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Digital Analysis of Immunostaining of ZW10 Interacting Protein in Human Lung Tissues

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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Department of Human Anatomy, Histology and Embryology, Peking Union Medical College, Beijing, People's Republic of China

Cover letter

Dear Editors,

We would like to submit the manuscript entitled “Utilization of digital techniques to analyze the immunostainning of ZW10 interacting protein in human lung tissues” and wish it is qualified to be published in “Journal of Visualized Experiments”.

Our manuscript is approved by all authors for publication. No conflict of interest exits in the submission of this manuscript. The re-use of figure 1 was permitted by the journal “OncoTargets and Therapy”. The paper was cited in the present manuscript. All the authors listed have approved the manuscript that is enclosed. Ethics approval and written informed consent have been obtained.

In this work, we presented a methodology of immunostaining of human lung cancer tissues, digitized scanning of whole slides by Pannoramic MIDI and image analysis by QuantCenter software. We found that the H-SCORE of tumor was significantly higher than that of adjacent non-tumor tissues. In addition, we observed that the expression level of ZWINT in squamous cell carcinomas (SCC) was significantly higher than that in adenocarcinomas (ADC). Notably, we figured out the most critical steps, existing limitations and possible utilization areas of this method.

Besides, we demonstrated the virtues of this method than conventional method. I hope this paper is suitable for "Journal of Visualized Experiments".

We deeply appreciate your kind consideration of our manuscript, and we look forward to receiving comments from the reviewers. If you have any enquiries, please don't hesitate to contact me at the address as below.

Thank you!

Yours sincerely,

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TITLE:

Digital Analysis of Immunostaining of ZW10 Interacting Protein in Human Lung Tissues

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KEYWORDS:

Automatic digital technique, lung cancer, ZW10 interacting protein, immunostaining, digitized whole-slide imaging, H-score

SUMMARY:
ZW10 interacting protein (ZWINT) participates in the mitotic spindle checkpoint and the pathogenesis of carcinoma. Here, we introduce a methodology of the immunostaining of ZWINT in human lung cancer tissues, followed by the digital scanning of whole slides and image analysis. This methodology can provide high-quality digital images and reliable results.

ABSTRACT:
The purpose of this study is to introduce a methodology of the immunostaining of human lung tissues, followed by whole-slide digital scanning and image analysis. Digital scanning is a fast way to scan a stack of slides and produce digital images with high quality. It can produce concordant results with conventional light microscopy (CLM) by pathologists. Furthermore, the availability of digital images makes it possible that the same slide can be concurrently observed by multiple people. Moreover, digital images of slides can be stored in a database, which means the long-term deterioration of glass slides is avoided. The limitations of this technique are as follows. First, it needs high-quality prepared tissue and the original immunohistochemistry (IHC) slides without any damage or excess sealant residue. Second, tumor or nontumor areas should be specified by experienced pathologists before the analysis using software, in order to avoid any confusion about the tumor or nontumor areas during scoring. Third, the operator needs to control the color reproduction throughout the digitization process in whole-slide imaging.

INTRODUCTION:
ZW10 interacting protein (ZWINT) is a necessary component of the kinetochore complex which is involved in the mitotic spindle checkpoint[1-3]. It has been reported that the depletion of ZWINT leads to aberrant premature chromosome segregation[1-3]. Recent studies have suggested that ZWINT is involved in the pathogenesis of multiple tumors by promoting the proliferation of tumor cells[4,5]. We previously reported the overexpression of ZWINT in lung cancer[5]. It has been widely accepted that the analysis of slides by pathologists using CLM is time-consuming and not quantitative[6-8]. Moreover, the deterioration of stored glass slides might make it impossible to retract previously created slides. The emerging method of computer-based, digital whole-slide imaging (WSI) may overcome these limitations[6-8].

To this end, we describe a methodology of the immunostaining of ZWINT in human lung cancer tissues, coupled with whole-slide digital scanning and software-based image analysis. The main advantage of this methodology is the production of concordant results with CLM. This technology can be widely used in the areas of pathological scoring of hematoxylin-eosin staining (H&E) and IHC, fluorescence *in situ* hybridization (FISH), tissue microarrays (TMA), and drug discovery and development.

PROTOCOL:
All methods described here have been approved by the Ethical Committee of Zhongnan Hospital of Wuhan University and Renmin Hospital of Wuhan University.

1. Preparation of IHC Slides

1.1. Fix the lung tissue sample by immersing the human lung tissue fragment (about 3 x 3 cm) in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at room temperature (RT).

1.2. Dehydrate the tissue in 80%, 95%, and 100% ethanol for 15 min, 20 min, and 20 min, respectively, at room temperature. Finally, immerse the tissue in 100% xylene 3x for 20 min each at room temperature.

1.3. For embedding, immerse the tissue in paraffin (63 °C) for 30 min. Change the paraffin and immerse the tissue again for another 45 min. Finally, change the paraffin again and immerse the tissue in it for an additional 1 h.

1.4. Use a rotary microtome to cut the tissue into 5 µm-thick sections and place them on 20 µg/mL poly-L-lysine-coated slides.

1.5. For dewaxing, place the slides in a 65 °C oven for 2 h and, then, immerse the sections in dimethylbenzene for 15 min, followed by soaking in 100% xylene for 15 min.

1.6. Hydrate the slides with the tissue sections 2x for 5 min in 100% ethanol, followed by a treatment with serially diluted ethanol of 5 min in 95% ethanol, 5 min in 80% ethanol, 5 min in 70% ethanol, and 5 min in 50% ethanol.

1.7. Perform antigen repair.

1.7.1. Dilute citric acid repair liquid (20x) to 1x with double-distilled H_2O (ddH_2O).

1.7.2. Place the slides with the tissue sections and the 1x citric acid repair liquid (300 mL) in an antigen repair box and, then, heat them in the microwave oven at high power for 2 min, followed by heating at low power to sustain boiling for 6 min.

1.7.3. Allow the sections to cool down naturally to room temperature.

1.7.4. Replace the citric acid repair liquid with 0.01 M PBS, wash the sections 3x, each time for 5 min, in the rotary shaker at room temperature.

1.8. Eliminate the endogenous peroxidase.

1.8.1. Dilute 30% of H_2O_2 to 3% with ddH_2O, add it to the slides (make sure it covers all tissues), and incubate the slides in a wet box at room temperature for 30 min.

Note: The wet box is a plastic box of 10 x 15 cm and can contain water.

1.8.2. Wash the slides 3x, each time for 5 min, with 0.01 M PBS in the rotary shaker at room temperature.

1.9. Block the nonspecific antigen.

1.9.1. Dilute the concentrated normal goat serum to 10% goat serum with PBS with 0.1% Tween 20 (PBST).

1.9.2. Add 10% goat serum to the slides and incubate them in the wet box at 37 °C for 30 min.

Note: The goat serum should cover all tissues in the slides.

1.10. Stain the tissues.

1.10.1. Incubate the slides with rabbit anti-human anti-ZWINT antibody (1:50) at 4 °C overnight.

1.10.2. Wash the slides 3x, each time for 5 min, with 0.01 M PBST (1 mL of 0.1% Tween 20 in 1,000 mL PBS) in the rotary shaker at room temperature.

1.10.3. Incubate the slides with secondary antibody (anti-rabbit detection system, 1:200) for 30 min at 37 °C.

1.10.4. Wash the slides 3x, each time for 5 min, with 0.01 M PBST in the rotary shaker.

1.11. To visualize the immunostaining, add the slides to 300 µL of fresh 3,3-diaminobenzidine and, then, wash the slides with tap water at room temperature.

Note: The degree of dyeing is monitored under a microscope (magnification 10X - 40X).

1.12. Counterstain the tissue. Stain the slides with hematoxylin staining solution for 2 min at room temperature and, then, wash the slides with tap water for 15 min.

Note: The nucleus appears blue under the microscope (magnification 10X - 40X). If the degree of blue staining is more potent, hydrochloric alcohol differentiation for 3 - 5 s can be used, followed by rinsing the tissue back to blue with tap water for 15 min to facilitate the prominent staining of the tissue.

1.13. Dehydrate the slides for 5 min in 50% ethanol, 5 min in 70% ethanol, 2x in 80% ethanol for 5 min, 5 min in 95% ethanol, and finally, 2x for 5 min each in 100% ethanol at room temperature.

1.14. For transparency, immerse the tissue sections in a tank containing 100% xylene for 15 min and transfer the sections to another tank containing 100% xylene for 15 min at room temperature.

1.15. For sealing, take 50 - 100 µL of neutral gum and add 5% xylene to the mixture (avoid mixing in bubbles). Add 15 µL of the above-mentioned mixture to the lung tissue sections. Seal the film with cover glass and gently press the bubbles out.

2. Automatic Whole-slide Scanning of IHC Slides

2.1. Allow the sealed slides to dry for 12 h at room temperature to desiccate them.

2.2. Select the **Bright field manually** option and enter the manual scanning interface.

2.3. Revise the name of the slides and select a storage path for the images.

2.4. Set the scanning area and choose the option **Scan samples using user-set thresholds**. The threshold is about 50 with a scanning area expansion value of 200 µm.

2.5. Set the scanning focus value, select the focus automatically—it ranges from 1050 to 2650—and finally, select **Single-layer mode**.

2.6. Load the IHC slides on the workstation and start the automatic scanning.

Note: Make sure the slides are not damaged. Keeping the slides clean and translucent and avoid dust, confetti, and excess sealant residue on the slides. The glass slide and coverslip should be devoid of marks. Do not clean the slides with xylene. The size of the slides is given in **Table 1**.

2.7. Automatically scan the entire tissue section on a slide with 20X, 40X, or 100X magnification.

2.8. Collect and store the images at the end of the whole-slide scanning.

3. Analysis of the Slides by Imaging Software

3.1. Start the histopathological image analysis software and select **Local computer**.

3.2. Open the file which stores the scanning data.

3.3. Select the appropriate zoom level from range 2X to 40X.

3.4. Adjust the color to set the optimal contrast *via* **Toggle color adjust**.

3.5. Manually circle and name the portions of interest on each image.

Note: For instance, you can circle one area and name it as **Tumor area**.

3.6. Select **Plugins** and **QC**. At this time, **Scenario builder** pops up.

3.7. Select **Density quant** and adjust the **Detection** bar.

Note: This process should be performed carefully. The bar of **Blue tolerance** is used to adjust the color of the cellular nucleus. The bar of **Brown tolerance** is used to adjust the saturation of the fill color in the image. The results of the images after adjustments should be consistent with the original counterparts.

3.8. Adjust the **Score levels** to make the staining intensity of the circled areas similar to that of the original images.

Note: Dark brown indicates strong positive, brownish yellow is moderately positive, light yellow is weakly positive, and white is negative.

3.9. Store and name the model as a file. Apply the model to other slides and select **Selected annotation**".

3.10. Let the software automatically calculate the H-score for the circled areas in the image.

4. Quantification of the Scores Using the H-scoring System[9,10]

4.1. Calculate the percentage of immunostaining and the staining intensity (0: negative, 1+: weak, 2+: moderate, 3+: strong).

4.2. Use a scoring system (H-score) to calculate the pathological scoring of the tumor and adjacent nontumor areas.

Note: H-score = (% of cells with weak intensity x 1) + (% of cells with moderate intensity x 2) + (% of cells with strong intensity x 3). Therefore, the maximum H-score is 300 if 100% of the cells are quantified with strong intensity.

REPRESENTATIVE RESULTS:

We measured the expression levels of ZWINT in 28 pairs of non-small-cell lung cancer (NSCLC) specimens (tumor and adjacent nontumor tissues), including 14 squamous cell carcinomas (SCCs) and 14 adenocarcinomas (ADCs), by IHC. The whole-slide digital scanning of the slides provided digital images of high quality (**Figure 1A**). The results showed that the H-score of the lung cancer was significantly higher than that of adjacent noncancer tissues ($P < 0.0001$, two-tailed *t*-test)

(**Figures 1A** and **1B**). Additionally, we found that the expression level of ZWINT in SCCs was significantly higher than that in ADCs (**Figure 1C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Immunohistochemical staining for lung cancer tissues. This figure has been modified from Yuan *et al.*[5] with permission from Dove Press. (**A**) This panel shows representative immunohistochemical images of lung cancer patients (magnifications: 10X and 40X). (**B**) This panel shows the H-score of lung cancer and adjacent noncancer tissues. (**C**) This panel shows the H-score of ADCs and SCCs. The error bars indicate standard deviation. * $P < 0.05$; *** $P < 0.001$.

Table 1: Slide sizes.

DISCUSSION:

Whole-slide scanning is becoming a hot topic for its robust scanning and production of high-quality images for clinical and research purposes[11-13]. Images can be produced by slide-scanning microscopes within minutes[11-13]. By applying this methodology, we obtained high-quality images for ZWINT IHC slides and compared the H-score between tumor and nontumor tissues. In the protocol presented here, the most critical steps are the preparation of the high-quality IHC slides, the image acquisition, and the specification of the tumor and nontumor areas before the pathological scoring[13-16].

The current method has some advantages compared to (CLM). Digital scanning is fast enough (~ 20 slides/hour) to scan a stack of slides and produce high-quality digital images. It can produce concordant results with CLM but takes relatively less time. The availability of digital images makes it possible that the same slide can be concurrently observed by multiple people. Digital images of slides can be stored in a database, which means the long-term deterioration of glass slides is avoided.

The limitations of this technique include the need for high-quality tissue and IHC slides[6,8,11] and technical expertise to specify the tumor or nontumor tissue samples before the analysis using imaging software, in order to avoid any confusion about the tumor or nontumor areas during scoring[6,7]. The operator also needs to monitor the color reproduction throughout the digitization process in whole-slide imaging[17].

This method can be actively applied in FISH, TMA, and drug discovery and development[6,18]. Tabata *et al.* have investigated the use of WSI in primary pathological diagnoses[19]. They retrospectively analyzed 1070 WSI specimens from nine hospitals in Japan and confirmed the validation of the use of WSI in primary diagnoses. One of the main advantages of WSI is that it allows simultaneous viewing of the slides by multiple students[20]. Therefore, the validation of whole-slide scanning images might contribute to the development of digital diagnostics for educational and research purposes[6,18,21].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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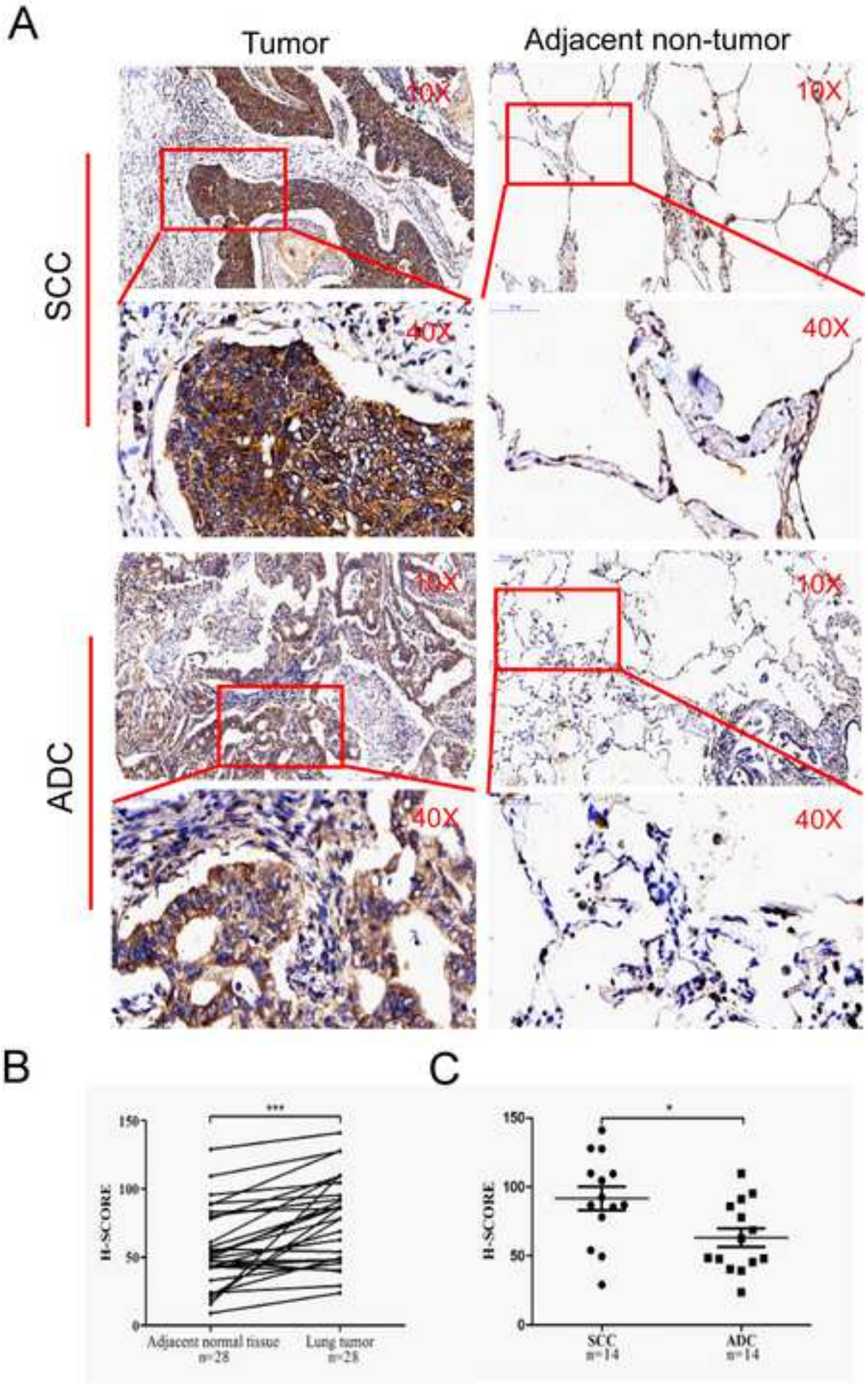

Table

	MIN (mm)	MAX (mm)
WIDTH	25	26
LENGTH	75	76
THICKNESS	0.9	1.2

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company
Pannoramic MIDI	3D HISTECH
QuantCenter	3D HISTECH
LEICA RM2235	Leica Microsystems
Rabbit anti-human Anti-ZWINT antibody	Abcam
Anti-rabbit secondary antibody	Wuhan Goodbio Technology
Phosphate-buffered saline	Wuhan Goodbio Technology
OLYMPUS CX23	OLYMPUS
Dimethylbenzene	Shanghai Lingfeng Chemical Reagent
Hematoxylin Staining Solution	Wuhan Servicebio technology
Tween 20	Baitg
Citric acid repair liquid	Wuhan Servicebio technology
LEICA ASP200s	Leica
LEICA Arcadia H	Leica
LEICA Arcadia C	Leica
CaseViewer Software	3DHISTECH

Catalog Number	Comments/Description
Cat: PMIDI-040709	An automated digital slide scanner with a rema
Downloaded from the official website of the company	The framework for 3DHISTCH's image analysis
Cat: 14050038604	The enhanced precision of the new accessories
Cat: ab197794	Immunohistochemical analysis of ZWINT in hu
Cat:GB23303-1	Secondary antibody for IHC staining.
Cat:G0002	A solution containing a phosphate buffer.
Cat:6M87620	Microscope for detection of H&E or IHC slides.
Cat:1330-20-7	A colorless, flammable fluid used as a solvent a
Cat:G1039	It is commonly used for histologic studies, ofte
Cat:2005-64-5	It is a polysorbate-type nonionic surfactant for
Cat:G1202	d to repair antigen after fixation during IHC pro
Cat: 14048043626	for routine and research histopathology of up t
Cat: 14039354103	simple operation and precise control, resulting
Cat: 14039354102	was designed with an environment adaptive cc

arkable feature set :12-slide capacity, fluorescence scanning, and many more.

applications.

s will add convenience to block to knife approach as well as specimen orientation.

man lung tissue.

and clarifying agent in the preparation of tissue sections for microscopic study.

rn colors the nuclei of cells blue.

med by the ethoxylation of sorbitan before the addition of lauric acid. It is used as a deterent and emulsifier in p

cedure.

o 200 cassettes.

; in improved quality, a smooth workflow and reliability.

ntrol module to make sure the operating temperature is always stabilized at -6℃.

ɔharmacological applications.

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Title of Article: Utilization of digital techniques to analyze the immunostaining of ZW10 interacting protein in human lung tissues

Author(s): Yuan Wen, Xie Song-ping, Liu Pan, Liu Xiao-yan, Pan Shan, Yin Qian, ~~Sun Meng, Huang Xiao-xing, Xiao Rui-jing, Xiong Jie, Zhang~~ Qiu-ping, Shao Liang

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