain what the blue samples are, and please explain the distribution of the signals more thoroughly.

The blue signal stands cell nucleus . We have annotated the image
b) Please explain more thoroughly how these results arise from the given protocol.

c) Please include and explain your positive and negative controls.
Have done.
d) Please include and explain additional types of tumors you have done this staining on.
We had done oral cancer tissues and used them as positive and negative controls..

12. Figures 1 and 2: Please explain what type of tumors these are. Please also more explicitly (e.g., with additional labeling and borders drawn in) point out important features of these figures (e.g., the exact boundaries of the tumors and tumor-adjacent stroma). Please do this for any new Figures included as well.
Have revised.

Editorial comments:

1. The manuscript still requires editing by a fluent English speaker. If you require more time for this, please let me know; I can revise the due date.

The manuscript is edited by Elixigen.

2. Results: Please explain Figures 3 and 4 here, in particular how exactly they are 'negative' and 'positive' controls; i.e., how did you know beforehand the presence or lack of presence of TIL cells in these tissues?

Figure 3 and 4 are oral cancer tissues. At first, we used tonsil tissues as positive control, and replaced anti-human CD8 antibody with PBS as negative control. We found tissues of Figure 3 and 4 can be 'negative' and 'positive' controls in our later experiments.

3. Figure 2: You mention an interface of tumor and tumor-adjacent stroma in the legend, but have only labeled the tumor in the Figure. Please either clearly indicate where the tumor-adjacent stroma is or revise the legend.

We have labeled the tumor and tumor-adjacent stroma in the Figure 2.