

Immunofluorescence Microscopy of Renal Biopsies

Ritambhara Nada ,Nitu Bhatia, Kusum Joshi

Department of Histopathology,PGIMER, Chandigarh

Microscopic evaluation of renal biopsies under immunofluorescence microscope using a panel of antisera is integral part of complete evaluation of renal biopsies. Direct immunofluorescence microscopy on renal tissue detects and localizes immunoglobulins, complement components, and fibrin related antigens within renal tissue by first reacting tissue sections with FITC-labeled antisera, followed by observation of specific fluorescent staining.

Antisera specific for immunoglobulins, kappa and lambda light chains, complement components and fibrinogen form the baseline panel useful for categorization of renal pathologies. However under special circumstances, antisera specific for other components help in making/refining the diagnosis eg C1q for specific type of FSGS and Lupus nephritis, C4d and HLA-DR in transplant setting.

Transport media for renal biopsy

Renal biopsies can be submitted for analysis as fresh tissue, frozen tissue, or in a transport medium. Biopsy specimens may be needle, wedge, or excisional biopsy. Tissue should not be fixed with cross-linking fixatives. Dried tissue can be rehydrated in PBS but will not give optimal results.

Biopsy specimens for immunofluorescence microscopy should be collected in Michel's Fixative. Michel's Fixative can be purchased commercially (Poly Scientific) or can be made in the laboratory. The combination of chemical components, allows tissue to be transported at ambient temperature for up to 5 days

Principle: Michel's Fixative is not considered a true fixative, like formalin and other fixatives routinely used in histological examination. Michel's Fixative contains an anti-autolytic agent N-ethylmaleimide and $(\text{NH}_4)_2\text{SO}_4$ to precipitate tissue-bound immunoglobulins without losing their antigenicity .

Specimen Handling

When the laboratory receives tissue submitted in Michel's Fixative, the tissue must be washed in multiple (2-4 times, each washing of at least 10 minutes with frequent shaking) washings in a wash solution to reverse the precipitation of immuno-globulins. Number of washings depends upon how long the tissue was in Michel's solution.

Specimens may be examined under a stereomicroscope at this time to assess the presence of glomeruli, however this is optional since all tissue samples will be sectioned and examined by routine light microscopy at a higher magnification.

All tissues should be treated as potentially infectious.

Tissue processing and sectioning

The tissues should be processed and slides prepared according to the procedure that follows.

Prepare and label a base mold with cello tape and fill the base mold with O.C.T.

After the third tissue wash, pour off all remaining wash solution into the beaker. With forceps, remove the biopsy tissue and allow it to drain well on a tissue paper.

Use forceps with a fine tip to place the tissue on the superficial surface of the OCT block.

Freezing the Tissue

Keep the mould that contains OCT and the biopsy in the cryostat and let it freeze.

Or Snap freeze Biopsy (preferential)

1. Take a dewar flask and thermal resistant gloves to obtain liquid nitrogen. Remove the lid to the dewar flask and gently place the long dispensing hose inside the flask. Take extra care not to bang the metal hose on the glass interior of the dewar flask. While wearing eye/face protection and thermal resistant gloves, open the tank valve and carefully fill the dewar flask with liquid nitrogen to approximately one-half. Close the valve, remove the dispensing hose and cover the dewar flask.
2. Once the liquid nitrogen is ready for use, place the dewar flask inside the chemical fume hood.

3. While working inside the chemical fume hood, pour 80-100ml of 2-methylbutane (isopentane) into a beaker with a handle attached to it. Gently lower the beaker of 2-methylbutane into the dewar flask filled with liquid nitrogen and partially submerge the beaker.
4. Chill the 2-methylbutane for about 3-4 minutes, or until the white vapors disappear and the bottom of the beaker begins to frost. The 2-methylbutane should be cold enough for the tissue freezing process to begin.
5. Using the long handled forceps, quick freeze the tissue by submerging the entire base mold into the beaker of chilled 2-methylbutane. The O.C.T. should begin to freeze and turn white instantly.
6. Leave the base mold in the beaker for approximately 20-30 seconds. Do not exceed 30 seconds. If the base mold remains in the 2-methylbutane longer it may begin to crack and the tissue imbedded inside will be damaged.
7. Remove the base mold and place it inside the Cryostat for sectioning.
8. After the freezing process is complete, the remaining 2-methylbutane can be recycled and used again by allowing it to warm to a safe handling temperature and placing it in a labeled brown glass recycle bottle located inside the chemical fume hood. Very small amounts can be allowed to evaporate inside the hood if not being reused.

Specimens can be placed in a -70°C freezer, as time does not always allow same day sectioning.

Sectioning of the Tissue

1. Before sectioning allow the tissue block to warm to the proper cutting temperature inside the Cryostat, approximately 15-20 minutes.
2. If peaks have formed on the bottom of the tissue block add a small drop of O.C.T. between the tissue and base mold and press down firmly on the block. Allow the drop to freeze. This will flatten the cutting surface and allow for easier cutting, especially if tissue samples are very small (equivalent to rough cutting of paraffin block).

3. Cut sections of the frozen O.C.T until the tissue is reached. Cut a section of the tissue and place it on a slide. Check for the presence of glomeruli under the light microscope.
4. When glomeruli have been located, total of 8-10 sections are cut. Three to five slides with 2 sections each will be obtained for staining and storage.
5. Allow slides to air-dry for 5 minutes. Label six slides with the assigned case number and stain to be used. One of the six slides will serve as a negative PBS control. Two-three slides will be labeled with the case number only and frozen for additional studies as needed. (If slides will not be stained that day, place all slides in a covered box in the -20°C freezer overnight).
6. Place a drop of O.C.T. on the remaining frozen tissue block and remove it from the chuck upon completion of tissue sectioning. Wrap the block in foil for storage at -70°C.

Staining of the Prepared Slides

1. Place slides in a staining jar containing PBS and rinse for 5 minutes.
2. Wipe excess PBS from slides. Avoid wiping the tissue within the wells.

Without allowing sections to dry, apply 50ul (Approximately 1 drop from a transfer pipet of the stains as follows:

Slide #

∅ IgG / IgA

∅ IgM/ C3,

Ø Kappa/lambda

Ø Fibrinogen

Ø C1q

Ø PBS Negative Control

3. Incubate the slides for 30 minutes in a humidity chamber.
4. Rinse slides twice in PBS for at least 10 minutes.
5. Remove slides from staining jars and wipe excess PBS from slide without allowing sections to dry. Avoid wiping the tissue within the wells. Coverslip each slide with Fluoromount G/ glycerine. Be careful not to create bubbles under the coverslip.
6. Allow the slides to dry prior to placing them in a labeled slide folder and wipe the back of each slide with a water moistened wipe or towel to remove excess Fluoromount G. If slides will not be reviewed for several days, place the entire slide folder in the refrigerator to prevent fading of the fluorescence.

Examination of Slides

Slides are examined by conventional immunofluorescence microscopy using reflected light fluorescence vertical illumination.

Interpretation of Slides

- Slides are evaluated qualitatively and semi-quantitatively on a 0-4+ scale.

- A semi-quantitative assessment of the intensity of staining is given as none (0), trace (0.5+), mild (1+), moderate (2+), moderately severe (3+), and severe (4+).
- Photographic records are obtained for pertinent positive staining and case specific positive staining.

Reporting of Slides

∅ Immunofluorescence results are reported in the immunofluorescence microscopy section of the final report.

∅ Negative results are reported as such.

∅ Positive results are reported with

- Distribution (e.g. glomerulus, tubulointerstitial, and/or blood vessels),
- Glomerular localization(capillary ,Mesnagial, podocytes)
- Pattern (linear, granular, nodular, etc.)
- Intensity (1-4+).
- Which Antisera Positive

Distribution (e.g. glomerulus, tubulointerstitial, and/or blood vessels)

Presence of extra-glomerular granular staining in a case could be clue disease specific diagnosis eg

i.Full house pattern with staining along tubular basement membranes suggest lupus

ii.Linear staining with IgG and C3 along glomerular basement membrane along with tubular basement membranes suggest anti GBM disease

iii.Granular staining with IgG ,IgM and C3 which is polyclonal and limited to glomerulus only think of Fibrillar Glomerulopathy to be confirmed by electron microscopy

iv. Immunoglobulins positive in glomerular podocyte in absence of any immunoglobulin deposition in the glomerulus suggests FSGS.

Which Antisera Positive

1. If C1q is in routine panel of staining, it is positive in glomerulus with any type of pattern ie membranous/ subendothelial it would suggest a possibility of lupus nephritis.
- 2 C1q positivity in two opposing commas pattern classic of C1q nephropathy

Limitations

Specimens submitted for analysis may not always be adequate. Biopsies are assessed upon receipt for presence of glomeruli. Samples received may contain adipose (fat), medulla, skeletal muscle, or rarely cortex with no glomeruli. Sections of this material will be processed, cut, and Stained with Toluidine blue and reported for any relevant information. Depending upon clinical situation pathologists can ask for processing for kappa/lambda in medullary tissue alone in setting suspicious of Plasma cell dyscrasias or C δ d can be done in transplant setting..

Quality Control

Quality control material is not available commercially. Biopsy material is in very small quantity and not available for daily quality control staining. As recommended by the Renal Pathology Society, quality control should be performed upon receipt of new lots of antibody reagents. A previously stained kidney biopsy specimen that is positive for the target antigen of the new antibody reagent, will be pulled for testing reactivity of the new lot in comparison with the old lot of antibody reagents. The pathologist will read the slides after staining to determine the quality of the new reagents and their acceptability for use. A Kidney Quality Control Worksheet will be used for recording the results of this testing.

Each biopsy is evaluated on a case-by-case basis and examined for internal staining patterns (internal controls). Each biopsy will have a tissue section stained with a drop of PBS to act as the negative staining control. If the immunofluorescence findings do not correspond to the expected findings based

on light microscopy and electron microscopy results, the immunofluorescence procedure will be repeated and the reactivity of the antibody in question tested against a known positive control.

References

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2. Jennette JC: Immunohistology of Renal Disease, in Immunohistopathology in Diagnostic Pathology, Jennette JC (ed), CRC Press, Boca Raton, chap 2, 1989; 29-84

3. Jennette JC, Olson JL, Schwartz MM, Silva FG (eds): Heptinstall's Pathology of the Kidney, Volumes 1 and 2, 5th Edition, Lippincott-Raven, Philadelphia, 1998.

Appendix A

Michel's Solution.

I. Materials for Transport media and wash solution

- A. Citric Acid Monohydrate
- B. N-Ethylmaleimide (Sigma)
- C. Magnesium Sulfate Crystals
- D. 1M Potassium Hydroxide
- E. Deionized Water

F. pH Meter

G. Graduated Cylinders (100ml, 250ml and 1000ml)

H. Balance

I. Large 4L Graduated Cylinder for Mixing

J. 500ml, 1L, and 2L Covered Bottles

II. Preparation of Stock Chemical Reagents

A. 10M Potassium Hydroxide

1. Using a balance inside the chemical fume hood weigh 56.1 grams of potassium hydroxide.
2. Add the potassium hydroxide SLOWLY in 100mls of deionized water while stirring.
3. Cool and bring to a final volume of 100mls.
4. Label with proper identification and hazard information.

B. 1M Potassium Hydroxide (KOH)

1. Using a balance inside the chemical fume hood weigh 5.6 grams of potassium hydroxide.

2. Add the potassium hydroxide SLOWLY in 1000mls of deionized water while stirring.
3. Cool and bring to a final volume of 100mls.
4. Label with proper identification and hazard information.

C. 1.0M Potassium Citrate Buffer (pH 7.0)

1. Using a balance weigh 21.0 grams of citric acid monohydrate (or 19.2g of citric acid anhydrous can be substituted).
2. Add the citric acid slowly to only 30-40mls of deionized water leaving enough room in the mixing container for the 10M KOH. Stir until dissolved.
3. Adjust the pH to 7.0 with 10M KOH (about 35mls) and allow to cool. This will add quite a bit of volume to your mixing container and it is very important not to go over the final volume of 100mls.
4. Once cooled bring the final volume to 100mls with deionized water.
5. Label with proper identification and hazard information. Store at room temperature up to 1 year.

D. 0.1M N-ethylmaleimide

1. Using a balance weigh 12.5 grams of N-ethylmaleimide.
2. This chemical does not go into solution easily and will need to stir for a long time. Begin stirring the deionized water before adding the solute to prevent clumping at the bottom of the cylinder. Add the N-ethylmaleimide to deionized water up to a final volume of 1L and stir until dissolved.

3. Label with proper identification and hazard information. Store at 2-4°C for up to 1 year.

E. 0.1M Magnesium Sulfate (MgSO₄·7H₂O)

1. Using a balance weigh 24.65 grams of magnesium sulfate.
2. Add the magnesium sulfate to deionized water up to a final volume of 1L and stir until dissolved.
3. Label with proper identification and hazard information. Store at room temperature up to 1 year.

III. Wash Concentrate Stock Solution

- A. To make 1L of wash concentrate add the following to a 1L graduated cylinder and mix well:

200mls 1.0M Potassium Citrate Buffer (pH 7.0)

400mls 0.1M Magnesium Sulfate

400mls 0.1M N-ethylmaleimide

- B. Divide 1L volume of the concentrate into two 500ml bottles, label appropriately (pH, storage temperature, date prepared, expiration and initials) and store at 2-4°C for up to 1 year.

IV. Dilution of Wash Concentrate to Make Working Wash Solution (4 liters)

- A. Using a large mixing container add one 500ml bottle of wash concentrate to 3500mls of deionized water.

- B. Stir well until the pH of the working wash solution equilibrates.
- C. Adjust pH to 7.0 with 1M KOH.
- D. Pour into two 2000ml bottles, label appropriately (pH, storage temperature, date prepared, expiration and initials) and store at 2-4°C for up to 3 months.
- E. Wash solution is ready for use at this point and should require no further dilutions or adjustments.
- F. Smaller volumes of the wash solution may be made by using the following volumes:

1L	2L	
25mls	50mls	1.0M Potassium citrate buffer
50mls	100mls	0.1M MgSO ₄ .7H ₂ O
50mls	100mls	0.1M N- ethylmaleimide
875mls	1750mls	Deionized water

Adjust pH to 7.0 with 1M KOH if needed

Transport media.

Transport Michell's solution is (NH₄)₂SO₄. Added to wash solution.

Add Ammonium sulfate (55mg) to 100ml of washing solution while slowly stirring it.

Adjust pH to 6.9 with 1M potassium hydroxide.

Appendix B

I Materials for Freezing ,Sectioning and Staining

Reagents

1. O.C.T Compound
2. 10mM Phosphate Buffered Saline (PBS) pH 7.4
3. FITC IgG antiserum (DAKO diluted 1:20)
4. FITC IgA antiserum (DAKO diluted 1:20)
5. FITC IgM antiserum (DAKO diluted 1:20)
6. FITC C3 antiserum (DAKO diluted 1:5)
7. FITC Fibrin-Fibrinogen (DAKO diluted 1:20)
8. FITC C1q antiserum (DAKO F254, diluted 1:20)

9. FITC Kappa Light Chains (DAKO, diluted 1:20)

10. FITC Lambda Light Chains (DAKO, diluted 1:20)

Equipment and Supplies

1. Wash Cups /Petridishes

2. Fine Tip Forceps

3. Stereomicroscope

4. Aluminum Foil

5. 3" x 1" glass slides

6. Cryostat with -19° to -24°C range (Leica CM 1800)

7. Thin Tissue papers

8. Dropper Bottles

9. Staining Jars

10. Humidity Chamber

11. Fluoromount G Mounting Medium /glycerine

12. 24 x 50 mm Coverslips

Snap freezing biopsies

1. Liquid Nitrogen
2. 2-Methylbutane (Isopentane)
3. Gloves that are Thermal Resistant and Impermeable to Liquid
4. Face Visor with Safety Glasses/Goggles
5. Chemical Fume Hood
6. Dewar Flask
7. Beaker With Handle Attachment
8. Long Handle Forceps
9. Recycle Bottle for 2-Methylbutane