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Application of modified PASM-Masson staining in renal biopsy

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TITLE:

Modified PASM-Masson Staining in a Renal Biopsy

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SUMMARY:

In this study, we present a protocol of PASM-Masson dual staining to identify the location and extent of glomerular immune complex deposition in renal diseases.

ABSTRACT:

Special histochemical staining is an important method for a renal biopsy, which includes PAS staining, periodic acid silver methenamine (PASM) staining, and Masson staining. In this study, we aim to develop and modify the method of PASM and Masson double staining (PASM-Masson) in a renal biopsy. One hundred and fifty-two cases of renal biopsy were detected by PASM-Masson staining in our hospital. The results showed that the background of the slices was clean, and the renal structures were more visible under the microscope. The glomerular basement membrane, renal tubular basement membrane, and capillary basement membrane were stained black or brown; the mesangium and collagen fibers were stained blue black; the renal tubular epithelial cells and immune complexes were dyed red. These findings indicate that the glomerular immune complex deposition can be accurately identified and positioned by modified PASM-Masson staining, which might have important effects on the pathological diagnosis of renal diseases.

INTRODUCTION

Special histochemical staining has been used by pathologists to assist in tissue-based diagnoses for over 100 years[1,2]. The application of special staining in the diagnosis of renal diseases has been recognized as an important method for distinguishing the types of renal diseases and displaying some special components in renal tissues[3].

To date, a variety of special stains have been applied to renal biopsies, which includes periodic acid silver methenamine (PASM) staining and Masson's trichrome staining. Lesions of the glomerular capillary walls are the main pathological changes, which helps to characterize and differentiate various glomerular diseases. PASM staining is mainly used to observe the degree of abnormalities of the glomerular capillary walls in glomerular basement membrane[4,5]. Masson staining is utilized to investigate an extremely wide variety of glomerular diseases[6,7] and has been found to be effective in identifying the accumulation of the immune complex as well as the proliferation of basement membranes and renal interstitial collagen fibers[8]. For example, chronic graft damage in both the medulla and the cortex in renal transplant biopsies was measured by PASM and Masson histochemistry, respectively[9]. However, if PASM and Masson staining are done separately, it is difficult to accurately detect the localization of the basement membrane and immune complex, and to judge whether these two lesions exist at the same site. On the contrary, PASM-Masson dual staining can clearly distinguish the staining and localization of the basement membrane and the immune complex in the same section. Moreover, it can shorten the staining time and improve the work efficiency. Therefore, PASM-Masson staining plays an important role in the diagnosis of glomerular diseases.

In order to identify the location and extent of glomerular immune complex deposition in different types of renal diseases, 152 cases of renal biopsy were collected and PASM-Masson dual staining was performed in the present study.

PROTOCOL:

The protocol follows the guidelines of our hospital's human research ethics committee. One hundred and fifty-two cases of renal biopsy specimens were collected from the Department of Pathology at the Shenzhen Third People's Hospital (The Second Affiliated Hospital of Southern University of Science and Technology) from January 2018 to May 2019.

1. Material preparation

1.1. Fix all tissues with 4% neutral buffered formaldehyde (pH 7.0) for 24 h.

1.2. Dehydrate the fixed tissues in an ethanol series (100%, 95%, 80% and 75%) for 1 min in each percentage, clear in xylene and then embed in paraffin wax.

1.3. Cut the slices into 1-1.5 μm slices and incubate for 40 min in a 70 °C oven.

NOTE: The main chemical reagents include Bouin's fixed solution (saturated picric acid:

formaldehyde: glacial acetic acid = 15: 5: 1), Masson kit (iron hematoxylin, bright red acid fuchsin working fluid, aniline blue, phosphomolybdic acid), and Periodic acid-sliver methenamine (PASM) kit (periodate, borax solution, silver nitrate hexamethylenetetramine powder).

2. Staining

2.1. After preparing the tissues in step 1, fix the tissues immediately in 10% formalin.

NOTE: The 1-1.5 μm thick tissue sections are strongly recommended.

2.2. Deparaffinize each slice in dimethylbenzene for 5 min and then repeat once. Oxidize the slices with 1% periodate for 30 min and rinse with distilled water for 5 s.

2.3. Immerse the sections into hexamine silver (working solution) for 35 min (water bath 70 °C). After the color turns golden yellow, terminate the staining and wash the slides with distilled water for 5 s.

NOTE: The reaction time can be adjusted according to the temperature of Bouin's solution, hexamine silver working solution, and Masson solution (Bouin's solution, 60 °C water bath for 1 h, 37 °C for 4 h and 26 °C for 24 h; Hexamine silver solution, 70 °C for 35 min and 60 °C for 50 min; Masson solution, 37 °C for 20 min and 26 °C for 30 min).

2.4. Decolorize the sections with 100 μL of 0.1% gold chloride for 1 min and observe the staining under light microscopy. When the golden-yellow staining disappears and basement membranes show an obvious black staining, wash the sections with distilled water three times.

NOTE: The optimal time of PASM-Masson staining is the critical step in the protocol. The staining needs to be controlled under light microscopy.

2.5. Fix the tissues with 3% sodium thiosulfate for 1 min, replenish in Bouin's solution (37 °C water bath for 4 h) and wash the sections with distilled water for 5 min.

2.6. Stain the sections with Mayer hematoxylin solution for 15 min, and incubate in 45 °C water for 30 s.

2.7. Dye the specimens with 100 μL of Masson solution for 30 min and rinse with distilled water for 5 s. Differentiate the sections with 100 μL of 1% phosphomolybdic acid for 5 min.

2.8. Remove and shake off the phosphomolybdic acid by hand. Dye the sections with 100 μL of 1% aniline blue for 5 min.

2.9. Rinse the sections with distilled water. Fix with 1% acetic acid for 2 min.

2.10. Dehydrate the sections lastly by 95% for 10 s and then 100% alcohol for 1 min. Seal.

REPRESENTATIVE RESULTS:

PASM-Masson staining before modification (**Figure 1A-D**) showed that the hexamine silver staining was dyed deep black, and Masson staining showed a light dyeing on the immune complex in the glomerulus. In addition, the contrast between the basement membrane and the immune complex was not distinct. As a whole, the structures of the glomeruli and renal tubules were indistinct, and the background colors displayed hyperchromasia.

On the contrary, PASM-Masson staining after modification (**Figure 2A-F**) showed that the slice background and structure were clean and clear, and the color contrast was distinct in the renal biopsy. As shown in **Figure 2**, we found that the glomerular basement membrane, the tubular basement membrane, and the capillary basement membrane were dyed black, the stroma and hyperplastic collagen fibers were stained dark blue, and the colors of the red blood cells, renal tubular epithelial cells and immune complexes were stained red. Thus, the glomerular immune complexes and basement membranes can be differentiated accurately by modified PASM-Masson staining.

In order to show the different components of lesions in renal disease clearly, H&E staining, PAS staining, Masson staining and PASM-Masson staining were performed individually on the same section. As shown in **Figure 3,** diffuse thickening of the glomerular basement membrane was observed through H&E staining and PAS staining (**Figure A, B**). There was protein deposition on the peripheral capillary wall by Masson staining (**Figure C**) and spike formation by PASM-Masson staining (**Figure D**).

FIGURE LEGENDS

Figure 1. The PASM-Masson staining before modification. As indicated in **Figure 1A-D**, the color of glomerular basement membrane was deep black (hexamine silver staining). The color of the immune complexes was dyed light by Masson staining in the glomerulus. In addition, the structures of kidney glomeruli and renal tubules were indistinct. All images were with 400x magnification (scale bar is 50 μm).

Figure 2. The PASM-Masson staining after modification. Membranous nephropathy (Phase 3), Many "spiking formations" on the outside of the basement membrane of glomerulus. PASM-Masson double staining showed that the basement membrane was black, and the immune complex was red (**A, B**). Lupus glomerulonephritis: Double staining showed that the proliferated mesangial cells and capillary endothelial cells are dark red (**C, D**). Hypertensive glomerulonephritis: the wall of afferent glomerular arteriole was dyed light blue, the fibrosis of the stroma and the glomerulus were dark blue, the color of the basement membrane was black (**E, F**). All images were with 400x magnification (scale bar is 50 μm).

Figure 3. Different stains in serial sections of membranous nephropathy. H&E and PAS staining showed diffuse thickening of the glomerular basement membrane (**A, B**). Masson

staining showed protein deposition on the peripheral capillary wall (**C**). PASM-Masson staining showed spike formation (**D**). All images were with 400x magnification (scale bar is 50 μm).

DISCUSSION:

Pathological diagnosis is an important tool for the diagnosis and differential diagnosis of renal diseases, and it is of great significance for the guidance of treatment and predicted value of renal diseases[10-12]. Special staining is an essential method for renal biopsy including PAS staining, PASM staining and Masson staining[3,13-15]. For routine work, there will be increased workloads for pathologists if the tissues were individually stained by PASM staining and Masson staining. Moreover, it is unfavorable for pathologists to observe and clarify the deposition and location of immune complexes under the microscope. In contrast, PASM-Masson dual staining can show the changes of glomerular capillaries and renal microcapsule basement membrane in inflammatory injuries, and it can also detect the deposition of the immune complex clearly.

We observed that silver hexamethylene staining was usually deep dyeing, and Masson staining was too weak on the immune complex using the conventional method of PASM-Masson staining. Furthermore, the view contrast between the basement membrane and the collagen fibers was indistinct in the glomeruli. To overcome these problems, several improvements were made in this study. For instance, the time of periodate oxidation was increased to fully expose the aldehyde component in the mucopolysaccharide of the basement membrane. A key step is fixing the renal tissues with Bouin's solution in a 37 °C water bath for 4 h. The picric acid in the Bouin's solution softens the fiber tissues. Therefore, macromolecular dyes (e.g., Ponceau Red) can easily penetrate. Meanwhile, the pH environment was reduced, and the acid dye can be easily colored. From these measures, the stained collagen fibers, red cells and immune complexes can be distinguished easily from the dyeing of the basement membrane.

There are critical steps in the process of PASM-Masson dyeing. Firstly, the working solution of silver hexamethylamine must be used as soon as possible after preparation. In addition, the solution needs to be preheated in a 60 °C water bath for 15 min before application. Of note, it cannot be preheated prematurely or be used repeatedly; otherwise, a deep background coloring will be produced. Secondly, the thickness of renal sections is critical. If the section is too thick, the structure of basement membrane and pathological changes including the glomerulus structure and the "spike formation" were indistinct for observation. In our experience, the thinner the slice is, the better the staining is (a thickness about 1-1.5 μm is suggested). Additionally, monitor the time of PASM-Masson staining. The dyeing time must be observed and adjusted under the microscopy. PASM staining should be terminated when the glomerular basement membrane is stained black under the microscope. In order to prevent non-specific staining of the basement membrane, do not stain for too long. Finally, the length of the high concentration alcohol dehydration needs to be controlled; otherwise, it will cause the decolorization of the Masson stains.

In conclusion, the location and extent of glomerular immune complex deposition are clearly observed by the modified PASM-Masson staining, which might provide reliable evidence for accurate pathological diagnoses.

DISCLOSURES:

The authors have nothing to disclose

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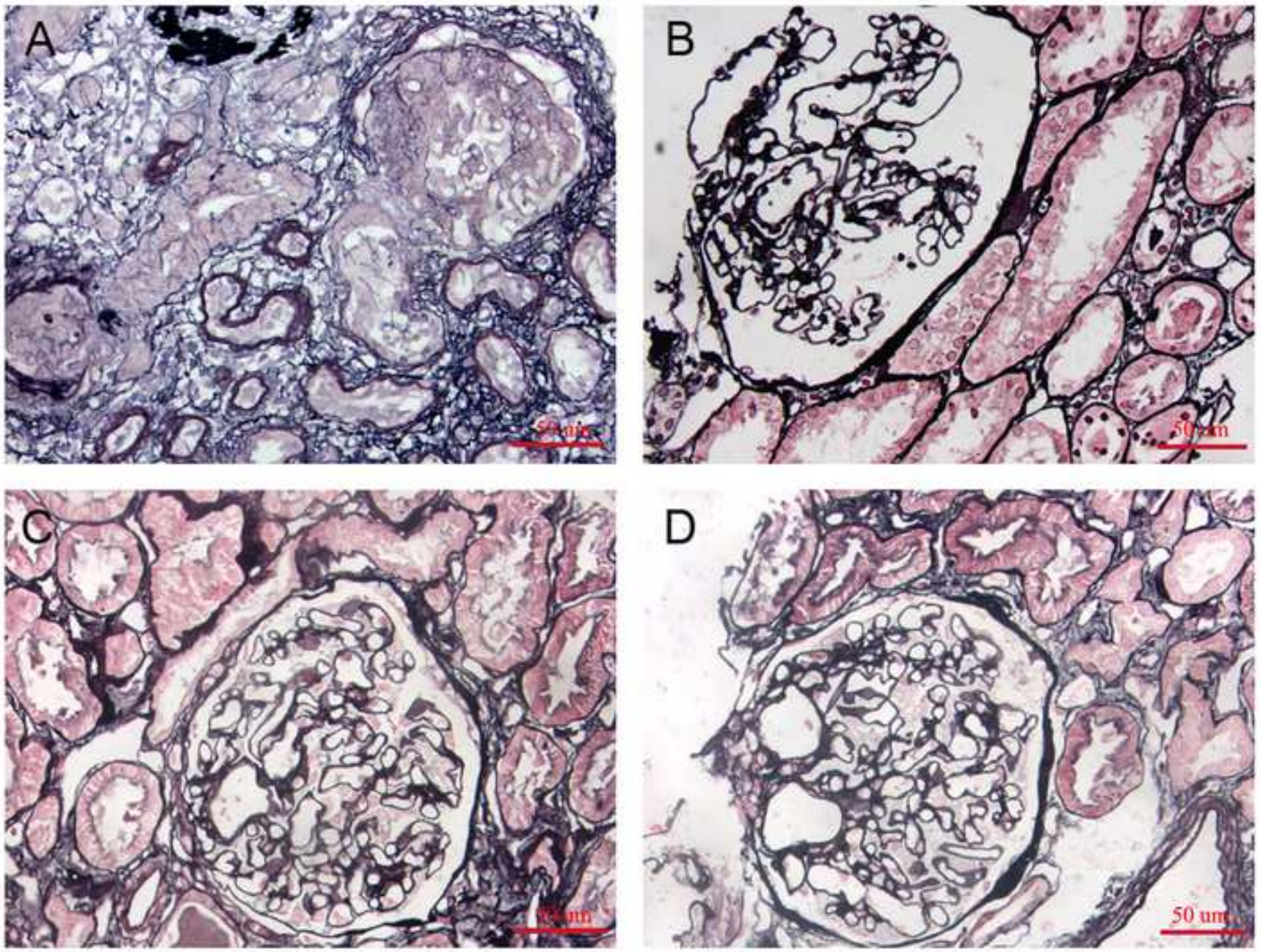

Figure 2

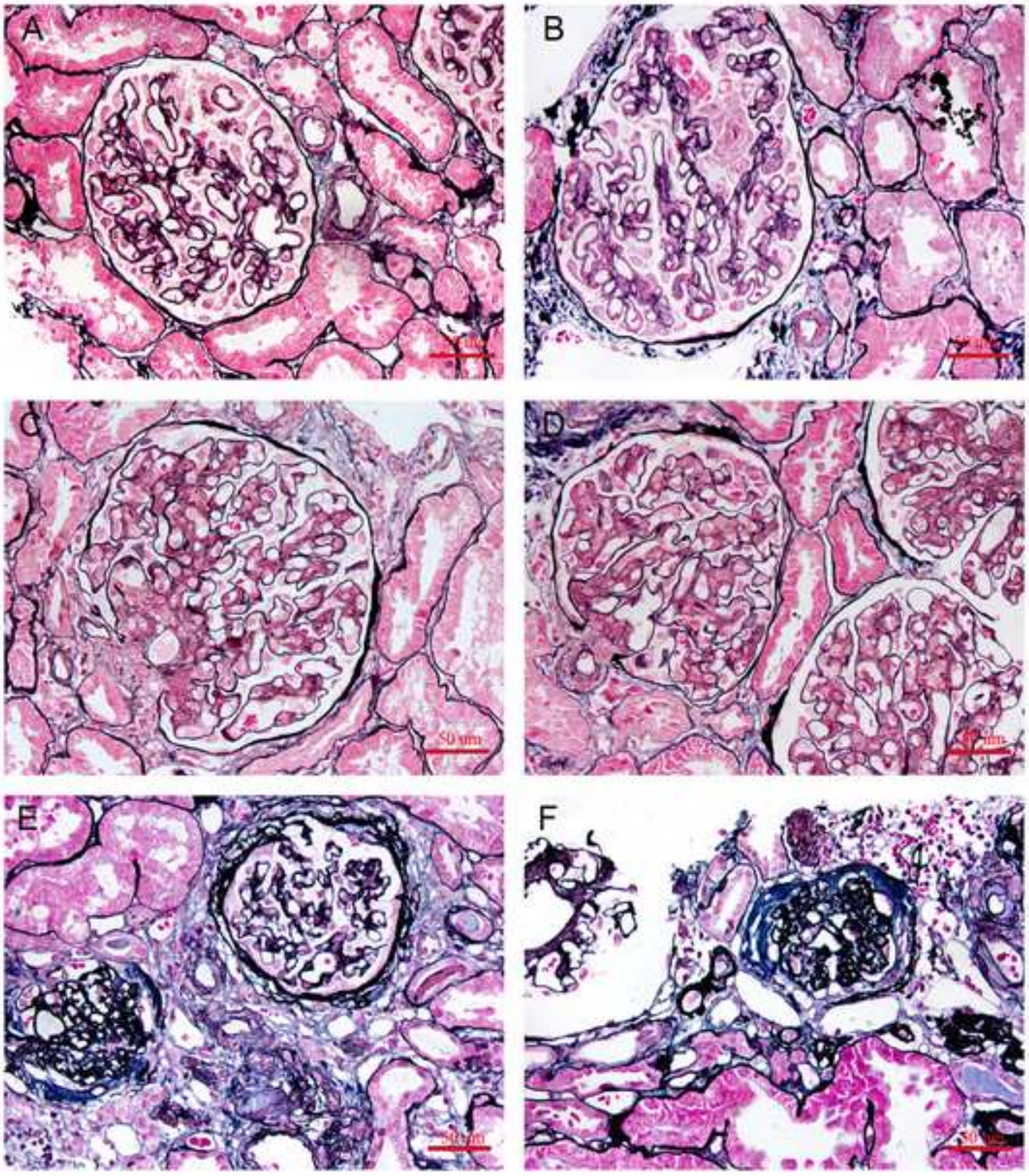

Figure 3

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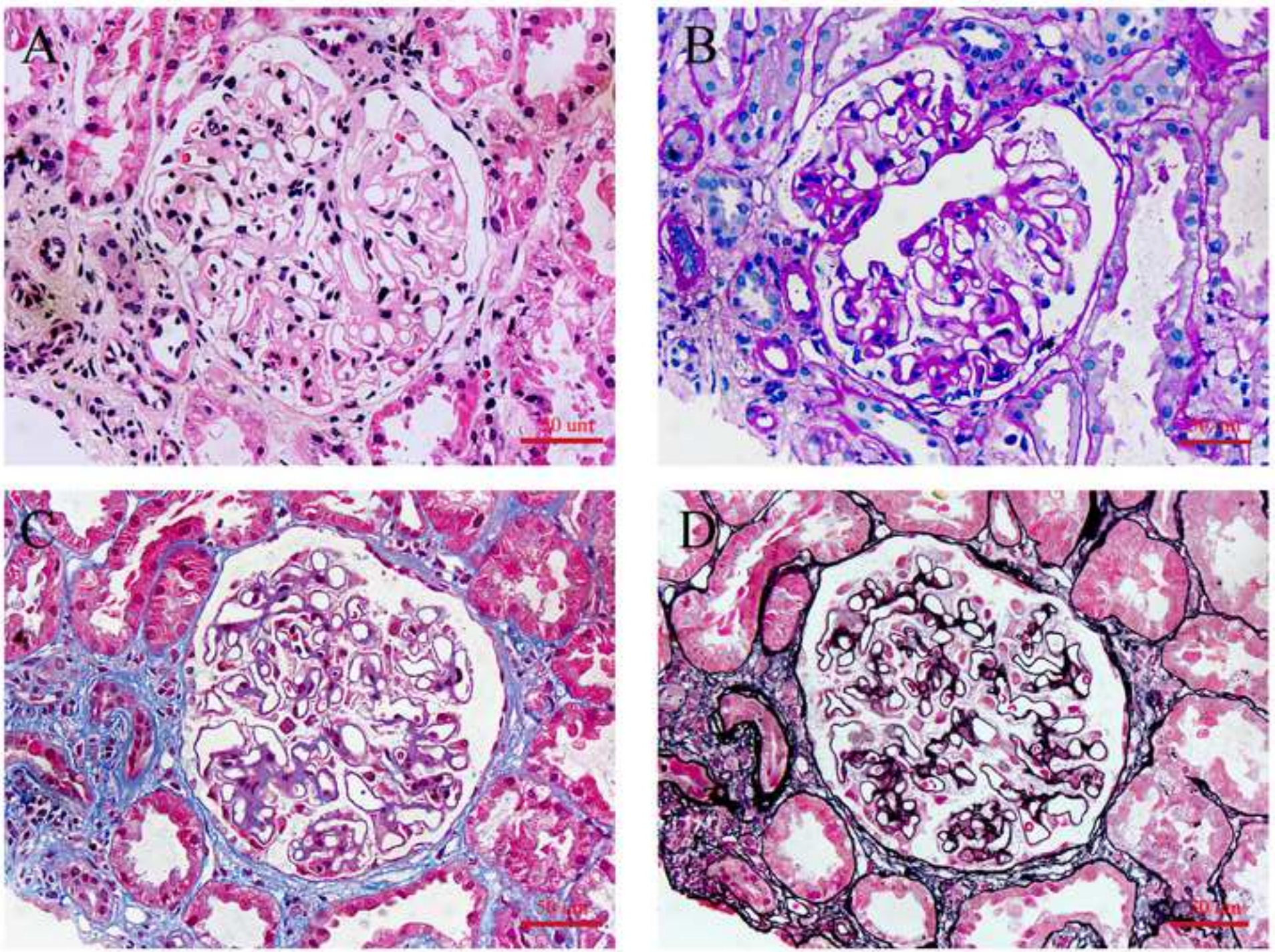

Name of Material/ Equipment	Company
Absolute ethyl alcohol	Guanghua company (Guangzhou city, China)
Bouin's fixed solution	Kaixiu company (Guangzhou city, China)
Dimethylbenzene	Sinopharm Chemical Reagent limited corporation
Ethyl alcohol (75%)	Likang company (Nanchang city, China)
Ethyl alcohol (95%)	Likang company (Nanchang city,China)
Glacial acetic acid	Lingfeng company (Shanghai city, China)
Hematoxylin staining solut	BASO company (Zhuhai city, China)
Masson kit	Weigxi company (Guangzhou city, China)
PASM kit	BASO company (Zhuhai city, China)

Catalog Number	Comments/Description
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20180915	
20181005	
20170912	
718101	
18121101	Store at 4℃
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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: We have thoroughly proofread the manuscript

2. Please provide at least 6 keywords or phrases.
Answer: (line 18-20) KEYWORDS
Special staining, PASM staining, Masson staining, PASM-Masson staining, renal biopsy, immune complexes, basement membrane, glomerular diseases

3. Step 1.1: Please write this step in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: (line 71-73) Collect one hundred and fifty two cases of renal biopsy specimens from Department of Pathology, Shenzhen Third People's Hospital (The Second Affiliated Hospital of Southern University of Science and Technology) from January 2018 to May 2019.

4. Step 1.2: Please write this step in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: (line 75-78) Fix all tissues with 4% neutral buffered formaldehyde (pH = 7.0) for 24h. Dehydrate the fixed tissues in an ethanol series (100%, 95%, 80% and 75%) for 1 min in each percentage, clear in xylene and then embed in paraffin wax. Finally, cut the slices into 1 μm-1. 5 μm and incubate for 40 min in 70 ℃oven.

5. Step 1.3: Please write this step in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: (line 80-83) The main chemical reagents include Bouin's fixed solution (saturated picric acid: formaldehyde: glacial acetic acid = 15: 5: 1). Masson kit (iron hematoxylin, bright red acid fuchsin working fluid, aniline blue, phosphomolybdic acid). Periodic acid-sliver methenamine (PASM) kit (periodate, borax solution, silver nitrate hexamethylenetetramine powder).

6. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Answer: (line 232-268)
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7. 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.

Answer: (line 87) The protocol follows the guidelines of our hospital’s human research ethics committee.