ADVANCING KIDNEY HEALTH WORLDWIDE. TOGETHER.

TECHNICAL MANUAL FOR RENAL PATHOLOGY

Methodologies on Specimen Handling, Processing, and Staining of Renal Biopsy Specimens for Light Microscopy, Immunohistochemistry and Immunofluorescence Microscopy, and Electron Microscopy

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INTRODUCTION AND OVERVIEW OF THE TECHNICAL MANUAL

Requirements for optimum renal biopsy results:

- Adequate specimen obtained by renal biopsy
- Technically well-prepared specimen for all methods of evaluation
- Complete relevant clinical history
- Pathologist who is knowledgeable and experienced in renal pathology
- Accurate and informative report and diagnosis
- Nephrologist who understands the report and how it should influence the patient's management

Prioritization with limited resources:

This manual makes recommendation for optimal processing and testing of renal biopsies. However, we recognize that many areas have limited resources. Useful information may still be obtained by more limited testing than described in this manual. Our suggested priorities when resources are limited, include light microscopic (LM) sections, 2-3 microns, with testing with immunofluorescence (less background than immunohistochemistry, IHC) as indicated below.

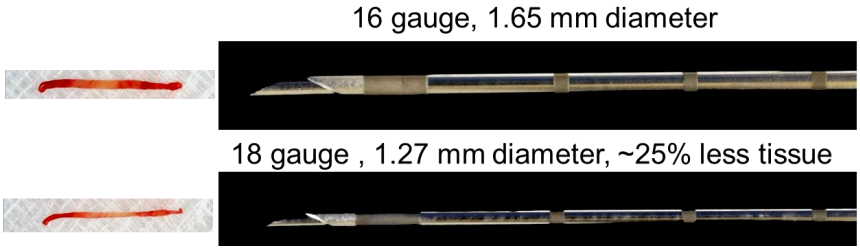
Stain priorities for LM: periodic acid Schiff (PAS) and hematoxylin and eosin (H&E), followed, if possible, by trichrome, silver stain and Congo red (the latter as indicated).

Immunofluorescence (or IHC) stain priorities for native kidneys: IgG, IgA, C3, followed, if possible, by kappa and lambda, and then in order of priority C1q, then C4, fibrin and others.

Immunostaining for transplant: C4d as priority, followed by SV40 for polyoma virus, and IF as for native kidneys, when there is suspicion of glomerular/monoclonal-related diseases.

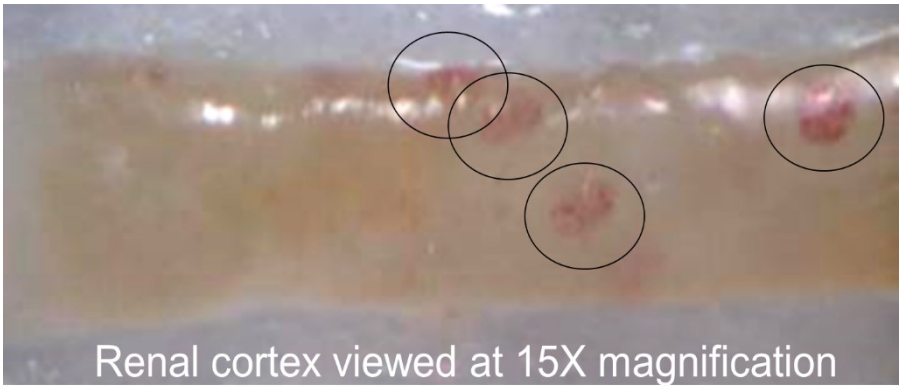
Electron microscopy: With limited resources, EM may be done only in selected cases, based on LM/IF findings, and/or clinical suspicion, or may not be available, recognizing that EM can be helpful to diagnose some diseases (e.g., thin basement membranes, immunoactoid glomerulonephritis).

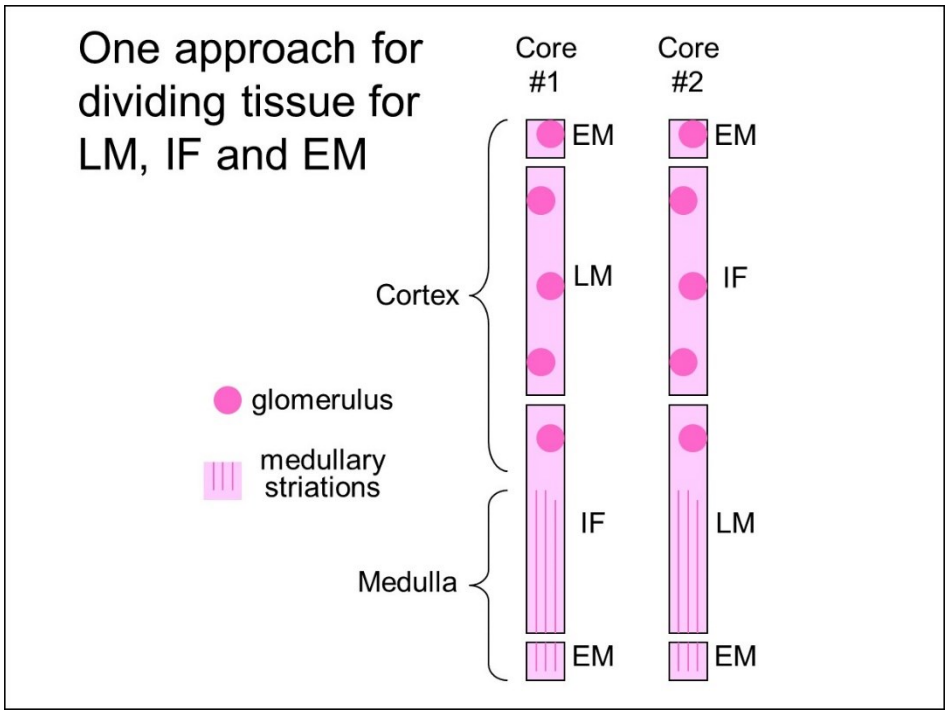
Recommended biopsy needle size: 16-gauge needles provide more glomeruli, more diagnostically adequate tissue, fewer cores, and fewer repeat biopsies with no increase in complications compared with 18-gauge needles (Am J Transplant. 2005; 5:1992-6; Am J Nephrol. 2013; 37:249-54; Nephrology. 2013; 18:525-30).



Recommendations for light microscopy:

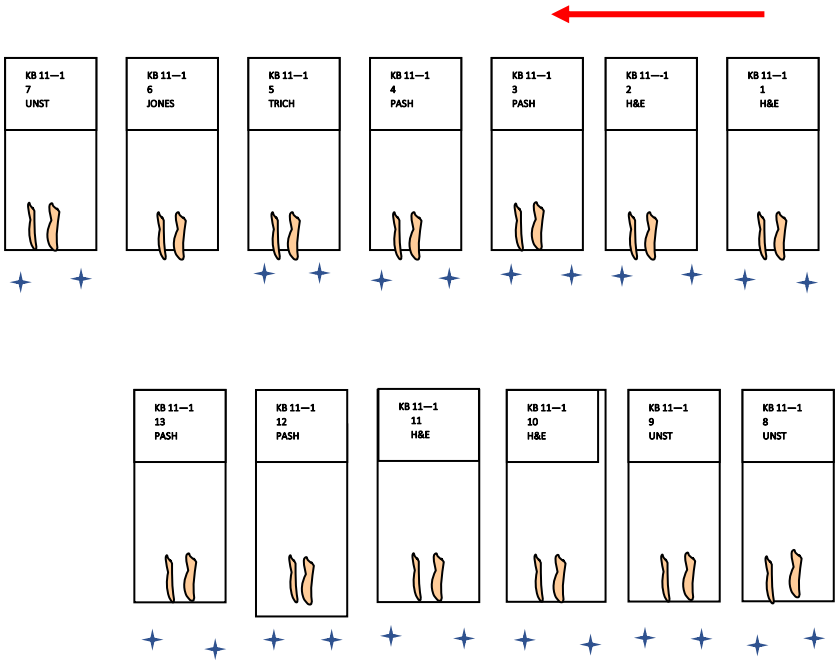
- Fix: fresh 10% neutral buffered formalin
- Cut: 2-3 micrometer thick sections, 10-20 consecutive levels
- Stain: alternating H&E, PAS, Masson trichrome, Jones silver

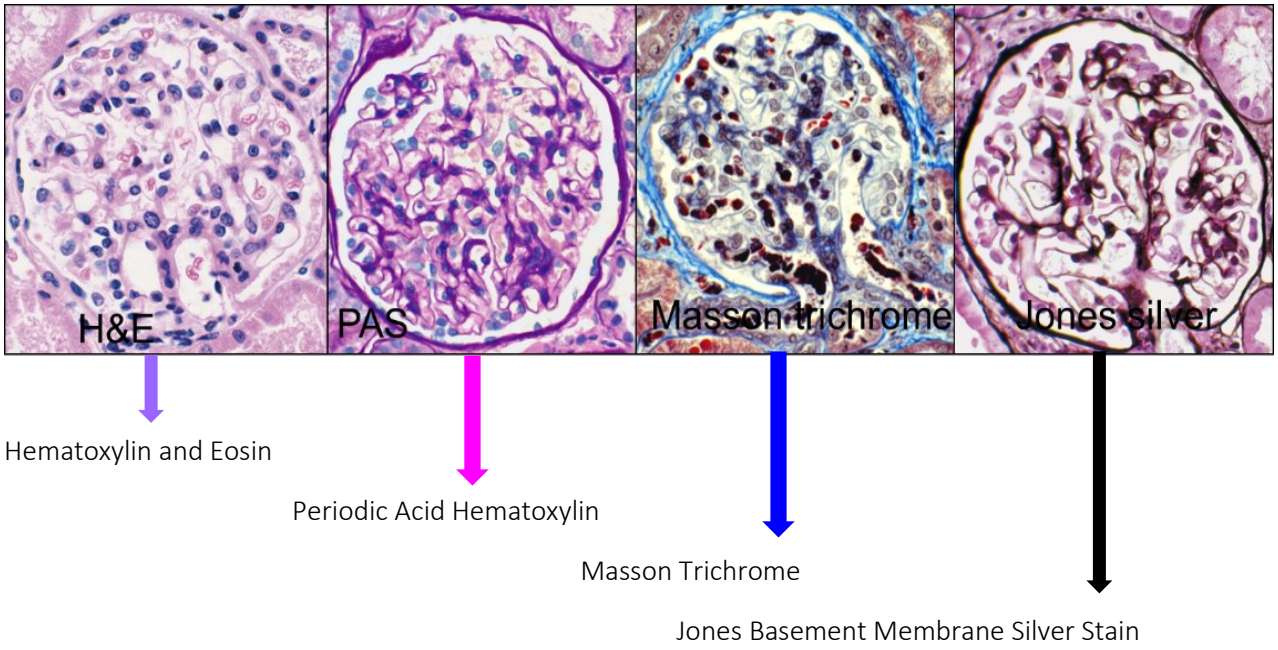




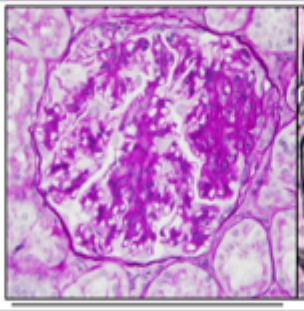
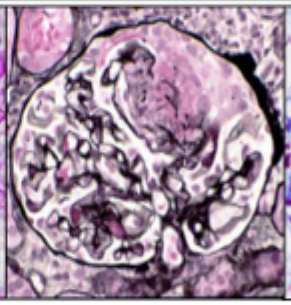
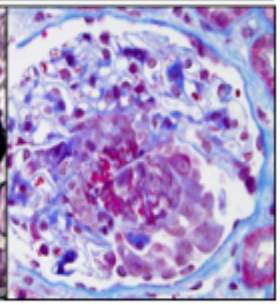
Cut the required number of slides and sections

Notes: This arrangement/requirement varies from one institution to the other.



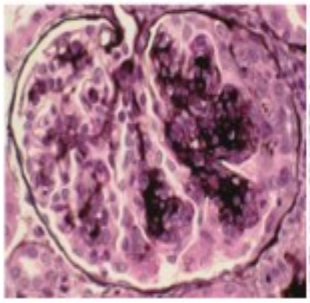


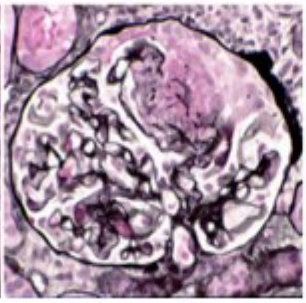
Three histochemistry stains commonly used in Renal Pathology:

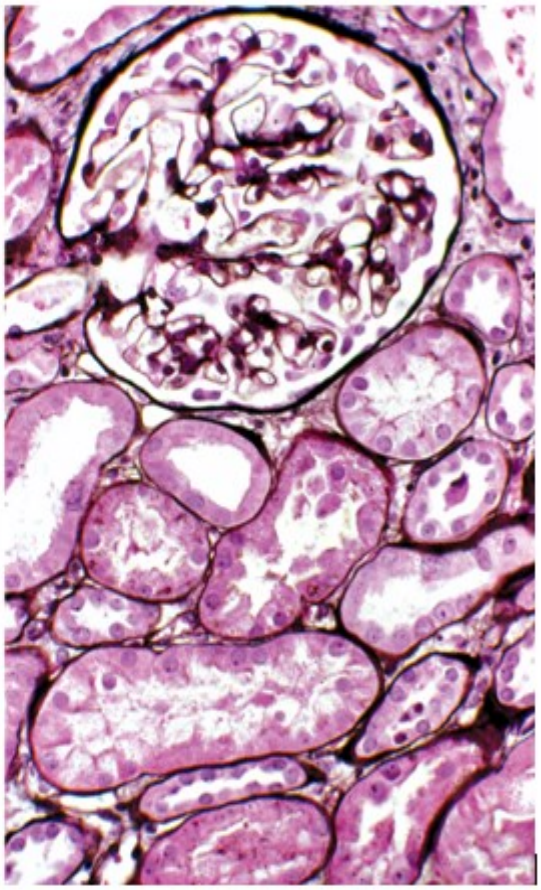
	PAS	JONES	MASSON
Basement membrane	purple	black	blue
Mesangial matrix	purple	black	blue
Interstitial collagen	negative	negative	light blue
Normal cell cytoplasm	negative/faint	negative	off red
Immune complex deposits	negative/faint	negative	red (not sensitive)
Insudative lesions (hyaline)	purple	negative	red
Sclerosis/fibrosis	purple	black	blue
Fibrin/fibrinoid	weakly positive	negative	red
Amyloid	negative/weakly positive	negative/weakly positive	light blue
Tamm-Horsfall protein casts	purple	negative	light blue
			

Jones Basement Membrane Silver Stain:

JONES	
Basement membrane	black
Mesangial matrix	black
Interstitial collagen	negative (i.e. silver-negative)
Normal cell cytoplasm	negative
Immune complex deposits	negative
Insudative lesions (hyaline)	negative
Sclerosis/fibrosis	black
Fibrin/fibrinoid	negative
Amyloid	negative/weakly positive
Tamm-Horsfall protein casts	negative

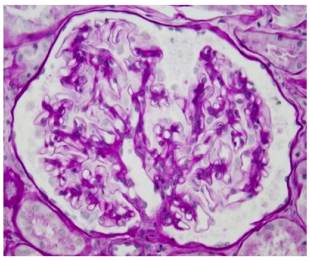


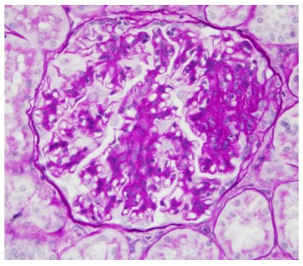


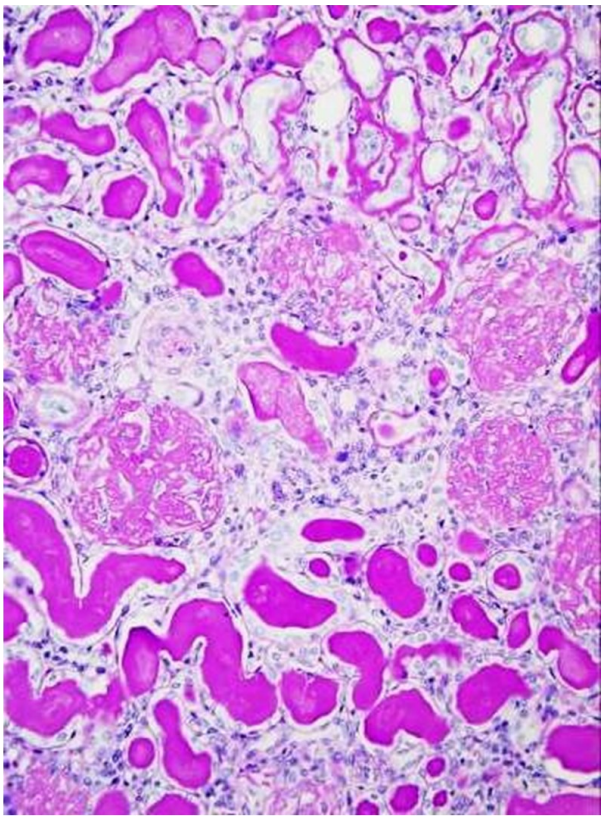


Periodic Acid Schiff Stain:

	PAS
Basement membrane	purple
Mesangial matrix	purple
Interstitial collagen	negative
Normal cell cytoplasm	negative/faint
Immune complex deposits	negative/faint
Insudative lesions (hyaline)	purple
Sclerosis/fibrosis	purple
Fibrin/fibrinoid	weakly positive
Amyloid	negative/weakly positive
Tamm-Horsfall protein casts	purple

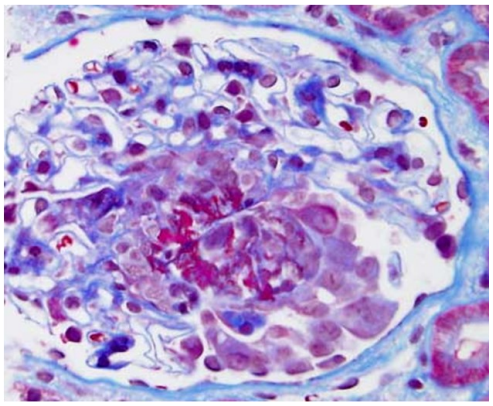


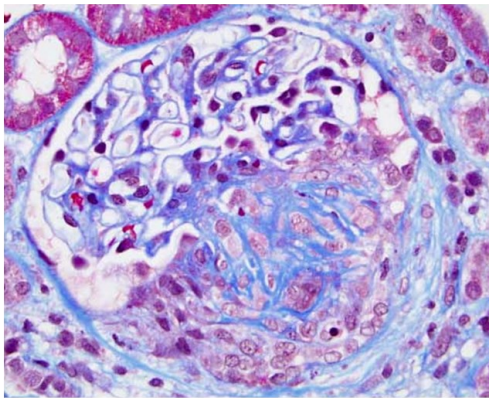


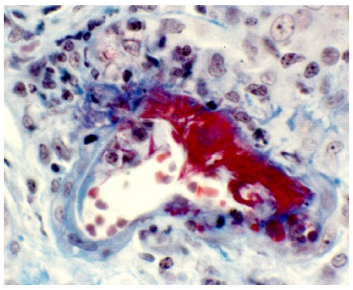


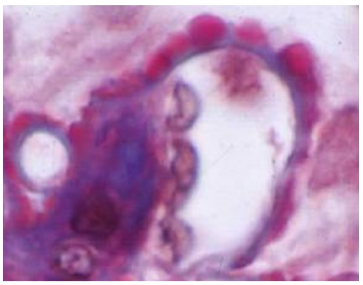
Masson Trichrome Stain:

MASSON	
Basement membrane	blue
Mesangial matrix	blue
Interstitial collagen	light blue
Normal cell cytoplasm	off red
Immune complex deposits	red (not sensitive)
Insudative lesions (hyaline)	red
Sclerosis/fibrosis	blue
Fibrin/fibrinoid	red
Amyloid	light blue
Tamm-Horsfall protein casts	light blue









Immunofluorescence Microscopy (IF)/ Immunohistochemistry (IHC)

Pros and Cons of Immunofluorescence Microscopy (IF) versus Immunohistochemistry (IHC)

Immunofluorescence Microscopy (IF)

- Pro: Faster
- Pro: Very good contrast (good signal to noise)
- Pro: Easier to semi-quantify reliably
- Con: Freezing required for optimum results
- Con: Special fluorescence microscope required

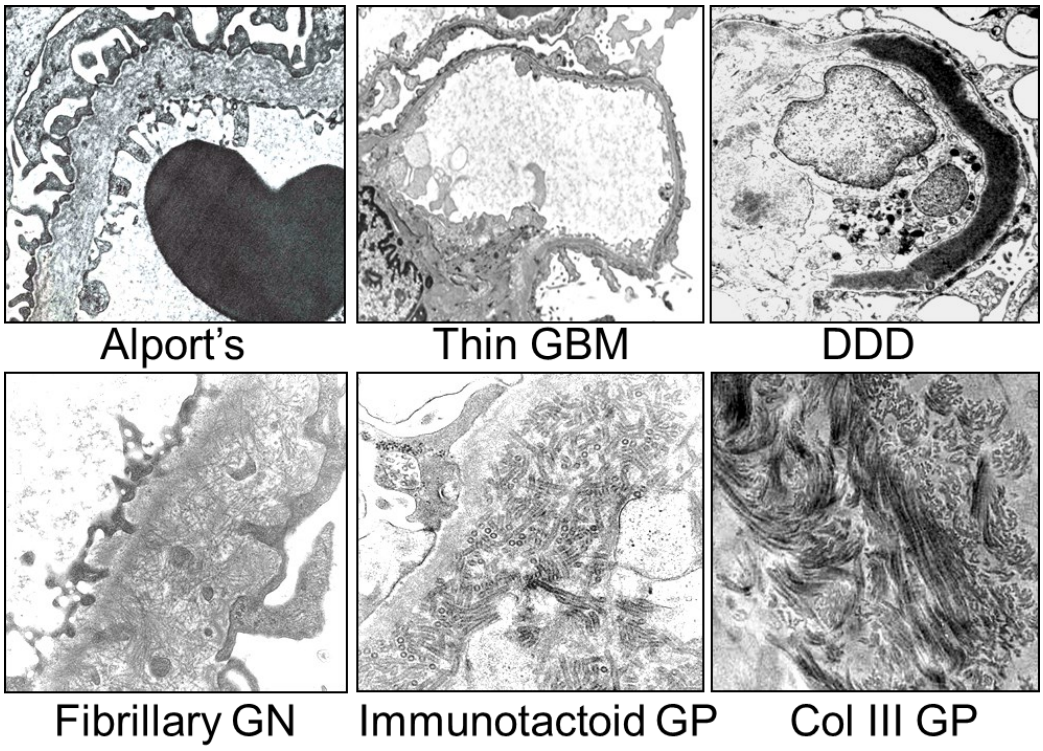
Add IF on pronase-digested paraffin sections as alternative opportunity with its “pro” and “contra” in comparison with IF on frozen sections, namely:

- Pro using IF for external(referral) cases) second opinion)
- Pro: necessary for diagnosis of some variants of MGRS
- Pro: useful in cases of non-informative second (IF) core of kidney tissue
- Con: Usually higher background
- Con: Some antigen-retrieval unreliable (first, C3-component)
- Con: Needs cryostat

Immunohistochemistry (IHC)

- Pro: Can use same fixative for LM and IHC
- Pro: Easy to correlate IHC stain results with LM histologic structures in same block
- Con: Usually higher background
- Con: Some antigen-retrieval unreliable
- Con: More difficult to semi-quantify reliably

Recommended routine antibody specificities: IgG, IgA, IgM, C3, C1q, kappa light chains, lambda light chains, fibrin and albumin. Additional IF/IHC Special Stains: Amyloid proteins, IgG subclasses, COL IV α chains, COL III, Fibronectin, PLA2R, myoglobin, hemoglobin, C4d (transplants), viruses (Polyoma, CMV, EBV, Adenovirus, etc.), DNAJB9.

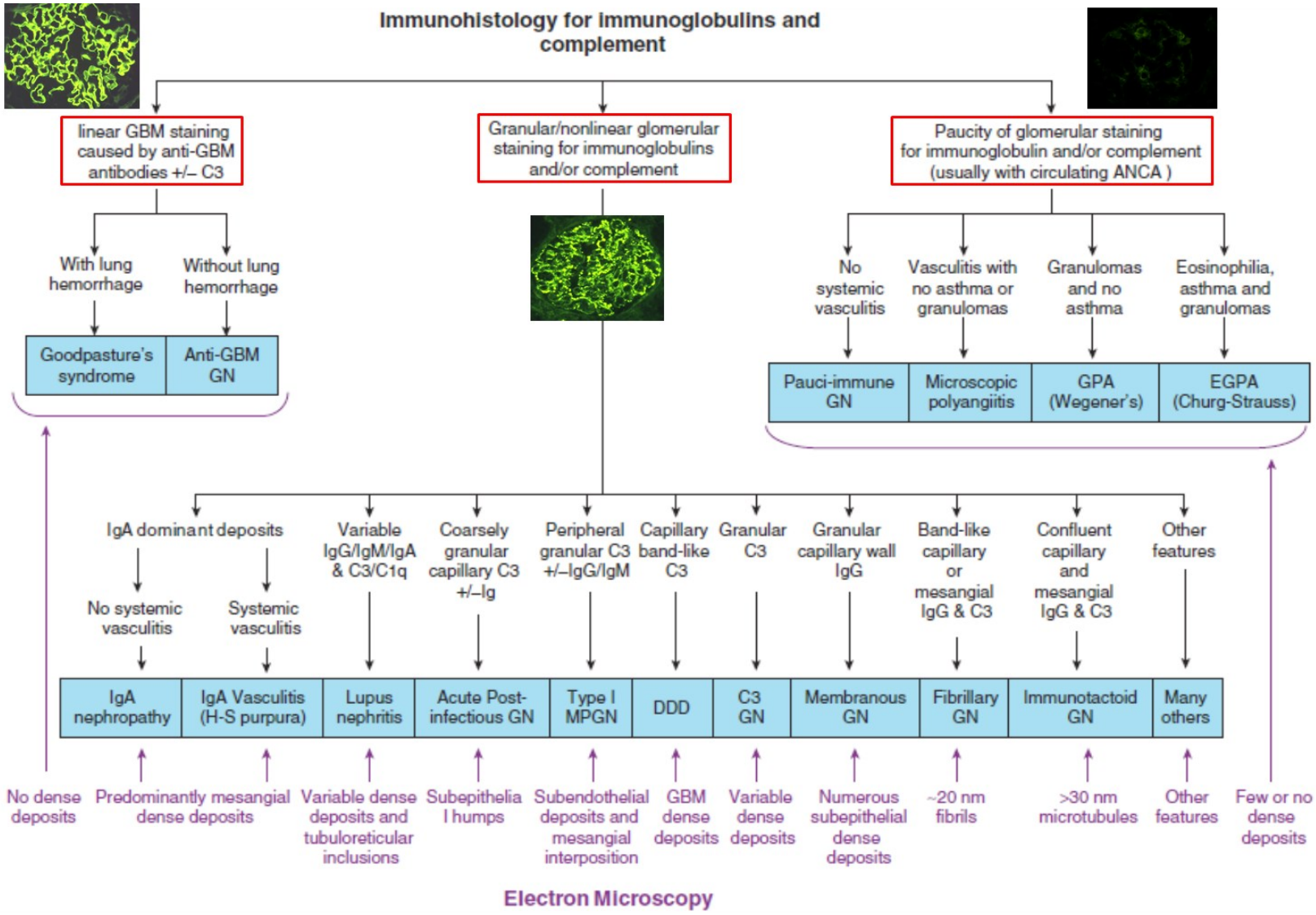


Transmission Electron Microscopy (EM)

EM is valuable for renal biopsy evaluation.

In a study of 233 native renal biopsies (Haas M. J Am Soc Nephrol 8:70,1997), EM was critical or important for diagnosis in approximately half of the specimens:

- Critical for diagnosis 21%
- Important contribution to diagnosis 21%
- Not required for diagnosis 58%



Primer on the Pathologic Diagnosis of Renal Disease *in* Heptinstall's Pathology of the Kidney, 7th Edition, Wolters Klewer, 2015

LIGHT MICROSCOPY

Specimen Handling of Renal Biopsies

Purposes

To describe the process of collection, transportation of renal tissue sample as fresh, in transport medium, or in fixative solutions. Proper identification of the specimen, getting the correct optimum sample, using the right equipment as these are the foundations of successful laboratory testing.

Principle

The process of collecting specimen from a patient, transporting it in a holding solution in as short as possible time for laboratory testing to obtain optimum results.

Specimen Fixatives

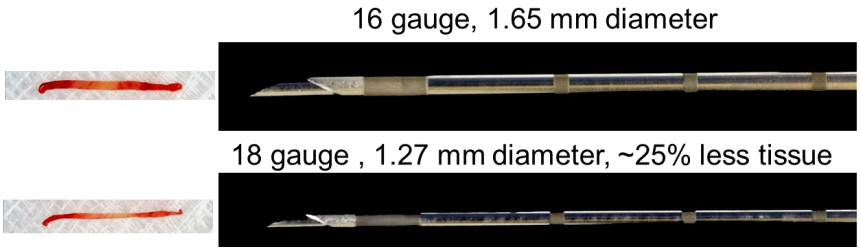
- Neutral buffered formalin,10%
  Cat# 3800757
  Leica Biosystems
- Alternative fixatives:
  Paraformaldehyde, 4% in phosphate buffer solution (PBS) pH 6.9-7.4
  Cat3 s2303
  Poly Scientific R & D
- Carson's Phosphate Buffered Formalin (Millonig's fixative)
  Cat # 12445A
  Newcomer Supply
- Michel's transport media (Stat Lab)
  Cat# SKU#MS0507/24
  StatLab
- Fresh, wrapped in gauze wet with normal saline solution
  Cat#S5812
  Teknova

Notes: All reagents are stored per manufacturer's instructions.

If renal biopsies are received in any of the fixatives/solutions, representative sections are eventually transferred to 10% neutral buffered formalin for light microscopy studies.

Collection of Renal Biopsies

Recommended biopsy needle size: 16-gauge needles provide more glomeruli, more diagnostically adequate tissue, fewer cores, and fewer repeat biopsies with no increase in complications compared with 18-gauge needles (Am J Transplant. 2005; 5:1992-6; Am J Nephrol. 2013; 37:249-54; Nephrology. 2013; 18:525-30).



Materials

- Telfa, individually packed, sterile, non-woven smooth gauze, dissecting microscope, petri dish, nitrile gloves, tweezers, handheld lens, plastic pipette, biohazard plastic bag,100-ml plastic container, labels

Stereozoom microscope

Handheld lens

Plastic transfer pipette (Uline)

100- ml plastic container

Resealable plastic biohazard bag

Non-woven, individually packed,

Telfa, sterile, non-adherent pads

Equipment and Maintenance

Dissecting microscope:

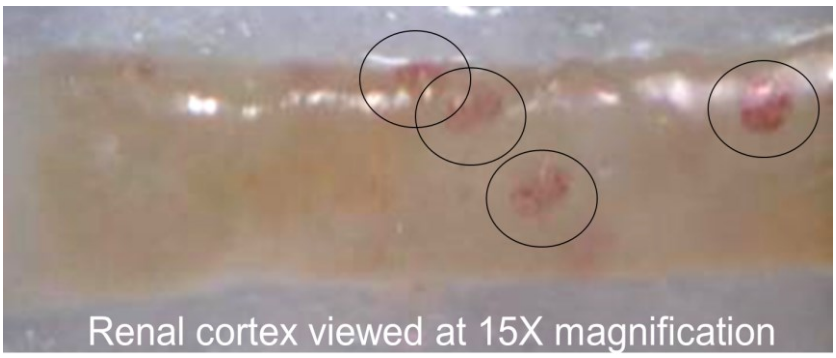
- Always keep clean and well-maintained
- Provide a quality control chart for documentation purposes of the regular maintenance.

Quality Control

During triaging, recommended number of glomeruli for immunofluorescence and electron microscopy studies are submitted and the remaining tissues of the renal biopsies are submitted for light microscopy studies.

Triaging Procedure

1. Transfer the tissue core from the biopsy instrument needle to telfa or gauze wet with normal saline solution to prevent drying out.
   1.1. May be transported in Michel's solution or Zeus.
2. Send the specimen immediately to the laboratory for processing along with a completed requisition form indicating that the specimen is a transplant or native.
3. Apply a few drops of normal saline solution on the dissecting dish.
   3.1. Layer the cores into this solution
   3.2. If received in Michel's solution, the glomeruli may not be clearly visible.
      3.2.1. Applying a few drops of normal saline solution to renal tissue may increase the visibility of glomeruli.
4. Check under the microscope for glomeruli (circles below).

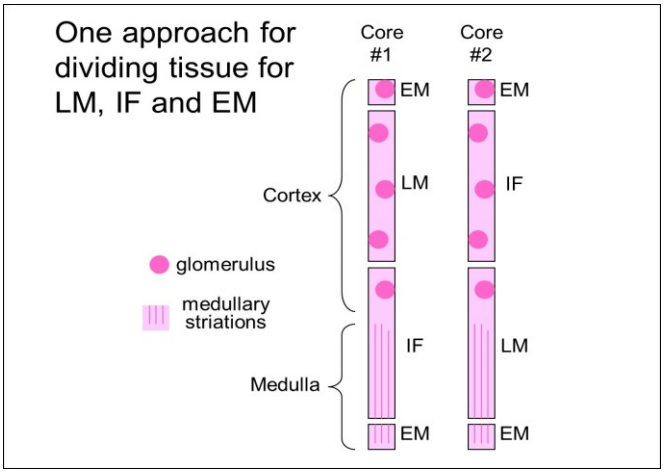


   4.1. If dissecting microscope is not available, a handheld magnifying glass may be used, although this does not allow optimal visualization.
   Further, if tissue is very ischemic, or sclerosed, glomeruli may not be readily visualized. In nonscarred tissue, glomeruli are small red circular areas under the dissecting scope.
5. When glomerular count is adequate (2 cores are usually required), divide using thin razor blade.
6. The ideal representative sections during triaging of renal biopsy are as follows:
   6.1. For electron microscopy, submit 1-2 glomeruli in paraformaldehyde, 4%
      6.1.1. Ideal size of tissue for electron microscopy studies is 1 mm^3
   6.2. For immunofluorescence, submit 3-4 glomeruli in Michel's solution
   6.3. For light microscopy, submit the remaining renal tissue in neutral buffered formalin, 10%
7. If a very small amount of tissue is obtained, divide according to clinical history, and after consulting with the renal pathologist and nephrologist on which studies are key for diagnosis.

7.1. Examples:

7.1.1. IF and LM are key for diagnosis of IgA nephropathy.

7.1.2. EM and IF are key for diagnosis of Alport syndrome and thin basement membrane lesion.



Safety

- Standard precautions apply
  - Wear appropriate personal protective equipment
    - Impervious laboratory coat
    - Nitrile gloves
    - Mask
  - Discard in red bag the following: gloves, dissecting dish, and any material that the specimen was transported in and not used in the storage.
  - Discard used blade in the sharp's container.
  - Return dissecting utensils to disinfecting solution.
  - Discard specimen containers in biohazard metal container

References

- Standard Operating Procedures in the Department of Pathology and Laboratory Medicine, Renal Division, Weill Cornell Medicine.
- Vaughn Jones, S.A., et al. The use of Michel's transport medium for immunofluorescence and immunoelectron microscopy in autoimmune bullous diseases. Journal of Cutaneous Pathology, 1995 August; 22(4): pages 365-370
- https://www.statlab.com/pdfs/ifu/FXFOR37.pdf
- https://biotium.com/wp-content/uploads/2017/10/PI-22023.pdf
- https://www.rabkindermpath.com/docs/fixative_qa_6.18.98.pdf
- https://www.mblintl.com/assets/JM-2113-500.pdf -
- Amazon.com (telfa, non-woven gauze)
- Uline (plastic bottle)
- Patrick Walker (picture of fresh renal biopsy)

Processing of Renal Biopsies

Purpose

To provide materials for a wide range of testing for light microscopy studies e.g., routine hematoxylin and eosin staining, histochemical, and immunohistochemical studies, etc.

Principle

Tissues contained in cassettes are submitted for processing. Tissue processors being used are closed systems to prevent harmful vapors from being released into the environment. While tissues remain in the retort, the solutions are delivered automatically into it by a computer -controlled mechanism. The initial process is the complete fixation of tissues; followed by gradual removal of water from the tissue by using ascending grades of alcohol (dehydration); then the tissues are cleared with xylene (removal of alcohol) making tissues translucent. The final step is the infiltration with paraffin to give support to the internal structures. The following added features are introduced to facilitate processing time: heat, pressure, vacuum, and agitation. The program is carefully designed, tested, and validated for each group of tissues based on sizes.

Specimens

Fix biopsies in 10% neutral buffered formalin (most frequently used) for at least 6 hours i.e., the fixation time begins when the specimen is initially placed in formalin (not when the specimen is sectioned during gross examination) and ends when the cassettes are no longer in formalin (Letter from CAP dated 12/12/2007) and not for more than 72 hours. Minimum fixation time for 1mm3 renal biopsies is 1 hour. However, prolonged (> one week) in fixative may lead to drier, more difficult to handle tissue. Immediate fixation is necessary to get optimum results. Rate of

penetration of fixative is 1mm per hour. Volume of fixative is 20 x the volume of the tissue. Cut wedge shaped at 3 microns, renal core tissues at 2 microns.

Quality Control

- The tissue processor is inspected to ensure that it is ready for processing
- Reagents are in their optimum levels of performance
- The final check of the quality is the microscopic review of stained slides.

Equipment and Supplies

- Examples of automatic tissue processor (shown right and below) and its accessories,
- Graduated cylinders, paraffin dispenser, metal pitcher for transferring melted paraffin, hydrometer

Standing

HistoCore PELORIS 3 Premium Tissue Processing System (Leica)

Tabletop

Sakura Tissue-Tek VIP E150 Tissue Processor, Bench

Reagents

- Neutral buffered formalin (NBF), 10%, commercially available from by Leica Biosystems,
  Cat #3800757
  Leica Biosystems,

- Paraformaldehyde, 4% in PBS pH 7.4.
  Cat #s2303
  Poly Scientific R&D

Notes: Tissue may be kept in PF or NBF for days before processing. However, prolonged (> one week) in fixative may lead to drier, more difficult to handle tissue.

Immediate fixation is necessary to get optimum results.

- Reagent alcohol, 100% (200 proof)
  Cat #RS4029
  Avantik

- Xylene
  Cat #RS4050
  Avantik
- Blue Ribbon Paraffin
  Cat #s3801360
  Leica Biosystems

Notes: All reagents are stored per manufacturer's instructions.

Processing Schedule for Renal Biopsies

AUTOMATIC TISSUE PROCESSING

- Load kidney biopsies in a 2-hour program
- Do not add eosin.
- The instruction on how to operate is included in every manual of a tissue processor

Station Number	Solution	Time in Station	Temp	Vacuum
1	Neutral buffered formalin, 10%	60 minutes	45° C	Ambient
2	Alcohol, 70%	1 minute	40° C	Ambient
3	Alcohol, 100%	1 minute	Ambient	Ambient
4	Alcohol, 100%	1 minute	Ambient	Ambient
5	Alcohol, 100%	1 minute	Ambient	Ambient
6	Alcohol, 100%	1 minute	Ambient	Ambient
7	Alcohol, 100%	18 minutes	45° C	Ambient
8	Xylene	1 minute	Ambient	Ambient
9	Xylene	1 minute	Ambient	Ambient
10	Xylene	14 minutes	45° C	Ambient
11	Paraffin	2 minutes	65° C	Vacuum
12	Paraffin	1 minute	65° C	Vacuum
13	Paraffin	14 minutes	65° C	Vacuum

Schedule for solution changes: Formalin and Alcohol series

Solutions	Monday	Tuesday	Wednesday	Thursday	Friday
NBF, 10%	Change			Change	
Alc, 50%	Change			Change	
Alc, 95%	Change		Rotated		Rotated
Alc, 100%, 1	Rotated		Rotated		Rotated
Alc, 100%, 2	Rotated		Rotated		Rotated
Alc, 100%, 3	Change		Rotated		Rotated
Alc, 100%, 4	Change		Change		Change

Notes:

- Solution changes is adjusted according to the number of cassettes loaded each day.
- Rotated means: Monday: Alcohol 1 and 2 are discarded, alc 3 moves to alc 1 position and alc 4 moves to alc 2 position, then the empty containers are refilled with fresh100% alcohol solutions.
- Wednesday and Friday: discard 95%, move alc 1 to 95%, alc 2 to alc 1, alc 3 to alc 2, alc 4 to alc 3 positions, then the last empty container is refilled with fresh 100% alcohol.

Schedule for solution changes: Xylene series

Solutions	Monday	Tuesday	Wednesday	Thursday	Friday
Xylene	Rotated			Rotated	
Xylene	Change			Rotated	
Xylene	Change			Change	

Schedule for solution changes: Paraffin series

Solutions	Monday	Tuesday	Wednesday	Thursday	Friday
Paraffin	Rotated			Rotated	
Paraffin	Rotated			Rotated	
Paraffin	Change			Rotated	
Paraffin	Change			Change	

Schedule for solution changes: cleaning solutions

Solutions	Monday	Wednesday	Friday
Cleaning alcohol, 100%	Change	Change	Change
Cleaning xylene	Change	Change	Change

Notes: Solutions for processing and cleaning changes depend on how many cassettes and how often the tissue processor is used

- Testing the Paraffin for xylene contamination
    - Dip your finger into the paraffin bath
    - Results:
        - Solid, dry…. Good, not saturated with xylene
        - Glossy, wet, and mushy paraffin--- not good. This means the solution is saturated with xylene. The last change of paraffin should be free of xylene

Notes: These schedules for solution changes are for tissue processors that do not indicate when to change the solutions according to saturation level of carry over.

- Offline measures like using a hydrometer to determine the concentration of alcohol series may help the user decides of when to change the alcohol.
- Testing paraffin for xylene saturation

There are, however, new tissue processors that have functionalities to indicate when to change the solutions, e.g., Leica Peloris, new Sakura Tissue Tek, and others.

Manual Method of Processing Biopsies

	FLEX ALCOHOL***		ETHYL ALCOHOL***	
Steps	Solutions	Time	Solutions	Time
1	Neutral buffered formalin, 10%	30' to 1 'at 40° C	Neutral buffered formalin, 10%	30' to 1 'at 40° C
2	Flex alcohol,70%	15 minutes	Ethyl alcohol, 70%	10 minutes
3	Flex alcohol,80%	15 minutes	Ethyl alcohol, 80%	10 minutes
4	Flex alcohol,95%	15 minutes	Ethyl alcohol, 95%	10 minutes
5	Flex alcohol,100%	20 minutes	Ethyl alcohol, 100%	10 minutes
6	Xylene	5 minutes	Xylene	5 minutes
7	Xylene	5 minutes	Xylene	5 minutes
8	Paraffin**	30 minutes	Paraffin	30 minutes
9	Paraffin**	30 minutes	Paraffin	30 minutes

Notes:

- Check if the tissues are translucent before infiltration*.
- For renal biopsies, 30 minutes for each paraffin change. **
- The above schedule works for tissues with thickness 0.1 cm or less. ***
- The time is adjusted according to the size of the tissues
- To increase the visibility of white tissue, put a drop of hematoxylin to the tissue during grossing
- Two protocols depending upon the kind of alcohol (see table) is being used.

Materials

- Forceps, chemical waste container, paraffin oven, a small paraffin bath to hold melted paraffin, paraffin pellets, timer, paper towel, thermometer, processing capsule, processing basket. Technicon Tissue Processor 2A Mono Duo Fixation Dehydration Staining Basket For reagents, use the reagents listed in the above table.
- Perform under the hood
    - Volume of each solution being used for processing is about 20 x the volume of the tissue

Procedural Notes

- Notes: Perform the process under the fume hood or a downdraft hood.
- For the length of time on each processing container, refer to the tabulation above.
- Use forceps to transfer cassettes from one container to the next.
- Use a 1-liter beaker to contain processing solutions (one may use equivalent containers). Cover the beaker to prevent evaporation. Or pour used solution to an amber container for storage purposes. The paraffin bath is set at 2 degrees temperature above the melting point of the paraffin in used. Use metal basket to hold cassettes being processed.

Processing capsule
Holds the submitted tissue

Holds the cassettes

Procedure

1. Put the basket of cassettes in the first beaker in a waterbath set at 40^0 C
2. Then transfer the basket from one container to the next manually
3. Beakers 2 through 7 at room temperature
4. The paraffin bath is set at 2 degrees above the melting point of paraffin wax used.

10% NBF
1

Waterbath

70% alcohol
2

80% alcohol
3

95% alcohol
4

100% alcohol
5

Xylene 1
6

Xylene 2
7

1st paraffin change
8

2nd paraffin change
9

Schedule for Solution Changes (for rush cases)

- All solutions are changed on Mondays
- Alcohol is changed on Wednesdays and Fridays
    - Discard 70% alcohol
    - Transfer the 95% alcohol into the empty 70% alcohol container
    - Transfer the 100% alcohol into the empty 95% alcohol container
    - Refill the empty container with fresh 100% alcohol.
- Xylene are changed on Wednesdays and Fridays
    - Discard xylene from the first container.
    - Transfer the content of the second xylene to empty xylene container
    - Refill the empty container with fresh xylene.
- Paraffin- Testing the Paraffin for xylene contamination
    - Dip your finger into the paraffin bath
        - Results:
            - Solid, dry…. Good, not saturated with xylene
            - Glossy, wet, and mushy paraffin--- not good. This means the solution is saturated with xylene. The last change of paraffin should be free of xylene

Troubleshooting the Most Common Processing Problems

- Tissues feel soft and spreads on the waterbath
    - Reason: Incomplete dehydration/clearing hence the presence of residual dehydrating/clearing agents in tissues will not allow complete infiltration resulting to soft tissues.
    - Resolution: Dry the paraffin block thoroughly, return to the embedding caddy, melt the paraffin, discard it then replace with fresh paraffin, re-infiltrate for 30 minutes, repeat, then embed.

- Tissues smell xylene and tissue spread on the waterbath
    - Reason:The paraffin is saturated with xylene.
    - Resolution: If the block has been trimmed/cut, dry it first. Return to the embedding caddy, melt the paraffin, discard it then replace with fresh paraffin, re-infiltrate for 30 minutes, repeat, then embed.
    - Resolution: If the paraffin block is soft, do not cut. Return to the embedding caddy, melt the paraffin, discard it then replace with fresh paraffin, re-infiltrate for 30 minutes, repeat, then embed.

Safety

- Apply standard precautions
- Discard used reagents according to federal, local, and state regulations
- Can combine alcohol and xylene wastes, another waste container for formalin,
- May discard solidified paraffin waste in the regular waste

References

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Embedding of Renal Biopsies

Purpose

To provide support to paraffin infiltrated tissues to preserve the relationships of the morphological structures within the tissue. The paraffin block as the product makes it possible to cut thin sections for microscopical examinations.

Principle

Representative tissues submitted for processing are grossed in a precise cut to capture the exact orientation during embedding for conclusive diagnostic purposes. Embedding also known as casting, or blocking, involves enclosing infiltrated tissue in the embedding medium (paraffin) and then allowing the medium to solidify forming a square or rectangular paraffin block of tissue attached to a wooden block or bottom part of the plastic cassette

Specimens

Any processed, well-infiltrated tissue specimen.

Reagents

Blue ribbon paraffin (Leica Biosystem Cat # 3801360) with a melting point of 56° C-58° C.

Equipment/Materials

- Curved non-serrated embedding forceps, pointed non-serrated forceps, plastic/metal molds, gauze, paper towel, thermometer
- Embedding center (set at 600 C), e.g.,
- General Data Company Tissue Embedding Center TEC™-II

Quality Control

- The temperature of the following equipment needed during embedding is recorded on appropriate quality control form daily:
    - Embedding Center form to record the temperature of the embedding center
    - The paraffin reservoir is set at 600C as recommended by the manufacturer.
- The forceps wells are cleaned before and after embedding

- Temperature Chart to record the temperature of the paraffin dispenser
    - The temperature range is from 58°C -65°C
    - Set at 60°C after melting paraffin pellets at 65°C.
- To maintain the integrity of the specimen, the following policies must be followed:
    - Each tissue cassette is opened and embedded one at a time i.e., only one cassette in the embedding platform at a time.
    - Forceps wells and embedding platform are cleaned before and after embedding
    - Wipe forceps with gauze and/or wash cloth in between cassettes to avoid contaminations/carry over.
    - Non-serrated curved forceps are the forceps of choice as the embedding tools.

Procedure

USING AN EMBEDDING CENTER

1. Turn cold plate on/off switch to on.
    1.1. Allow about 30 minutes for cold plate to reach operating temperature
2. Adjust hot plate temperature. Control to setting appropriate for molds being used. Typical operating temperature is 60°-70° centigrade.
3. Turn on the orientation platform lights.
4. Place the processed tissue cassettes in the embedding caddy
5. Select the pre-warmed appropriate size plastic/metal base mold from the embedding caddy.
6. Notes on embedding renal core biopsies
    6.1. Kidney needle biopsies: must be embedded in a straight line parallel to the longer side of the embedding mold.

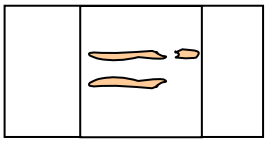


Illustration 1

    6.2. Cores should be parallel to each other, 1 mm apart.
    6.3. Two polar ends must have clearances of 1-2 mm of paraffin to avoid folding of the sections during sectioning.
    6.4. Small fragments are embedded on either of the extreme ends of the cores.
7. To determine the appropriate size of mold: there should be about 1-2 mm margin of paraffin around the tissue; the tissue must not touch the inner wall of the base mold.
8. Fill the base mold with molten paraffin, by placing the mold under the dispensing spigot, press and hold the manual dispense touch plate until the mold is filled to the rim.
9. Place the mold on the hot plate to prevent the paraffin from solidifying.
10. Open the cassette
    10.1. Orient the specimen (see illustration 1)
    10.2. Press the core lightly with a tamper.
11. Slide the mold to the cold plate section (adjacent to the hot plate) holding the specimen in position with the tamper. Wait for a few seconds for the paraffin to change from clear to whitish.

11.1. It is very important that light pressure be placed over the entire specimen during the orientation and initial chilling so that the tissue is embedded flat; otherwise, a complete section cannot be obtained during sectioning.

12. Place the cassette on top of the base mold and fill to the rim with molten paraffin.
13. Place the block on the cold plate of the embedding center to complete solidification process.

Manual Method of Embedding

EQUIPMENT/MATERIALS

- Blue ribbon paraffin (Surgipath), non-serrated curved forceps, paper boats, wooden blocks, metal spatula, tamper, 2 x 1 cm. pieces of paper for labeling, pencil

Dulytek® Cooling/Cold Plate Kit 9" x 6" Aluminum Plate [6]

Metal Pitcher [4] 1-liter capacity

Paraffin dispenser [8]

PROCEDURE

1. Prepare a paper boat (equivalent to a cassette), the size of which is adjusted to the size of the specimen.

Paper boat

Cassette [7]

2. Adjust the temperature of the hot plate to 60 degrees centigrade.

Hot plate from Fisher Scientific[5]

3. Dispense some melted paraffin from the paraffin dispenser into a metal pitcher.

Paraffin bath [8]

Metal pitcher [4]

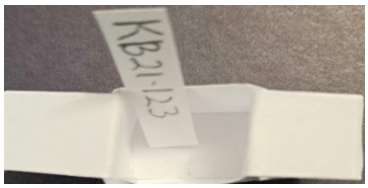
4. Put the paper boat on the hot plate
5. Pour melted paraffin into the paper boat just enough to cover the tissue
6. Transfer and orient the cores, parallel to each other
7. Slowly transfer to the cold plate to solidify the paraffin that will anchor the tissues in their position.

Dulytek® Cooling/Cold Plate Kit [6]

8. Use a metal tamper, to flatten the cores, but not squeeze the tissue.

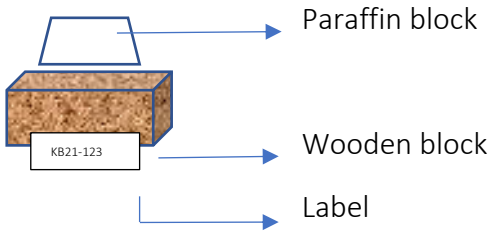
Tamper

9. Top off the paper boat with melted paraffin
10. Insert a piece of paper with the identifying case number.



11. Complete solidification on the cold plate.
12. Peel off paper boat around the paraffin block.
13. Trimmed tissue paraffin block and the wooden block are placed side by side on the hot plate. (This allows the paraffin block to melt, creating a flat surface while simultaneously heating the wooden block.
14. Quickly place the two heated surfaces together, applying enough pressure to ensure good adhesion
15. Pour melted paraffin around the along the sides of attachment.
16. Let the paraffin completely solidify by immersing in ice cold water.

17. Attach the label on the side of the block.
18. Results:



Side view of the paraffin block

Top view of the paraffin block

Troubleshooting of the Commonly Encountered Problems in Embedding

- Problem: Tissues not flat
  - Reason: Failure to use a tamper to flatten the tissue
  - Resolution: Do not attempt to cut the tissue. Return the block to the embedding caddy and re-embed.
- Problem: Bubble underneath the tissue and within the paraffin block. If cut will cause collapse on the tissue.
  - Reasons: Abruptly dispense the melted paraffin to the cassette layered on the tissue. Solidified paraffin surrounding the tissue
  - Resolution: Dispense the melted paraffin on one corner of the cassette making sure that the cassette and paraffin are warm.
- Problem: The tissue separates from the paraffin observed during tissue flotation.
  - Reason: Paraffin surrounding the tissue has solidified
  - Resolution: Embed tissues while the paraffin surrounding it is melted to ensure proper fusing of the paraffin in the mold and the tissue once solidified.

Safety

- Apply standard precautions
- Discard used reagents according to federal, local, and state regulations.

References

- Histotechnology A Self-Instructional Text: Freida L. Carson/Department of Pathology, Baylor University Medical Center/Dallas, Texas, Copyright by the American Society of Clinical Pathologist, Chicago, page 37
- A Manual for Histologic Technicians Third Edition Ann Preece, H.T. (ASCP) Copyright1972, by Ann Preece Library of Congress catalog card No. 73- 155042
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Sectioning of Paraffin Embedded Renal Biopsy

Purpose

To describe the sectioning guidelines as to thickness of the sections, number of sections per slide, number of slides, type of the slides, and the order of the tests.

Principle

Sectioning is performed using a rotary microtome to cut thin sections of paraffin infiltrated tissues thin enough for examination with a light microscope.

Specimens

Paraffin infiltrated kidney tissue block.

Materials

- Camel hairbrush, Disposable microtome blades, cleaning brush, #2 lead pencil Plus, and vision slides, metal sharps container, Frostbite, forceps: pointed/curved, Gauze, tissue blocks cold plate, waste receptacle, paper towel/wash cloths

Equipment

Rotary microtome, floatation bath, thermometer, lamp

Leica- Rotary microtome

Boekel waterbath

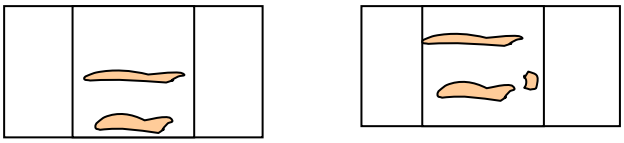
Gooseneck lamp

Quality Control

The kidney is cut at 2 microns and stained with established staining protocols.

NOTES: Cores should be parallel to each other, 1 mm apart. Two polar ends must have clearances of 1-2 mm of paraffin to avoid folding of the sections during sectioning. Small fragments are embedded on either of the extreme ends of the cores.

Different institutions have different methodologies as to the number of sections, slides, and tests ordered for examinations of kidney biopsies.



Cut sections as follows: (Weill Cornell Protocol)

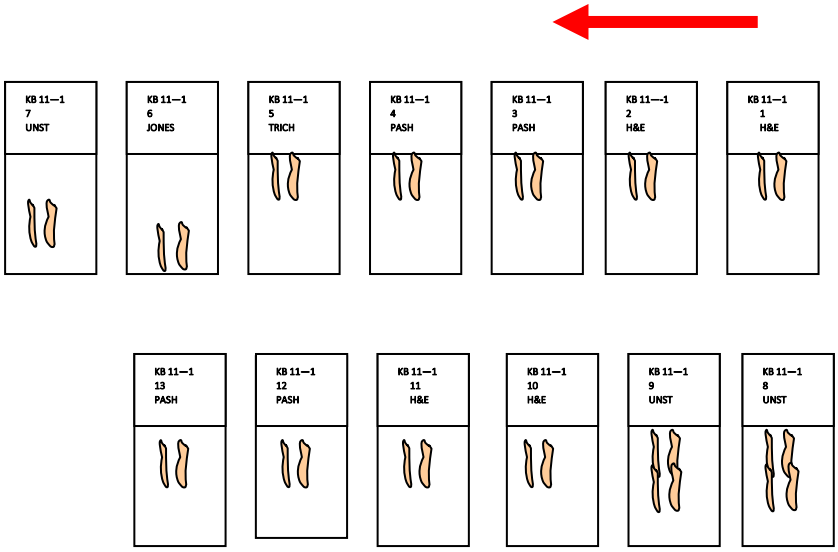


Illustration 1

- Type of sections: Serial
- Total number of slides 13 slides
- Thickness of the section: 2 microns
- Sections per slides: one section per slide
- Stains: H&E, PAS, Trichrome, Jones, and unstained slides
- Grouping of Slides
    - H&E placed in rack
    - Special stains placed in rack
    - Unstained slides are placed on a folder
- Drying of the slides in the oven: 30 minutes between 80-90 degrees Centigrade

Procedure

1. Two-micron serial sections are obtained by ribboning sections of the tissue block mounted on the microtome. Ribbons are floated in a standard water bath and divided to allocate and pick up on serial slides. It is important to maintain the serial orientation of the sections.
2. Using super frost charged slides, start picking up sections when tissue is first visible. Check each slide under the microscope and proceed with the sequence of slides allocated for the protocol of serial section staining after the first glomerulus is identified.
3. Section tissue with identified glomeruli to allocate to consecutive slides:
    3.1. One approach is to cut 13 slides, with levels 7,8 and 9 on + charged or plus slides left unstained for possible additional needed immunohistochemistry.
    3.2. Levels 1,2, 10, and 11 each stained with H&E while levels 3,4 12, and 13 are stained with PASH

3.3. Jones on level 6

3.3.1. Note: Variations of the sequence and number of slides may vary in different laboratories. The sectioning is based on the principle of a sequence of special stains, with one or two interspersed unstained slides, followed by another set of the sequence of special stains, and in most laboratories, another set of unstained slides, and another set of special stains. This approach allows optimal examination of the tissue by varying stains.

3.4. Dry slides thoroughly to prevent tissue from washing.

3.5. Blot waterbath with paper towel to remove any portions of sections between sectioning of each paraffin block to avoid cross contamination.

Troubleshooting

- Problem: Tissue sections wrinkle and curl
  - Reason: Too much paraffin surrounding the tissue
  - Resolution: Leave just 1-2 mm of paraffin surrounding the tissue. Trim off excess.

- Tissues cut had chatters
  - Reason: Over dehydrated
  - Resolution: Reduce the length of time in alcohol for the next batch of processing. However, validate first the new processing schedule before use.
  - Resolution: Trim the block, soak in icy water for a few minutes or soak in 5% ammonia water for 1-2 minutes, or soak in warm water for 30 seconds. Chill the block then cut.

Safety

- Discard chemical waste according to federal, state, and local regulations
- Apply standard precautions

References

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- A Manual for Histologic Technicians Third Edition Ann Preece, H.T. (ASCP) page 66-69. Copyright 1972, by Ann Preece Library of Congress catalog card No. 73- 155042
- Laboratory Methods in Histotechnology. Edited by Edna B. Prophet, Bob Mills, Jacquelyn B. Arrington, Leslie H. Sobin, M.D., Prepared by the Armed Forces Institute of Pathology, Washington, D.C., Published by the American Registry of Pathology, Washington, D.C.
- https://us.vwr.com/store/product/22049797/microtome-rm-2245-leica#gallery-1

Routine Hematoxylin and Eosin Staining

Purpose

To visualize the different cells and tissues as to shapes, pattern, types, and structures microscopically.

Principle

Hematoxylon is oxidized into hematein by using chemicals e.g., mercuric oxide, sodium iodate, or potassium permanganate that take shorter time or by natural process i.e., exposure to air and sunlight which will take about 3 to 4 weeks. Hematoxylin (as commonly called) is a basic dye that has affinity for acidic structures in the nucleus, calcium, organelles, extra cellular components, and mucin making them appear as purple blue or greyish blue respectively. However, when preparing hematoxylin, it is important to add a mordant e.g., aluminum to form a tissue -mordant -hematoxylin bond which is called “lake”. Hematoxylin is often used as a primary color. A secondary or counterstain is applied e.g., eosin, that is acidic in reaction that gives tritonal color to basic structures like red blood cells, muscles, collagen, elastic fibers, and cytoplasm.

Specimen

Any mounted tissue sections on slide e.g., smears, paraffin sections, or frozen sections.

Equipment/Materials

- Automated Stainer with attached coverslipping machine

Leica Autostainer XL with attached coverslipping machine

- Drying oven, funnel, flasks, filter paper, cover glass, forceps

Reagents

- Nuclear Stains: Gill’s 111 hematoxylin
  Cat #3801541
  Leica Biosystems
- Counterstain: Eosin 515 LT
  Cat #3801619
  Leica Biosystems

- Bluing agent: Blue buffer (pH8.0)
  Cat #s3802916
  Leica Biosystems
- Differentiating solution: Define MX aqueous (0-15. % Concentration of hydrogen chloride
  Cat #3803595
  Leica Biosystems
- Xylene
  Ca# 3803665
  Leica Biosystems
- Reagent alcohol
  Cat #3800757
  Leica Biosystems,
- Mountant
  Cat #3801731
  Leica Biosystems,

Note: All reagents are stored per manufacturer's instructions.

Quality Control

- Solutions are changed according to suggested frequency
- The integrity of the staining solution is tested by running a control (composed of a piece of tonsil and GI) at the beginning of the shift before staining patient slides
- Stained H&E control slides are checked microscopically by a supervisor

Procedure

AUTOMATIC METHOD OF HEMATOXYLIN and EOSIN STAINING

1. Drying slides in the oven:
   1.1. The slides are dried in an oven at 620 C for 30 minutes
   Notes: It is important to position the slides with sections in an upright position to drain off excess water.
2. De-paraffinization- removal of paraffin from the tissue sections
   2.1. Xylene- 3 minutes
   2.2. Xylene- 3 minutes
   2.3. Xylene- 3 minutes
   Notes: Guideline: 1 micron thickness for one minute.
3. Descending grades of alcohol for hydration purposes:
   3.1. Dehydrant, 100%- 1 minute
   3.2. Dehydrant, 100%- 1 minute
   3.3. Dehydrant, 95% - 30 seconds
   3.4. Running water - 1 minute
4. Hematoxylin 560 MX – 1 minute 15 seconds
5. Running water – 1 minute
6. Define- 1 minute 15 seconds
7. Running water – 2 minutes
8. Blue buffer for 30 seconds

9. Running water for 2 minutes
10. Dehydrant, 80% - 10 dips
11. Eosin LT – for 1 minute 30 seconds
12. Dehydration
    - 12.1. Dehydrant, 100%- 5 seconds
    - 12.2. Dehydrant, 100%- 10 seconds
    - 12.3. Dehydrant, 100%-10 seconds
    - 12.4. Dehydrant, 100%-10 seconds
13. Clearing or De-alcoholization
    - 13.1. Xylene- 10 seconds
    - 13.2. Xylene- 10 seconds
    - 13.3. Xylene- 10 seconds
    - 13.4. Last xylene container- to temporary hold-stained slides before coverslipping.~
14. Some stainers are attached to automatic coverslipping machines, hence from the last xylene container, the rack is transferred automatically for coverslipping.
    - 14.1. Coverslip under the hood if done manually.

Results

- Nuclei stain dark purple to blue
- Cytoplasm stain pink
- Muscle fibers: deep red
- RBCs: orange red
- Calcium: Dark blue
- Mucin: Grey blue

To Test for the Efficiency of Hematoxylin

- Add several drops of the hematoxylin solution to tap water.
    - If it turns purplish immediately, it contains the proper ratio of hematoxylin to hematein.
    - If it changes slowly or stays reddish brown, it has too much hematein or the hematein has been further oxidized and the solution should be discarded.

Overoxidized hematoxylin
(Reddish brown)

Good hematoxylin
(Purplish)

- A simple paper test may also be performed.
    - Put a drop of hematoxylin on a paper towel.
        - Purplish coloration indicates that the solution is still good.
        - A reddish brown result means that hematoxylin is overoxidized

Overoxidized hematoxylin
(Reddish brown)

Good
(Purplish)

Manual Method of Hematoxylin and Eosin Staining

1. Drying slides in the oven:
   1.1. The slides are dried in an oven at 620 C for 30 minutes
   Notes: It is important to position the slides with sections in an upright position to drain off excess water.
2. De-paraffinization- removal of paraffin from the tissue sections
   2.1. Xylene- 3 minutes
   2.2. Xylene- 3 minutes
   2.3. Xylene- 3 minutes
3. Descending grades of alcohol for hydration purposes:
   3.1. Dehydrant, 100%- 1 minute
   3.2. Dehydrant, 100%- 1 minute
   3.3. Dehydrant, 95% - 30 seconds
   3.4. Running water - 1 minute
4. Hematoxylin 560 MX – 1 minute 15 seconds
5. Running water – 1 minute
6. Define- 1 minute 15 seconds
7. Running water – 2 minutes
8. Blue buffer for 30 seconds
9. Running water for 2 minutes
10. Dehydrant, 80% - 10 dips
11. Eosin LT – for 1 minute 30 seconds
12. Dehydration
    12.1. Dehydrant, 100%- 5 seconds
    12.2. Dehydrant, 100%- 10 seconds
    12.3. Dehydrant, 100%-10 seconds
    12.4. Dehydrant, 100%-10 seconds

13. Clearing or De-alcoholization
    13.1. Xylene- 10 seconds
    13.2. Xylene- 10 seconds
    13.3. Xylene- 10 seconds
    13.4. Last xylene container- to temporary hold-stained slides before coverslipping
14. Mount in synthetic resin.

Routine Hematoxylin and Eosin Staining

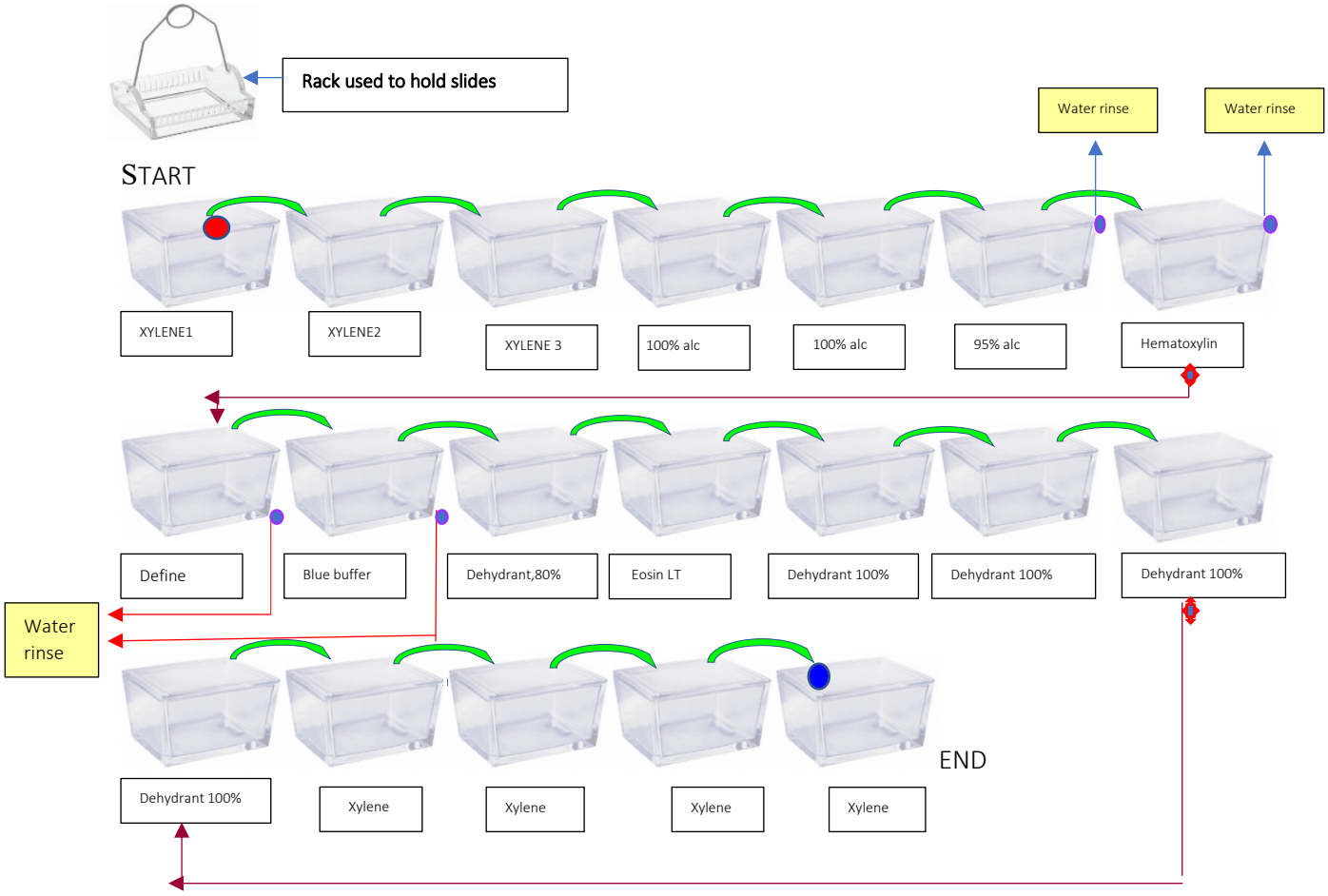

Illustration of Hematoxylin and Eosin Manual Staining

Note: There are other staining dishes available in the market.

Safety

- Apply standard precautions
- Dispose used reagents according to federal, local, and state regulations.

Notes on Troubleshooting

- Guideline in removal of paraffin from tissue sections: 1 micron thickness for one minute.
- Xylene should always be followed or preceded by 100% alcohol. The presence of water in alcohol will create whitish cloudiness when mixed with xylene.
- It is important to completely remove alcohol from the tissue before coverslipping.
- The presence of alcohol in the tissue will cause cloudiness when mixed with mounting medium

References/Reading Materials

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- https://www.leicabiosystems.com/knowledge-pathway/he-staining-overview-a-guide-to-best-practices/
- Staining dish from Grainger

Periodic Acid Hematoxylin Stain

Purpose

The demonstration of polysaccharides, neutral mucosubstances, and basement membranes.

Principle

Tissue sections are treated with periodic acid solution. The carbon bonds on the carbohydrate molecules are oxidized to an aldehyde state. The formed aldehydes and Schiff's reagent then react to form a colorless aldehyde addition group. Washing the tissue sections in running tap water results in the loss of sulfonic acid group on the Schiff's molecule. The loss of bisulfite group restores the bright pink color and indicates the presence of carbohydrates in the tissue sections. Metabisulfite rinses are used to remove excess Schiff reagent and prevent false colorization of the tissue elements due to oxidation of any absorbed reagent. Nuclei are stained blue with hematoxylin.

Diagnostic Applications

- Demonstrates the PAS-positive reticulum fibers and basement membranes.
- Indicates the presence of PAS-positive glycolipids in certain disease states
- Distinguishes a secreting adenocarcinoma (PAS positive) from an undifferentiated squamous cell carcinoma (PAS - negative). Demonstrate fungus in tissue sections.
- Worm cuticles may be demonstrated with PAS technique.

Specimen

Renal tissue fixed in 4% paraformaldehyde or 10% neutral buffered formalin (NBF) and embedded in paraffin and sectioned at 2-micron sections and placed on positive charged slides .

Quality Control

A section of kidney is the most sensitive control. Kidney tissue has its own internal control e.g. tubular brush border and mesangial areas). If the procedure is used to demonstrate glycogen use a section of liver containing glycogen.

Equipment and Materials

- Balance, spatula, reagent bottles, labels, graduated cylinder, Coplin jars, beakers, magnetic stirrer, forceps, glass slides, cover glass, staining dishes. Paper towel.

Reagents

- PERIODIC ACID, 1.0%
    - Periodic acid 1.0 gram
    - Deionized water 100.0 ml.

      Cat #s1861

      Poly Scientific R&D

- SCHIFF'S REAGENT
    - Dissolve 1.0 gm basic fuchsin in 200.0 ml hot distilled water. Bring to boiling point. Cool to 50 degrees C. Filter and add 20.0 ml normal hydrochloric acid. Cool further and add 1.0 gm anhydrous sodium bisulfite, or sodium metabisulfite. Keep in the dark for 48 hours until solution becomes straw colored

      Cat #s272
      Poly Scientific R&D
    - Test for Schiff Reagent
        - Pour a few drops of Schiff reagent solution into 10 ml of 37% to 40% formaldehyde in a watch glass. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resulting color deep blue purple, the solution is breaking down.
    - Commercially prepared Schiff's reagent is available from Poly Scientific.
- HARRIS' HEMATOXYLIN SOLUTION:
    - Hematoxylin crystals 5.0 gm
    - Alcohol, 100% 50.0 ml
    - Ammonium or potassium alum 100.0 gm
    - Deionized water 1000.0 ml
    - Mercuric oxide (red) 2.5 gm

      Cat #s212
      Poly Scientific R&D
- Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as RAPIDLY as possible. (Limit this heat to less than 1 minute and stir often).
  Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2 to 4 ml of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use
- Commercially prepared reagents from Poly Scientific Company are available for manual use. These were pre-validated by the company before they are released to the consumer. These are again re-validated by running known positive and/or negative control on site.
    - Xylene (Avantik))
    - Ethyl alcohol or Dehydrant (Leica Biosystems)
    - Hematoxylin 560 MX commercially prepared by Surgipath (Leica)
    - Ready- made reagents in dispensers are available from automated special staining machine
    - Mountant medium (Surgipath)
    - The GHS signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.
    - This information is all indicated on individual label of the solution.

Materials

- Coplin jars, forceps, glass slides and cover glass, staining dishes, graduated cylinder, paper towel

Procedure

- Manual procedure
  1. DPHW (See procedure notes)
     1.1. Wash well in water – 10x
  2. Oxidize in 1.0 %periodic acid solution for 10 minutes.
  3. Rinse in Deionized water -10x
  4. Schiff reagent solution for 15 minutes
     4.1. For kidney biopsies - treat with potassium metabisulfite for1 minute
  5. Wash in running water for 10 minutes for pink color to develop
  6. Hematoxylin for 6 minutes, or light green counterstain for a few seconds
     6.1. Light green is recommended for counterstaining sections in which fungi are to be demonstrated. Omit steps 7 through 11 if light green is used.
     6.2. For kidney biopsies, Hematoxylin 560 MX- 10 minutes
  7. Wash in tap water- 10 x.
  8. Differentiate in 1.0% (HCL) acid alcohol-three to ten quick dips.
     8.1. Use Define for kidney biopsies– 15 dips
  9. Wash in water -10x
  10. Dip in blue buffer- 20 dips
  11. Wash in tap water for 10x
  12. DCM (See procedure notes below)
      12.1. It is important that the alcohol used for dehydration is free from eosin contamination.

Automatic Method of Staining

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists. Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine.

Results

Glycogen, mucin, reticulin, fibrin or thrombi, colloid droplets, hyaline of arteriosclerosis, hyaline deposits in glomeruli, granular cells in the renal arterioles where preserved most basement membranes, colloid of pituitary stalks and thyroid, amyloid infiltration may show a positive reaction-rose to purplish red.

Nuclei.....- blue
Fungi.....- red
Hematoxylin background.....- blue

Procedure Notes

The intensity of staining in the routine PAS reaction is due to a combination of the following factors:

- The number of available 1:2 glycol groups
- The reactivity of Schiff reagent with the reaction product
- The structure of the polymer oxidized
- Troyer, Lillie and Fullmer, and Vacca consider the sulfite rinses essential to remove any uncombined leucofuchsin following exposure to the Schiff reagent.
- Highly chlorinated water is capable of oxidation, and if the sections are transferred directly to tap water, any loosely absorbed Schiff reagent may be re-oxidized to basic fuchsin, which may then non-specifically stain the section.
- For color development, washing in tap water is very important after the sulfite rinses.
- Glutaraldehyde is not recommended as a fixative if PAS reactions are to be performed. Glutaraldehyde is a dialdehyde, and one aldehyde group may not be involved in protein cross-linking during fixation but may be left free to react with the Schiff reagent
- Stain may be removed by placing sections in 0.5% aqueous potassium permanganate for 5 minutes. Wash in tap water for 5 minutes and place in 5% aqueous oxalic acid for 5 minutes. Wash well with tap water.
- DPHW means:

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute

Note: If the next step is silver solution, rinse in deionized water

- DCM means

  D-Dehydration means removal of water from the tissue sections
  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds

C-Clearing or De-alcoholization means removal of alcohol

Xylene- 10 seconds
Xylene- 10 seconds
Xylene- 10 seconds
Xylene- to temporary hold-stained slides before coverslipping
M- Mount in synthetic medium

Troubleshooting

- Problem: Pink background staining
  - Caused by:
    - Aging solution
    - Carry over of periodate
    - Insufficient washing after formalin fixation
  - Resolutions:
    - Discard old solution, use fresh ones.
    - Rinse very well after oxidation with periodic acid
    - Rinse well after formalin fixation

Safety

- Apply standard precautions.
- Discard chemical waste according to federal, local, and state regulations.

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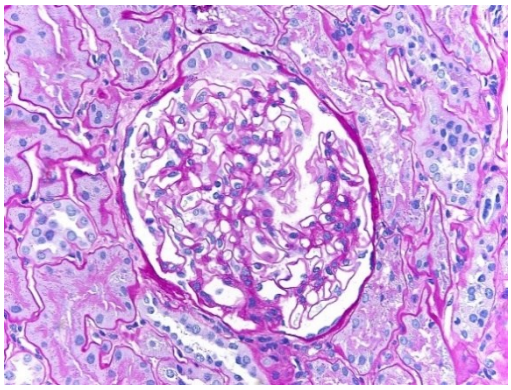
Pictures

Periodic Acid Schiff -Hematoxylin of a normal glomerulus



Normal Glomerulus

Magenta staining for PAS on all glomeruli and tubular basement membranes, mesangium matrix, and Bowman's capsule

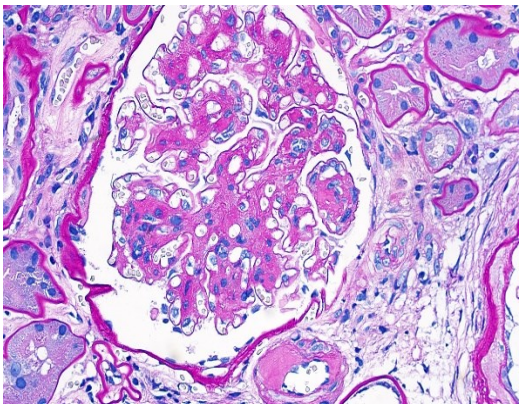
Nuclei…….. blue

Periodic Acid Schiff -Hematoxylin of a Diabetic kidney

Diabetic kidney

Glomerulus sclerosing showing staining positive on basement membrane and expanded mesangium matrix. Also, note pink staining on segmental arteriolar intimal hyalinosis

Nuclei…….. blue



Masson Trichrome Stain

Purpose

Trichrome stains are frequently used to differentiate between collagen and smooth muscle and to identify increases in collagenous tissue.

Principle

The tissue is first stained with an acid dye i.e., Biebrich scarlet; all acidophilic tissue elements such as cytoplasm, muscle and collagen will bind with the acid dye. Phosphomolybdic-phosphotungstic acid will cause the Biebrich to diffuse out of the collagen but not out of the cytoplasm. The phosphomolybdic/phosphotungstic acid acts as a link between the collagen and the aniline dye.

Diagnostic Applications

This stain is used to differentiate between collagen and smooth muscle in tumors, and to identify increases in collagenous tissue in diseases such as cirrhosis of the liver. Motility disorders, scleroderma, alternative stain for collagenous colitis, muscle tumors.

Specimen

Bouin's solution is preferred, but 10% neutral buffered formalin may be used, however slides must be post-mordant with Bouin's solution. Provide pre-cut kidney paraffin sections at 2 microns.

Quality Control

Known positive. Nearly all normal tissues contain collagen. Examples of tissues with abundant collagen include uterus and scarred kidney.

Equipment and Materials

- Water bath, balance, glass slides and cover glass, staining dishes, graduated cylinder, paper towel, Coplin jars, forceps, GHS labels, reagent bottles

Reagents

- BOUIN'S SOLUTION
    - Picric acid, saturated solution 75.0 ml.
    - Formaldehyde.37-40% 25.0 ml.
    - Glacial acetic acid 5.0 ml.
    - Commercially prepared available.
    - Storage: Room temperature
    - Poly Scientific, Cat # s129)
- WEIGERT'S IRON HEMATOXYLIN
    - Stock Solution A:
        - Hematoxylin 2.0 g
        - Alcohol, 90% 100.0 ml.
          Cat #s212B
          Poly Scientific R&D,

- Stock Solution B:
  - Ferric chloride, 62% aq solution 4.0 ml.
  - Deionized water 95.0 ml.
  - Hydrochloric acid 1.0 ml.

    Cat #s180B

    Poly Scientific R&D,

- WORKING SOLUTION OF WEIGERT'S
  - Solution A 25.0 ml.
  - Solution B 25.0 ml.
  - Shelf life: 1 week
- BIEBRICH SCARLET/ACID FUCHSIN SOLUTION
  - Biebrich scarlet 4.5 g
  - Acid fuchsin 0.5 g
  - Deionized water 450 .0 ml.
  - Acetic acid., glacial 5.0 ml.
  - Filter before use.

    Cat #s2601

    Poly Scientific R&D,
- PHOSPHOTUNGSTIC/PHOSPHOMOLYBDIC ACID
  - Phosphomolybdic acid 10.0 g
  - Phosphotungstic acid 10.0 g
  - Deionized water 500.0 ml.

    Cat #s255

    Poly Scientific R&D,
- ANILINE BLUE SOLUTION
  - Aniline blue, C.I. 42755 10.0 g
  - Acetic acid, glacial 8.0 ml.
  - Deionized water Type II 400.0 ml.

    Cat # s2627

    Poly Scientific R&D,
- GLACIAL ACETIC ACID,1%
  - Acetic acid, glacial 5.0 ml.
  - Deionized water 495.0 ml.

    Cat #s100

    Poly Scientific R&D,

Notes

- Commercially prepared reagents from Poly Scientific Company are available for manual use. These were pre-validated by the company before they are released to the consumer. These are again re-validated by running known positive and/or negative control on site
- Xylene (Avantik, Cat# RS4050)
- Alcohol, (Avantik, Cat #RS4029)
- Hematoxylin 560 MX commercially prepared by Surgipath (Leica)
- This stain is done manually to meet the optimum staining of structures by the renal pathologist

- The GHS signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.
- This information is all indicated on individual label of the solution.
- Ready- made reagents in dispensers are available from Dako
- The GHS signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.

Procedure

MANUAL METHOD

1. DPHW (See procedure notes below))
2. Rinse well in Deionized water.
3. Mordant sections in Bouin's solution for 1 hour at 56°-600 C or overnight at room temperature
   3.1. Change Bouin's solution as follows:
      3.1.1. Monday: pour 50 ml of solution into Coplin jar
      3.1.2. Wednesday and Friday: Discard 25 ml and replace with 25 ml of fresh solution
4. Remove slides from oven; allow cooling; washing in running water until the yellow color disappears
5. Rinse in Deionized water.
6. Stain sections in Wiegert's hematoxylin for 10 minutes
   6.1. Discard at the end of the day.
7. Wash in tap water
8. Differentiate in 0.5% acid –alcohol for 2-3 dips.
   8.1. Prepare 0.5 % acid –alcohol from 1% acid alcohol:
      8.1.1. 1% acid-alcohol ….25 ml
      8.1.2. Dehydrant, 70%.....25 ml
   8.2. Discard at the end of the week or change as needed.
9. Rinse in Deionized water
10. Blue in ammonia water for 10 dips
    10.1. Preparation of ammonia water:
       10.1.1. Add 5 drops of concentrated ammonium hydroxide in 50 ml of water. Mix
       10.1.2. Discard at the end of the day.
    10.2. Wash ten times in Deionized water
11. Stain sections in Biebrich' s scarlet-acid fuchsin solution for 2 minutes
    11.1. Discard this solution at the end of the week.
12. Rinse in Deionized water.
    12.1. Place the slides in phosphomolybdic-phosphotungstic acid solution for 15 minutes.
    12.2. Discard solution at the end of the day
13. Without washing, stain sections in aniline blue solution for 10 minutes.
    13.1. Change aniline blue solution as follows:
       13.1.1. Monday: pour 50 ml of solution into Coplin jar
       13.1.2. Wednesday and Friday: Discard 25 ml and replace with 25 ml of fresh solution
14. Rinse in Deionized water
15. Place slides in 1% acetic acid solution for 3 minutes.
    15.1. Discard this solution at the end of the week.
16. Without washing, dehydrate and clear as follows:

16.1. Dehydrant, 95% 5 dips
16.2. Dehydrant, 95% 5 dips
16.3. Dehydrant, 100% 5 dips
16.4. Dehydrant, 100% 10 dips
16.5. Xylene 20 dips
16.6. Xylene 20 minute
16.7. Xylene Holding solution
16.7.1. Filter xylene if droplets of water are visible.

17. Mount in synthetic medium.

Automatic Procedure

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists. Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

Results

- Nuclei black
- Cytoplasm, keratin, muscle fibers red
- Collagen and mucus blue

Procedure Notes

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute

  Note: If the next step is silver solution, rinse in deionized water.

- DCM means

  D-Dehydration means removal of water from the tissue sections£
  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds

C-Clearing or De-alcoholization means removal of alcohol

Xylene- 10 seconds
Xylene- 10 seconds
Xylene- 10 seconds
Xylene- to temporary hold-stained slides before coverslipping

M- Mount in synthetic medium

- If desired, collagen may be counterstained with light green instead of aniline blue. The following changes are made:
- Place the sections in a 5% aqueous phosphotungstic acid solution.
- Stain 5 minutes in 2% LIGHT GREEN:
    - Light green SF yellowish 2.0 gm
    - Deionized water 98.0 ml
    - Glacial acetic acid 1.0 ml
- Light green is a better counterstain when collagen is predominant, but when only small amounts are to be demonstrated; the aniline blue is the better counterstain.
- Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded. If blue staining of connective tissue appears faded, the section has probably been over differentiated in the acetic acid solution. Pathologically altered collagen, such as that seen in burns, may lose its affinity for aniline blue and bind the acid dye instead.
- Picric acid containing less than 10% water is very explosive; therefore, it is important that solution is not spilled in the oven and then allowed to evaporate. For this reason, the staining jar containing picric acid should be placed inside of another container while in oven.
- An iron hematoxylin solution is used for nuclear staining in the trichrome procedures because iron hematoxylin is more resistant than aluminum hematoxylin to decolorization in subsequent acidic dye solutions.

Troubleshooting

- Problem: The entire tissue looks greenish.
    - Reason: Failure to completely remove the yellow color of Bouin's fixative
    - Resolution: Wash tissue in warm running tap water until the yellow color completely disappears.

Safety

- Apply standard precautions.
- Discard chemical waste according to federal, local, and state regulations.

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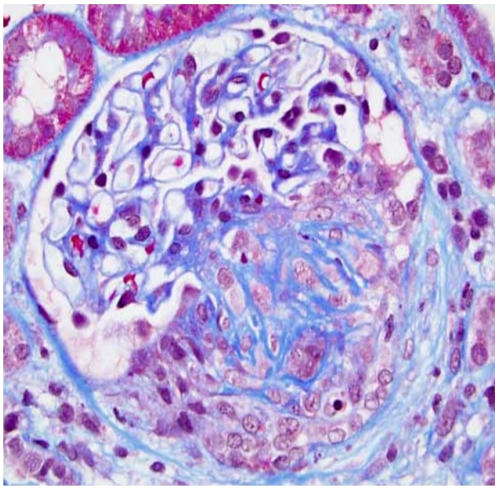
Picture



Nuclei	Black blue
Cytoplasm, muscle fibers	red
Collagen and mucus	blue

Jones Basement Membrane Silver Stain

Purpose

Silver stains carbohydrates black to demonstrate extracellular matrix and basement membranes.

Principle

Methenamine silver methods rely on oxidation of carbohydrates to aldehydes. In the technique for staining the basement membrane, silver ions from the methenamine silver complex is bound to carbohydrate components of the basement membrane and reduced to visible metallic silver by the aldehyde groups. Toning is with gold chloride and any unreduced silver are removed by sodium thiosulfate.

Specimen

Kidney tissue processed cut at 2 microns

Fixative

10% neutral buffered formalin is preferred.

Equipment

Microwave oven or 50 to 60 ° centigrade water bath, plastic staining jars graduated cylinders, Erlenmeyer flasks. Automatic special staining machine

Quality Control

Kidney tissue (as well as other tissue with extracellular matrix) has its own internal control. Basement membranes of glomeruli and tubules stain black/brown.

Reagents

- Periodic acid solution,1%
    - Periodic acid 1.0 gm
    - De-ionized water 100.0 ml

      Cat #s1861

      Shelf life:Maximum 2 years Store in a dry, cool, and well ventilated place. Keep container closed when not in use. Store locked up.

      Poly Scientific
- Silver nitrate solution,5%
    - Silver nitrate.....5.0 gm
    - De-ionized water.....100.0 ml

      Cat #s1890

      Poly Scientific

- Methenamine solution,3%
    - Hexamethylenetetramine (methenamine).....3.0 gm
    - De-ionized water.....100.0 ml
      Cat #s240A
      Poly Scientific
- Methenamine-silver nitrate solution (stock):
    - Silver nitrate, 5% solution.....5.0 ml
    - Methenamine, 3% solution.....100.0 ml
    - A white precipitate forms but immediately dissolves on shaking. Clear solution remains usable for months.
      Cat #s240a
      Poly Scientific
- Borax solution,5%
    - Borax.....5.0 gm
    - De-ionized water.....100.0 ml
      Cat #s128
      Poly Scientific
- Methenamine-silver nitrate solution (working):
    - Methenamine-silver nitrate solution (stock).....25.0 ml
    - De-ionized water.....25.0 ml
    - Borax, 5% solution.....2.0 ml
    - Make solution fresh
- Gold chloride solution, 0.2%
    - Gold chloride, 1% solution.....10.0 ml
    - De-ionized water.....90.0 ml
      Cat #s202
      Poly Scientific,
- 1% GOLD CHLORIDE
    - Gold chloride.....1.0 gm
    - De-ionized water.....100.0 ml
    - Cat #s202
    - Poly Scientific
    - Note: This solution may be used repeatedly until it turns turbid
- Sodium thiosulfate (hypo) solution,2%
    - Sodium thiosulfate.....2.0 gm
    - De-ionized water.....100.0 ml
      Cat # s280
      Poly Scientific
        - 0.2% light green solution (stock)
            - Light green SF yellowish.....0.2 gm
            - De-ionized water.....100.0 ml
            - Glacial acetic acid.....0.2 ml
              Cat #s232
              Poly Scientific

- Light green solution (working)
    - Light green (stock).....10.0 ml
    - De-ionized water.....50.0 ml
    - Storage: Room temperature

- Normal hydrochloric acid solution:
    - Hydrochloric acid, sp. gr. 1.19.....83.5 ml
    - De-ionized water.....9l6.5 ml
    - Add acid to water and mix well

      Cat #s1824

      Poly Scientific,
- Ammonia water: (Freshly prepared)
    - Ammonium Hydroxide, concentrated .....15 drops
    - De-ionized water.....500 m
- Commercially prepared reagents from Poly Scientific Company are available for manual use. These were pre-validated by the company before they are released to the consumer. These are again re-validated by running known positive and/or negative control on site.
- The GHS signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.

Procedure

MANUAL PROCEDURE

1. DPHW (See procedure notes)
    1.1. This procedure is for five slides. If you do not have five slides, then include blank slides
    1.2. This procedure should be followed exactly for optimum results.
2. Place sections in 1% periodic acid solution for 15 minutes at room temperature
3. Rinse in de-ionized water.
4. Place slides (five) in a plastic Coplin jar containing 50 ml of methenamine working solution.
5. Loosely apply the screw cap and place in the microwave oven. Also place a loosely capped plastic Coplin jar containing 50 ml (measured) of distilled water in the oven.
    5.1. Expose for exactly 70 seconds. Remove both jars from the oven and let stand on the counter. Check the slides frequently until the desired staining intensity is achieved.
    5.2. For 4 patient slides and 1 control will take 10 minutes.
6. Rinse slides in warm de-ionized water.
7. Tone sections in 0.2% gold chloride for 1 minute
8. Rinse slides in de-ionized water.
9. Treat sections with 2% sodium thiosulfate for 1 minute
10. Wash in tap water
11. Counterstain with hematoxylin – 1 minute and 15 seconds
    11.1. Counterstain of choice by renal pathologist
12. Running water – 1 minute
13. Define- 1 minute 15 seconds
14. Running water – 2 minutes
15. Blue buffer for 30 seconds
16. Running water for 2 minutes
17. Dehydrant, 80% - 10 dips

18. Eosin LT – for 1 minute 30 seconds
19. DCM. (See procedure notes)

Automatic Method of Staining

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists. Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

Results

- Basement membrane, extracellular matrix- black
- Nuclei - blue
- Cytoplasm - pink
- Red blood cells- red

Troubleshooting

- To destain overstained methenamine, use a 0.5% solution of potassium ferricyanide. Quickly dip, 1-2 dips at a time, until results are satisfactory. (This must be done before toning in gold chloride!).
- Sharper staining of the basement membrane and less background staining can be obtained with the use of the microwave oven for silver techniques.
- The temperature is critical and should be just below boiling or approximately 95° centigrade immediately after removal from the oven. Each oven should be calibrated for the time required to reach the correct temperature.

Procedure Notes

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute
  Note: If the next step is silver solution, rinse in deionized water

- DCM means

D-Dehydration means removal of water from the tissue sections

Dehydrant, 100%- 5 seconds
Dehydrant, 100%- 10 seconds
Dehydrant, 100%-10 seconds
Dehydrant, 100%-10 seconds

C-Clearing or De-alcoholization means removal of alcohol

Xylene- 10 seconds
Xylene- 10 seconds
Xylene- 10 seconds
Xylene- to temporary hold-stained slides before coverslipping

M- Mount in synthetic medium

If for some reason the microwave oven cannot be used, substitute the following silver solution and staining time:

- Stock Methenamine Silver Solution 50 ml
- 5% borax 5 ml

Preheat the solution and stain slides at 56° to 60° C for 40 to 90 minutes.

Safety

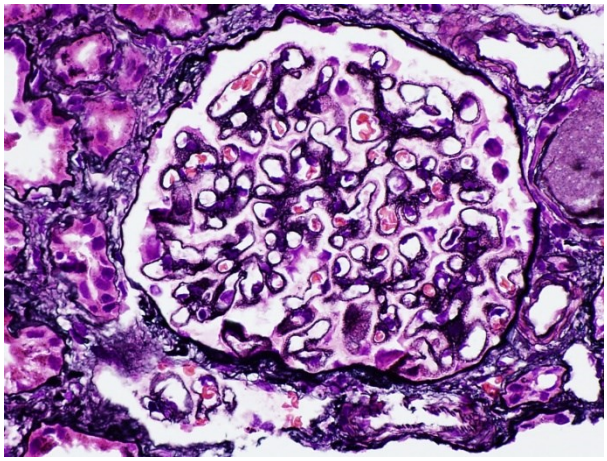
- Apply standard precautions.
- Discard chemical waste according to federal, local, and state regulations.

Reference

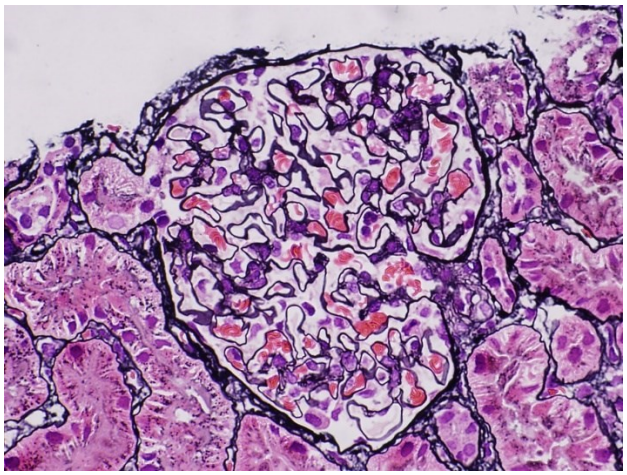
- Histotechnology A Self-Instructional Text: Freida L Carson/Department of Pathology Baylor University Medical Center/Dallas, Texas Copyright 1990 by the American Society of Clinical Pathologist Chicago, page 152
- Antonio, LB., Suriawinata, A, Thung, SN.: Liver Tissue Processing Technique In Surgical Pathology of GI Tract, Biliary Tract, and Pancreas, Odze Robert D, Goldblum, John, Crawford, James, Saunders, 1st ed., 2004, chapter 32. pp739-756,

Pictures

Jones methenamine silver stain of a kidney with Membranous Glomerulonephritis



Jones Silver Methenamine staining of a glomerulus with membranous glomerulopathy, where the basement membranes are variably thickened appearing irregular and stiff, showing basement membrane spikes on the outer aspect, with narrowed capillary lumina. Silver staining is absent in the areas of subepithelial and intramembranous deposits. X400



Jones Silver Methenamine stain highlighting the basement membranes of a normal glomerulus and tubules having a smooth contour, and the mesangial matrix. The capillary lumina contain eosin stained RBC, while all the cellular nuclei stain purple. X400

Jones methenamine silver stain of a normal kidney

Alkaline Congo Red Stain

Purpose

To demonstrate the presence of amyloid in tissues.

Principle

Amyloid is an abnormal protein material with or without carbohydrate components arising from an immune reaction. In light microscopy, it is eosinophilic and homogenous. In transmission microscopy it reveals fibrils, in polarized microscopy, it exhibits green birefringence. To determine this protein, Congo red stain is applied. There are two important factors in the Congo red reaction i.e., the linearity of the dye molecule and the B- pleated sheet configuration, if the spatial configuration is altered, even though the chemical groupings are left intact, the reaction fails. The reaction has been established i.e., the attachment is through non- polar hydrogen bonds.

Diagnostic Application

Demonstrates the presence of amyloid (glycoprotein) in tissue section. It can be primary (ex. arising from predisposing disease) or secondary (due to chronic inflammation). Amyloid can be deposited in the following areas: tongue, heart, gastrointestinal tract, skeletal and smooth muscle, nerve, skin, carpal ligaments, liver spleen, kidney, adrenal

Specimen

Tissues are fixed in 10 % neutral buffered formalin, Bouin's, Carnoy's or absolute alcohol. Cut at 5-10 microns. (Minute deposits are best demonstrated at 12 microns.) Prolonged storage in 10% formalin will cause a gradual decrease in staining intensity. Can be done on frozen tissue cut at 2 microns and fixed in acetone for 2 minutes, when paraffin tissue is not available.

Quality Control

- The control slide consists of a piece of tissue known to be positive for amyloid. The control tissues are fixed in 10% neutral buffered formalin.
- Each set of tests is run with a control. Bancroft and Stevens stated that it is better not to keep too many control sections cut, as the staining intensity has been reported to decrease with the age of the sections. Also, massive, presumably long-standing deposits give less intense histochemical reaction than small, newly formed deposits.

Equipment and Materials

- Mechanical stirrer, Coplin jars, filter paper, Erlenmeyer flask, graduated cylinders,balance, spatula, reagent bottles, labels, cylinder.

Solution Preparations

- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation

- Date of expiration
- Storage information
- Protective equipment
- Hazardous label
- Preparer's initials

- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- Sodium chloride- alcohol solution
  - Sodium chloride 2.5 g
  - De-ionized water 50 .0 ml.
  - Absolute alcohol 50 .0 ml.
  - Storage: Room temperature
- Sodium hydroxide, 1%
  - Sodium hydroxide 1.0 g
  - De-ionized water 100.0 ml.

  Cat #s1918A

  Poly Scientific R&D
- Congo red working solution
  - Congo Red (C.I. 22120) 0.1 gram
  - Sodium chloride-alcohol 50.0 ml.
  - Sodium hydroxide, 1% 0.5 ml.

  Cat #s167

  Poly Scientific R&D
- Wiegert's iron hematoxylin
  - Stock Solution A
    - Hematoxylin 10.0 gm
    - Alcohol, 95% 1000.0 ml

    Cat #s212B

    Poly Scientific R&D
  - Stock Solution B
    - De-ionized water 475.0 ml
    - Hydrochloric acid, concentrated 5.0 ml
    - Ferric chloride, 29% solution 20.0 ml

    Cat #s180B

    Poly Scientific R&D
- Working solution:
  - Mix equal parts of solution A and B.
  - Storage: Room temperature
  - Shelf life: One week for working solution.

- 0.5% Acid -Alcohol solution:
    - Hydrochloric acid, concentrated 0.5 ml
    - 70% Alcohol 99.5 ml
      Cat #s2194
      Poly Scientific R&D

Procedure

Manual Method

1. DPWH - control and test slides to water. (See procedure notes)
2. Wiegert's iron hematoxylin for 10 seconds. Wash in water.
3. Differentiate in acid -alcohol for 5 dips. Wash in running tap water for 1 minute.
4. Rinse in de-ionized water.
5. Rinse in 95% ethanol for 5 dips
6. Stain in Congo red for 20 minutes.
7. DCM. (See procedure notes)

Results

- Amyloid -pink-red under standard light microscopy and with polarized light apple green birefringence
- Nuclei ----------blue
- At times other hyaline materials may stain red.

Automatic Procedure

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists.

- Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

Procedure Notes

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute

Note: If the next step is silver solution, rinse in deionized water

- DCM means

  D-Dehydration means removal of water from the tissue sections

  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds

  C-Clearing or De-alcoholization means removal of alcohol

  Xylene- 10 second
  Xylene- 10 seconds
  Xylene- 10 seconds
  Xylene- to temporary hold-stained slides before coverslipping

  M- Mount in synthetic medium

Notes

- Commercially prepared reagents from Poly Scientific Company are available for manual use. These were pre-validated by the company before they are released to the consumer. These are again re-validated by running known positive and/or negative control on site.
- The GHS signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.
- This information is all indicated on individual label of the solution
- Sections of suspected amyloidosis may be cut at 12 microns if a brighter color is desired. This thick section technique is also being used to advantage to demonstrate minute amyloid deposits. Twelve-micron sections can also be used to advantage in the crystal violet and Sirius red methods for amyloid.

Troubleshooting

- Eliminate background staining by using modified Wiegert's iron hematoxylin.
- Staining of elastic, collagen, and muscle is suppressed by incorporating sodium chloride in the preparation of Congo red solution.
- Eosin contaminated alcohol that has been used during dehydration in Hematoxylin and Eosin staining may cause contamination and false positive Congo red stain.
- Prolonged stay in xylene may cause fading of stain.

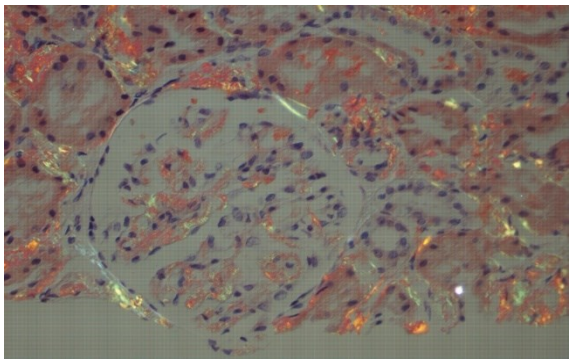
Safety

- Apply standard precautions.
- Discard used reagents according to federal, local, and state regulations

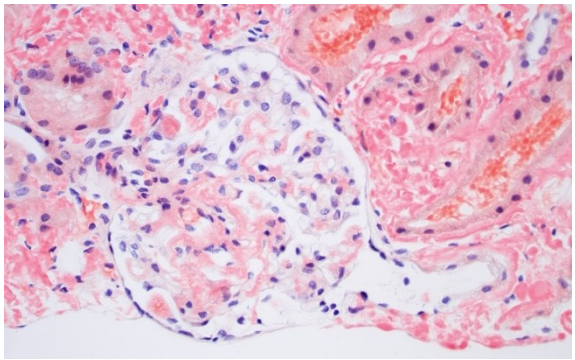
References

- Putt, Frederick. A.: Manual of Histopathological Staining Methods.; John Wiley and Sons, New York, 1972; p163-167.
- Sheehan, H.: Theory and Practice in Histotechnology, Battelle Press, Columbus, second ed., 1980, pp: 175, 176.
- Mallory, F. B. Pathological Technique New York, Hafner Publishing Co. 1961, p 33-134 (AFIP modification) Manual of Histological Staining Methods of the Armed Forces Institute of Pathology (third Edition) page 153
- Histotechnology A Self-Instructional Text Freida L. Carson/Department of Pathology Baylor University Medical Center/Dallas, Texas Copyright 1990 by the American Society of Clinical Pathologist Chicago, page 132
- https://www.pathologyoutlines.com/topic/kidneyamyloidosis.html
- https://ajkdblog.org/2018/07/26/pathpointer-congo-red-positive-always-means-amyloid-deposits-right
- Antonio, LB., Suriawinata, A, Thung, SN.: Liver Tissue Processing Technique In Surgical Pathology of GI Tract, Biliary Tract, and Pancreas, Odze Robert D, Goldblum, John, Crawford, James, Saunders, 1st ed., 2004, chapter 32. pp739-756,

Pictures



Amyloid in polarized light microscopy appears apple green birefringence



Amyloid in kidney glomerulus. Hematoxylin and Eosin Stain

Amyloid -pink-red under standard light

Nuclei ----------blue

Iron-Prussian Blue Stain

Purpose

To demonstrate the presence of iron in tissues.

Principle

This reaction is an anion substitution method in which the section is exposed to a freshly prepared acidified solution of potassium ferrocyanide. The acid released ferric ions from the tissue anions (generally the protein apoferritin and aposiderin). The ferric ions on liberation immediately form a complex with ferrocyanide anion:

K + [Fe (CN) 6] 4- + Fe +3 - - - - K [Fe 2 (CN) 6]

The resultant highly insoluble complex is called **Prussian blue**.

Clinical Significance

- Excessive amount of ferric iron is found in:
- Hemochromatosis where iron deposits are found mainly in the liver and pancreas
- Hemosiderosis found in the liver, spleen, and lymph node, which may be due to: increase destruction of red cells, excessive iron intake, local hemorrhages, chronic congestive conditions

Specimen

Tissue is fixed in 10% neutral buffered formalin for 6 to 72 hours at room temperature. The volume of the fixative should be 20 x the volume of the tissues. Sections are cut at 5 micrometers.

Quality Control

A multi-control slide consists of tissues one of which is a piece of liver known to be positive for iron. Cut at 5 microns. Each set of tests is run with a positive control. Record the results of the control slide on the Special stain control worksheet as adequate, over-stained, or under-stained

Equipment/Materials

Balance, spatula, reagent bottles, GHS labels, graduated cylinder, Coplin jars, beakers, deionized water.

Manual Solution Preparations

- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation and expiration
    - Storage information
    - Protective equipment
    - Globally Harmonized System of Labeling Chemicals
    - Preparer's initials

- All laboratory prepared reagents must be prepared with Type II water.
- Store all reagents according to manufacturer's instructions.

Reagents

- HYDROCHLORIC ACID SOLUTION 2%
  - Hydrochloric acid, concentrated HCL 2.0 ml.
  - Deionized water 98.0 ml.
  - Note: Add acid to water slowly
  - Cat # s2112
  - Poly Scientific
- POTASSIUM FERROCYANIDE SOLUTION 2%
  - Potassium ferrocyanide K [Fe (CN)] 2.0 g.
  - Deionized water 100.0 ml.
  - Note: Do not mix on a magnetic stirrer. Iron ions are magnetic.
  - Poly Scientific
- WORKING IRON SOLUTION
  - Hydrochloric acid 2% 25.0 ml.
  - Potassium ferrocyanide 2% 25.0 ml.
  - Note: Prepare fresh
- NUCLEAR FAST RED SOLUTION, 0.1%
  - Aluminum sulfate 5.0 g.
  - Deionized water 100.0 ml.
  - Mix well then add:
  - Nuclear fast red (Kernechtrot) 0.1 g.
  - Dissolve with heat. Cool and filter. Add a grain of thymol as a preservative.
  - Storage: Room temperature
  - Poly Scientific Cat # s248

Procedures

Manual Method

1. DPHW (See procedure notes)
2. Place in working solution for 10 minutes. Be sure that the control slide is facing opposite the test slides.
3. Rinse well in deionized water.
4. Counterstain in Nuclear fast red solution for 5 minutes.
5. Rinse thoroughly in deionized water
   Notes: If the slides are not washed thoroughly in water, then a white hazy background will be seen on the slide after mounting.
6. DCM. (See procedure noted)

Automatic Method of Staining

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists. Thereafter, the machine is validated

using known positive and/or negative controls for the most requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

Results

- Iron pigments ------bright blue
- Nuclei ---------- red
- Cytoplasm ----------light pink

Procedure Notes

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute
  Note: If the next step is silver solution, rinse in deionized water

- DCM means
  D-Dehydration means removal of water from the tissue sections
  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds
  C-Clearing or De-alcoholization means removal of alcohol
  Xylene- 10 seconds
  Xylene- 10 seconds
  Xylene- 10 seconds
  Xylene- to temporary hold-stained slides before coverslipping
  M- Mount in synthetic medium
- The optimal pH for Prussian blue reaction centers around pH 1.5. At high pH level (pH 5-7) iron bearing pigment may retain their natural yellow-brown color. At pH 3-4 that reaction is variably green to blue. At pH 3-4 low concentration of iron appear less intense and greener in color.
- Omit counterstain, to demonstrate lipid pigments and lipofuscin simultaneously, stain with Oil Red O.

Limitation of the Procedure

- Extended reaction time causes non-specific Prussian blue formation.
- Accumulation of asbestos fiber, calcium deposits bind with ferric ion may give Prussian blue positive.
- Iron bound in chelate form (e.g., Hgb) does not react, one can try to destroy the organic portion, e.g., by treating with hydrogen peroxide.
- Poorly soluble iron or dust (e.g., iron particle, introduced into tissues in accident) also fail to react with Prussian or Turnbull's blue. Acidifying the reagent (4N HCl) and raising the temperature to 60-80 degrees Centigrade may help sometimes.

Safety

- Discard used reagents according to federal, local, and state regulations.
- Always apply standard precautions

References

- AFIP Manual: p. 183
- Mallory, F.B. and et al: Pathological Techniques, 8th ed. Philadelphia, W.S. Saunders Co., 1924 p. 207.
- Sheehan. Theory and Practice in Histotechnology, 2nd ed., Columbus, Battelle Press, 1980.
- Vacca, L. Laboratory Manual in Histochemistry. Raven Press, New York, 1985.
- Luna, L.: Histopathologic Methods, American Histolabs, Inc. Johnson Printers, Il., 1992, pp333-334.
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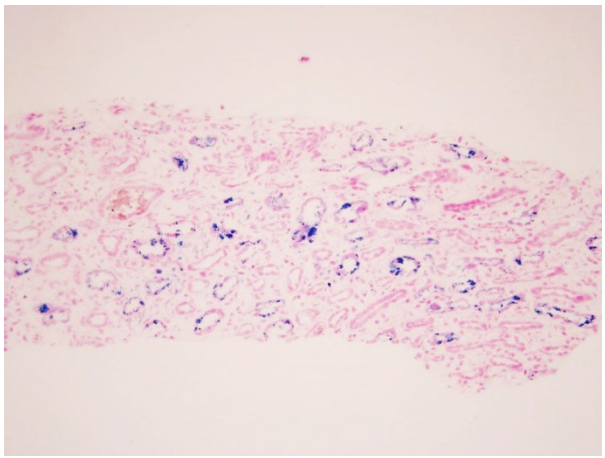
Picture



Blue deposits- positive for iron

Von Kossa Calcium Stain

purpose

To demonstrate the presence of calcium in tissues.

Principle

Calcium in tissue sections is indirectly detected through substitution process. First, tissue sections are treated with silver nitrate. If calcium is present, silver is substituted for calcium then the bound silver is reduced by light forming black, metallic silver.

Clinical Significance

Abnormal deposits of calcium may be found in areas including arterial elastic lamellae, foci of infection with Mycobacterium tuberculosis or Histoplasma capsulatum, hyaline cartilage, lymph nodes, fibroids, etc. Determines the presence of calcium in hyperthyroidism.

Specimen

Tissues fixed in absolute alcohol, 10% neutral buffered formalin. Alcoholic fixatives are preferred. Minimum fixation time is for 6-72 hours. Volume of fixative is 20x the volume of the tissue. Cut paraffin sections at 5 microns.

Quality Control

A control slide consists of a piece of tissue known to be positive for calcium. The control tissues are fixed in 10% neutral buffered formalin. Cut at 5 micrometers. Each set of tests is run with a control. Record the result of the control slide on the special stain quality control sheet as adequate, over-stained, or under-stained.

Equipment and Materials

Automatic stainer, balance, spatula, reagent bottles, GHS labels, graduated cylinder, Coplin jars, beakers, magnetic stirrer, UV light or 60-watt bulb, deionized water, plastic tweezers.

Manual Solution Preparations

- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Dates of preparation and expiration
    - Storage information
    - Protective equipment
    - Globally Harmonized System of Labeling Chemicals
    - Preparer's initials
- All laboratory prepared reagents must be prepared with a Type II water. Follow manufacturer's storage instructions.

Reagents

- SILVER NITRATE, 5%
  - Silver nitrate 5.0 g.
  - Deionized water 100.0 ml.

    Cat # s1890

    Poly Scientific
- SODIUM THIOSULFATE, 5%
  - Sodium thiosulfate 5.0 g.
  - Deionized 100.0 ml.

    Cat # s1895

    Poly Scientific
- NUCLEAR FAST RED SOLUTION, 0.1.%
  - Aluminum sulfate 5.0 g
  - Deionized water 100. 0 ml.
  - Nuclear fast red (Kernechtrot) 0.1 g

    Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate with the aid of heat. Cool, filter, then add a grain of thymol as a preservative.

    Cat # s248

    Poly Scientific

Procedures

Manual Method

1. DPHW (See procedure notes)
2. Impregnate in 5% silver nitrate solution for 30 minutes under UV light.
3. Rinse well in deionized water.
4. Place in 5% sodium thiosulfate for 5 minutes.
5. Rinse well in deionized water.
6. Counterstain in nuclear fast red for 5 minutes.
7. Rinse thoroughly in deionized water.

   Notes: If the slides are not washed thoroughly in water, then a white hazy background will be seen on the slide after mounting.
8. DCM (See procedure notes)

Automatic Method of Staining

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure being adjusted according to the preferences of the user and/or pathologists.

Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

results

- Calcium salts - black
- Nuclei - red
- Cytoplasm - light pink

Limitations of the Procedure

- Calcium oxalate may not be detected as formed silver oxalate is soluble.
- Uric acid and urates also reduce silver ions and may be mistaken for calcium deposits.
- The presence of melanin can mimic a positive von Kossa as can carbon particles.

Procedure Notes

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute

  Note: If the next step is silver solution, rinse in deionized water

- DCM means

  D-Dehydration means removal of water from the tissue sections
  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds

  C-Clearing or De-alcoholization means removal of alcohol
  Xylene- 10 seconds
  Xylene- 10 seconds
  Xylene- 10 seconds
  Xylene- to temporary hold-stained slides before coverslipping

  M- Mount in synthetic medium

- Use saturated lithium carbonate before substitution to remove uric acid and urates.
- Two forms of calcium in the tissue: Free ions and calcium precipitated as constituent of carbonate, phosphate, oxalate, soaps.

- Important biological calcium salts: Carbonate, phosphate, and oxalate
- Calcium phosphate occurs as inclusion in bacteria, and protozoa, bone, dentine, and tooth enamel.
- Calcium oxalate demonstration may be performed by adding hydrogen peroxide to the silver nitrate by prior microincineration
- Additional residue can be obtained by showing that there is no staining when the calcium reaction is preceded by treatment with weak acid or 2-5 % EDTA.
- The pure crystalline forms of calcium carbonate and calcium phosphate do not react with von Kossa.

Safety

- Discard used reagents according to federal, local, and state regulations.
- Always apply standard precautions.

References

- Mallory, F.B.: Pathological Techniques, Hafner Publishing Co., NY. 1961.
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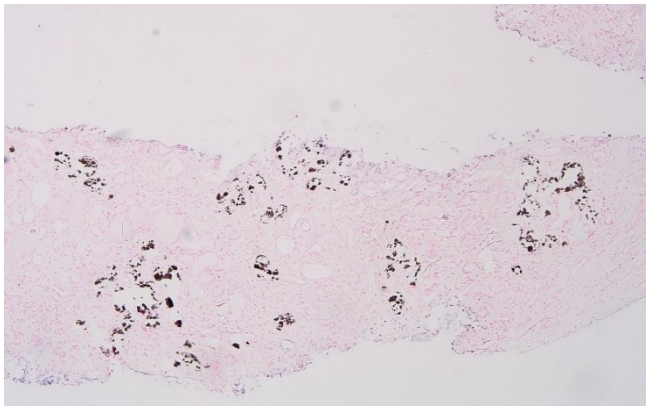
Picture

Von Kossa stain for Calcium on kidney sample



Black deposit- positive for calcium

Sirius Red for Collagen Staining

Purposes

- It is intended for use in the histological visualization of collagen I and III fibers in addition to muscle in tissue sections.
- To observe fibrosis levels in a lot of cases of inflammation induced by cancer, vascular or metabolic pathologies

Principle

Sirius Supra Red F3BA is an elongated molecule containing six sulphonic acid groups and its length is approximately 46 A. As collagen is a basic protein, it is probable that the sulphonic groups of the dye may interact at a low pH (pH2.0) with the amino groups of lysine and hydroxylysine, and the guanidine groups of arginine. It is believed that Sirius Red is specific for collagen and stains only collagen types I, II, III, and component of C1q of the complement protein similar in structure to collagen).

The role of the picric acid is still unknown. However, if this chemical is omitted from the stain, all the tissues will be stained red.

Diagnostic Application

The formulation in this procedure determines the presence of collagen. Sirius Red when used as a simple stain (0.05 g/100 ml.) is a good counterstain for hematoxylin. It reveals collagen, reticulum, basement membrane. It can be used as a substitute for acid fuchsin in the Van Gieson technique.

Quality Control

The multi- control tissues include small intestine which are fixed in 10% neutral buffered formalin and are cut at 5 micrometers. Each set of tests is run with a control. Record the result of the control slide on the special stain quality control sheet as adequate, over-stained, and under-stained. If the control slide results are not acceptable, the stain will be repeated in accordance with the established procedure. All control slides are reviewed by the supervisor or her designated licensed. histotechnologist before the test slides is given out. The final review of the control slides and signing of the quality control are done by a pathologist

Equipment and Supplies

Graduated cylinder, spatula, balance, square boats, reagent bottles, diamond label, label

- Sirius Red.....0.1 gm.
- Picric acid, saturated aq. soln..... 100.0 ml.
- Storage: Room temperature
- Shelf life: 1 year.
- The pH should be pH2.0, the optimum pH for staining collagen.

Procedure

1. Deparaffinize and hydrate tissue sections to water
2. Stain in picrosirius stain for 30 minutes
3. Rinse briefly in tap water (2 dips) to remove excess stain
4. Dehydrate clear and mount.

Procedural Notes

- Collagen birefringence has been reported to increase 700% over control sections.
- Amyloid has been known to stain lightly with Sirius Red with a slight increase in its birefringence in polarized light. However, the amorphous structure of amyloid allows it to be easily distinguished from collagen.
- Sirius red can be eluted from stained tissue sections by 0.1 N sodium hydroxide at 37 0 Centigrade for 30 minutes.
- A counterstain with hematoxylin was frequently employed after Picrosirius staining.
- To study the collagen of cartilage by the Picrosirius -polarization method, sectionsof this tissues were treated by previous digestion at 37 degrees centigrade, for 90 minutes, in 0.5% papain (Papain IVF VIII from Difco) dissolved in 0.02 M phosphate buffer at pH 4.7, containing 0.005M sodium bisulfite and 0.0005M EDTA. This step removes the proteoglycan bound to collagen; hence its birefringence effect is enhanced.

- DPHW means

  DP -Deparaffinize means the removal of paraffin from the tissue sections
  Xylene- 3 minutes
  Xylene- 3 minutes
  Xylene- 3 minutes

  H -Hydrate in descending grades of alcohol
  Dehydrant, 100%- 1 minute
  Dehydrant, 100%- 1 minute
  Dehydrant, 95% - 30 seconds

  W-Running warm water - 1 minute

  Note: If the next step is silver solution, rinse in deionized water

- DCM means

  D-Dehydration means removal of water from the tissue section
  Dehydrant, 100%- 5 seconds
  Dehydrant, 100%- 10 seconds
  Dehydrant, 100%-10 seconds
  Dehydrant, 100%-10 seconds

C-Clearing or De-alcoholization means removal of alcohol

Xylene- 10 seconds
Xylene- 10 seconds
Xylene- 10 seconds
Xylene- to temporary hold-stained slides before coverslipping

M- Mount in synthetic medium

Results

Sirius Red stains collagen red under normal light microscopy and gives an enhanced birefringence to collagen under polarized microscopy. All other tissue components are stained shades of bright yellow.

References

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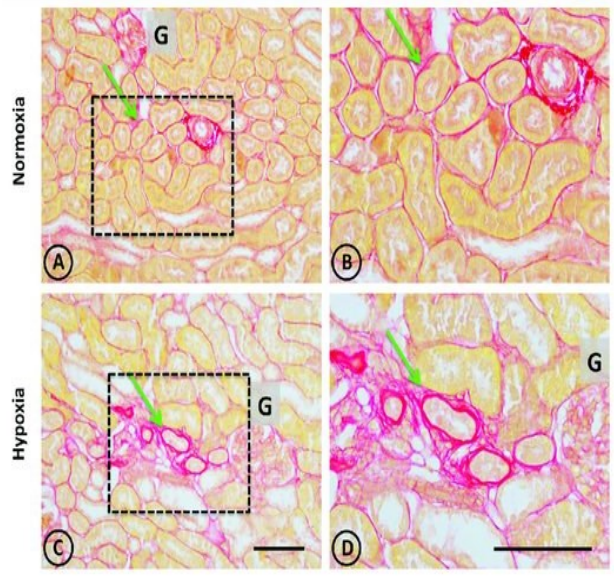
- https://creativecommons.org/licenses/by/4.0/

Picture



Picture from creative commons 6

Collagen Staining with Picrosirius red stain in renal tissue from normoxic and hypoxic animals.

Frames A and B correspond to the normoxia group, C and D correspond to hypoxia group. The left panel shows a panoramic view of the cortical and medullary zones of both groups. The dotted area indicates images with higher magnification on the right column. The arrows indicate the location of collagen stained in red. Note the peritubular collagen in scarce in all images. However, in the hypoxia it is possible to observe small areas of fibrosis, characterized by increase in local collagen. In A, B an arterial blood vessel is observed with perivascular collagen, which does not constitute fibrosis. All structures other than collagen are stained in yellow. G corresponds to the glomerulus. 100 µm.

Verhoeff's Van Gieson Elastic Stain

Purpose

Elastic fiber techniques are used for the demonstration of pathologic changes in elastic fibers. These include atrophy of the elastic tissue, thinning or loss that may result from arteriosclerotic changes, and reduplication, breaks, or splitting that may result from other vascular diseases. The techniques also may be used to demonstrate normal elastic tissue, as in the identification of veins and arteries, and to determine whether or not the blood vessels have been invaded by tumor.

Principle

The tissue is overstained with a soluble lake of hematoxylin-ferric chloride iodine. Both ferric chloride and iodine serve as mordants, but they also have an oxidizing function that assist in converting hematoxylin to hematein. The mechanism of dye binding is probably by formation of hydrogen bonds but the exact chemical groups reacting with the hematoxylin have been identified. This method requires that the sections be overstained and then differentiated, so it is regressive. Differentiation is accomplished by using excess mordant, or ferric chloride, to break the tissue-mordant-dye complex. The dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The elastic tissue has the strongest affinity for the iron-hematoxylin complex and will retain the dye longer than the other tissue elements. This allows other elements to be decolorized and the elastic fibers to remain stained. Sodium thiosulfate is used to remove excess iodine. Van Gieson solution is the most used counterstain, but others may also be used.

Specimen

Any well-fixed tissue may be used.

Cut paraffin sections at 4 microns.

Reagents, Materials, and Equipments

Coplin jars, graduated cylinders, pipettes and filter paper, commercially prepared reagents for Poly Scientific.

Quality Control

- Use a section of aorta embedded on edge, or a cross section of a large artery.
- All control slides for staining procedures are pre-checked every day:
    - By the supervisor or a delegated technologist
    - An attending gives the final approval
    - The controls are viewed also by the technician-on-duty with the pathologist on-duty.
- If the control slide did not work the technician must check all solutions pertaining to the staining procedure to ensure the integrity of the reagents.
- Re-run the staining protocol in the automated machine.
- A new patient and control slide must be used in the repeated staining procedure. If the problem is due to an inadequate control slide, a known control slide must be used, and the procedure repeated

- Controls are selected by the supervisor or recommended by an attending, approved by an attending; then sections are cut. The first and the last slides are stained to determine the positive structures are still present repeated

Manual Preparation of Reagents

These ingredients are made available for users to know the constituents of each reagent used. This laboratory prefers commercially prepared reagents (mostly purchased from Poly Scientific) to be used during machine downtime for the following reasons: pre-validated/manufacturer tested, elimination of preparation time, etc.

- Lugol's iodine:
    - Iodine.....2.0 gm
    - Potassium iodide.....4.0 gm
    - Filtered water.....100.0 ml
- 2% Ferric chloride
    - Ferric chloride.....2.0 gm
    - Filtered water.....100 ml
- 10% Ferric chloride:
    - Ferric chloride.....50.0 gm
    - Filtered water.....500.0 ml
- VERHOEFF'S elastic stain:
    - Dissolve 2 gm. hematoxylin in 44 ml of absolute alcohol in a Pyrex beaker on a hot plate.
    - Cool, filter and add 16 ml of a 10% aqueous solution of ferric chloride and 16 ml. of iodine solution.
    - Prepare fresh
    - Van Gieson's solution:
    - Picric, saturated solution (14g/l) .....380.0 ml
- 5% Sodium thiosulfate:
    - Sodium thiosulfate.....50.0 gm
    - Filtered water.....1000.0 ml

    - Commercially prepared reagents from Poly Scientific Company are available for manual use.
    - These were pre-validated by the company before they are released to the consumer. These are again re-validated by running known positive and/or negative control on site.
    - The diamond signs, lot number, storage, and shelf life for each solution are dictated by the manufacturer.
    - This information is all indicated on individual label of the solution

Procedure

Manual Procedure

1. DPHW (see Procedure Notes)
2. Verhoeff's solution for 20 minutes
3. Prepare fresh solution of Verhoeff's
4. Rinse in tap water.
5. Differentiate individually in 2% ferric chloride while controlling microscopically. An acceptable result should show black elastic fibers and grey background.
6. Wash in tap water
7. Treat with 5% sodium thiosulfate for 1 minute. Discard solution after use.
8. Wash in running tap water for 5 minutes
9. Counterstain in van Gieson solution for 30 seconds; one dip in water.
10. Blot and air-dry for 10 minutes.
11. Clear in xylene and mount.

Automatic Procedure

There are automatic machine available examples: Leica Autostainer XL, Artisan Link Pro, Ventana Benchmark Special Stainer, Sakura Tissue Tek DRS -601, to mention a few. Each of this machine is equipped with built-in protocols for the most used special stains. The procedure is being adjusted according to the preferences of the user and/or pathologists. Thereafter, the machine is validated using known positive and/or negative controls for the commonly requested special stains. If the results of the validation were found to produce satisfactory and acceptable results for patient tests, and is approved by the Director of the Laboratory, then the laboratory may start using the machine

Results

- Elastic fibers blue-black to black
- Nuclei blue to black
- Collagen red
- Other tissue elements yellow

Procedural Notes

- It is easy to over-differentiate this stain. If the background is completely colorless,
- so that a clear yellow counterstain is obtained, the section may be over-differentiated. If is probably better to err on the side of under-differentiation.
- Over-differentiated sections may be re-stained at any step provided they have not been treated with alcohol.
- Do not prolong staining with Van Gieson's solution as picric acid will differentiate the stain further.
- It is not necessary to remove mercury deposits before staining as they will be removed by the staining solution.
- The preparation of Van Gieson's solution is critical for proper differentiation of muscle and collagen. If the picric acid is not saturated, collagen will not stain red, and cytoplasm, muscle, and collagen may all stain the same color.

- To prepare the Verhoeff's elastic staining solution, the reagents must be added in the order given, with mixing after each addition, or poor staining may result.
- The staining jar that contained the Verhoeff's solution may be cleaned easily by transferring the 2% ferric chloride to the jar for a few minutes before discarding the solution.
- For optimum results, slides must be individually differentiated, as the time of differentiation is somewhat dependent on the amount of elastic tissue present. Do not depend on the control for timing the differentiation of all sections.
- DPHW means

DP -Deparaffinize means the removal of paraffin from the tissue sections
Xylene- 3 minutes
Xylene- 3 minutes
Xylene- 3 minutes

H -Hydrate in descending grades of alcohol
Dehydrant, 100%- 1 minute
Dehydrant, 100%- 1 minute
Dehydrant, 95% - 30 seconds

W-Running warm water - 1 minute

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- Revision adopted by: Yao-Tseng Chen, MD, PhD, Director of Surgical Pathology, Michele Maimone-Schoen, Laboratory Manager, Lilian Antonio, Histology Day supervisor
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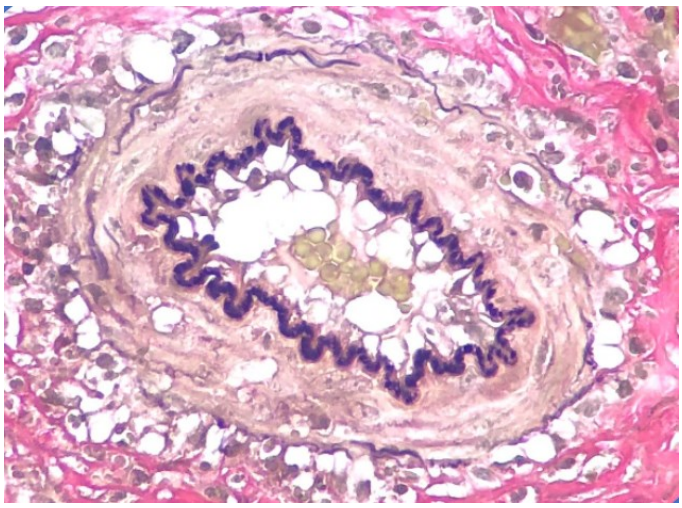
Picture



VERHOEFF'S VAN GIESON ELSTIC STAIN [3]

- Elastic fibers — blue-black to black
- Nuclei — blue to black
- Collagen — red
- Other tissue elements — yellow

IMMUNOFLUORESCENCE

Specimen Handling of Renal Biopsies

Purposes

To describe the process of collection, transportation of renal tissue sample as fresh, or in a transport medium Proper identification of the specimen, getting the correct optimum sample, using the right equipment as these are the foundations of successful laboratory testing.

Principle

The process of collecting specimen from a patient, transporting it in a holding solution in as short as possible time for laboratory testing to obtain optimum results.

Specimen Transport Medium

For Immunofluorescence

- Michel's transport media (Stat Lab)
  Cat# SKU#MS0507/24
  StatLab
- Zeus Transport medium
- Zeus Scientific
- Fresh, wrapped in gauze wet with normal saline solution
  Cat#S5812
  Teknova

For Light Microscopy

- Neutral buffered formalin,10% (fixative of choice)
  Cat# 3800757
  Leica Biosystems

For Electron Microscopy Studies

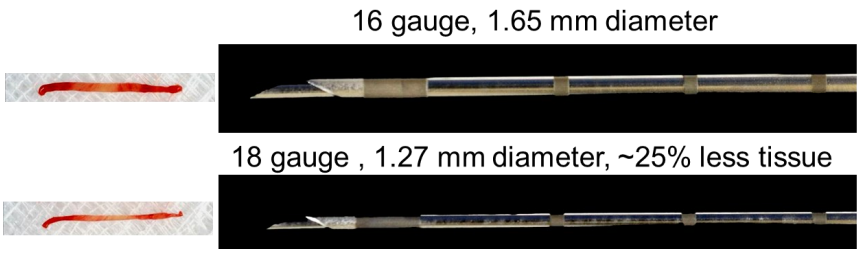
- Paraformaldehyde, 4% in phosphate buffer solution (PBS) pH 6.9-7.4
  Cat3 s2303
  Poly Scientific R & D

- Glutaraldehyde
  Electron Microscopy Sciences

Notes: All reagents are stored per manufacturer's instructions.

Collection of Renal Biopsies

Recommended biopsy needle size: 16-gauge needles provide more glomeruli, more diagnostically adequate tissue, fewer cores, and fewer repeat biopsies with no increase in complications compared with 18-gauge needles (Am J Transplant. 2005; 5:1992-6; Am J Nephrol. 2013; 37:249-54; Nephrology. 2013; 18:525-30).



Materials

- Telfa, individually packed, sterile, non-woven smooth gauze, dissecting microscope, petri dish, nitrile gloves, tweezers, handheld lens, plastic pipette, biohazard plastic bag, 100-ml plastic container, labels

Stereozoom microscope

Handheld lens

Plastic transfer pipette (Uline)

Telfa, sterile, non-adherent pads

Resealable plastic biohazard bag

Non-woven, individually packed, sterile gauze

100- ml plastic container

Equipment and Maintenance

Dissecting Microscope

- Always keep clean and well-maintained
- Provide a quality control chart for documentation purposes of the regular maintenance.

Quality Control

During triaging, the adequate number of glomeruli submitted for light microscopy, immunofluorescence, and electron microscopy and the use of proper holding/transporting solutions.

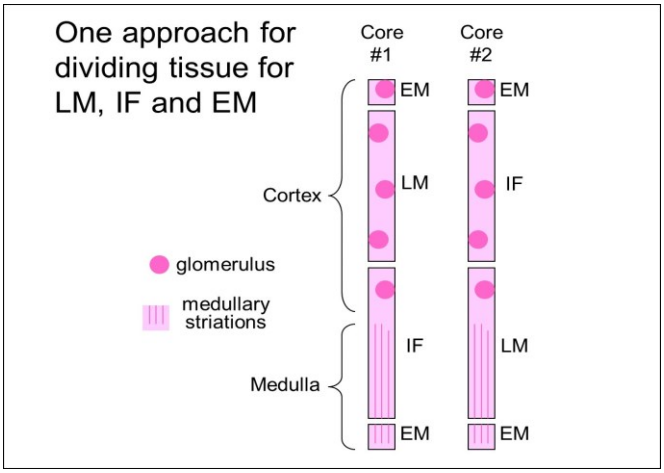
Triaging Procedure

1. Transfer the tissue core from the biopsy instrument needle to telfa or gauze wet with normal saline solution to prevent drying out.
   1.1. May be transported in Michel's solution or Zeus.
2. Send the specimen immediately to the laboratory for processing along with a completed requisition form indicating that the specimen is a transplant or native.
3. Apply a few drops of normal saline solution on the dissecting dish.
   3.1. Layer the cores into this solution
   3.2. If received in Michel's solution, the glomeruli may not be clearly visible.
      3.2.1. Applying a few drops of normal saline solution to renal tissue may increase the visibility of glomeruli.
4. Check under the microscope for glomeruli (circles below).

   4.1. If dissecting microscope is not available, a handheld magnifying glass may be used, although this does not allow optimal visualization.

   Further, if tissue is very ischemic, or sclerosed, glomeruli may not be readily visualized. In non-scarred tissue, glomeruli are small red circular areas under the dissecting scope.
5. When glomerular count is adequate (2 cores are usually required), divide using thin razor blade.
6. The ideal representative sections during triaging of renal biopsy are as follows:
   6.1. For electron microscopy, submit 1-2 glomeruli in paraformaldehyde, 4%
      6.1.1. Ideal size of tissue for electron microscopy studies is 1 mm^3
   6.2. For immunofluorescence, submit 3-4 glomeruli in Michel's solution
   6.3. For light microscopy, submit the remaining renal tissue in neutral buffered formalin, 10%
7. If a very small amount of tissue is obtained, divide according to clinical history, and after consulting with the renal pathologist and nephrologist on which studies are key for diagnosis.
   7.1. Examples:

7.1.1. IF and LM are key for diagnosis of IgA nephropathy.
7.1.2. EM and IF are key for diagnosis of Alport syndrome and thin basement membrane lesion.



Safety

Standard precautions apply

- Wear appropriate personal protective equipment
  - Impervious laboratory coat
  - Nitrile gloves
  - Mask
- Discard in red bag the following: gloves, dissecting dish, and any material that the specimen was transported in and not used in the storage.
- Discard used blade in the sharp's container.
- Return dissecting utensils to disinfecting solution.
- Discard specimen containers in biohazard metal container

References

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- https://www.mblintl.com/assets/JM-2113-500.pdf -
- Amazon.com (telfa, non-woven gauze)
- Uline (plastic bottle)
- Patrick Walker (picture of fresh renal biopsy)

Immunofluorescence Microscopy on Renal Biopsy

Purpose

To detect the presence of antigens in tissues in as short as possible time.

Principle

Antigens are visualized in frozen tissue sections by using antibodies conjugated with fluorochromes e.g., fluorescein isothiocyanate. Its sensitivity, specificity, and simplicity make the method very useful. This is for direct immunofluorescence studies.

Diagnostic Application

The main applications known are as follows: detection of antigens in tissues, in cells and cell membranes and detection of immune circulating autoantibodies or immune cells.

Specimen

Fresh tissue wrapped in gauze wet with normal saline solution, tissues transported in Michel's or Zeus solutions.

Quality Control

In accordance with the Renal Pathology Society Recommendation on the following:

Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Antibodies used in Direct Immunofluorescence

Antibodies	Dilutions
1. IgG	1:20
2. IgA	1:80
3. IgM	1:80
4. Kappa	1:40
5. Lambda	1:100
6. Albumin	1:80
7.C3	1:200
8.C1q	1:30
9. Fibrinogen	1:40

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits. Multiple specimens for immunofluorescence are typically done in a complete run.
    - Technical adequacy of staining can be assessed by detection of positivity in any slides for each antiserum.

- Internal controls, such as staining of intratubular casts or vessels or sclerotic glomeruli for immunoglobulins, light chains and/or complement, which is frequently present, also serve as quality controls. If staining patterns are not observed as expected, based on these procedures, the immunofluorescence staining may be repeated.

Equipment/Materials

- Cryostat requires daily cleaning after use with no calibrations necessary.
- Phosphate buffered saline (PBS), 1.0M pH 7.4
  Cat: 211248
  Manufacturer: BD
- Ethyl alcohol, 95%
- De-ionized water
- Anti-human FITC-conjugated antibodies
- Vacuum chamber requires neither calibration nor maintenance.
- Moisture chamber refreshed daily or as needed with warm tap water.
- Michel's transport media (Cat# 1242) Newcomer Supply
- OCT Compound (Cat# 4583) Richard Allan Scientific
- Glycerin mounting medium or
- VECTASHIELD® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Microtome blade

Solution Preparations

Label all laboratory prepared reagents with the following:

- Name of the solution
- Concentration of the solution
- Date of preparation
- Date of expiration
- Storage information
- Protective equipment
- Hazardous label
- Preparer's initials

All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- [PBS buffer] (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water
- Fluorescein conjugated antisera (**See Quality Control)
- Prepare flattened OCT on chucks, stored inside the cryostat chamber.

The structures, both normal and abnormal, which are used to act as controls for the labeled antibodies are:

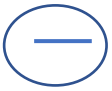
Positive internal controls:

Antibodies	Tissue structure
1. IgG	All basement membranes in diabetic patients
2. IgA	Tubular casts
3. IgM	Sclerotic glomeruli
4. Kappa	Tubular casts
5. Lambda	Tubular casts
6. Albumin	All basement membranes
7.C3	Arterial walls
8.C1q	Sclerotic glomeruli
9. Fibrinogen	High background/artefact of procedure

Negative controls: Each run will include a section with primary antibody omitted.

Procedure

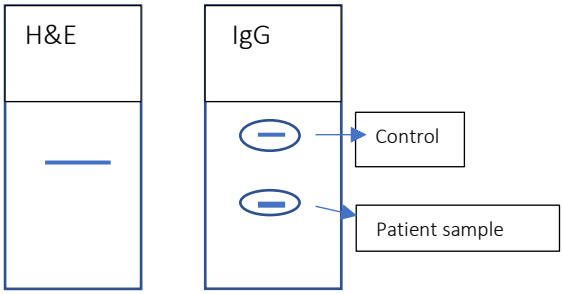
1. Renal tissues are received as follows:
   1.1. If received fresh in Michel's transport solution wash with Michel's wash solution, 3 x10 minutes each [14.6]
   1.2. If received fresh in Zeus transport medium wash with Zeus wash solution, 3 x 10 minutes each
   1.3. If received fresh wrapped in gauze wet with normal saline solution.
2. Place the tissue flat on a pre-frozen, flattened OCT (Optimum cutting temperature) embedding medium on a chuck



Top view of the OCT

Side view of the flattened OCT

3. Cover the tissue with OCT, apply the heat extractor
4. Freeze by placing the chuck in the quick freeze bar.
5. Place the chuck in the cryostat chuck holder
6. Section renal tissue at 2 microns as follows:
   6.1. H&E 1- 1 section on 1 positively charged slide
   6.2. 14 unstained slides, 1 section per positively charged slide



7. When all pre-labeled slides for all antisera and for H&E have one section, circle the back of each slide with the tissue by scoring with diamond knife or appropriate marking pen
8. Air dry sections at room temperature for 10 minutes
9. Wash sections in phosphate buffered saline solution (PBS) for 10 minutes
10. Fix sections in 95% alcohol for 10 minutes
11. Wash sections in PBS – 2 X 5 minutes each,
12. Wipe slide dry around tissue sections to remove excess PBS
13. Lay each slide in the moisture chamber and stain with 1 to 2 drops of recommended diluted FITC conjugated antisera for 30 minutes.
14. Incubate in the dark at room temperature
15. Use wash bottle to rinse each slide with PBS to remove antibodies
16. Put all the slides in a Coplin jar with PBS for additional 10 minutes washing. Wipe slide dry around tissue sections to remove excess PBS
17. Mount with aqua mount anti-fading medium.

Results

Positive result is indicated by greenish fluorescence under fluorescent microscope Background- clear

Notes

- If slides are not read immediately, refrigeration in a slide folder is required.
- Do not dry the tissue at any stage of staining.
- DIF being not permanent is limited due to fading, photobleaching, and autofluorescence.

Safety

- The tissue for immunofluorescence microscopy must be assumed to be unfixed tissue and therefore biohazardous.
- Standard precautions for handling frozen tissue/biohazard apply, no other special safety requirements.
- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have TB, hepatitis, HIV, or another highly infectious agent, dispose of knife immediately following sectioning in the sharp container and disinfect cryostat with absolute alcohol!

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IMMUNOHISTOCHEMISTRY

C4d: Immunohistochemistry

Purpose

C4d is a stable split product of the classical complement pathway activation which is covalently bound in endothelium and basement membrane. The presence of C4d deposition has been identified in antibody-mediated rejection and denotes an inferior graft outcome.

Principle

Antigen can be detected in tissues and cells. The primary antibody binds to its specific epitope. An enzyme labeled polymer binds to the primary antibody. The detection of the bound antibody is evidenced by a colorimetric reaction.

Reagents

- Xylene – 100%
- Ethyl alcohol – 100%, 95%, 70%, 50%
- Citrate-based Antigen Unmasking Solution
  Cat# H3300
  Vector Laboratories
- Blocker™ BSA, (10X) in PBS
  Cat # 37525, 1% dilution
  Thermo Fisher
- Polyclonal, Rabbit anti-human C4d,
  Cat# LS-B3921, 1:50 dilution
  Lifespan Biosciences
- HRP
  Cat # K8002
  Agilent
- Pierce™ DAB Substrate Kit
  Cat# 34002
  Thermo Fisher
- Hematoxylin
  Cat # H3136
  Sigma-Aldrich
- Richard-Allan Cytoseal XYL Mounting Medium
  Cat# 8312-4
  Thermo Scientific
- Wash buffer
  Cat# K800721-2CN
  Agilent
- Antibody diluent
  Cat# U3510

Sigma-Aldrich

- De-ionized water

Materials

- Superfrost slides
- Coplin jar
- Moisture chamber
- Forceps
- Microwave or Water bath

Specimen

Formalin-fixed paraffin-embedded tissue

Procedure

1. Cut paraffin-embedded tissue sections and mount sections on slides. Place slides in vertical plastic holders.
2. Heat slides for 20 minutes at 50-60□C in a dry oven to facilitate attachment of tissue and soften the paraffin.
3. Remove paraffin by submersing slides in 4 changes of 100% xylene for 5 minutes each. Rinse in graded alcohols by submerging in 2 changes of absolute alcohol, 1 change of 95% alcohol, 1 change of 70% alcohol, and 1 change in 50% alcohol for 2 minutes each. Dunk slides up and down several times at the beginning of each incubation.
4. Rinse well in double de-ionized water.
5. Place slides in microwave or water bath and completely submerge slides in excess amount of pre-heated antigen retrieval solution (Citrate-based Antigen Unmasking Solution, Vector Laboratories Cat # H3300) at 100□C for 20 minutes.
6. Remove slides, and place immediately into tap water bath. Do not let sections dry out.
7. Rinse slides in wash buffer for 5 minutes.
8. Cover the tissue with blocking agent (Blocker□ BSA, (10X) in PBS Thermo Fisher Cat# 37525, 1% dilution) and incubate in humidified, light-protected chamber for 20 minutes at room temperature (100□L/tissue section).
9. Remove slides and place in Coplin jar containing wash buffer. Rinse in wash buffer 3 times for 5 minutes each, changing the wash buffer solution in between washing.
10. Remove slides and overlay with C4d antibody (Polyclonal, Rabbit anti-human, Cat# LS-B3921, 1:50 dilution). Incubate in a humidified, light-protected chamber for 20 minutes at room temperature.
11. Rinse slides in wash buffer, 3 times for 5 minutes each.
12. Overlay horseradish peroxidase (HRP, Agilent Cat # K8002) on the tissue. Incubate in a humidified, light-protected chamber for 20 minutes at room temperature.
13. Gently rinse tissue 3 times in wash buffer for 5 minutes each, changing wash buffer after each wash.
14. Incubate with fresh DAB solution for 5 minutes.
15. Rinse with de-ionized water.
16. Counterstain with hematoxylin
17. Dehydrate with alcohol and xylene. Dip slides up and down in solutions 20 times:

- 50% ethyl alcohol – 1 min
- 70% ethyl alcohol – 1 min
- 95% ethyl alcohol – 1 min
- 100% ethyl alcohol – 1 min
- 100% xylene – 3 min
- 100% xylene – 5 min

18. Mount permanent coverslip: Leave slides in xylene until they are cover slipped. Use forceps to pull slides out of the rack. Coverslip with Cytoseal XYL, or similar mounting media. Allow coverslips to harden overnight, with the slides flat.

Results

Brown color, linear, circumferential stain of > 0% of glomerular and peritubular capillaries of any intensity

Procedural Notes

Incubations are to be at room temperature or a 4□C humidified chamber. Antibody concentrations here may not hold for all sources. These concentrations should be titrated for each source.

Special Safety Precaution

Reagents are considered hazardous. Use disposable mask and gloves.

Never pipette reagents by mouth and avoid contact with skin and mucus membranes.

Quality Control

1. Immunoperoxidase results are validated by correlation with clinical and serologic findings, light microscopy, immunofluorescence microscopy, and electron microscopy.
2. Positive control – glomerular mesangium (internal control) or a prior case of antibody-mediated rejection
3. Negative control – peritubular capillary staining in native kidney (except lupus nephritis)

Relative Sensitivity/Specificity

Specificity – 98-100%

Sensitivity – 81-87.5%

False positive

- Plasma in lumen of capillaries
- Plasma fixation of C4d along endothelium
- Granular staining of peritubular capillaries
- Duplicated glomerular basement membranes in native kidneys

False negative

- Poor tissue fixation or processing
- Areas of necrosis
- Some antibody-mediated rejections have negative or minimal C4d deposition

Limitations of the Procedure

- Plasma C4d in capillaries can give high background that obscures or mimics positive endothelial staining
- Some fixatives destroy immunoreactivity (e.g., Blouin's)
- Optimal antigen retrieval is important
- Over dilution of primary antibody affects results
- Granular peritubular capillary C4d deposits correlate with decreased graft survival and delayed graft function but not with other evidence of antibody-mediated rejection

References

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- Collins AB, Schneeberger EE, Pascual MA, et al. Complement Activation in Acute Humoral Renal Allograft Rejection: Diagnostic Significance of C4d Deposits in Peritubular Capillaries. J Am Soc Nephrol 10: 2208–2214, 1999.
- Seemayer CA, Gaspert A, Nickeleit V, et al. C4d staining of renal allograft biopsies: a comparative analysis of different staining techniques. Nephrol Dial Transplant (2007) 22: 568–576.
- Santos A, Viana H, Galvão MJ, et al. C4d detection in renal allograft biopsies immunohistochemistry vs. immunofluorescence. Port J Nephrol Hypert 2012; 26(4): 272-277.
- BioCare Medical (Bio-Optica) product insert on C4d (RM).
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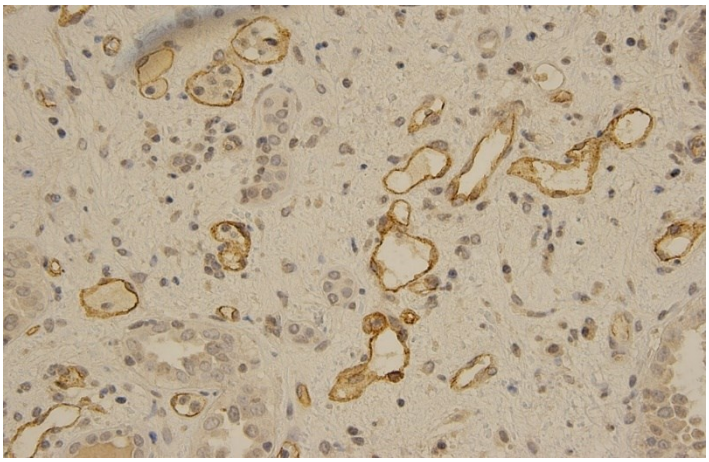
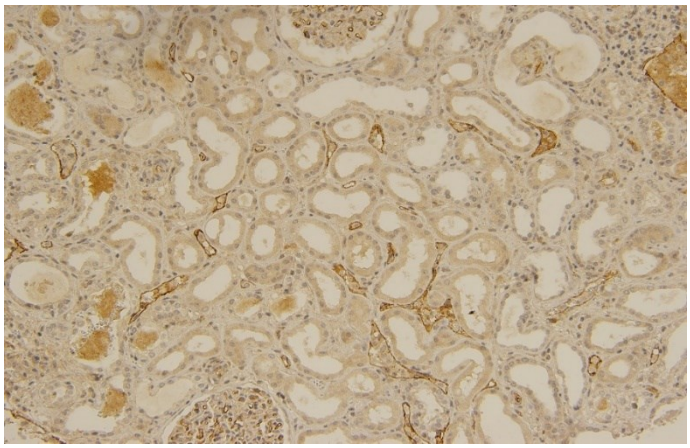
- Vector Laboratories User Guide.

High power magnification



Low power magnification



Brown color, linear, circumferential stain of > 0% of glomerular and peritubular capillaries of any intensity.

DNAJB 9: Immunohistochemistry

Purpose

Formalin fixed paraffin embedded tissue sections may be stained with antibody for detection of DNAJB 9 (strong biomarker for fibrillary GN)

Principle

Antigens are visualized in paraffin embedded tissue sections by using unconjugated primary antibody and secondary ultra-View Universal DAB Detection Kit

Diagnostic Application

DNAJB9 (DnaJ Heat Shock Protein Family [Hsp40] Member B9) was recognized as the fourth most abundant protein (by normalized spectral counts) in glomeruli of patients suffering from fibrillary GN. Antibody against DNJAB9 are used to help diagnose fibrillary GN.

Specimen

Formalin fixed paraffin embedded tissue sections cut at 4 microns

Quality Control

In accordance with the Renal Pathology Society recommendation each new lot of antisera is checked to determine the correct working dilution.

Equipment/Materials

- BenchMark ULTRA system
  Fully automated immunohistochemistry slide staining system offering workflow efficiency, the VENTANA BenchMark ULTRA system enables to process samples as they arrive and easily manage urgent requests. The fully integrated staining solution delivers medical value through continuous and random processing of patient samples.

Primary Antibody

- DNAJB9 Polyclonal Antibody, unconjugated, diluted 100x in Antibody Diluent Dako S2022
  Invitrogen, Thermo Fisher Scientific; PA5-59621

Detection Kit and Reagents

BenchMark ULTRA bulk reagents

- EZ Prep (10x)
  Catalog Number: 950-102 Quantity: 2 L bottle
  Ordering Code: 05279771001 Format: Concentrate
- ULTRA Cell Conditioning (ULTRA CC1)
  Catalog Number: 950-124 Quantity: 1 L bottle
  Ordering Code: 05279801001 Format: Ready to Use
- ULTRA LCS (Predilute)
  Catalog Number: 650-210 Quantity: 2 L bottle
  Ordering Code: 05424534001 Format: Ready to Use
- REACTION BUFFER (10x)
  Catalog Number: 950-300 Quantity: 2 L bottle
  Ordering Code: 05353955001 Format: Concentrate
- Ultra -View Universal Dab Detection Kit
  Catalog Number760-500 Quantity:250 tests
  Ordering Code: 05269806001 Format: Ready to Use
  Notes:
  Ultra -View Universal DAB Detection Kit is an indirect, biotin-free system for detecting mouse IgG, mouse IgM and rabbit primary antibodies. The kit is intended to identify targets by immunohistochemistry in sections of formalin-fixed, paraffin-embedded tissue
- Hematoxylin II
  Catalog Number790-2208 Quantity:250 tests
  Ordering Code: 05277965001 Format: Ready to Use
  Notes:
  Hematoxylin II is a modified Mayer's hematoxylin intended for laboratory use in staining cellular nuclei on slides containing cells from formalin fixed, paraffin-embedded tissue on a BenchMark IHC instrument. This reagent is intended as a counterstain to immunohistochemistry
- Bluing Reagent
  Catalog Number760-2037 Quantity:250 tests
  Ordering Code: 05266769001 Format: Ready to Use
  Notes:
  Ventana Medical Systems' Bluing Reagent is an aqueous solution of buffered lithium carbonate. This reagent is intended for use on Ventana automated slide stainers and for bluing hematoxylin stained sections on glass slides.

  Negative controls: Each run will include a section with primary antibody omitted.

Solution Preparations

Label all laboratory prepared reagents with the following:

- Name of the solution
- Concentration of the solution
- Date of preparation

- Date of expiration
- Storage information
- Protective equipment
- Hazardous label
- Preparer's initials

All laboratory prepared reagents must be prepared with Type II water5. Store all reagents in amber colored bottles unless otherwise indicated.

Processing of Samples in BenchMark ULTRA System

Online:

1. Baking 66°C; 4minutes
2. Deparaffinization 72°C
3. Pretreatment Ultra Conditioner CC1 94°C "Short" 8 minutes
4. Apply Primary Antibody and Incubate for 32 minutes at 37°C
5. Counterstain Hematoxylin II 8 minutes
6. Blue in Bluing reagent for 4 minutes
7. Offline:
   7.1. Put a few drops of Dawn liquid detergent in a dish of tap water (1-liter).
   7.2. Wash well
   7.3. Rinse well with tap water
   7.4. DCM means[6]

D-Dehydration means removal of water from the tissue sections

Dehydrant, 100%- 5 seconds
Dehydrant, 100%- 10 seconds
Dehydrant, 100%-10 seconds
Dehydrant, 100%-10 seconds

C-Clearing or De-alcoholization means removal of alcohol

Xylene- 10 seconds
Xylene- 10 seconds
Xylene- 10 seconds
Xylene- to temporary hold-stained slides before coverslipping

M- Mount in synthetic medium

Results

Positive result is indicated by brownish color

Background- clear

Safety

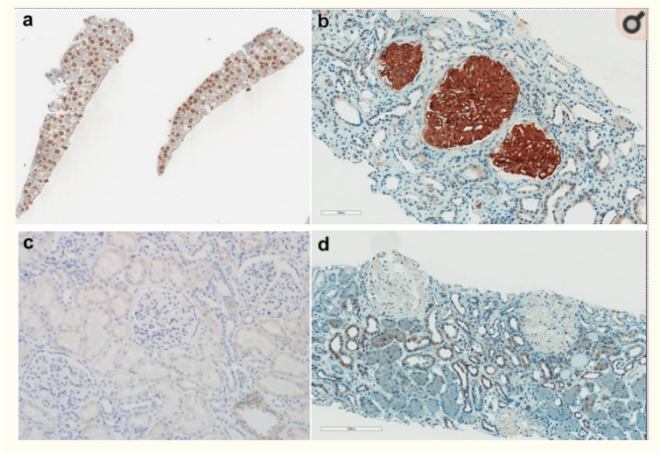
Apply standard precautions.

References

- Nasr SH, Vrana JA, Dasari, et al. DNAJB9 Is a Specific Immunohistochemical Marker for Fibrillary Glomerulonephritis. Kidney Int Rep. 2018 Jan; 3(1): 56–64.
- Dasari S, Alexander MP, Vrana JA, et al. DnaJ Heat Shock Protein Family B Member 9 Is a Novel Biomarker for Fibrillary GN. J Am Soc Nephrol. 2018 Jan; 29(1): 51–56. doi: https://doi.org/10.1681/ASN.2017030306
- Klomjit N, Alexander MP, and Zand L. Fibrillary Glomerulonephritis and DnaJ Homolog Subfamily B Member 9 (DNAJB9). KIDNEY360 1: 1002–1013, 2020. doi: https://doi.org/10.34067/KID.0002532020
- Said SM, Rocha AB, Royal V et al. Immunoglobulin-Negative DNAJB9-Associated Fibrillary Glomerulonephritis: A Report of 9 Cases. AJKD. 2021: (77) 454-458. doi.org/10.1053/j.ajkd.2020.04.015
- https://medium.com/@merckpr1/types-of-laboratory-water-and-its-usages-c2aa3c477e70
- Antonio, LB., Suriawinata, A, Thung, SN.: Liver Tissue Processing Technique In Surgical Pathology of GI Tract, Biliary Tract, and Pancreas, Odze Robert D, Goldblum, John, Crawford, James, Saunders, 1st ed., 2004, chapter 32. pp739-756

Pictures

Glomeruli Positive for DNAJB 9 (1)
(Taken from Nasr SH, et.al., 2018)



Immunohistochemistry (IHC) of DNAJB 9 exclusively highlights glomerulonephritis (FGN) glomeruli.

(A &B). IHC shows strong glomerular staining of DNAJB 9 in 2 different cases of FGN.

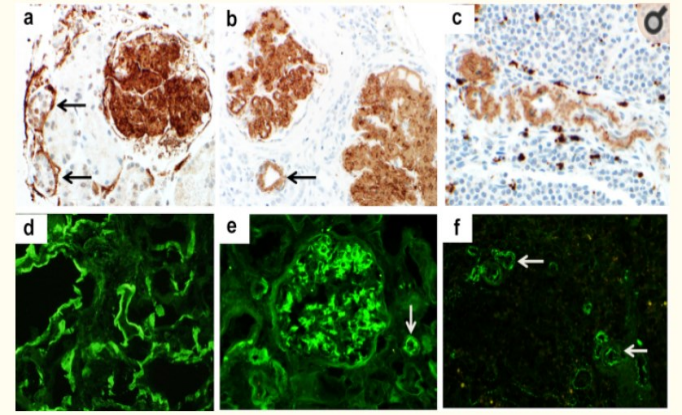
(C) Normal

(D) k light chain amyloidosis does not show glomerular staining for DNAJB9

A, original magnification x 20

B-D original magnification 200

(Taken from Nasr SH, et.al., 2018)



Extraglomerular deposits of DNAJB9 in fibrillary glomerulonephritis (FGN).

a. Focal smudgy staining of tubular membrane (arrows) like the glomerular staining.

b. Smudgy staining of an arteriole (arrow).

c. Smudgy staining of splenic arterioles

d. Linear to smudgy staining of tubular basement membranes of IgG

e. Smudgy staining of an arteriole (arrow) for IgG

f. Splenic arterioles from the same specimen as in (c) show smudgy staining for IgG by pronase IF (arrows).

a-e original magnification x 400

f. original magnification x 200)

SV40: Immunohistochemistry

Purpose

Polyomavirus includes BK, JC, and SV40. Around 85% of polyomavirus infection/nephropathy is caused by BK and 15% by JC. Large T antigen is a protein of early phase of polyomavirus infection. The antibody to Simian Virus 40 (SV40) large T antigen detects BK and JC viruses. The presence of these viruses can cause loss of allograft kidney.

Principle

Antigen can be detected in tissues and cells. The primary antibody binds to its specific epitope. An enzyme labeled polymer binds to the primary antibody. The detection of the bound antibody is evidenced by a colorimetric reaction.

Specimen

Formalin-fixed paraffin-embedded tissue

Procedure

1. Cut paraffin-embedded tissue sections and mount sections on slides. Place slides in vertical plastic holders.
2. Heat slides for 20 minutes at 50-60°C in a dry oven to facilitate attachment of tissue and soften the paraffin.
3. Remove paraffin by submersing slides in 4 changes of xylene for 5 minutes each. Rinse in graded alcohols by submerging in 2 changes of absolute alcohol, 1 change 95% alcohol, 1 change of 70% alcohol, and 1 change in 50% alcohol for 2 minutes each. Dunk slides up and down several times at the beginning of each incubation.
4. Rinse well in double de-ionized water.
5. Place slides in microwave or water bath and completely submerge slides in excess amount of pre-heated antigen retrieval solution (Citrate-based Antigen Unmasking Solution, Vector Laboratories Cat # H3300) at 100°C for 20 minutes.
6. Open lid, remove slides, and place immediately into tap water bath. Do not let sections dry out.
7. Rinse slides in wash buffer for 5 minutes.
8. Cover the tissue with blocking agent (Blocker BSA, (10X) in PBS Thermo Fisher Cat# 37525, 1% dilution) and incubate in humidified, light-protected chamber for 20 minutes at room temperature (100 L/tissue section).
9. Remove slides and place in Coplin jar containing wash buffer. Rinse in wash buffer 3 times for 5 minutes each, changing the wash buffer solution in between washing.
10. Remove slides and overlay with SV40 antibody (SV40 Large T Antigen Pab 101 Cat# 554149, 1:100 Dilution, Pharmingen). Incubate in a humidified, light-protected chamber for 20 minutes at room temperature.
11. Rinse slides in wash buffer, 3 times for 5 minutes each.
12. Overlay horseradish peroxidase (HRP Agilent Cat # K8002) on the tissue. Incubate in a humidified, light-protected chamber for 20 minutes at room temperature.
13. Gently rinse tissue 3 times in wash buffer for 5 minutes each, changing wash buffer after each wash

14. Incubate with fresh DAB solution for 5 minutes.
15. Rinse with de-ionized water.
16. Counterstain with hematoxylin
17. Dehydrate with alcohol and xylene. Dip slides up and down in solutions 20 times:
    7.5. 50% ethyl alcohol – 1 min
    7.6. 70% ethyl alcohol – 1 min
    7.7. 95% ethyl alcohol – 1 min
    7.8. 100% ethyl alcohol – 1 min
    7.9. 100% xylene – 3 min
    7.10. 100% xylene – 5 min
18. Mount permanent coverslip: Leave slides in xylene until they are cover slipped. Use forceps to pull slides out of the rack. Coverslip with Cytoseal XYL, or similar mounting media. Allow coverslips to harden overnight, with the slides flat.

Results

Brown nuclear staining in renal tubular and parietal epithelial cells (1 nucleus)

Procedural Notes

Incubations are to be at room temperature or a 4C humidified chamber. Antibody concentrations here may not hold for all sources. These concentrations should be titrated for each source.

Special Safety Precaution

- Reagents are considered hazardous. Use disposable mask and gloves.
- Never pipette reagents by mouth and avoid contact with skin and mucus membranes.
- Apply standard precautions.

Quality Control

Immunoperoxidase results are validated by correlation with clinical and serologic findings, light microscopy, immunofluorescence microscopy, and electron microscopy.

- Positive control – a prior case of polyomavirus infection
- Negative control – omit primary antibody

Relative Sensitivity/Specificity

- Sensitivity: True positive (true positive + false negative cases) = 14/18 = 77.7%
- Specificity: True negative (true negative + false positive cases)) = 257/257 = 100%
- Positive Predictive Value (true positive + false positive cases) = 14 (14 + 0) = 100%
- Negative predictive value (true negative + false negative cases) = 257/261 = 98.4%

Limitations of the Procedure

- Can be due to either BK or JC polyomavirus
- Casts sometime stain due to shed epithelial cells and viruses
- Staining possible due to decoverslipped slides stained with H and E
- Optimal antigen retrieval is important

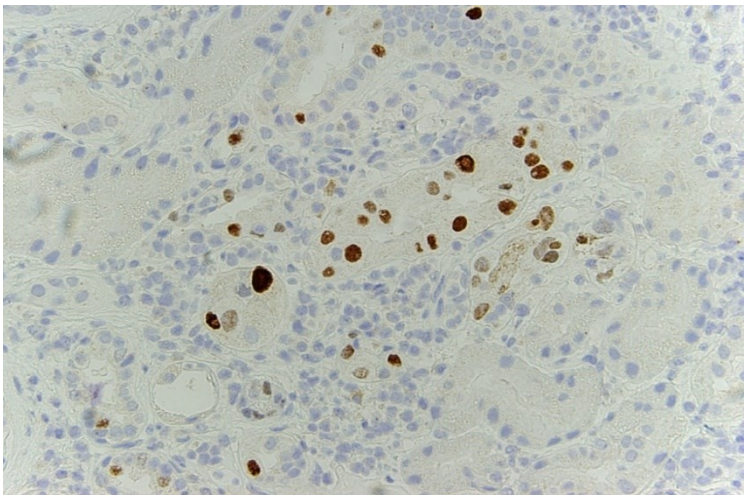
- Over dilution of primary antibody affects results

References

- Chang A, Farkash E (2019). Polyomavirus Nephritis. In Colvin RB, Chang A. Diagnostic Pathology Kidney Diseases, third edition (pp. 810-817). Elsevier, Philadelphia, 2019.
- Nili F, Mohammadhoseini M, Khatami SM, et al. Routine immunohistochemistry study for polyomavirus BK nephropathy in transplanted kidney biopsies, is it recommended? BMC Nephrology (2021) 22:226.
- IHC World – Life Science Products and Services.
- Vector Laboratories User Guide.
- Immunoperoxidase Protocol for Dako Autostainer.

Picture

Tumor cells shows strong staining of SV40 (brown color) without polyomavirus replication in the tissues without tumor.



Alport's Syndrome: Immunofluorescence

Purpose

This immunofluorescence is used to detect the absence or reduction of the collagen α5(IV) chain in inherited Alport's syndrome in areas like: glomerular basement membrane (GBM), tubular basement membrane (TBM), and Bowman's capsular membrane (BCM).

Principle

Frozen section of tissues may be stained with fluorescein conjugated antisera to detect the absence or reduction of the collagen α5(IV) buy using anti-collagen 4cocktail mAb (cat, # SGE-CFT45325), a cocktail of three different rat mAbs for easy and rapid staining of human kidney and skin biopsy sections to distinguish Alport syndrome from normal tissue. It uses two different fluorescein conjugated antisera (clones H53 and B51) that will reveal Alport-affected collagen α5(IV) chain in glomerular, tubular, and Bowman's capsular basement membranes and internal positive control Texas Red- conjugated mAb (clone H25) that targets α2(IV) to reveal endothelial basement membrane structure.[4]

Reagents

- OCT Compound (Fisher Scientific Cat. # 22-110-617)
- Phosphate buffer solution (PBS), pH. 7.3 (BD, Cat. # 211248)
- Vectashield® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Product name: Fluorochrome-conjugated MoAbs for Alport's syndrome
  Fluorochrome-conjugated MoAbs for Alport's syndrome: FITC-Anti-Collagen IV α5(IV) Chain, Human (Mono) + Texas Red-Anti Collagen IV α2(IV) Chain, Human (Mono)[3]
  - Code: SGE-CFT45325
  - Quantity: 1.0 ml
  - Appearance: Solution. Monoclonal antibodies were purified by affinity chromatography. The monoclonal antibodies against α5(IV) were conjugated with FIFC (H53& B51), and the monoclonal antibody against α2(IV), with Texas Red (H25). 0.1% NaN_3 is added for preservation.
  - Clone names: H53 (rat IgG2a/kappa, B51 (rat IgG2a), & H25 (rat IgG1/kappa)
  - Specificity of antibodies:H53 is specific to imperfection III of α5(IV) (Reference 1)
  - Preparation of antibodies: Monoclonal antibodies were prepared by the rat lymph node method developed by Shigei Medica Research Institute with synthetic peptides and native NC1 domain of type IV collagen as immunogens.
  - Use Staining of human cryostat sections by indirect immunofluorescence (No acid-urea treatment is necessary)
  - Storage: In dark at 2-4° C, or in freezing at below -30° C. Stable at this condition for several years.
  - Producer[3]: Shigei Medical Research Institute
    2117 Yamada, Okayama 701-0202, Japan
    Tel: +81-86-282-3113; Fax: +81-86-282-3115
    E-mail: inat@shigei.or.jp

Materials

- Coplin jar
- Forceps
- Cryostat
- Moisture chamber
- Charged slides
- Cover slip
- Microtome blade
- Micropipette

Specimen

SGE-CFT45325 is prepared to stain human frozen sections of renal biopsies and skin biopsies by direct immunofluorescence

Procedure for Kidney Biopsy [9]

Put 20-50 µl of the staining solution on frozen sections, incubate for 30 minutes in a moisture chamber at room temperature. Wash with PBS, mount, observe under fluorescence microscope.

- Cautions:
  - Do not use stale frozen sections
  - Do not fix sections with fixatives
  - Do not dilute the staining solution

Results

In case that a section is not stained or stained with mosaic pattern with FITC-anti-: α5(IV) but the background staining of Texas Red-anti- α2(IV) is normally stained.

The disease is diagnosed as Alport's syndrome.

Notes: For accurate diagnosis, it is important to consider clinical symptoms. In case the staining is not clear, it is necessary to make additional staining with other monoclonal antibodies against type IV collagen.[9]

Reporting of IF Double Staining for Alpha 2 & 5 Chains of Type IV Collagen on Kidney Biopsies[4]

- Normal pattern of staining (i.e., preserved linear alpha 5 staining of glomerular basement membranes, Bowman's capsule, and distal tubular basement membranes). The pattern of staining is seen normal individuals and patients with this glomerular basement membrane disease but does not exclude the diagnosis of hereditary nephritis/Alport 's syndrome.
- Consistent with X-linked hereditary nephritis (Alport's syndrome). There is global or segmented loss of alpha 5 staining of glomerular basement membranes, Bowman's capsule, and distal tubular basement membranes. This pattern of loss of staining is usually due to mutation in the COL4A5 gene on the X chromosomes.
- Consistent with autosomal hereditary nephritis (Alport's syndrome). There is global or segmental loss of alpha 5 staining of glomerular basement membranes but preserved alpha 5 staining of

Bowman's capsule and distal tubular basement membranes. The pattern of loss of staining is usually due to mutations in the COL4A3 and COL4A4 genes on chromosome 2.

Notes[4]

- Approximately one-third of patients with established hereditary nephritis based on typical ultrastructural findings and family history show loss of glomerular basement membrane staining for the alpha 5 chain of type IV collagen. Therefore, a normal staining pattern does not exclude the diagnosis of hereditary nephritis.
- In patients with hereditary nephritis, preserved alpha 5 staining indicates small mutations (e.g., missense, splice site) and is generally associated with a better renal outcome, while loss of alpha 5 staining indicates larger mutations (e.g., deletion, nonsense, frameshift) and a worse renal outcome
- Alpha 3 and alpha 4 chains of type IV collagen are not preserved in the epidermal basement membranes, patients with autosomal hereditary nephritis have preserved staining for alpha 5 on epidermal basement membranes and, therefore, skin biopsy cannot exclude autosomal hereditary nephritis.

Special Safety Precaution

- The tissue for immunofluorescence microscopy must be assumed to be unfixed tissue and therefore a biohazard. Universal precautions for handling frozen tissue/biohazard apply – no other special safety requirements.
- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have tuberculosis, hepatitis, HIV, or another highly infectious agent, dispose of the knife immediately following sectioning and disinfect cryostat.

Quality Control

- Each new lot of antisera is checked to determine the correct working dilution.
- Immunofluorescence results are validated by correlation with clinical and serologic findings, light microscopy, immunofluorescence microscopy, and electron microscopy.
- Positive control – previous positive cases.
- Negative control –normal kidney

Limitations of the Procedure

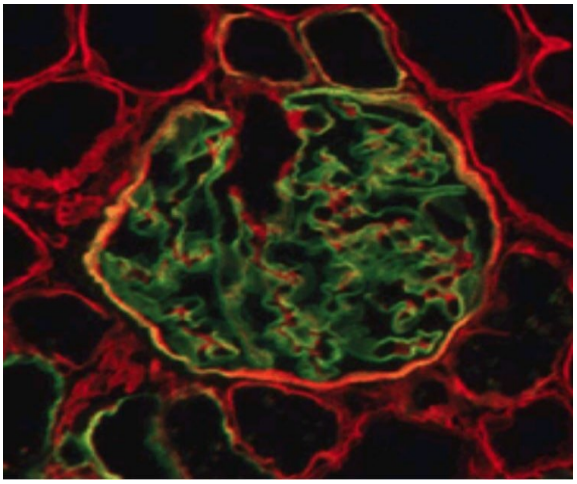
- Requires frozen tissue.

References

- Kagawa et al. (1997) Nephrol. Dial. Transplant. 12: 1238-1241
- Borza DB et al. (2001) J. Biol. Chem. 276:28532-28540
- Shigei Medical Research Institute, 2117 Yamada, Okayama 701-0202, Japan
  Tel: +81-86-282-3113; Fax: +81-86-282-3115; E-mail: inat@shigei.or.jp
- Kagawa M et al. (1997) Epitope-defined monoclonal antibodies against type-IV collagen for diagnosis of Alport's syndrome. Nephrol. Dial. Transpalnt.12:1238-1241
- Yoshioka K et al. (1994) Type IV collagen a5 chain: Normal distribution and abnormalities in X-linked Alport syndrome revealed by monoclonal, antibody. AM J. Pathol. 144-986-996

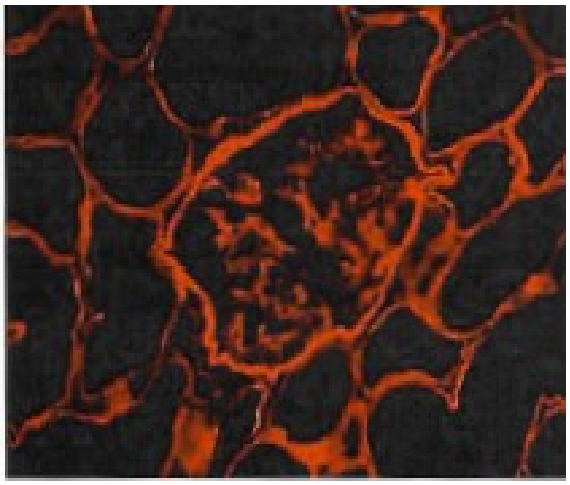
- Ninomiya Y et al. (1995) Differentia expression of the basement membrane collagen genes, COL 4 A6 and COL4A5, demonstrated by immunofluorescence staining using peptide-specific monoclonal antibodies. J. Biol. 130: 1219-1229
- Naito I et al. (1996) Relationship between COL 4A% gene mutation and distribution of type IV collagen in male X-linked Alport syndrome. Kidney Int.50:304-311
- https://www.cosmobiousa.com/products/anti-collagen-4-coctail-mab-clones-h53-b51-h25
- Yoshikazu Sado, PhD, Shigei Medical Research Institute, 2117 Yamada, Okayama 701-0202, Japan, Tel: +81-86-282-3113; Fax: +81-86-282-3115; E-mail: sado@shigei.or.jp

Immunofluorescence; Collagen IV- ALPHA 2 (CONTROL-RED) and ALPHA 5 Chain-Green



Double exposure of normal human kidney section stained with SGE-CFT45325 [3]

FITC fluorescence (α5 chain) is observed in the GBM, part of the TBM and Bowman's capsular BM. BMs were two fluorochromes are present look orange to yellow.



Double exposure of Alport human kidney section stained with the product [3]

Because no FITC fluorescence (α5 chain) is observed in the kidney of the patient with X-linked Alport's syndrome, only Texas Red fluorescence

PLA2R: Immunofluorescence Microscopy From a FFPE Tissue Section

Purpose

To detect the presence of PLA2R antigens with anti-PLA2R antibodies on deparaffinized tissue sections of renal tissues

Principle

PLA2R is detected in deparaffinized tissue sections using rabbit polyclonal anti-PLA2R antibodies (Sigma-Aldrich) at a dilution of 1:50 followed by highly cross-adsorbed Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) at a dilution of 1:100. Run each case with a positive and negative (secondary antibody only) control.

Positive results are indicated by granular capillary loop staining in the glomeruli and negative if there is no staining. Each stain scoring method is on a scale of 0 to 3+. This is an indirect immunofluorescence study.4

Diagnostic Application

Detection of M-type phospholipase A2 receptor (PLA2R) expressed on glomerular podocytes which was identified as a major target antigen of the autoantibodies involved in the membranous nephropathy

Specimen

Deparaffinized FFPE tissue sections cut at 3 microns

Quality Control

In accordance with the Renal Pathology Society recommendation on the following:

Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Antibodies used in Indirect Immunofluorescence

Antibodies	Dilutions	Diluent	Vendor	Catalog number
Anti-PLA2R1*	**1:50**	PBS	Sigma -Aldrich	Cat# HPA012657
Anti-PLA2R2**	**1:100**	PBS	Life Technologies	Cat # A32731

For Primary antibody*: Rabbit polyclonal anti-PLA2R1 antibody (Sigma -Aldrich, 1:50)

For Secondary antibody **Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, 1:100)

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits
- Technical adequacy of staining can be assessed by detection of positivity in any slides for antiserum.

Reagents/Equipment

- Rotary microtome.
- Phosphate buffer (PBS) 1. 0M pH7.4
- Rabbit polyclonal anti-PLA2R antibody (Sigma -Aldrich, 1:50)
- Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, 1:100)
- De-ionized water
- Moisture chamber refreshed daily or as needed with warm de-ionized water.
- Superfrost glass slides (VWR)
- Glycerin mounting medium or
- Vectashield® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Cover glass (VWR)
- Diamond pen
- Commercially prepared:
- Invitrogen Ultrapure 1 M Tris-HCL buffer, pH 7.5 (Cat # 15 -567-027, Fisher Scientific)
- Protease (from Bacillus licheniformis, Sigma, Cat # P5459-5G)

Solution Preparations

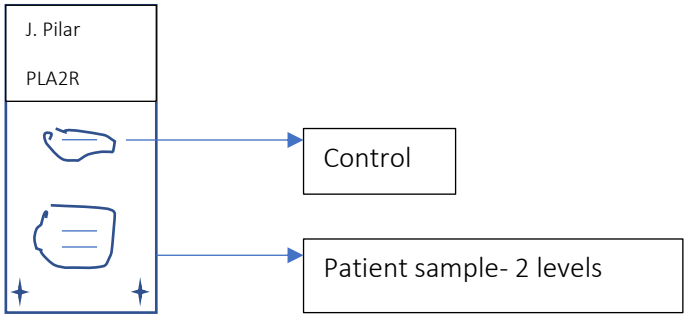
- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation
    - Date of expiration
    - Storage information
    - Protective equipment
    - Hazardous label
    - Preparer's initials
- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- PBS buffer] (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water or
- One part concentrate PBS buffer and 9 parts of de-ionized water, mix well.
- Prepare all FITC-conjugated antibodies at their desired dilutions with PBS
- There are no positive structures in the sample which can be used to act as controls for the labeled antibodies. Previously identified positive cases are being used as controls.

A. Deparaffinization of tissue section:

1. Dry 3-micron sections of renal tissue in an oven 65°C for 45 minutes.
   1.1. 1 unstained slide, 2 step sections on a positively charged slide
   1.2. Circle the back of slide with the tissue by scoring with diamond knife or appropriate marking pen



   1.3. At the same time, heat the waterbath at 37°C
2. Hydrate the tissue section as follows:[5]

DP -Deparaffinize means the removal of paraffin from the tissue sections
Xylene- 3 minutes
Xylene- 3 minutes
Xylene- 3 minutes

H -Hydrate in descending grades of alcohol
Dehydrant, 100%- 1 minute
Dehydrant, 100%- 1 minute
Dehydrant, 95% - 1 minute
Dehydrant, 95%- 1 minute

Note: At this point pour 50 ml of Tris buffer into a Coplin jar and place in a 37°C waterbath

2.1. Wash slides in tap water for 5 minutes then rinse in de-ionized water.

Note: At this point, mix protease in a 50 ml tris buffer

2.2. Put all slides in Coplin jar with protease and leave them for 5 minutes
2.3. Wash in tap water for 5 minutes
2.4. Then place all slides in the moisture chamber then put1-2 drops rabbit polyclonal PLA2R #1 antibody to each slide and incubate for 30 minutes in the dark room.
2.5. While inside the chamber, wash with PBS using a plastic bottle
2.6. Transfer the slides into a Coplin jar with PBS for 10 minutes
2.7. Transfer the slides back into the moisture chamber
2.8. Then place all slides in the moisture chamber then put1-2 drops Alexa flour 488 goat anti-rabbit IgG PLA2R #2 antibody to each slide and incubate for 30 minutes in the dark room.
2.9. While inside the chamber, wash with PBS using a plastic bottle
2.10. Transfer all slides into a Coplin jar with PBS for 10 minutes
2.11. Coverslip as follows:
   2.11.1. Get one slide at a time, with tissue paper remove excess PBS around the tissue, then apply antifade mounting medium, then coverslip

Results

Positive result is indicated by greenish fluorescence under fluorescent microscope

Notes

- If slides are not read immediately, refrigeration in a slide folder is required.
- Do not dry the tissue at any stage of staining.
- IF being not permanent is limited due to fading, photobleaching, and autofluorescence.

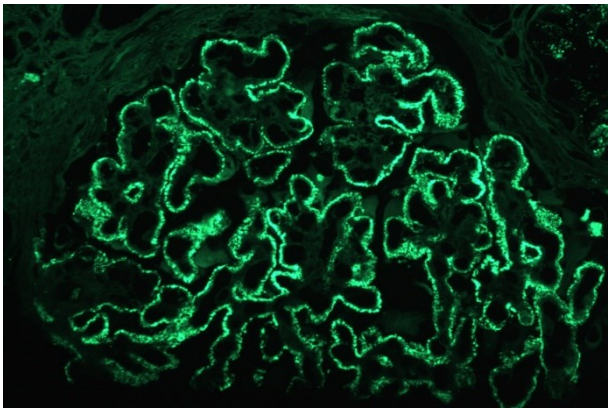
Safety

- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have TB, hepatitis, HIV, or another highly infectious agent, dispose of knife immediately following sectioning in the sharp container and disinfect cryostat with absolute alcohol!
- Apply standard precautions.

References

- Beck LH, Jr., Bonegio RG, Lambeau G, Beck DM, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 361:11-21, 2009.
- Fresquet M, Jowitt TA, Gummadova J, et al. Identification of a Major Epitope Recognized by PLA2R Autoantibodies in Primary Membranous Nephropathy. J Am Soc Nephrol, 2014 Oct 6 pii: ASN2014050502. PMID:25288605.
- Glassock RJ: Antiphospholipase A2 receptor autoantibody guided diagnosis and treatment of membranous nephropathy: a new personalized medical approach. Clin J Am Soc Nephrol 9:1341-1343, 2014.
- Larsen. Larsen, Messias Nidia C., Silva, Fred G., Messias, Erick, and Walker, Patrick D. Determination of primary versus secondary membranous glomerulopathy utilizing phospholipase A2 receptor staining in renal biopsies. Modern Pathology, (2013) 26,709-715, doi:10.1038/modpathol.2012.207; published online 30 November 2012.
- Antonio, LB., Suriawinata, A, Thung, SN.: Liver Tissue Processing Technique In Surgical Pathology of GI Tract, Biliary Tract, and Pancreas, Odze Robert D, Goldblum, John, Crawford, James, Saunders, 1st ed., 2004, chapter 32. pp739-756

Picture



Positive result is indicated by greenish fluorescence for PLA2R under fluorescent microscope

PLA2R: Immunofluorescence Microscopy From Frozen Section

Purpose

To detect the presence of PLA2R antigens with anti-PLA2R antibodies on frozen sections of renal tissues.

Principle

Antigens are visualized in frozen tissue sections by using antibodies conjugated with fluorochromes e.g., fluorescein isothiocyanate. Its sensitivity, specificity, and simplicity make the method very useful.

Diagnostic Application

Detection of M-type phospholipase A2 receptor (PLA2R) expressed on glomerular podocytes which was identified as a major target antigen of the autoantibodies involved in the membranous nephropathy.

Specimen

Fresh tissue wrapped in gauze wet with normal saline solution, tissues transported in Michel's or Zeus solutions.

Quality Control

In accordance with the Renal Pathology Society recommendation on the following:

Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Primary Antibodies used in Indirect Immunofluorescence

Antibodies	Dilutions	Diluent	Vendor	Catalog number
Anti-PLA2R1	**1:200**	PBS	Sigma-Aldrich	HPA012657
Anti-PLA2R2	**1:200**	PBS	Invitrogen by Thermo Fisher	A21206

Secondary antibody used in IF
For Primary antibodies: Rabbit anti-mouse FITC, DAKO, F0261, dilute using PBS

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits
- Technical adequacy of staining can be assessed by detection of positivity in any slides for antiserum.

Reagents/Equipment

- Cryostat requires daily cleaning after use with no calibrations necessary.
- Phosphate buffer (PBS) 1.0M pH7.4
- Ethyl alcohol, histological grade (95%) for fixation
- Anti-human FITC- conjugate antibodies
- De-ionized water
- Moisture chamber refreshed daily or as needed with warm de-ionized water.
- Michel's transport media (Cat# 1242) Newcomer Supply or Zeus solution (Zeus Scientific, Inc.)
- Wash solution for either Michel's or Zeus solution
- OCT Compound (Cat# 4583) Richard Allan Scientific
- Superfrost glass slides (VWR)
- Glycerin mounting medium or
- VECTASHIELD® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Cover glass (VWR)

Solution Preparations

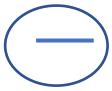
- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation
    - Date of expiration
    - Storage information
    - Protective equipment
    - Hazardous label
    - Preparer's initials
- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- PBS buffer] (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water or
- One part concentrate PBS buffer and 9 parts of de-ionized water, mix well.
- Prepare all FITC-conjugated antibodies at their desired dilutions with PBS
- Prepare flattened OCT on chucks, stored inside the cryostat chamber.
- Negative controls: Each run will include a section with primary antibody omitted.
- There are no positive structures in the sample which can be used to act as controls for the labeled antibodies. Previously identified positive cases are being used as controls.

Cryotomy and Immunofluorescence Staining Procedure

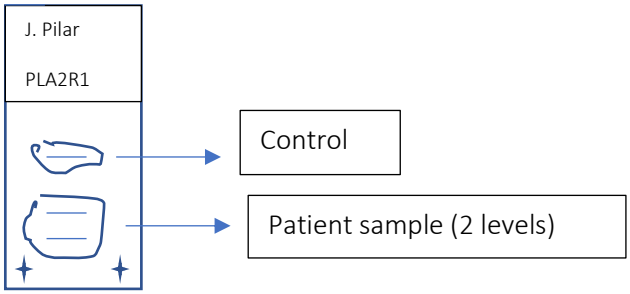
1. Renal tissues are received in any of the following solutions:
   1.1. If received fresh in Michel's transport solution wash with Michel's wash solution, 3 x10 minutes each14.6
   1.2. If received fresh in Zeus transport medium wash with Zeus wash solution, 3 x 10 minutes each
   1.3. Fresh wrapped in gauze wet with normal saline solution.
2. Place the tissue flat on a pre-frozen, flattened OCT (Optimum cutting temperature) embedding medium on a chuck



Top view of the OCT

Side view of the flattened OCT

3. Cover the tissue with OCT, apply the heat extractor
4. Freeze by placing the chuck in the quick freeze bar.
   4.1. Place the chuck in the cryostat chuck holder
   4.2. Section renal tissue at 4 microns as follows:
      4.2.1. unstained slide, 2 step sections on a positively charged slide



Purpose

To detect the presence of PLA2R antigens with anti-PLA2R antibodies on frozen sections of renal tissues

Principle

Antigens are visualized in frozen tissue sections by using antibodies conjugated with fluorochromes e.g., fluorescein isothiocyanate. Its sensitivity, specificity, and simplicity make the method very useful.

Diagnostic Application

Detection of M-type phospholipase A2 receptor (PLA2R) expressed on glomerular podocytes which was identified as a major target antigen of the autoantibodies involved in the membranous nephropathy

Specimen

Fresh tissue wrapped in gauze wet with normal saline solution, tissues transported in Michel's or Zeus solutions.

Quality Control

In accordance with the Renal Pathology Society recommendation on the following:
Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Primary Antibodies used in Indirect Immunofluorescence

Antibodies	Dilutions	Diluent	Vendor	Catalog number
Anti-PLA2R1	**1:200**	PBS	Sigma-Aldrich	HPA012657
Anti-PLA2R2	**1:200**	PBS	Invitrogen by Thermo Fisher	A21206

Secondary antibody used in IF
For Primary antibodies: Rabbit anti-mouse FITC, DAKO, F0261, dilute using PBS

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits
- Technical adequacy of staining can be assessed by detection of positivity in any slides for antiserum.

Reagents/Equipment

- Cryostat requires daily cleaning after use with no calibrations necessary.
- Phosphate buffer (PBS) 1.0M pH7.4
- Ethyl alcohol, histological grade (95%) for fixation
- Anti-human FITC- conjugate antibodies
- De-ionized water
- Moisture chamber refreshed daily or as needed with warm de-ionized water.
- Michel's transport media (Cat# 1242) Newcomer Supply or Zeus solution (Zeus Scientific, Inc.)
- Wash solution for either Michel's or Zeus solution
- OCT Compound (Cat# 4583) Richard Allan Scientific
- Superfrost glass slides (VWR)
- Glycerin mounting medium or
- VECTASHIELD® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Cover glass (VWR)

Solution Preparations

- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation
    - Date of expiration
    - Storage information
    - Protective equipment
    - Hazardous label
    - Preparer's initials

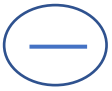
- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- PBS buffer] (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water or
- One part concentrate PBS buffer and 9 parts of de-ionized water, mix well.
- Prepare all FITC-conjugated antibodies at their desired dilutions with PBS
- Prepare flattened OCT on chucks, stored inside the cryostat chamber.
- Negative controls: Each run will include a section with primary antibody omitted.
- There are no positive structures in the sample which can be used to act as controls for the labeled antibodies. Previously identified positive cases are being used as controls.

Cryotomy and Immunofluorescence Staining Procedure

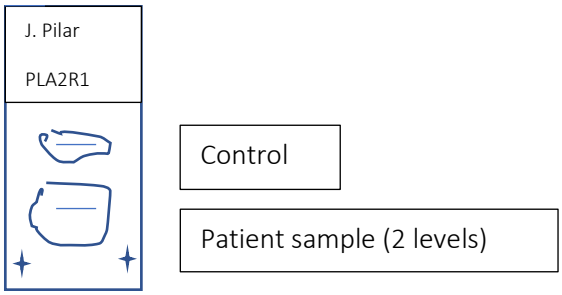
1. Renal tissues are received in any of the following solutions:
   1.1. If received fresh in Michel's transport solution wash with Michel's wash solution, 3 x10 minutes each 14.6
   1.2. If received fresh in Zeus transport medium wash with Zeus wash solution, 3 x 10 minutes each
   1.3. Fresh wrapped in gauze wet with normal saline solution.
2. Place the tissue flat on a pre-frozen, flattened OCT (Optimum cutting temperature) embedding medium on a chuck



Top view of the OCT

Side view of the flattened OCT

3. Cover the tissue with OCT, apply the heat extractor
4. Freeze by placing the chuck in the quick freeze bar.
5. Place the chuck in the cryostat chuck holder
6. Section renal tissue at 4 microns as follows:
   6.1. unstained slide, 2 step sections on a positively charged slide



   6.2. Circle the back of slide with the tissue by scoring with diamond knife or appropriate marking pen
7. Air dry sections at room temperature for 15 minutes or 5 minutes using small electric fan
8. Place the slides in an appropriate size Coplin jar
9. Wash sections in phosphate buffered saline solution (PBS) 3 X for 5 minutes
10. Fix sections in 95% ethyl alcohol for 10 minutes

11. Wash sections in PBS 2x for 5 minutes
12. Remove slides from the Coplin jar one at a time and wipe dry around tissue sections to remove excess PBS
13. Lay each slide in the moisture chamber and put 1 to 2 drops of FITC conjugated anti-PLA2R1 and incubate for 45 minutes in the dark at room temperature
14. After incubation, return the slides back to a Coplin jar containing PBS buffer.
15. Rinse slides with PBS buffer three times, five minutes each
16. Remove slides one at a time and wipe the excess PBS buffer from each slide without disturbing the tissue sections
17. Lay each slide in the moisture chamber and put 1 to 2 drops of FITC conjugated anti-PLA2R2 and incubate for 30 minutes in the dark at room temperature
18. After incubation, return the slides back to a Coplin jar containing PBS buffer.
19. Rinse slides with PBS buffer three times, five minutes each
20. Remove slides one at a time and wipe the excess PBS buffer from each slide without disturbing the tissue sections
21. Mount with aqua mount anti-fading medium.

Results

Positive result is indicated by greenish fluorescence under fluorescent microscope

Notes

- If slides are not read immediately, refrigeration in a slide folder is required.
- Do not dry the tissue at any stage of staining.
- IF being not permanent is limited due to fading, photobleaching, and autofluorescence.

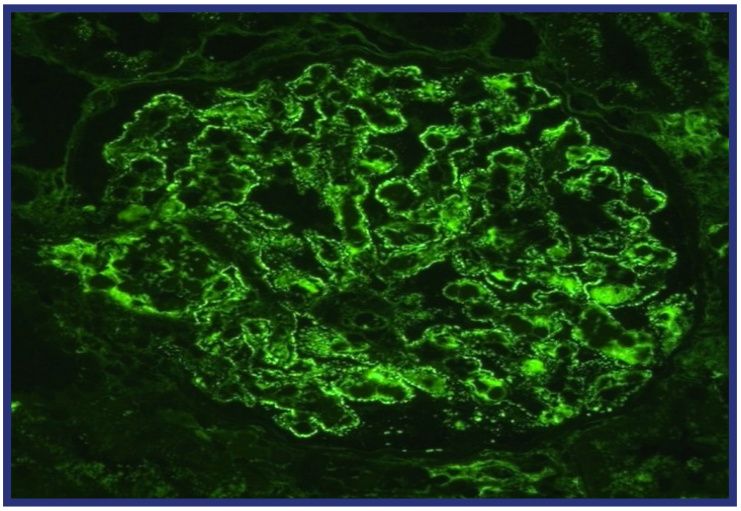
Safety

- The tissue for immunofluorescence microscopy must be assumed to be unfixed tissue and therefore biohazardous.
- Standard precautions for handling frozen tissue/biohazard apply, no other special safety requirements.
- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have TB, hepatitis, HIV, or another highly infectious agent, dispose of knife immediately following sectioning in the sharp container and disinfect cryostat with absolute alcohol!

References

- Beck LH, Jr., Bonegio RG, Lambeau G, Beck DM, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 361:11-21, 2009.
- Fresquet M, Jowitt TA, Gummadova J, et al. Identification of a Major Epitope Recognized by PLA2R Autoantibodies in Primary Membranous Nephropathy. J Am Soc Nephrol, 2014 Oct 6 pii: ASN2014050502. PMID:25288605.
- Glassock RJ: Antiphospholipase A2 receptor autoantibody guided diagnosis and treatment of membranous nephropathy: a new personalized medical approach. Clin J Am Soc Nephrol 9:1341-1343, 2014.

Picture



Positive result is indicated by greenish fluorescence for PLA2R under fluorescent microscope

Pronase- Digested FFPE Tissue Section: Immunofluorescence Microscopy

Purpose

When no glomeruli are available on the frozen tissue sections, formalin fixed paraffin embedded tissues may be used. Hence, to detect the presence of antigens on deparaffinized tissue sections of renal tissues in as short as possible time one may apply pronase-digestion method

Principle

Direct immunofluorescence by applying FITC-conjugated polyclonal rabbit antibodies directed against IgG, IgM, IgA, C3, C1q, kappa, lambda on a pronase-digested formalin fixed embedded tissue section.

Diagnostic Application

Detection of granular global glomerular capillary wall positivity for IgG in a case of MGN; global mesangial positivity for IgA in a case of IgAN; intracapillary protein thrombi staining for IgM in a case of cryoglobulinemic glomerulonephritis; smudgy mesangial deposits of kappa in a patient with primary amyloidosis, AL kappa type. Linear tubular basement membrane deposits of kappa light chain in a case of kappa-light-chain deposition disease, and intracellular proximal tubular crystals of kappa light chain in a case of kappa LCFS.

Specimen

Deparaffinized FFPE tissue sections cut at 3 microns

Quality Control

In accordance with the Renal Pathology Society recommendation on the following:

Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Antibodies used in Direct Immunofluorescence (Dako, Carpinteria, CA, USA)

Antibodies	Dilutions	Dako, Carpinteria, CA, USA
IgG, IgM, IgA, kappa, lambda	1:10	

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits
- Technical adequacy of staining can be assessed by detection of positivity in any slides for antiserum.
- Results for C3 and C1q are not reliable

Reagents/Equipment

- Rotary microtome.
- De-ionized water
- Moisture chamber refreshed daily or as needed with warm de-ionized water.
- Superfrost glass slides (VWR)
- Glycerin mounting medium or
- Vectashield® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Cover glass (VWR)
- Diamond pen
- Commercially prepared:
- Tris buffer pH 7.4-7.8
- Pronase (Streptomyces griseus, Sigma Aldrich, St. Louis, MO, USA)

Solution Preparations

- Label all laboratory prepared reagents with the following:
  - Name of the solution
  - Concentration of the solution
  - Date of preparation
  - Date of expiration
  - Storage information
  - Protective equipment
  - Hazardous label
  - Preparer's initials
- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

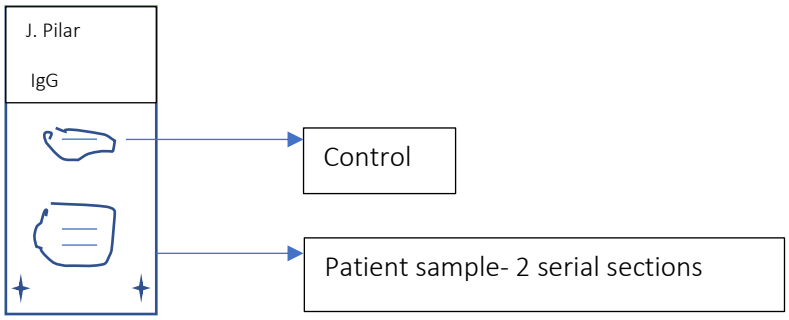
Manual Preparation of Reagents

- PBS buffer] (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water or
- One part concentrate PBS buffer and 9 parts of de-ionized water, mix well.
- Prepare all FITC-conjugated antibodies at their desired dilutions with PBS
- There are no positive structures in the sample which can be used to act as controls for the labeled antibodies. Previously identified positive cases are being used as controls.

Procedure

Deparaffinization of tissue section:

1. Cut 3-micron serial sections on a charged slide
2. 1 slide with 2 serial sections on a positively charged slide
3. Circle the back of slide with the tissue by scoring with diamond knife or appropriate marking pen
4. Oven dry at 370 C overnight (or 600 C for 15 minutes



5. Deparaffinize then hydrate the tissue section as follows:5

   DP -Deparaffinize means the removal of paraffin from the tissue sections

   Xylene- 3 minutes
   Xylene- 3 minutes
   Xylene- 3 minutes

   H -Hydrate in descending grades of alcohol

   Dehydrant, 100%- 1 minute
   Dehydrant, 100%- 1 minute
   Dehydrant, 95% - 1 minute
   Dehydrant, 95%- 1 minute

6. Rinse briefly in de-ionized water (about 20 dips)
7. Rinse in Tris buffer pH 7.6 at 370 C for 30 minutes
8. Incubate with pronase (Streptomyces griseus) i.e., 75 mg /100 of Tris buffer at 37° C for 60 minutes. Note: Use waterbath set at 37°C
9. Stop enzymatic digestion with Tris buffer at 4° C for 40 minutes
10. Rinse in PBS for 10 minutes
11. Incubate in a wet chamber at 40 C for 30 minutes with FITC-conjugated polyclonal rabbit antibodies directed against IgG (1:10), IgM (1:10), IgA (1:10), kappa (1:10), and lambda (1:10)
12. Rinse with PBS 40 C for 10 minutes (x 2)
13. Mount in Vector Shield Aqueous Mounting Medium (Vector Laboratories Burlingame, CA)
14. Examine slides under dark field ultraviolet (IF) microscope.

Results

Positive result is indicated by greenish fluorescence under fluorescent microscope.

Notes

- If slides are not read immediately, refrigeration in a slide folder is required.
- Do not dry the tissue at any stage of staining.
- IF being not permanent is limited due to fading, photobleaching, and autofluorescence.
- Results for C3 and C1q are not reliable

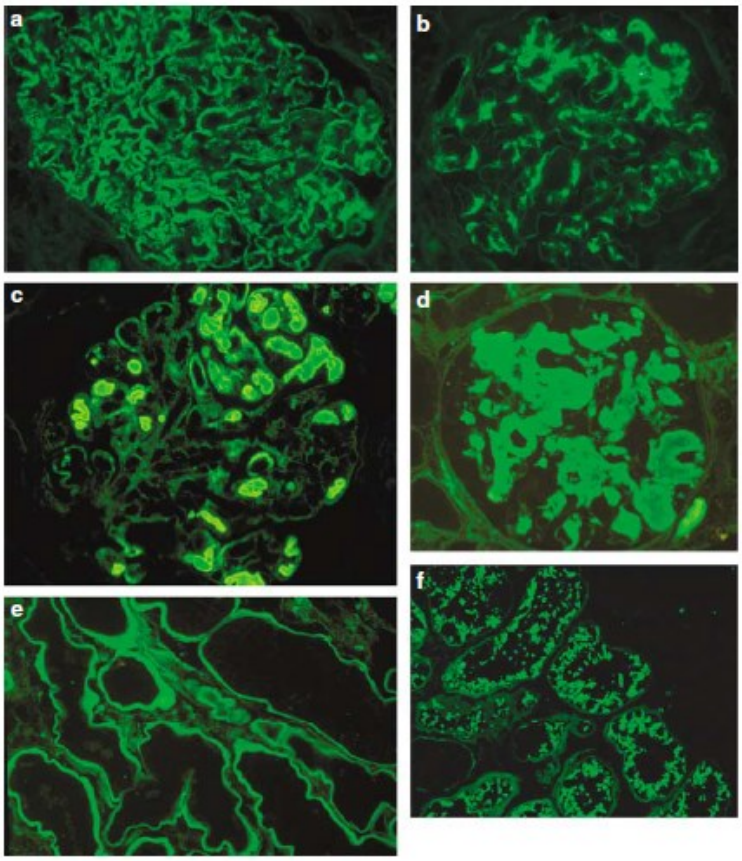
Safety

- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have TB, hepatitis, HIV, or another highly infectious agent, dispose of knife immediately following sectioning in the sharp container and disinfect cryostat with absolute alcohol!
- Apply standard precautions.

References

- SH Nasr, SJ Galgano, GS Markowitz, MB Stokes, and VD D'Agati. Immunofluorescence on pronase-digested paraffin sections: A valuable salvage technique for renal biopsies, Department of Columbia University, College of Physician and Surgeon, NY., NY USA, International Society of Nephrology, 2006, pp 1-4. doi:10.1038/sj.ki.5001990.
- Antonio, LB., Suriawinata, A, Thung, SN.: Liver Tissue Processing Technique in Surgical Pathology of GI Tract, Biliary Tract, and Pancreas, Odze Robert D, Goldblum, John, Crawford, James, Saunders, 1st ed., 2004, chapter 32. pp739-756,

Picture



Positive result is indicated by greenish fluorescence under fluorescent microscope

Captions:[1]

a) Granular global glomerular capillary wall positivity for IgG in a case of MGN.
b) Global mesangial positivity for IgA in a case of IgA
c) Intracapillary protein thrombi staining for IgM in a case of cryoglobulinemic glomerulonephritis
d) Smudgy mesangial deposits of kappa in a patient with primary amyloidosis, AL kappa type.
e) Linear tubular basement membrane deposits of kappa light chain in a case of kappa-light-chain deposition disease, and
f) Intracellular proximal tubular crystals of kappa light chain in a case of kappaLCFS.

IgG SUBSETS: Immunofluorescence Microscopy

Purpose

Frozen sections of tissue may be stained with antibodies for detection of IgG1, IgG2, IgG3, and IgG4, to identify specific IgG subset/subsets in immune complex mediated glomerular disease or establish monoclonal IgG deposits such as heavy chain deposition disease or with monoclonal kappa or lambda light chains.

Principle

Antigens are visualized in frozen tissue sections by using antibodies conjugated with fluorochromes e.g., fluorescein isothiocyanate. Its sensitivity, specificity, and simplicity make the method very useful

Diagnostic Application

Detection of the subclass of IgG antibody (IgG1, IgG2, IgG3, and IgG4(and determining if the deposits are monoclonal or monotypic.

Specimen

Fresh tissue wrapped in gauze wet with normal saline solution, tissues transported in Michel's or Zeus solutions (follow vendor instructions for rinsing out the medium).

Quality Control

In accordance with the Renal Pathology Society Recommendation on the following:

Each new lot of antisera is checked to determine the correct working dilution. Three dilutions are made (1:4, 1:8, 1:12) for staining a positive control and current working dilution is used for comparison. Usual dilution ranges are 1:8 to 1:12 or occasionally more dilute for standard IF antisera.

Dilutions of Primary Antibodies used in Immunofluorescence for IgG subsets

Antibodies	Dilutions	In (Diluent)	Company	Catalogue no.
1. IgG1	100x	5% rabbit serum	LS Bio	LS-C140927
2. IgG2	50x	5% rabbit serum	LS Bio	LS-C70266
3. IgG3	100x	5% rabbit serum	LS Bio	LS-C70321
4. IgG4	Ready to use		Cell Marque/Roche	06523854001

LSBIO means Life Span Biosciences Inc.
For all Primary antibodies: Rabbit anti-mouse FITC, Dako, F0261, dilution 10X in PBS
Secondary antibodies used in Immunofluorescence

- Immunofluorescence results are validated by correlation with light microscopy and electron microscopy for expected absence or presence of deposits. Multiple specimens for immunofluorescence are typically done in a complete run.
- Technical adequacy of staining can be assessed by detection of positivity in any slides for each antiserum.

Equipment/Materials

- Cryostat requires daily cleaning after use with no calibrations necessary.
- Vacuum chamber requires neither calibration nor maintenance.
- Moisture chamber refreshed daily or as needed with warm tap water.
- Michel's transport media (Cat# 1242) Newcomer Supply
- OCT Compound (Cat# 4583) Richard Allan Scientific
- Acetone reagent (Cat# 03704) VWR
- Glycerin mounting medium or
- VECTASHIELD® Vibrance™ Antifade Mounting Medium (Vector Laboratories)
- Albumin Dako Dilutions:

Solution Preparations

- Label all laboratory prepared reagents with the following:
    - Name of the solution
    - Concentration of the solution
    - Date of preparation
    - Date of expiration
    - Storage information
    - Protective equipment
    - Hazardous label
    - Preparer's initials
- All laboratory prepared reagents must be prepared with Type II water. Store all reagents in amber colored bottles unless otherwise indicated.

Manual Preparation of Reagents

- PBS buffer (Cat# 211248) BD 36.92 grams added to 4000 ml of de-ionized water
- Fluorescein conjugated antisera (**See Quality Control)
- Prepare flattened OCT on chucks, stored inside the cryostat chamber.

There are no positive structures in the sample, which can be used to act as controls for the labeled antibodies. However, for testing new lots of antisera a sample of SLE nephritis class IV or membranous glomerulonephritis represent a good control.

Negative controls: Each run will include a section with primary antibody omitted.

Cryotomy and Immunofluorescence Staining Procedure

1. Wash renal tissues as follows:
   1.1. If received fresh in Michel's transport solution wash with Michel's wash solution, 3 x10 minutes each
   1.2. If received fresh in Zeus transport medium wash with Zeus wash solution, 3 x 10 minutes each
   1.3. If received fresh wrapped in gauze wet with normal saline solution.
2. Place the tissue flat on a pre-frozen, flattened OCT (Optimum cutting temperature) embedding medium on a chuck

Top view of the OCT

Side view of the flattened OCT

3. Cover the tissue with OCT, apply the heat extractor
4. Freeze by placing the chuck in the quick freeze bar.
5. Place the chuck in the cryostat chuck holder
6. Section renal tissue at 6-8 microns as follows:
   6.1. 4 unstained slides, 2 sections (2 levels) per positively charged slide
   6.2. Dry slides well (15 min air-dry or 5 minutes using a small electric fan)
7. When all pre-labeled slides for all antisera have one section, circle the back of each slide with the tissue by scoring with diamond knife or appropriate marking pen
8. Place the slides in an appropriate size Coplin jar
9. Wash sections in phosphate buffered saline solution (PBS) 3 x for 5 minutes each.
10. 10. Remove slides from the Coplin jar one at a time and wipe dry around tissue sections to remove excess PBS
11. Lay each slide in the moisture chamber and put 1 to 2 drops of 5% normal rabbit serum at room temperature for 30 minutes.
12. Remove gently using gauze and without rinsing put 1 to 2 drops of appropriate diluted primary antibody
13. Cover the chamber with dark cover and incubate in the dark at room temperature for 30 minutes.
14. After incubation, return the slides back to a Coplin jar containing PBS buffer.
15. Rinse slides with PBS buffer three times, five minutes each
16. Remove slides one at a time and wipe the excess PBS buffer from each slide without disturbing the tissue sections.
17. Lay each slide in the moisture chamber and put 1 to 2 drops of appropriate diluted secondary antibody.
18. Cover the chamber with dark cover and incubate in the dark at room temperature for 30 minutes.
19. After incubation, return the slides back to a Coplin jar containing PBS buffer.
20. Rinse slides with PBS three times, five minutes each
21. Remove slides one at a time and wipe the excess PBS buffer from each slide without disturbing the tissue sections.
22. Mount with aqua mount anti-fading medium.

Results

Positive result is indicated by greenish fluorescence under fluorescent microscope

Notes

- If slides are not read immediately, refrigeration in a slide folder is required.
- Do not dry the tissue at any stage of staining.
- IF being not permanent is limited due to fading, photobleaching, and autofluorescence. Use a dedicated forceps for IF specimens only to avoid cross contamination of reagents.

Safety

- The tissue for immunofluorescence microscopy must be assumed to be unfixed tissue and therefore biohazardous.
- Standard precautions for handling frozen tissue/biohazard apply, no other special safety requirements.
- Gloves must be worn when sectioning anything in the cryostat. If a patient is suspected to have TB, hepatitis, HIV, or another highly infectious agent, dispose of knife immediately following sectioning in the sharp container and disinfect cryostat with absolute alcohol!

References

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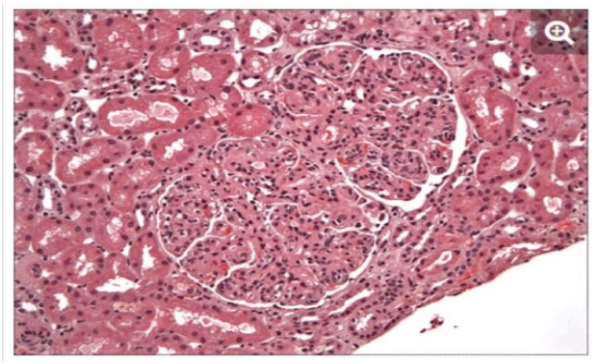
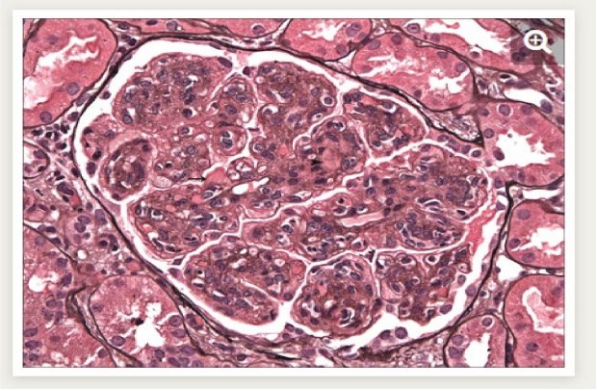
- Michel B. Milner Y. David K. Preservation of tissue-fixed immunoglobulins in skin biopsies of patients with lupus erythematosus and bullous diseases. A preliminary report. J. Invest. Dermatol. 59: 449-452 (1972).
- Storch W. Anwendungsmöglichkeiten der Immunfluoreszenz in der klinisch-immunologischen Diagnostik [Possibilities for the use of immunofluorescence in clinical immunologic diagnosis]. Acta Histochem Suppl. 1984; 30:89-92. German. PMID: 6425963.

Pictures

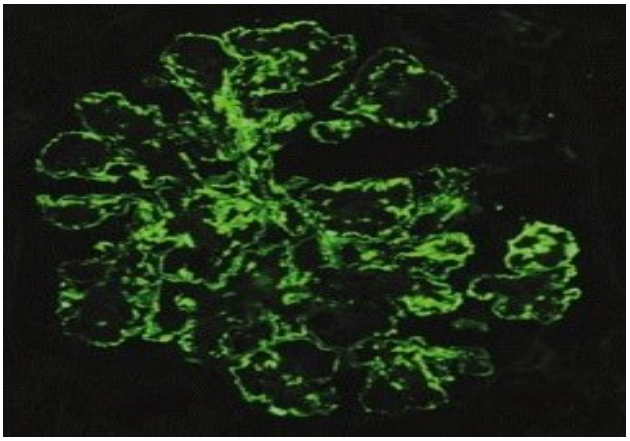
All pictures taken from Hemminger J, Nadasdy G, et.al., Sept. 2009



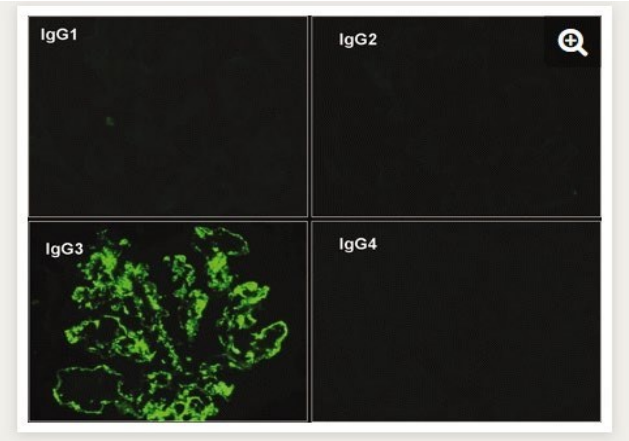
Glomerular capillary lumina are globally narrowed by mesangial and endocapillary proliferation including abundant infiltrating monocytes. Magnification n, x200 (hematoxylin and Eosin Stain)



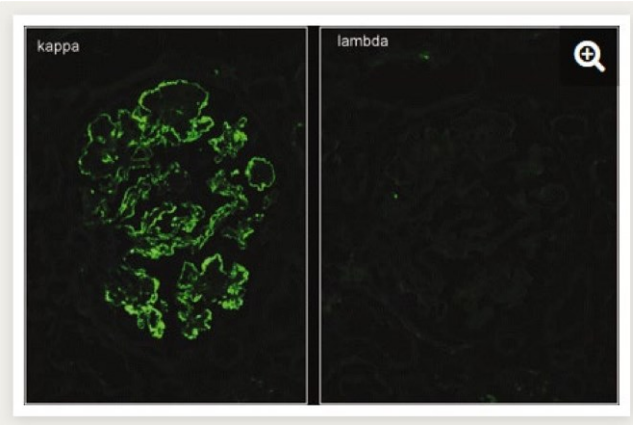
There are widespread double contours of the GBMs. Segmental subendothelial (arrow) and mesangial (arrowhead) nonargyrophilic deposits are seen. Magnification, x400 (Jones Methenamine Silver Stain).



There is a global granular to semi linear staining of GBMs for IgG. Fewer punctate granular deposits are also present in the Mesangium. No staining is observed in Bowman's capsule or the TBMs. Magnification, x200 (IF micrograph)



On IF staining for IgG subtypes, there is a strong glomerular positivity for IgG3 with negative staining for IgG1, IgG2, and IgG4.
Magnification x 400.



There is a strong global granular to semi linear staining for K light chain outlining the GCws with fewer deposits in the Mesangium.
There is no staining of Bowman's capsule or TBMs.
The stain for Lambda light chain is completely negative in glomerulus as well as tubules. Magnification, x 400 (IF micrographs)

ELECTRON MICROSCOPY

Specimen Handling of Renal Biopsies

Purpose

To describe the process of collection, transportation, processing, and staining of renal tissue sample with glomeruli in the cortex for submission to electron microscopy (EM) studies for optimal assessment. Proper identification of the specimen, getting the correct optimum sample, using the right equipment as these are the foundations of successful laboratory testing.

Principle

The process of collecting specimen from a patient, transporting it in a holding solution in as short as possible time for laboratory testing to obtain optimum results.

Specimen Fixatives

Fixatives for electron microscopy

- Paraformaldehyde,4% in phosphate buffer solution (PBS) pH 6.9 -7.4
  Cat# s2303
  Poly Scientific R&D
- Glutaraldehyde, 2.5%
  Electron Microscopy Sciences
- NOTES:
  - Tissues for electron microscopy must be in fixative in as little as 15 minutes after excision. Ideal size: 1 mm3
  - Specimens may be submitted in the following fixatives/transport medium or as fresh and after triaging, the representative 1mm tissues are placed in the fixative of choice (e.g., paraformaldehyde or glutaraldehyde, etc.)
- Neutral buffered formalin,10%
  Cat# 3800757
  Leica Biosystems
- Carson's Phosphate Buffered Formalin (Millonig's fixative)
  Cat # 12445A
  Newcomer Supply

Transport medium/ Holding medium for immunofluorescence

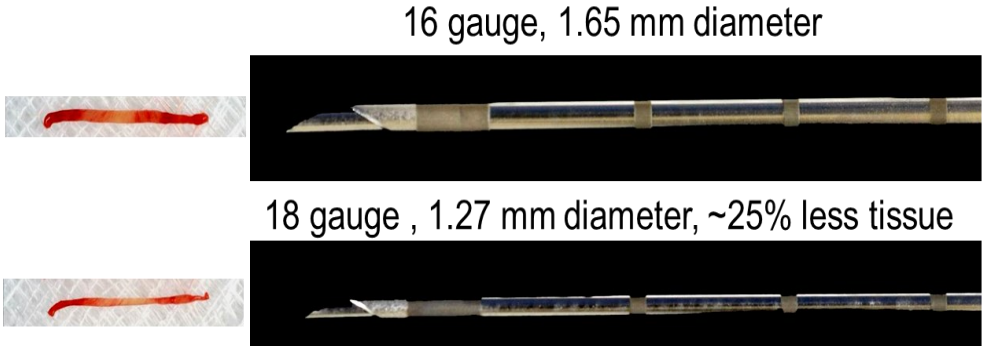
- Michel's transport media (Stat Lab)
  Cat# SKU#MS0507/24
- Snap frozen
- Millonig's buffer
  Cat # 100496-394
  VWR
- Fresh, wrapped in gauze wet with normal saline solution
  Cat#S5812
  Teknova

- Zeus transport medium
  Zeus Scientific
- Paraffin block
  If there are no glomeruli in the Epon block, glomeruli in the paraffin block are retrieved, deparaffinized, and submitted for electron microscopy studies. The morphology is not that ideal though but can be used for diagnostic purposes.

Notes: All reagents are stored per manufacturer's instructions.

Collection of Renal Biopsies

Recommended biopsy needle size: 16-gauge needles provide more glomeruli, more diagnostically adequate tissue, fewer cores, and fewer repeat biopsies with no increase in complications compared with 18-gauge needles (Am J Transplant. 2005; 5:1992-6; Am J Nephrol. 2013; 37:249-54; Nephrology. 2013; 18:525-30).



Materials

- Telfa, individually packed, sterile, non-woven smooth gauze, dissecting microscope, petri dish, nitrile gloves, tweezers, handheld lens, plastic pipette, biohazard plastic bag, 100-ml plastic container, labels

Stereozoom microscope

Handheld lens

Plastic transfer pipette (Uline)

100- ml plastic container

Resealable plastic biohazard bag

Non-woven, individually packed, sterile gauze

Telfa, sterile, non-adherent pads

Tissue Processing Log Sheet

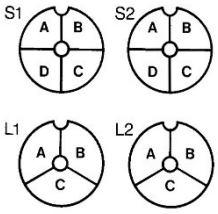
Date: 11.1.2021

Loaded by: _____

Embedded by: _____

Prepared labels: _____

Entered in Logbook _____



Circle the Baskets, Program, & Processor Used

Program: Renal /Muscle/ Nerve/Skin Processor: Lynx/ RMC

Basket	Pos.	Patient Name	Accession #	Pieces
S1	A	Doe, Jane	KB21-123	1
S1	B	Al, Growth	KB21-234	2
S1	C	Go, Merri	KB21-236	2
S1	D	Test, AL	KB21- 243	1
Cumulative total # of cases		*Start new stats at the beginning of each month*	# of daily blocks	6
4			Plus, cumulative from previous day	0
			TOTAL	6
Legends: Pos: is for position of the tissue in the basket A, B, C, or D Basket: Small (S) or Large (L) Circle basket(s) and program used			FORM 1	

Notes:
The Tissue Process Log is implemented to maintain and record the sequence of the specimens being grossed and submitted for processing.

Equipment and Maintenance

- Dissecting microscope
  - Always keep clean and well-maintained
  - Provide a quality control chart for documentation purposes of the regular maintenance.

Quality Control

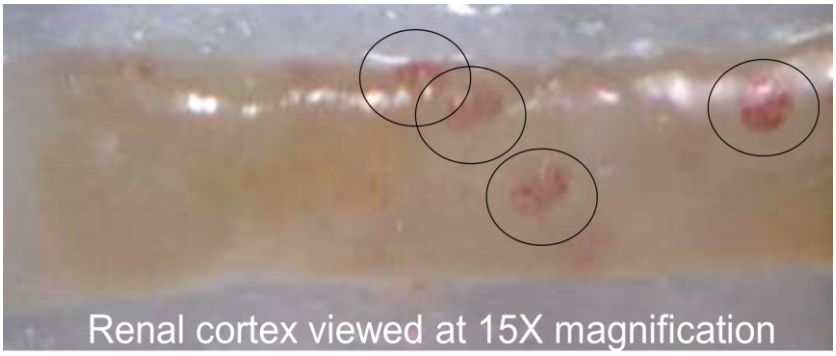
Proper selection and submission of the required number of glomeruli from the cortical part of the kidney sample for electron microscopy studies.

Triaging Procedure

1. Transfer the tissue core from the biopsy instrument needle to telfa or gauze wet with normal saline solution to prevent drying out.
   1.1. May be transported in Michel's solution or Zeus.
2. Send the specimen immediately to the laboratory for processing along with a completed requisition form indicating that the specimen is a transplant or native.
3. Apply a few drops of normal saline solution on the dissecting dish.
   3.1. Layer the cores into this solution
   3.2. If received in Michel's solution, the glomeruli may not be clearly visible.

3.2.1. Applying a few drops of normal saline solution to renal tissue may increase the visibility of glomeruli.

4. Check under the microscope for glomeruli (circles below).



PICTURE 1

4.1. If dissecting microscope is not available, a handheld magnifying glass may be used, although this does not allow optimal visualization. Further, if tissue is very ischemic, or sclerosed, glomeruli may not be readily visualized. In nonscarred tissue, glomeruli are small red circular areas under the dissecting scope.

5. When glomerular count is adequate (2 cores are usually required), divide using thin razor blade.
6. The ideal representative sections during triaging of renal biopsy are as follows:
   6.1. For electron microscopy, submit 1-2 glomeruli in paraformaldehyde, 4%
      6.1.1. Ideal size of tissue for electron microscopy studies is 1 mm^3
   6.2. For immunofluorescence, submit 3-4 glomeruli in Michel's solution
   6.3. For light microscopy, submit the remaining renal tissue in neutral buffered formalin, 10%
7. If a very small amount of tissue is obtained, divide according to clinical history, and after consulting with the renal pathologist and nephrologist on which studies are key for diagnosis.
   7.1. Examples:
      7.1.1. IF and LM are key for diagnosis of IgA nephropathy.
      7.1.2. EM and IF are key for diagnosis of Alport syndrome and thin basement membrane lesion.

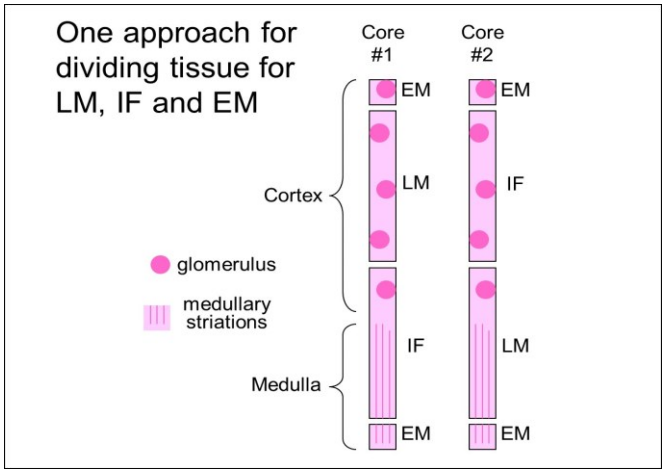
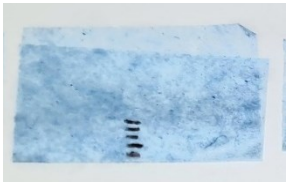


illustration 1

Wrapping of Renal Tissues for Electron Microscopy Processing

1



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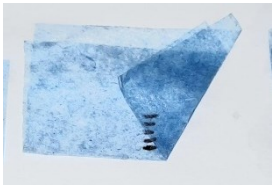

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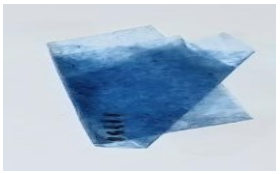

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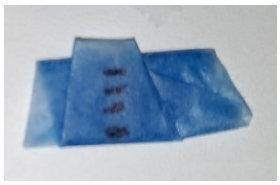

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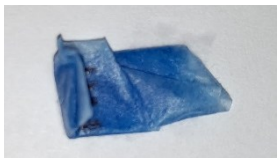
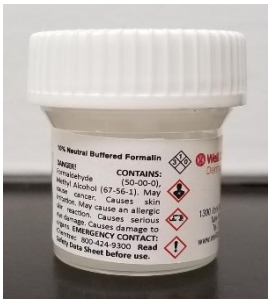


6

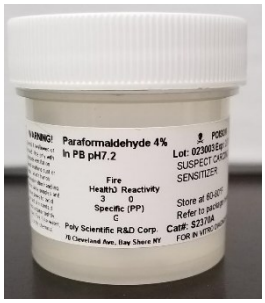
7

Pictures 2

Fixatives of Choice



Light Microscopy
10% Neutral buffered formalin



Electron Microscopy
4% Paraformaldehyde

Immunofluorescence
Michel's solution

Safety

- Standard precautions apply
    - Wear appropriate personal protective equipment
        - Impervious laboratory coat
        - Nitrile gloves
        - Mask
    - Discard in red bag the following: gloves, dissecting dish, and any material that the specimen was transported in and not used in the storage.
    - Discard used blade in the sharp's container.
    - Return dissecting utensils to disinfecting solution.
    - Discard specimen containers in biohazard metal container

References

- Standard Operating Procedures in the Department of Pathology and Laboratory Medicine, Renal Division, Weill Cornell Medicine.
- Vaughn Jones, S.A., et al. The use of Michel's transport medium for immunofluorescence and immunoelectron microscopy in autoimmune bullous diseases. Journal of Cutaneous Pathology, 1995 August; 22(4): pages 365-370
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- https://www.rabkindermpath.com/docs/fixative_qa_6.18.98.pdf
- https://www.mblintl.com/assets/JM-2113-500.pdf

Processing of Renal Biopsy in Electron Microscopy

Purpose

To describe the automatic and manual processing for renal biopsy specimens that will aid in the visualization of renal structures at higher magnifications.

Principle

Renal tissue, about 1mm3, is fixed in aldehyde fixative causing cross-linking of protein and between them, rinse in a buffer followed by secondary fixation in osmium tetroxide. The effect of which is better contrast and retention of lipids. Mordant effect of tannic acid causes the damaged cells to be more pronounced versus undamaged and enhancing the contrast and preserve antigenic properties of a cell or tissues. Furthermore, the subsequent sodium sulfate solution rinse enables to stabilize the normal electrolyte and maintain the isosmotic solution hence there will be no loss nor retention water or ions. Succeeding dehydrants cause coagulation effect and subsequent removal of water from the tissue making infiltration with resins possible.

Specimen

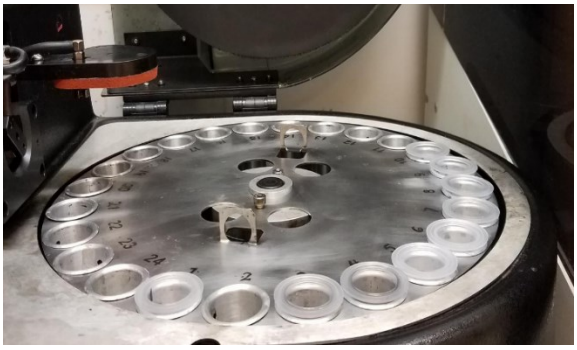
Renal tissues, 1mm3, 1-2 pieces wrapped in lens paper, fixed in paraformaldehyde, 4% pH 7.4. pieces submitted may be more as per renal pathologist's instructions. Others may use other fixatives e.g., glutaraldehyde.

Quality Control

- The solutions for processing are used within the manufacturer's instructions. The end-product
- Epon block feels hard after polymerization.
- Produces uniform sections in thickness
- The first microscopic view of the quality of processing is in thick section stained with toluidine blue. The section has different shades of orthochromatic and/or metachromatic colors.

Equipment/Materials

- Transfer plastic pipette, pair of tweezers and curved probe, aspirator pipette,50-ml syringe, paper towel, syringe tip,1-ml syringe with needle, disposable nitrile Gloves, waste containers for used reagents, graduated cylinder, personal protective equipment (Standard precaution), ampule breaker, tapes
- Automatic tissue processor and its accessories e.g., vials with cover, basket, and holder



Picture 1 Lynx tissue processor

Picture 2 Carousel of a tissue processor

Reagents

- Osmium tetroxide,4%, stock solution
  Cat #19170
  Electron Microscopy Sciences
  - The working solution is prepared last under a fume hood
    - Pour 15 ml of 0.1M sodium cacodylate into a 100-ml amber bottle
    - Break the ampule with an ampule breaker and with a transfer plastic pipette, add the entire content of the ampule (5 ml) to the amber bottle.
    - Cover and mix thoroughly
    - Measure 15 ml only for processing
    - Discard the rest into the waste bottle container with corn oil (The ratio of used osmium and corn oil is 50:50)
- Sodium cacodylate buffer 0.2M (Electron Microscopy Sciences)
  Cat # 11653
  Electron Microscopy Sciences
- Paraformaldehyde, 4% in PBS pH 7.4. (RTU - Poly Scientific R&D)
  - 1M Sodium Hydroxide
  - 10X phosphate-buffered saline (PBS)
  - 1M Hydrochloric acid
  - To prepare a 4% of paraformaldehyde (PFA) solution in PBS,
    - Add 4 grams of granular or prill paraformaldehyde to water (H2O)
    - Use stirring hot plate to heat the solution to 60-degree Celsius.
    - Add 1mL of 1M NaOH until the paraformaldehyde is fully dissolved.
    - Add 10 mL of 10x phosphate-buffered saline (PBS) and allow the mixture to cool. Adjust the solution pH with 1M HCL then top up the volume to 100 mL with H2O. Filter.
- Cat: s2370A
  Commercially prepared by Poly Scientific R&D
- Tannic acid, 8% in 0.05M sodium cacodylate
  - 0.05M Sodium cacodylate buffer _____ 500 ml
  - Tannic powder _____ 40 grams
  - Note: Prepare under a fume hood
    - Use a 1-Liter Pyrex beaker to dissolve tannic acid powder
    - Set the magnetic stirrer-heater at 400 rpm and 1400 C respectively
    - Filter using a large filter paper (33.0 cm grade 362) into a GHS pre-labeled bottle.
      Cat # 0377-01 (powder)
      J.T. Baker
- Propylene oxide
  Cat # 20411
  Electron Microscopy Sciences
- Ethyl alcohol, 200 proof
  Cat# V1016
  Koptec

- Prepare the following concentrations from this stock solution. 50%, 70%, and 95%

- Equal volume of ethyl alcohol 200 proof and propylene oxide
- Sodium sulfate, 1% in 0.1M sodium cacodylate (Electron Microscopy Sciences)
  - Add 5 grams of sodium sulfate to 500 ml of 0.1M HCL-sodium cacodylate buffer contained in a 1-liter beaker.
  - Mix in a magnetic stirrer at 300 rpm for 30 minutes
  - Filter and pour into a GHS pre-labeled 500 ml bottle
  - Sodium sulfate powder
    Cat #BSH8026-500g
    VWR
- Embed 812
  Cat# 14900
  Electron Microscopy Sciences
- Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride (NMA)
  Cat# 19000
  Electron Microscopy Sciences
- Dodecenyl Succinic Anhydride (DDSA)
  Cat # 13710
  Electron Microscopy Sciences
- 2,4,6-Tris(diethylaminomethyl)phenol) DMP-30)
  Cat# 13600
  Electron Microscopy Sciences
- Epon resin for processing is prepared as follows:
  - Prepare an extra syringe of Epon resin for embedding using the same formulation as in processing below

Combine in the order below using a 50-ml syringe			
Ingredients	**Embed 812**	**NMA**	**DDSA**
Amount required	25 ml	13 ml	12 ml
Mix, then add 0.7 ml of DMP 30, mix thoroughly again.			

TABLE 1

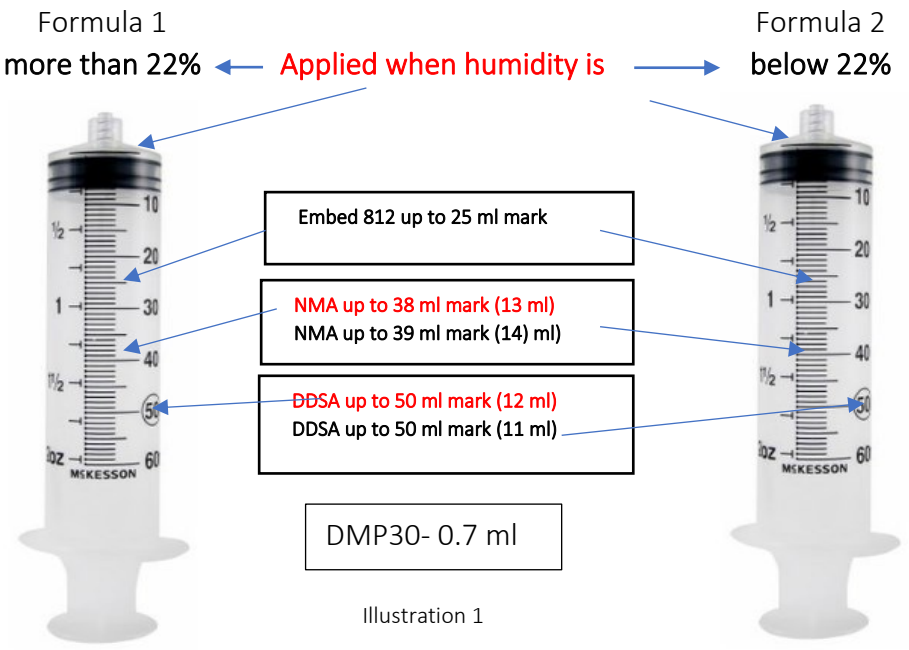


Illustration 1

Automatic Processing Schedule

Vial Number	Solutions	Length of time
1	Sodium cacodylate, 0.1M	10 min
2	Osmium tetroxide, 4% (**last solution to prepare**) Refer to 2.2.1.	55min
3	Sodium cacodylate, 0.1M	10 min
4	Sodium cacodylate, 0.1M	10 min
5	Tannic acid, 8%	20 min
6	Sodium sulfate, 1% in 0.1M sodium cacodylate	15 min
7	Ethyl alcohol, 50%	10 min
8	Ethyl alcohol, 70%	10 min
9	Ethyl alcohol, 95%	10 min
10	Ethyl alcohol, 95%	10 min
11	Ethyl alcohol, 200 proof	10 min
12	Ethyl alcohol, 200 proof	10 min
13	Equal volume of ethyl alcohol, 200 proof and propylene oxide	10 min
14	Propylene oxide	10 min
15	Propylene oxide	10 min
16	Diluted Epon (5 ml Epon + 10 ml Propylene oxide)- Table 1	15 min
17	Diluted Epon (7 ml Epon + 7 ml Propylene oxide) - Table 1	30 min
18	Diluted Epon (10 ml Epon + 5 ml Propylene oxide) - Table 1	60 min
19	Undiluted Epon	15 min
20	Undiluted Epon	15 min
	Total processing time	**5’ 75”**

TABLE 3

Procedure in Loading Tissue Processor

1. Load 1-10 pre-filled, pre-numbered vials to the processing cups
2. Followed by 11 and 12 vials filled with absolute alcohol up to 15 ml mark
3. Prepare Epon as seen in Table 2 for processing
4. Mix well as follows:
   4.1. Aspirate the solutions using a 50-ml syringe in this order:
      4.1.1. Embed 812 up to 25 ml
      4.1.2. NMA up to 38 ml mark
      4.1.3. DDSA up to 50 ml mark
      4.1.4. Pull the plunger to allow space near the nozzle
      4.1.5. Mix 10 times
      4.1.6. Seal with a cap tip, mix again
      4.1.7. Remove the cap tip
      4.1.8. Add 0.7 ml of DMP-30 using one- ml syringe.
      4.1.9. Put back the cap tip
      4.1.10. Mix 40x (10 taps per rotation)
      4.1.11. Dispense as follows:

Vial #	Amount of Epon	Amount of Propylene oxide
16	5 ml of Epon	10 ml
17	7 ml of Epon	7 ml
18	10 ml of Epon	5 ml

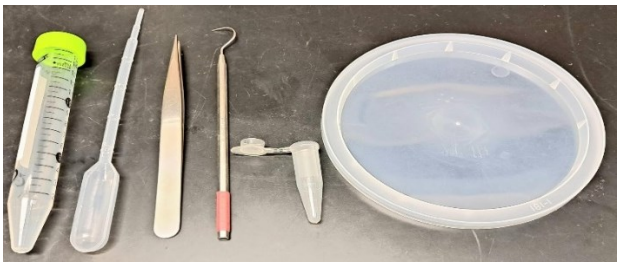
Picture 3

Mix in a rotator

5. Dispense 14-15 ml of the remaining Epon into vials 19 and 20
6. Fill vials 14 and 15 with propylene oxide up to 15 ml mark
7. Load the specimens in the baskets
8. Use the completed Tissue Processing Log as guide in the order of loading

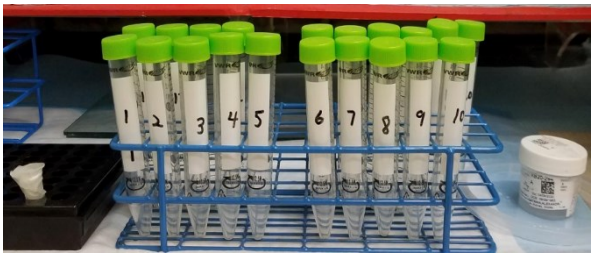
Manual Processing

Materials/Reagents



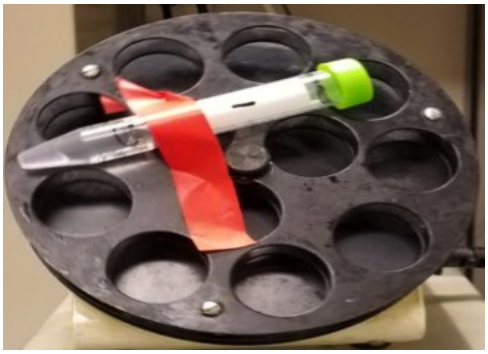
Conical tubes, transfer pipet, forceps, curved probe, Effendorf tubes, plastic dissecting dish,

Picture 4



Set-up in manual processing

Picture 5



Mixer

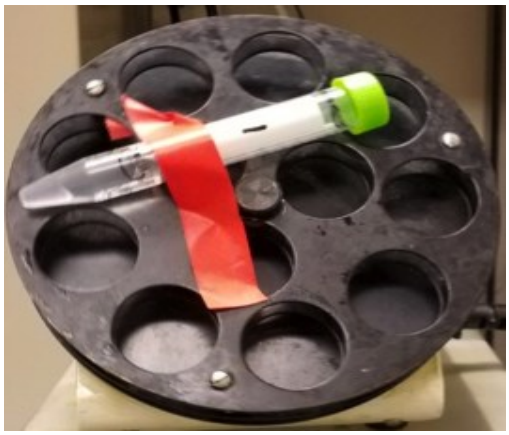
Picture 6

Line-up of Processing Reagents

1. 5 ml of sodium cacodylate 0.1M
2. Transfer the partially thawed osmium tetroxide 1.6 ml, to a conical shaped tube then tops it off to 5 ml mark with 0.1M sodium cacodylate buffer.
   - 2.1. Note: Aliquot the remaining osmium tetroxide from the vial as follows:
     - 2.1.1. Effendorf one -1.6 ml
     - 2.1.2. Effendorf two -1.6 ml
   - 2.2. Seal the lid of the Effendorf with parafilm
   - 2.3. Freeze the unused vials containing osmium tetroxide
3. 5 ml of sodium cacodylate, 0.1M
4. 5 ml of sodium cacodylate, 0.1M
5. 5 ml of 0.1M sodium cacodylate, with sodium sulfate, 1%
6. 5 ml of tannic acid, 8%
7. 5 ml of 50% ethyl alcohol
8. 5 ml of 70% ethyl alcohol
9. 5 ml of 95% ethyl alcohol
10. 5 ml of 95% ethyl alcohol
11. 5 ml of 100% ethyl alcohol
12. 5 ml of 100% ethyl alcohol
13. 5 ml of an equal volume of propylene oxide (2.5 ml) and absolute ethyl alcohol (2.5 ml)
14. 5 ml of propylene oxide
15. 5 ml of propylene oxide
16. 5 ml of mixtures of Epon and propylene oxide in 3 different proportions as follows:

	Propylene oxide	Mixed Epon
Tube 16	3.0 ml	2.0 ml
Tube 17	2.5 ml	2. 5 ml
Tube 18	2.0 ml	3. 0 ml

17. Use a plastic transfer pipette to add the correct amount of Epon from tubes 16 through 20
    - 17.1. The conical portion of the 15 ml tube is graduated 0.5 ml and 1.0 ml respectively
18. Then add propylene oxide to 5 ml. mark from tubes 16 through 18
19. Using the same plastic transfer pipette mix from tubes 16 through 18 in that order.
20. Tubes 19 and 20 each contain 5 ml of Epon.
21. Put the wrapped specimen inside tube # 1, cap it tightly then tape to the rotator, mix according to the recommended time. Use forceps to transfer the wrapped specimen to the next tube after each cycle.
    - 21.1. Mix in the rotator vials from1 through 19
    - 21.2. Do not put vial # 20 (with specimen) in the mixer. Just let it stand in the rack during the duration of infiltration.



Mixer

Picture 7

Manual Processing Schedule

Vial Number	Solutions	Length of time
1	Sodium cacodylate, 0.1M	10 min
2	Osmium tetroxide, 4% (**last solution to prepare)** Refer to 2.2.1	55min
3	Sodium cacodylate, 0.1M	10 min
4	Sodium cacodylate, 0.1M	10 min
5	Tannic acid, 8%	20 min
6	Sodium sulfate, 1% in 0.1M sodium cacodylate	15 min
7	Ethyl alcohol, 50%	10 min
8	Ethyl alcohol, 70%	10 min
9	Ethyl alcohol, 95%	10 min
10	Ethyl alcohol, 95%	10 min
11	Ethyl alcohol, 200 proof	10 min
12	Ethyl alcohol, 200 proof	10 min
13	Equal volume of ethyl alcohol, 200 proof and propylene oxide	10 min
14	Propylene oxide	10 min
15	Propylene oxide	10 min
16	Diluted Epon (5 ml Epon + 10 ml Propylene oxide)- Table 1	15 min
17	Diluted Epon (7 ml Epon + 7 ml Propylene oxide) - Table 1	30 min
18	Diluted Epon (10 ml Epon + 5 ml Propylene oxide) - Table 1	60 min
19	Undiluted Epon	15 min
20	Undiluted Epon	15 min
	Total processing time	**5’ 75”**

TABLE 4

Quality Control

- The tissue processor is inspected to ensure that it is ready for processing
- Reagents are in their optimum levels of performance
- The first microscopic review of the toluidine blue stained slides.

Safety

- Apply standard precautions.
- Discard used reagents according to federal, state, and local

Disposal of Used Reagents/Materials

Waste container labeled:	Used Reagents from Vials
1. Osmium tetroxide/buffer	Vial # 2,3,4
2. Fixatives/buffer	Vial # 1,5,6
3. Dehydrant/ Epon	Vials 7 through 20
Wastes from processing	
4. Syringes/needle/pipettes	Sharp containers
5. Paper towels/gauze	Regular trash
6. Empty fixative containers	Red biohazard bags
7. Used empty tubes with caps	Red biohazard bags

Table 5

References

- Standard Operating Procedure in Electron Microscopy, Department of Pathology Laboratory and Medicine, Weill Cornell Medicine.
- http://stmichaelshospitalresearch.ca/staff-services/research-facilities/facilities/histology-core/tissue-fixation-and-fixatives/
- https://www.emsdiasum.com/microscopy/products/sem/QX102_pdf/Osmium.TetroxideStainingFcells.pdf
- http://cshprotocols.cshlp.org/content/2006/1/pdb.rec9959.short _ (paraformaldehyde)
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. *Processing*, 3rd edition, CRC Press, Inc. Boca Raton, FL

Embedding Techniques for Renal Biopsy

Purpose

To provide support to resin infiltrated tissues to preserve the relationships of the morphological structures within the tissue. The resin block as the product makes it possible to cut ultrathin sections for heavy metal staining and imaging purposes using electron microscope.

Principle

Representative renal tissues with glomeruli are submitted for processing, properly embedded as exact orientation will provide targeted materials for conclusive diagnostic purposes. Embedding also known as casting, or blocking, involves enclosing infiltrated tissue in the embedding medium (resin) and then allowing the medium to polymerize in a 600C - 650C degree oven to form a solid Epon block.

Specimen

Properly processed renal tissue

Equipment/Materials

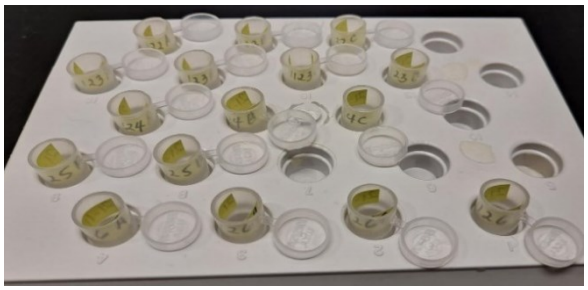
- Disposable plastic Petri dish, curved and pointed probes, tweezers, disposable nitrile gloves, acetone for cleaning used utensils after embedding, Embed 812, NMA DDSA, (Electron Microscopy Sciences) pre-labeled Beem capsules, pieces of paper, size 2 x 1 inches,
- 100-ml plastic cup for acetone

Procedure

1. Preparation prior embedding:
1. Label Beem capsules, see illustrations below:
   1.1. The Beem capsules and labels depend upon the number of pieces of tissues submitted for processing
   1.2. Cut labels individually

KB21-123 A	KB21-123 B	KB21-123 C	KB21-123 D

Embedding Techniques for Renal Biopsy



Picture 1

2. Put in the oven for at least 30 minutes
   2.1. Don disposable gloves, respirator, and laboratory apron
   2.2. Prepare Epon under a fume hood
   2.3. Refer to tabulation below

Combine in the order below using a 50-ml syringe			
Ingredients	Embed 812	NMA	DDSA
Amount required	25 ml	13 ml	12 ml
Mix, then add 0.7 ml of DMP 30, mix again.			

Table 1

3. Mix in a rotator for at least 2 hours.

Picture 2

4. Use the Tissue Processing Log as a reference (highlighted areas) during embedding

Tissue Processing Log Sheet

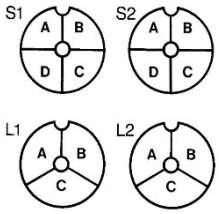
Date: 11.1.2021

Loaded by: _____

Embedded by: _____

Prepared labels: _____

Entered in logbook_____



Circle the program and processor to use

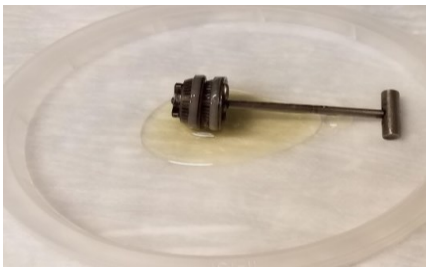
Program: Renal /Muscle/Nerve/Depar Processor: Lynx/ RMC EM 5160

Basket	Pos.	Patient Name	Accession #	Pieces
S1	A	Doe, Jane	KB21-123	1
S1	B	Al, Growth	KB21-234	2
S1	C	Go, Merri	KB21-236	2
S1	D	Test, AL	KB21- 243	1
S2	A	Am, Total	KB21-256	3
Cumulative total # of cases ↓		*Start new stats at the beginning of each month*	# of daily blocks	9
5			Plus, cumulative from previous day	0
			TOTAL	9
Legends: Pos: is for position of the tissue in the basket A, B, C, or D Basket: Small (S) or Large (L) Circle basket(s) and program used			FORM 1	

Notes:

The Tissue Process Log is implemented to maintain and record the sequence of the specimens being grossed and submitted for processing.

5. Embedding procedure
   5.1. Put the basket(s) on a Petri dish

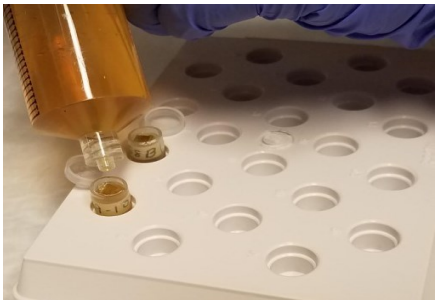


Picture 3

5.2. If there are 2 baskets, the bottom one is the first and the ones on top is the second basket

Picture 4

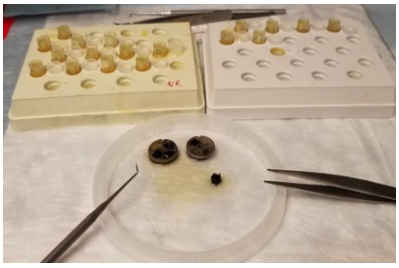
5.3. Fill each mold with Epon up to the bottom edge of the label.



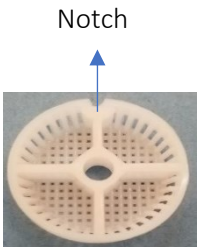
Picture 5

5.4. Use a pointed probe to dislodge any tiny bubbles at the bottom of the pre-I5 labeled Beem capsule.

6. Unwrap the lens paper starting from the left side of the notch indicating that it is the first sample in the first basket. Continue in a clockwise direction



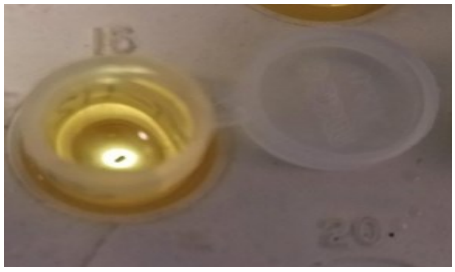
Picture 6



Picture 7

7. Embed one piece of tissue per capsule.
8. Continue the process until all tissues are embedded.

9. Use a straight probe to center the tissue

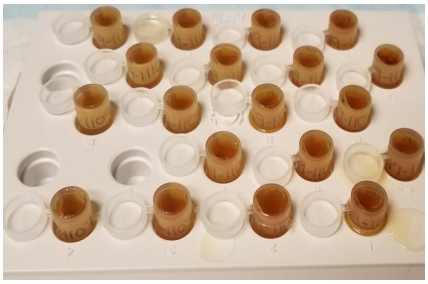


Picture 8

10. Top off each Beem capsule with Epon

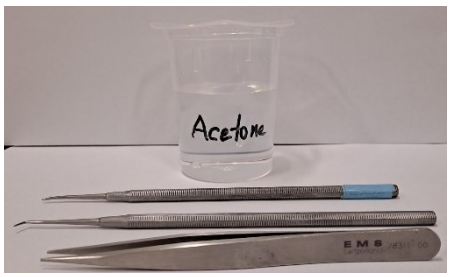
Picture 9

11. Remove excess Epon with a pair of forceps or with a 1-ml syringe
12. Once embedding is completed, put in a 60^0-65^0C oven to polymerize overnight.



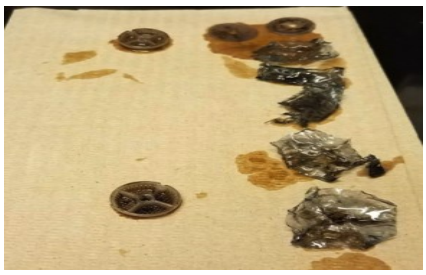
Picture 10

13. Put all embedding utensils into acetone container to dissolve traces of Epon, wash with soap and water then rinse.



Picture 11

14. Collect all Epon contaminated materials like processing vials, baskets, lids, and lens paper
15. Wrap in paper towel, then insert in a pair of gloves.
16. Discard in a dedicated container for residual Epon as hazardous materials.



Picture 12

Safety

- Standard precaution applies:
  - Nitrile gloves
  - Impermeable laboratory gowns
  - Plastic aprons
  - Masks (Respirator for embedding Epon based materials)
  - Face shields
  - Eye protection
  - Hand hygiene

References

- Standard Operating Procedure at Electron Microscopy, Department of Pathology Laboratory and Medicine, Weill Cornell Medicine
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. Rinsing, Dehydration, and Embedding, 3rd edition, CRC Press, Inc. Boca Raton, FL
- Photography: Lilian Antonio, Manager, Electron Microscopy, Weill Cornell Medicine

Thick Sectioning of Renal Biopsies

Purpose

To describe the process of preparing a 1-micron thick section then stain with an oversight stain to aid the pathologist in choosing glomerulus(i) for thin sectioning.

Principle

An ultramicrotome is used to cut a 1-micron thick Epon section; layered on a drop of water then dried on a hot plate set at 70 -100 degrees centigrade. Followed by staining with Toluidine blue for 15-20 seconds (or observe for the formation of a rim of metallic sheen around the drop of toluidine blue); rinse in water, blow dry with Dust off; complete drying on a hot plate for a few seconds; then coverslip using cover glass and synthetic medium applying the dry method of mounting.

Materials/Reagents

- Toluidine blue,1%
    - Constituents:
        - Toluidine blue-O ----------1 grams- 2 grams
            - (Powder from Cat# 22050- Electron Microscopy Science)
        - Sodium tetraborax ----------2 grams- 4 grams
            - Powder from Beantown Chemical, Cat# 225970-500g)
        - De-ionized water----------100 ml- 200 ml
- Steps:
    - Wear basic personal protective equipment: gloves, mask, and laboratory coat
    - Brush off any debris inside the balance before use.
    - Line the table with blue chuck
    - Carefully measure the required volume and quantity of the constituents
        - Use a separate 250 ml of Wheaton bottle
        - Put the magnetic stirrer in it
        - Pour 200 ml of de-ionized water
        - Add 4 grams of sodium tetraborate
        - Stir for 2 hours at 400 rpm
        - Add 2 grams of toluidine blue and stir for another 1 hour
        - Filter the solution into a bottle with proper GHS labels using Whatman no.1 filter
            - Attach filter to a syringe to re-filter toluidine blue solution each time it is used.
- Synthetic mounting medium e.g., Surgipath Micromount
  Cat# 3801731
  Leica Biosystems

- Cassette press, Epon blocks, glass rods bent at an angle, 10 ml syringe with filter regular Microscope, double-edge blade, de-ionized water, brush microtome and Histo Diamond Knife, glass slides and coverslips, Stereozoom Microscope, marking pens, paper towels, Renal

Ultra-thin Section Outlay Form Sheet, hot plate, plastic spray bottle, waste container for toluidine blue stain

Renal Ultra-Thin Section Outlay Form

Case #: _____

To: _____

Patient name: (Last) _____ First:_____

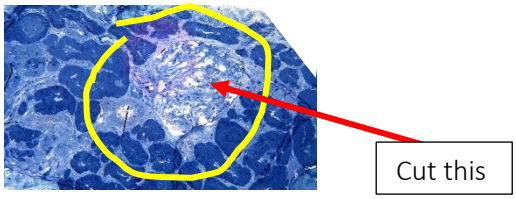
Form completed by:	Date:		Thin section by:	Date:
Thick section by:	Date:		Stained by:	Date:

Deparaffinization: _____ Transplant: _____

Digital Image Numbers: _____ Grid Box: _____ Row: ____ Block: ____

By: _____ Date: _____ Grid Box: _____ Row: ____ Block: ____

Please examine THICK SECTIONS submitted with this form and kindly sketch in pencil the area you are interested in for imaging with the electron microscope. Be sure to put the block number down on this form that corresponds with the appropriate thick section.

Block #: ____A_____ 	Descriptions of the toluidine blue sections Example: A- cortex, 1 glom
Block #: _____	

Form 1

Quality Control

Renal tissue will stain orthochromatic in varying shades of blue and/or metachromatic.

Procedure

1. After overnight polymerization, Epon blocks are taken out of the oven
2. Use cassette press to remove the blocks from the Beem capsules
   2.1. It is easier to remove Epon blocks from Beem capsules while still warm.
3. Prepare the Renal Ultra-thin Section Outlay form.
   3.1. One form per case.
4. Trim the apical portion of the Epon block as shown below.
5. Use razor blade to expose the renal tissue.
6. Once exposed, put a notch on one corner of the trimmed surface of the Epon block
7. Continue the process until all blocks are trimmed.
8. Put a pre-labeled slide under a Stereozoom microscope.
9. Dispense drops of water using a 1-ml syringe on the slide corresponding to the number of blocks per case.
10. Take the first block of the case and mount in the chuck of the microtome.
    10.1. Position the notch on top.

    NOTE: At this, point set the hot plate at 100 marks.

11. Gingerly trim the surface at 0.5 microns until a full face is obtained
    11.1. Do not rock the wheel up and down during this process as it will cause micro holes visible only during imaging.
    11.2. Always rotate the wheel around
12. Set the microtome at 1micron, then section until the desired color greenish gold is obtained.
13. Use a glass tubing with a slightly bend tip (see below) to transfer the section from the water trough of the diamond knife to the drops of water on a slide.
    13.1. Do not touch the edge of the knife with the glass tubing or brush.
14. Continue the process until all blocks for each case are cut.
15. Place the slide on a hot plate until the sections dry.
16. Put a few drops of 1% toluidine blue on sections for 15-20 seconds
17. Pick up the slide and rinse thoroughly in running water
18. Air dry (or use Dust off)
19. Complete drying of stained slides on a hot plate.
20. Use dry technique when mounting dried stained section.
    20.1. This technique does not use xylene.
21. For easy visualization of the sections, draw a blue line at the back of the slide as shown below

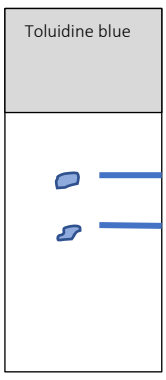


Illustration 1

22. Submit the slides together with the outlay forms to the renal pathologist
    22.1. One section per Epon block

Materials/Equipment

Stereozoom

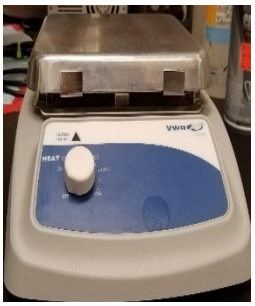
Picture 1

Ultramicrotome

Picture 2

Cassette press

Picture 3

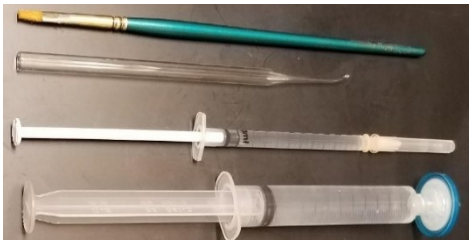


Hot plate

Picture 4

Block holder

Picture 5

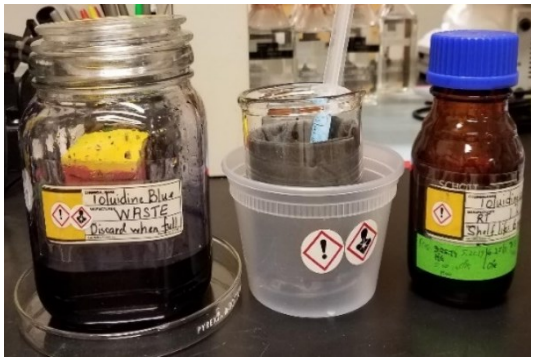


Syringes, bent glass tubing, brush

Picture 6

Dust Off and De-ionized water

Picture 7



Bottle for Toluidine water, Toluidine blue stain, 10-ml syringe with filter

Picture 8

Mounting kit

Picture 9

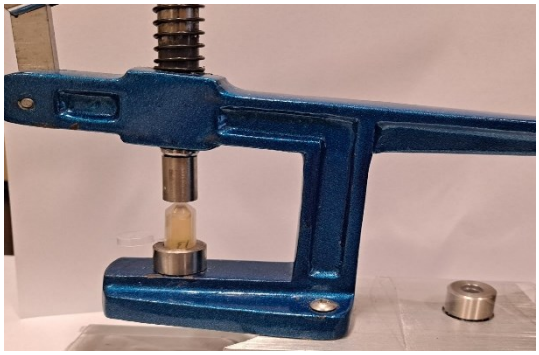
Diamond knife

Picture 10

1. Remove the polymerized Epon with tissue from the Beem capsule using a cassette press

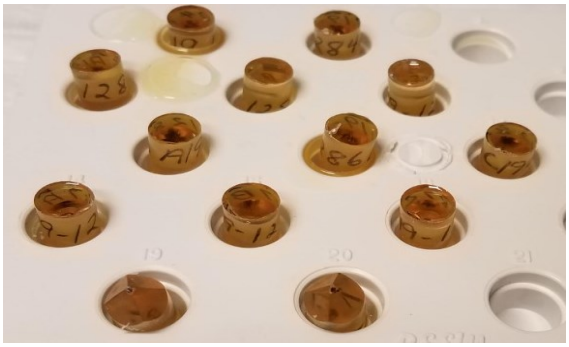
Picture 11

2. Position the block with the conical bottom right under the cassette press



Picture 12

3. Conical portion positioned down means not trimmed.



Picture 13

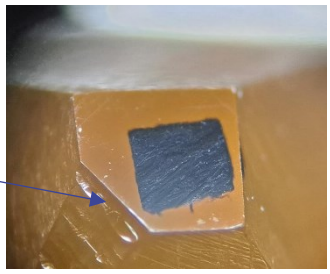
4. The trimming is aided by a dissecting microscope set at 2.0

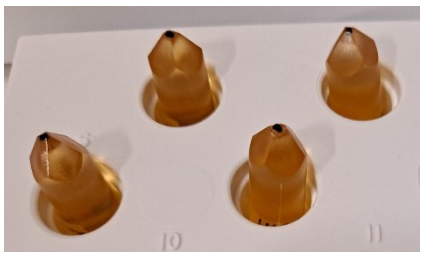
Picture 14

5. Use the thin razor blade to trim off excess Epon to expose the tissue.
   5.1. Put a notch in one corner
   5.2. The notch is used for re-orientation if a recut is needed.
6. After trimming, the trimmed portion in an upward position which means ready for thick sectioning.

Top view after trimming

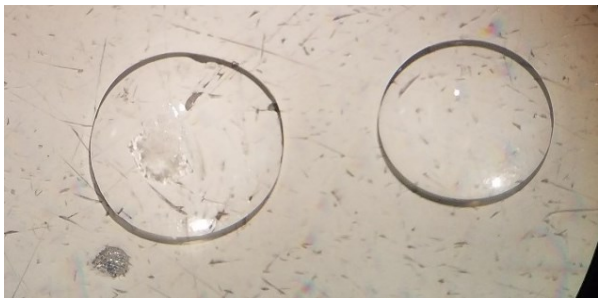
Notch





Pictures 15

7. Put drops of deionized water on the slide equivalent to the number of blocks per case for sectioning.



Picture 16

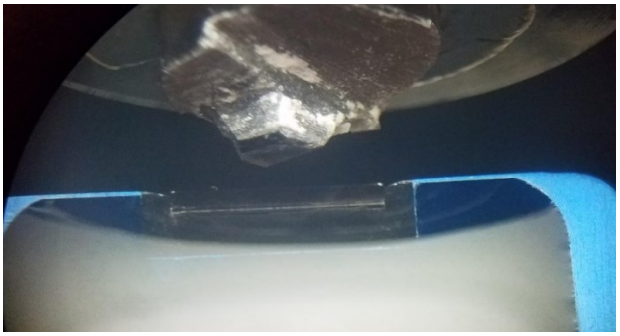
8. Mount the trimmed Epon block in the microtome.

Picture 17

9. The histo diamond knife (Diatome) or glass knife with attached boat (EMS) is filled with water and the block is now ready for rough trimming at 0.5 micron

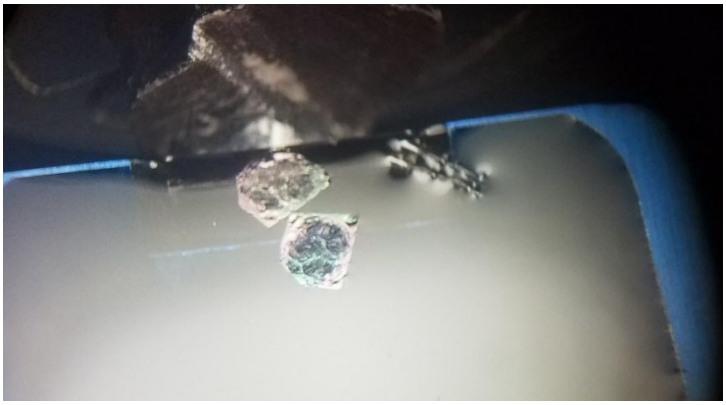
Picture 18

Picture 19



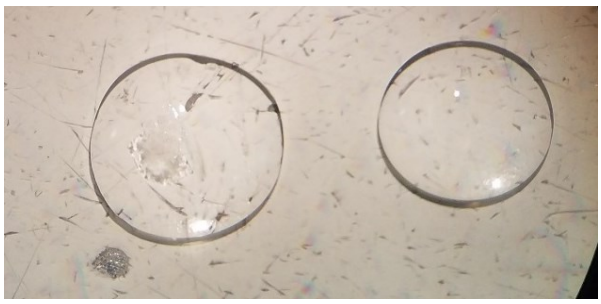
Picture 20

10. The section for submission should be cut at 1 micron thick



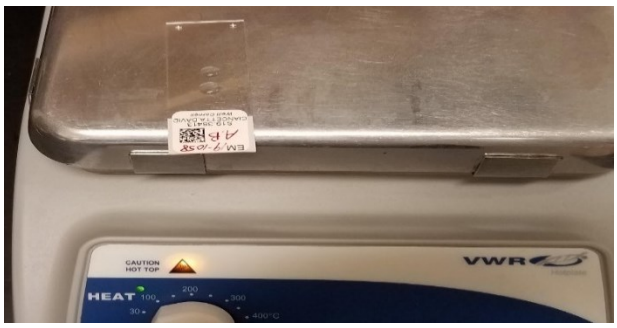
Picture 21

11. Pick up the section using a glass rod the tip of which is slightly angulated
12. Pick up the section in an upward stroke of the glass rod from the diamond trough
13. Layer the section unto the drop of water in an inward stroke of the glass rod



Picture 22

14. Dry on a hot plate set at 100° C



Picture 23

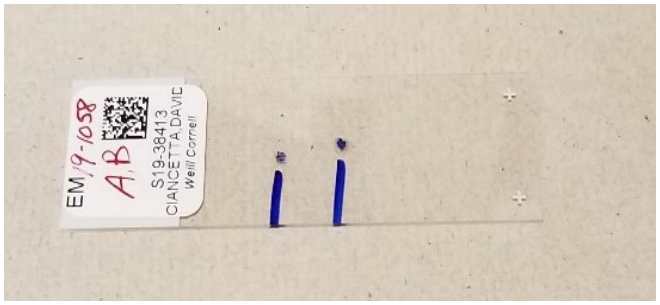
15. Put enough toluidine blue on the section and leave the stain for a few seconds (indicated by a metallic sheen around the drop of the stain around 15-20 seconds) (Picture24)

Staining Epon tissue sections

Picture 24

Picture 25

16. Wash with water until stain is not dripping anymore from the tissue.
17. Dry with dust off; then complete drying by returning to the hot plate for a few seconds
18. Below is the product of the process. It is now ready for microscopic examination by a renal pathologist.
19. A blue marker is drawn at the back of the slide to serve as a guide when viewing for the sections.

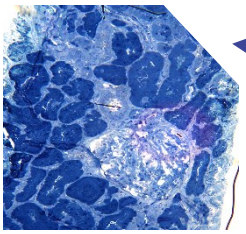


Picture 26

20. The renal pathologist uses this thick section to ensure the appropriate areas are selected.
21. Whichever tissue block is selected is the one to be trimmed and thinned for staining and eventually will be used for electron microscopy examination

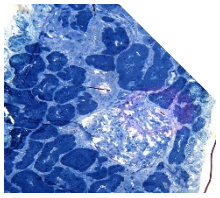
Different Types of Cut Sections

- Initial sections



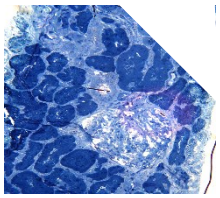
Picture 27

- Initial sections are the first cuts from the Epon blocks
    - Thick section requires one section per block
    - If there are two blocks, cut one section from each block and put individual sections on one slide.
    - Maximum number of blocks per case (2) unless the renal pathologist instructed to submit more.
- The recut section is like a replica of the initial section



Initial section

Picture 28

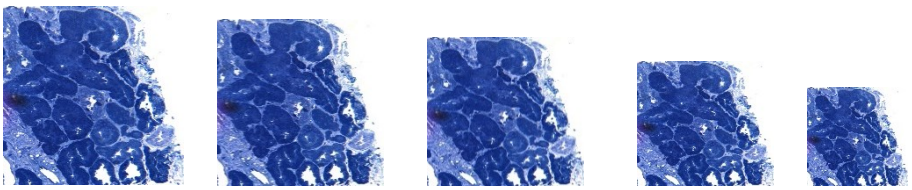


Recut

Picture 29

- Deeper sections:
    - The micrometer is set at 0.5 micron
    - Shallow cut: Discard five sections before picking up section for submission
    - Deeper one: Discard 20 sections before picking up section for submission
- Deeper two: Discard 30 sections before picking up section for submission
    - The number of deeper depends upon the instructions from the requesting
    - pathologist
    - The succeeding deeper cut: Discard 30 sections before picking up the section for submission
- Number of deeper:
    - If the tissue is medulla, maximum deeper section is 2.
    - If the tissue is cortex, continue to cut deeper sections as per pathologist
    - instructions
    - If the glomerulus is sclerosed, continue to cut deeper sections as per
    - pathologist instructions

- Cut thru the block- requested if there is no glomerulus
  - Discard 30 sections before picking up the section for submission.
    - Repeat this process until the tissue is exhausted.
    - Number slides in numerical order.



First Epon section → last Epon section

Pictures 30

Labeling of slides

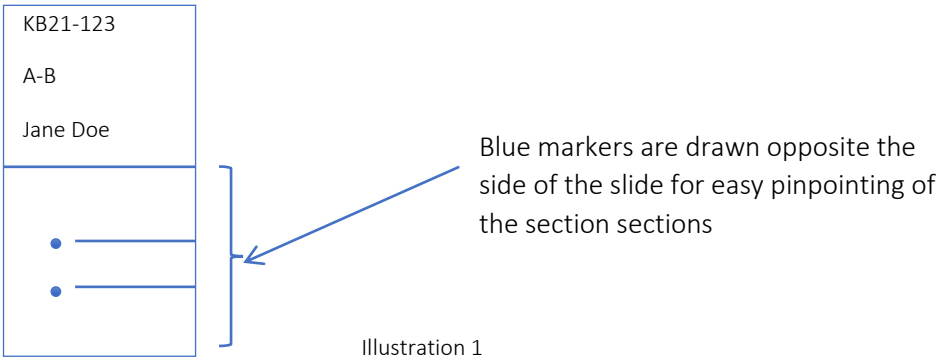


Illustration 1

Accountability

Affix one's signature and date beside the written instruction of the request to do recut

Review by the Renal Pathologist

- Give to the pathologist the stained sections accompanied with Renal Ultra-thin Outlay form. (Form1)
- The pathologist returns the slides and forms with instructions to the electron microscopy tech.
- Blocks with targeted structures chosen for thin sectioning are separated and placed in the thin sectioning area.
  - Include the slides and forms for the case.
  - The drawing on the Renal Ultra-thin Outlay form will be used to guide the tech during trimming for thin sectioning.
- File unused blocks.

Safety

- Standard precautions apply
- Dedicate a waste container to collect toluidine blue waste.

LKB Glass Knife Maker (Ted Pella)

Accessories

Glass knife fork tool (Ted Pella)

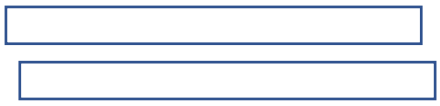
Picture 32

Glass knife station (Ted Pella)

Picture 33

Glass knife boat (EMS)

Picture 34



Glass strips (EMS)

Picture 35

Diamond glass cutter (EMS

Picture 36

Glass knife scoring jig (EMS)

Picture 37

Steps in Glass Knife Making

View glass knife making at this URL:

https://www.ronaldschulte.nl/en/making-glass-knives-with-the-lkb-7800-knifemaker.html

References

- Standard Operating Procedure at Electron Microscopy, Department of Pathology Laboratory and Medicine, Weill Cornell Medicine
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. Sectioning, 3rd edition, CRC Press, Inc. Boca Raton, FL
- https://en.wikipedia.org/wiki/Orthochromasia
- www.ihcworld.com/_protocols/em/em_thick_section.htm
- https://www.diatomeknives.com/product.aspx?pid=346
- Pictures by: Lilian Antonio, Electron Microscopy Manager, Weill Cornell Medicine

Thin Sectioning OF Trapezoidal-Shaped Epon Block

Purpose

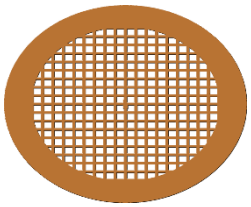
To describe the process of preparing ultrathin section for imaging

Principle

Ultrathin slices of Epon infiltrated tissues are sectioned less than 100 nanometer using ultramicrotome to allow electron beams penetrate the heavy metal-stained tissue and form images.

Materials/Reagents

- De-ionized water, double-edge blade, dust off, Epon blocks, regular microscope , 10 ml syringe with filter, 1 ml syringe, ultra-diamond knife, eyelash manipulator or eyelash brush, #5 tweezers, ultra-microtome and ultra-diamond knife, petri dish with mat inside, plastic spray bottle, cotton tip, filter paper (hardened), Sonicator, copper grids
- Copper grids
    - Comes in different number of squares e.g., 50, 100, 200,300, 400, 500, 600 mesh
    - Our laboratory uses the 200 mesh Cu/Rh



Cat# 200 Mesh Cu/Rh (Electron Microscopy Sciences)

Picture 1

- Chloroform
  Cat# 12540
  Electron Microscopy Sciences
- Ethyl alcohol, 200 proof (Koptec)
  Cat# V1016
  Koptec

Quality Control

Selection and mounting of medium gold tissue sections on copper grids.

Preparation Before Thin Sectioning

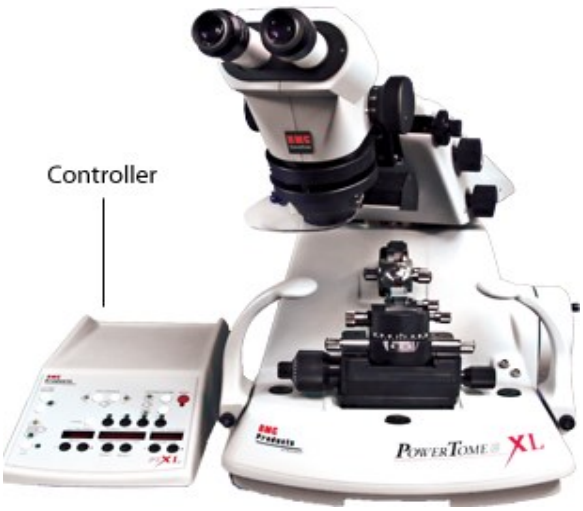
Cleaning of copper Grids

- Materials:
    - Sonicator, absolute alcohol, tap water, 2-100 ml beaker, plastic bottle dispenser, petri dish, filter paper (hardened), copper grids, plastic transfer pipette
- Process of sonication:

1. Pour 20 ml of water into the Sonicator
2. Put 10 ml of absolute alcohol in a 100 ml beaker
3. Dislodge 1-2 vials of copper grids into it
4. Put the beaker inside the Sonicator
5. Turn it on for 10 minutes
6. Thereafter, decant the absolute alcohol into the second beaker
7. Replace with fresh 10 ml of absolute alcohol
8. Sonicate for the second time for 10 minutes.
9. Drain the alcohol
10. Use the alcohol bottle dispenser if needed to remove all the copper grids from the beaker.
11. Into a clean Petri dish lined with 2 filter papers, layer the cleaned copper grids
    11.1. Aspirate excess absolute alcohol with plastic transfer pipette.
12. Dry in the oven for 10-20 minutes.
13. Cool to room temperature
14. Seal the edges of the Petri dish with parafilm (optional)

Procedure

1. Turn the ultra-microtome and control board on of an ultra-microtome



Picture 2

2. Match the case number on the form with the Epon block selected by the renal pathologist.
3. Mount it on the block holder

Block holder with Epon block mounted

Picture 3

4. Wet an applicator stick with chloroform
5. Apply it on the surface of the Epon block
   5.1. At this point, the glomerulus becomes visible to the cutter
6. Examine the toluidine blue stained slide under the microscope to match the glomerulus drawn on the Renal Ultra-thin Outlay form

Epon section stained with Toluidine blue

Picture 4

Renal Ultra-Thin Section Outlay Form

Case #: _____

To: _____

Patient name: (Last) _____ First: _____

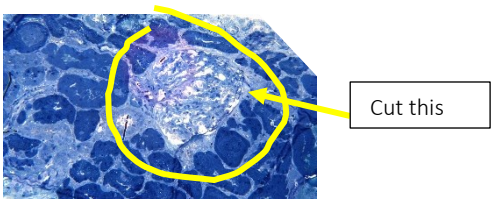
Form completed by:	Date:		Thin section by:	Date:
Thick section by:	Date:		Stained by:	Date:

Deparaffinization: _____ Transplant: _____

Digital Image Numbers: _____ Grid Box: _____ Row: ____ Block: ____

By: _____ Date: _____ Grid Box: _____ Row: ____ Block: ____

Please examine THIN SECTIONS submitted with this form and kindly sketch in pencil the area you are interested in for imaging with the electron microscope. Be sure to put the block number down on this form that corresponds with the appropriate THIN section.

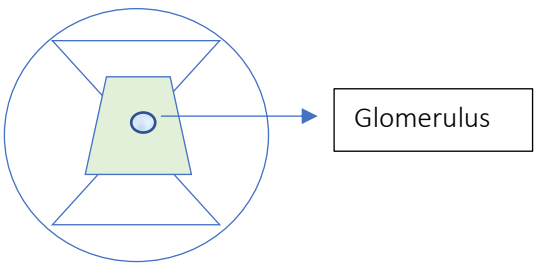
Block #: A  	Descriptions of the toluidine blue sections Example: A- cortex, 1 glom B- cortex, no glom

Form 1

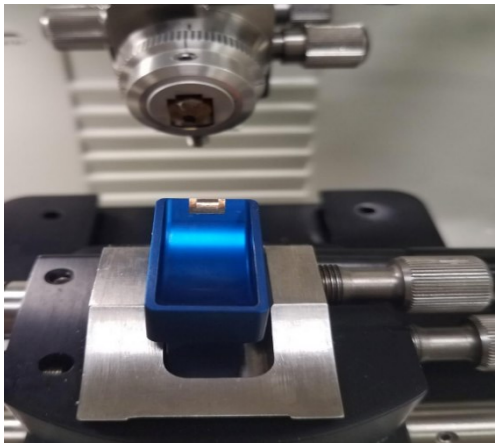
Illustration1

7. Trim around the glomerulus of the renal the tissue in a trapezoidal shape
   - 7.1. The leading face and two sides of the Epon block must be vertically trimmed
   - 7.2. The trailing side must be trimmed in a sloping manner
   - 7.3. It is imperative that sides touching the bottom and upper parts of the trimmed block have clean edges and are parallel to each other.

Illustration 2



8. Mount Epon block in the block holder or chuck (assembly)
9. Mount the unit (assembly) in the microtome



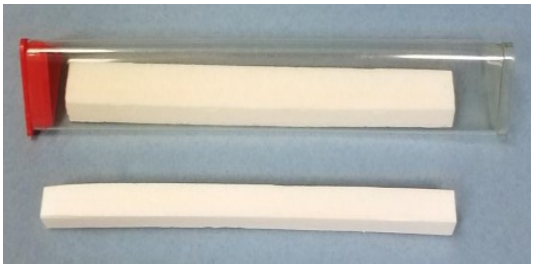
Picture 5

10. Tighten all screws in the block holder, knife holder, etc.
11. Align the block (with backlight on) parallel to the cutting direction
12. Align the upper and the lower side of the block (with the backlight on) parallel to the cutting edge
13. Set the upper and the lower cutting phase.
14. When everything has been set, put water in the trough using a 10 ml syringe
    14.1. The water should be level with the cutting edge and give a good reflection
15. Put the plastic guard around the block to prevent debris from the surrounding environment
16. Start the cut mode at 100 nm
17. Slowly move the knife with fine adjustment screw of the microtome towards the Epon block
18. Start trimming.
    18.1. Once full section is cut, change the thickness that will give medium gold sections (80-90 nm)
19. Dip an applicator stick into the chloroform bottle
20. Sections are flattened out by exposing to chloroform.
    20.1. Put the cotton tip on top of the sections floating on a water trough for a few seconds
21. The size of the trapezoidal Epon dictates the number of sections collected on the copper grid.
22. Use an eyelash manipulator to aid in the transfer of sections onto the copper side of the grid.
23. Use #5 tweezers to support the rim of the copper grid. Do not bend the rim of the copper grid
24. Gently blot the back of the grid on a filter paper to remove excess water.
25. Transfer to the mat contained in a plastic petri dish
26. Dry the sections on copper grids as follows:
    26.1. Forty minutes at room temperature
    26.2. Ten minutes in a 60-650Centigrade oven
    26.3. Forty minutes at room temperature
27. It is now ready for staining

Cleaning Diamond Knife (Diatome)

1. Method One
   1.1. Empty the trough of the diamond knife; wash the knife with deionized water (use a squirt bottle)
   1.2. Take one of the polystyrol rod and bevel it to an angle of approximately 600 using an oil free razor blade.

Polystyrol rods (Diatome)



Picture 6

   1.3. Dip the rod into 100% ethyl alcohol; shake off the excess.
   1.4. Pass the rod over the cutting edge without applying pressure
2. Method Two
   2.1. Remove all unused sections with a scotch tape
   2.2. Rinse the knife thoroughly with deionized water
   2.3. Blow the water off the knife with Dust Off.
3. Method Three- This method is used to remove dried sections from the back of the knife
   3.1. Place the knife in deionized water; add one or two drops of liquid detergent
   3.2. Let it spin for 1-2 hours
   3.3. Remove the knife and rinse with deionized water
   3.4. Complete the cleaning process by applying Method One

Safety

- Standard precautions apply
- Discard chemical wastes according to federal, state, and local institution

References

- Standard Operating Procedure at Electron Microscopy, Department of Pathology Laboratory and Medicine, Weill Cornell Medicine
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. Sectioning, 3rd edition, CRC Press, Inc. Boca Raton, FL
- http:// www.diatome.ch - Diatome, Handling and Use.
- https://www.deshbandhucollege.ac.in/pdf/resources/1585214390_PHY(H)-VI-NANO_MATERIAL-9-AJAYPRATAP.pdf
- http://www.grid-tech.com/catalog.htm
- https://www.emsdiasum.com /

Video of thin sectioning in electron microscopy from YouTube

https://www.google.com/search?q=video+of+thin+sectioning+of+renal+biopsies+embedded+in+epon&rlz=1C1GCEB_enUS951US951&oq=&aqs=chrome.0.69i59i450l8.478557267j0j15&sourceid=chrome&ie=UTF-8

Heavy Metal Staining: Uranyl Acetate and Lead Citrate

Purpose

To describe the method of heavy metals staining using uranyl acetate and lead citrate in preparation for electron microscopy studies.

Principle

The double contrast method of ultrathin sections with uranyl acetate and lead citrate is the standard contrasting technique for electron microscopy. Uranyl acetate stains nucleic acid while lead citrate stains the ribosomes.

Quality Control

An acceptable image with little or no contamination and good contrast.

Materials/Reagents

- Tweezers, petri dishes/ceramic staining dish, timer, staining mat-four 50-ml beakers toiled de-ionized water, copper grids with tissue sections, Kimwipes, plastic transfer pipette
- Lead citrate solution, 3%
    - Lead nitrate 1.33 grams
    - Sodium citrate 1.76 grams
    - 1 N Sodium hydroxide 8.0 ml
    - Boiled de-ionized water 42 ml.
      Commercially prepared by Electron Microscopy Sciences
      Cat# 22410 (dispenser) or 22410-01 (syringe)
- Preparation of lead citrate:
  Wear basic personal protective equipment: gloves, mask, and laboratory coat

1. Brush off any debris inside the balance before use.
2. Line the table with blue chuck
3. Carefully weigh the amounts of lead nitrate and sodium citrate
4. Pour the weighed powder of lead nitrate and sodium citrate into a 50-ml volumetric flask.
5. Add 30 ml of de-ionized water into it
6. Secure the lid with parafilm to minimize exposure to air
7. Cover with gloves. (See illustrations below).

   Storage: Room temperature
   Shelf life: 6 months

Steps 4 & 5

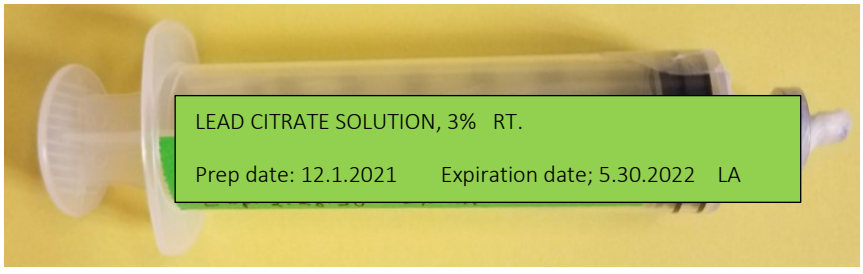
Step 6 & 7

8. Let stand with intermittently mixing for 30 minutes
9. Add 8.0 ml of 1N sodium hydroxide
10. Use plastic transfer pipette to bring the volume to a 50-mark.
11. Mix by inverting the volumetric flask until solution is clear.

Clear lead citrate after the addition of sodium hydroxide

Step 9

12. Quickly dispense the contents into a parafilm covered 50-ml beaker
13. Aspirate clear solution into a syringe
14. Replace needle with a cap tip



Step 13

- Uranyl acetate, 1% Commercially prepared
  - Uranyl acetate 1 gram
  - Ethyl alcohol, 50% 100 ml (may use de-ionized water instead)

    Cat# 22400-1

    Electron Microscopy Sciences
- Uranyl acetate powder

  Cat# 22400

  Electron Microscopy Sciences

Preparation of uranyl acetate:

Wear basic personal protective equipment: gloves, mask, and laboratory coat

1. Brush off any debris inside the balance before use.
2. Line the table with blue chuck
3. Carefully measure the require volume and quantity of the constituents
4. Pour 1 gram of uranyl acetate powder and 100 ml of 50% ethyl alcohol into a 100-ml volumetric flask

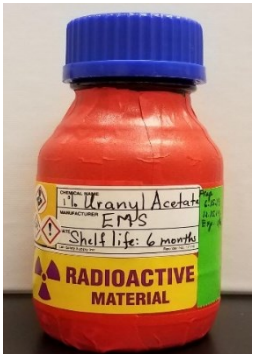
   4.1. Perform process under the hood
5. Put magnetic stirrer
6. Wrap volumetric flask with aluminum foil to avoid exposure to light
7. Stir for an hour at 400 rpm
8. QS to 100-ml mark
9. Transfer solution into a 250-ml amber bottle (properly labeled with
10. Wrap the entire bottle with tape or aluminum foil.

    10.1. Store at room temperature

    10.2. Let it stand overnight before use

    Storage: Room temperature

    Shelf life: 6 months



Picture 1

- Sodium hydroxide pellets

  Cat#21162

  Electron Microscopy Sciences

Preparation Before Staining

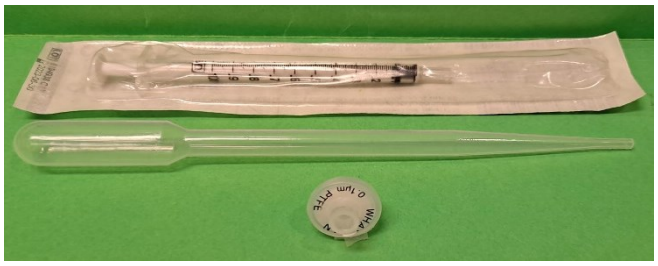
- Prepare a Petri dish with sodium hydroxide pellets, staining mat, and a lid to collect lead citrate waste
- Black Petri dish with ceramic dish for uranyl acetate
- Boil de-ionized water for 10 minutes for use for rinsing

Picture 2

Petri dish with staining mat and sodium hydroxide for lead citrate

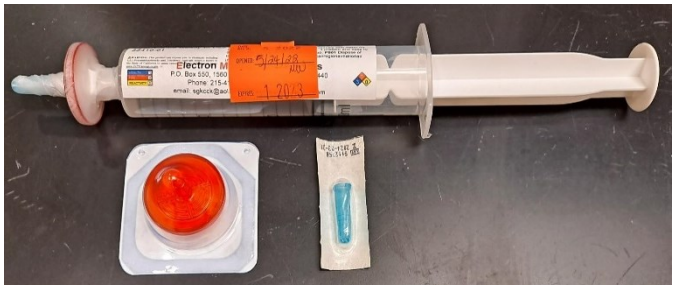
Picture 3

Ceramic staining dish for uranyl acetate



Picture 4

1-ml syringe, filter, 0.1. um, 13 mm diameter and plastic pipet



Picture 5

Commercially prepared lead citrate, 0.1 µm filter, tip cover

Set-up in uranyl acetate-lead citrate staining method

Picture 6

Procedure

1. Place the ceramic dish inside the uranyl acetate petri dish and staining mat inside the lead citrate petri dishes at the beginning of the process
   1.1. Dispense uranyl acetate i.e., 0.5 ml per well.
   1.2. Recommended: two copper grids for staining at a time
2. Use plastic pipette to dispense boiled de-ionized water in each well adjacent to uranyl acetat
3. Secure the copper grid at the point of the bent edge
   3.1. Put the copper grid (copper side up or down) under the surface of the uranyl acetate drop.
4. Put the "light cover" on top of the Petri dish with uranyl acetate and leave it there during the duration of staining time

Picture 7

5. Set the timer for 10 minutes
6. At this point, fill the four 50-ml beakers with boiled de-ionized water. (4th beaker for rinsing tweezers in between staining).
7. At 1minute mark, add a drop of water to the layer of sodium hydroxide.
8. Then get the syringe with lead citrate, discard first 2 drops of lead citrate on the waste container provided within the Petri dish.
9. Put two 1- 2 drops of lead citrate separately on a staining mat

9.1. The tip of the dispenser should be touching the mat while dispensing
10. At 30” mark, transfer the copper grids to the adjacent wells with water
11. Secure the first grid from uranyl acetate well on the same bent side of the copper grid and start rinsing as follows:
    11.1. Dip 40X in each of the 3 beakers; each dip is brought up for aeration
12. Insert the first washed grid inside the first drop of lead citrate. (See picture 8)
13. Set the timer for 2’15”, put back the glass weight on top of the lid
14. Rinse the tweezers
15. Repeat the process of rinsing on the second grid
16. Set the timer for 2’15”; secure the lid of the Petri dish with heavy glass
17. When the first grid time is up, pick the grid up and start rinsing
18. Dip 40X in each of the 3 beakers, all dips must be under the surface of the water.
    18.1. Repeat the process on the second grid
19. Dry the stained grid with folded Kimwipes
20. Put all stained copper grids on the mat contained inside a petri dish. (See picture 8 below)

Picture 8

Lead citrate staining

Picture 9

Beakers that contain boiled de-ionized water for rinsing

Pictures 10

Petri dishes with mat to hold cut (not stained) and stained copper grids

21. Assign the locations of the stained copper grids
    21.1. See illustrations i.e., the red colored number/letter below:

Renal Ultra-Thin Section Outlay Form

To: _____ Case #: _____

Patient name: (Last) _____ First: _____

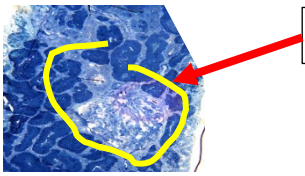
Form completed by:	Date:		Thin section by:	Date:
Thick section by:	Date:		Stained by:	Date:

Deparaffinization: _____ Transplant: _____

Digital Image Numbers: _____ Grid Box: 1097 Row: 1 Block: A

By: _____ Date: _____ Grid Box: _____ Row: ____ Block: ____

Block #: ____A_____



Descriptions of the toluidine blue sections

Example:

A- cortex, 1 glom

B- cortex, no glom

FORM 1

22. At the end of the staining process, discard uranyl acetate and lead citrate according to federal, local, and state regulations.

Record Keeping

1. First column: write 1 as the starting number on the Grid Storage File card
2. Under descriptions, write the relevant information needed as follows:
   2.1. Example of the information logged in the Grid Storage File card:

Row	Ref	Descriptions
1	AB	KB19-582
2	ABC	KB19-583

Table 1

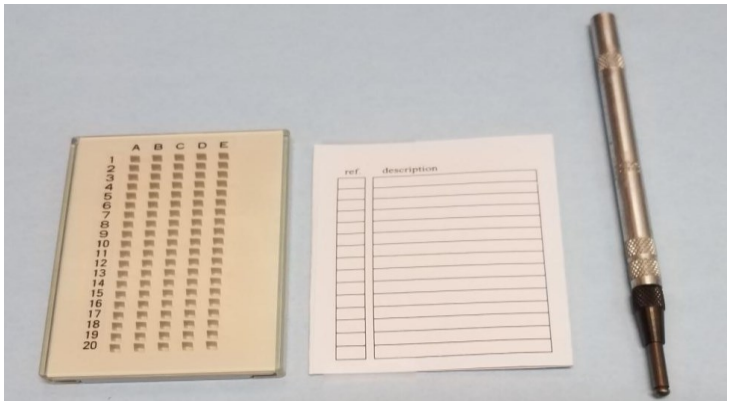
Location

KB accession number

Number of grids cut from a block

Storage of Stained Copper Grids

1. On row 1, under column A put the first stained grid
2. On the same row, put the unstained copper grid under column C
   2.1. The use of second grid: if there is a need to re-stain the case.
   2.2. Column B is where the second grid is placed if there is a need to stain it.



Picture 11

Grid box File Storage Card Diamond point

3. Put the stained copper grids sideways inside the grid box
   3.1. If there are more than two grids continue inserting to the next column

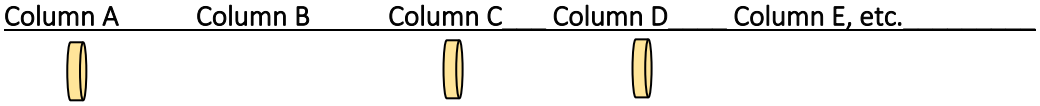


Illustration 1

3.2. If the second grid is stained the grid from column C is now in position B.

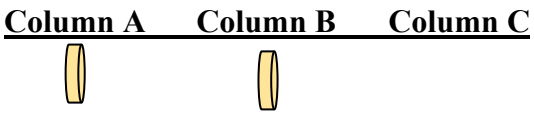
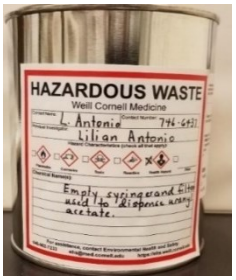


Illustration 2

Safety

- Discard uranyl acetate and lead citrate according to federal, local, and state regulations.
- Have separate waste bottles for each
- Collect used syringes and filters in a can or plastic container.



Picture 12

Holds empty used syringes

Picture 13

Holds used filters and tips

Picture 14

Separate waste containers for uranyl acetate and lead citrate

- Apply standard precautions.

References

- Standard Operating Procedure at Electron Microscopy, Department of Pathology Laboratory and Medicine, Weill Cornell Medicine
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. Positive Staining, 3rd edition, CRC Press, Inc. Boca Raton, FL
- Graham, L., Orenstein, J. Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat Protoc 2, 2439–2450 (2007). https://doi.org/10.1038/nprot.2007.304.
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- Marguerite A. Persi, J.C. Burnham, Use of tannic acid as a fixative-mordant to improve ultrastructure appearance of Candida albicans blastophores, Sabouraudia, Volume 19, Issue 1, January 1981, pp1-8, https://doi.org/10.1080/00362178185380021
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Troubleshooting in Electron Microscopy

NOTES: Imaging follows heavy metal staining. The steps in imaging vary differently according to the make of the electron microscope. But the principle consists of:

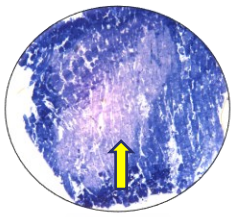
1. preparation of the camera and the microscope
2. identifying the sample
3. locating the sample
4. alignment of the scope
5. adjustment of the background
6. ironing the glomerulus(i)
7. ironing the adjoining proximal tubules
8. stabilization of the image
9. focusing the image
10. imaging of the sample at different magnification (dictated by the pathologist)
11. imaging of interesting parts of the glomerulus
12. putting all the images in one folder for the case for review and storage purposes.

H7500 Hitachi Electron Microscope

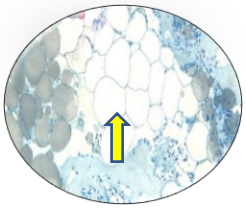
Troubleshooting of Some Commonly Encountered Artifacts

NOTES: Artifacts can be observed at different steps from processing through imaging.

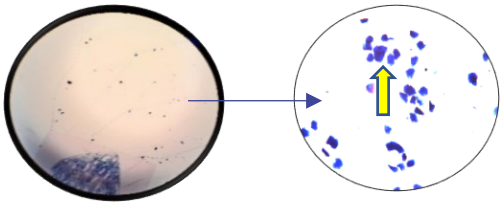
Artifacts from processing viewed during staining with toluidine blue



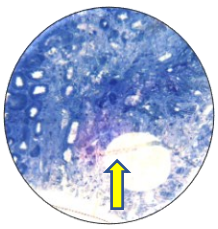
- Problem: Light staining of the inner side of the tissue on a toluidine blue stain
- Reason: Incomplete primary fixation of a relatively thick endomyocardial tissue
- Resolution: Submit 1mm3 size of the tissue and use a longer processing schedule



- Artifacts: The effects of dehydration are first seen in toluidine blue sections. Removal of lipids within the adipose tissue. These will not be stained with heavy metal hence appears as white ghost cells i.e., white empty stroma during imaging.

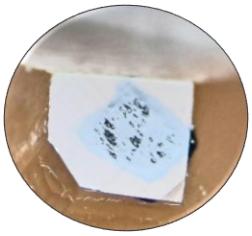


- Artifacts: Squamous epithelia dislodged from the skin
- Reason: Not wearing gloves. Touching the slides during labeling /Epon block during mounting on the block holder.
- Resolution: Wear gloves



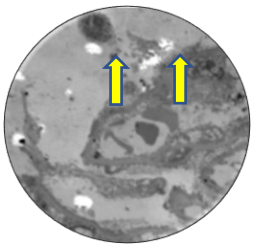
- Artifacts: Circular unstained area on toluidine blue section.
- Caused by: Drop of water after the stain has dried.
- Resolution: Re-stain the section to attain uniform stain of the section.

Artifacts from thick sectioning viewed during thinning of Epon block

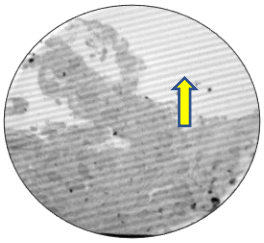


- Artifacts: Holes on the tissue section
- Reason: Due to aggressive trimming during thick sectioning or dull knife
- Resolution: Do fine trimming by rotating the wheel and/or replace with a new knife

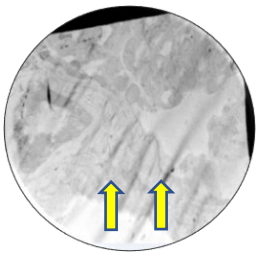
Artifacts from thick sectioning viewed during imaging



- Artifacts: Holes in the section as captured during imaging.
- Caused by: Aggressive trimming during thick sectioning
- Resolution: Do fine trimming

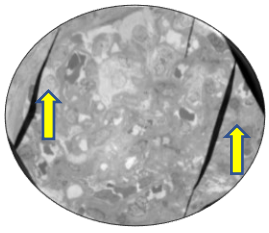


- Artifacts: Parallel undulation, thick and thin, venetian blind, skipping.
- Caused by Epon block loosely locked in the block holder, blades not secured, on tables supported by anti-vibration system using nitrogen gas is not substantially enough to support the table, fast sectioning.
- Resolutions:



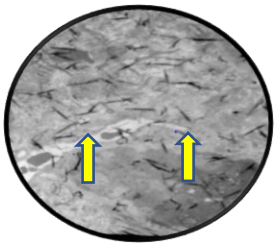
- Artifacts: Wrinkles
- Resolutions:
  - Stretch sections using chloroform before picking them up.
  - Position the copper grid at an angle position while lifting the grid

v

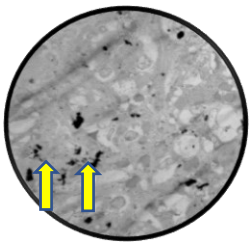


- Artifacts: Folds
- Resolutions:
  - Stretch sections using chloroform before picking them up.
  - Position the copper grid at an angle position.

Artifacts from staining viewed during imaging



- Artifacts: Uranyl acetate crystals on stained tissue section.
- Resolutions: Put the tissue facing down and within the drop of the solution. Wash well (40 x per beakers) Total 3 beakers. Use 0.1micron syringe filter



- Artifacts: Lead crystals
  Caused by component of staining solution (lead) reacting with carbon dioxide in the air producing lead carbonate deposited as black precipitate on sections.
- Recommended resolutions:
  - Use boiled de-ionized water during washing.
  - The grid must be under the level of water during washing to avoid interaction with carbon dioxide in air.
  - Line a part inside petri dish with sodium hydroxide pellets to absorb the carbon dioxide in the air during the process of staining
  - Put 1 drop of water on the layer of sodium hydroxide
  - Put the grid facing down inside the solution.
  - Wash 40 dips per beaker (total 3 beakers).
  - Wash forceps in between staining (4th beaker for washing forceps in between staining)

To minimize artifacts in electron microscopy, below is a list of some guidelines to follow:

- Washing of aldehyde fixatives before osmium tetroxide
- Use only boiled de-ionized water
- Use sodium hydroxide pellets with an assay of < 1.0% sodium carbonate
- Do not breathe on the grids during lead staining
- If white precipitate is visible in the lead citrate dispenser, discard the stain.
- Insert the copper grid upside down inside lead citrate

Safety

- Annual TEM radiation survey
- Monthly monitoring of the electron microscopy staffs by wearing radiation badges
- Apply standard precautions

References

- Standard Operating Procedure at Electron Microscopy, Department of PathologyLaboratory and Medicine, Weill Cornell Medicine
- Hayat, M.A. (1989). Principles and Techniques of Electron Microscopy. In. Positive Staining, 3rd edition, CRC Press, Inc. Boca Raton, FL
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- https://www.researchgate.net/post/Dark-lines-through-cells-embedded-in-epoxy-resins-for-TEM
- A simplified lead citrate stain for use in electron microscopy John H. Venable and Richard Coggeshall. From the Department of Anatomy, Harvard Medical School, Boston. Dr. Venable's present address is the Department of Veterinary Anatomy, Oklahoma State University, Stillwater, Oklahoma
- https://rupress.org/jcb/article/25/2/407/16564/A-simplified-lead-citrate-stain-for-use-in-Electron Microscopy
- The use of lead citrate at high pH as an electron-opaque stain in electron microscopy Edward S. Reynolds. From the Department of Anatomy, Harvard Medical School, Boston https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2106263/
- FELDMAN D. G. (1962). A method of staining thin sections with lead hydroxide for precipitate-free sections. The Journal of cell biology, 15(3), 592–595. https://doi.org/10.1083/jcb.15.3.592
- https://www.youtube.com/watch?v=NG44AEWHtRQ (Principles of TEM)