

Observational Study

**Immunofluorescence on paraffin embedded renal biopsies:
Experience of a tertiary care center with review of literature**

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Abstract**AIM**

To describe the technique of immunofluorescence on paraffin embedded tissue sections and discuss the potential pitfalls with an in depth review of literature.

METHODS

Immunofluorescence is integral to diagnostic renal pathology. Immunofluorescence on paraffin embedded renal biopsies (IF-P) after enzyme treatment has been described in literature, however has not found widespread use in renal pathology laboratories. In our laboratory proteinase K digestion of paraffin embedded renal biopsy material was standardized and applied prospectively in cases where immunofluorescence on fresh frozen tissue was non contributory or not possible. Diagnostic utility was assessed and in a cohort of cases comparison of intensity of staining with routine immunofluorescence was performed.

RESULTS

Over the 5-year study period, of the 3141 renal biopsies received IF-P was performed on 246 cases (7.7%) and was interpretable with optimal digestion in 214 cases (6.8%). It was of diagnostic utility in the majority of cases, which predominantly included glomerular disease. Non-diagnostic IF-P was found in membranous nephropathy (2 of 11 cases), membranoproliferative glomerulonephritis (2 of 32 cases), lupus nephritis (1 of 25 cases), post infectious glomerulonephritis (1 of 11 cases) and chronic glomerulonephritis (3 of 8 cases). Comparing cases with both routine IF and IF-P, 35 of 37 showed either equal intensity or a minor difference in intensity of staining

(1+) for the diagnostic immunoglobulin/complement. Technically assessment of immunofluorescence on the paraffin embedded tissue was found to be easier with clearly observed morphology, however a false positive staining pattern was observed in under-digested tissue.

CONCLUSION

As a "salvage" technique, immunofluorescence on paraffin embedded renal biopsies is of great diagnostic utility, however not without pitfalls.

Key words: Immunofluorescence on paraffin section; Renal biopsy; Salvage technique; Enzymatic digestion; Proteinase K

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Core tip: Immunofluorescence on formalin fixed paraffin embedded tissue is a useful "salvage" technique for renal diagnostic pathology, in case of non-availability of representative fresh frozen tissue. This article describes the technique of immunofluorescence on paraffin embedded tissue sections, discusses the potential pitfalls with an in depth review of literature.

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INTRODUCTION

Immunofluorescence (IF) is an indispensable technique for rendering an accurate diagnosis in renal pathology. Diseases such as IgA nephropathy (IgAN), C1q nephropathy (C1qN) and C3 glomerulopathy (C3G) cannot be diagnosed without IF. Direct immunofluorescence (DIF) on fresh frozen tissue (IF-F) is the most widely used IF technique. Not uncommonly, however IF-F is not satisfactory due to non-representative sampling (medulla) or is not possible due to unavailability of fresh unfixed tissue, such as in referral cases and archived tissue. This leads to incomplete diagnosis and suboptimal patient management. To overcome these hurdles a method of enzymatic digestion of formalin fixed paraffin embedded tissue was standardized and introduced in our laboratory in 2011.

Enzymatic digestion breaks the protein cross linkages formed during formalin fixation^[1] thereby exposing the antigenic immune complexes to staining with FITC (fluorescein isothiocyanate) labeled antibodies. Though this technique has been described in literature using different enzymes with the earliest report in 1976^[2], it is still not in widespread use in laboratories handling renal

biopsies.

We discuss our experience with this technique in day-to-day diagnostic renal pathology, its utility in reaching final diagnoses and compare it with usual IF-F where available. Technical and interpretation issues faced are described in detail, and may be helpful to any laboratory planning to introduce this technique.

MATERIALS AND METHODS

Standardization: In a case of diffuse proliferative lupus nephritis, proteinase K (Sigma Aldrich, United States) enzymatic digestion was standardized (at concentrations according to manufacturer's protocol) with variation in timing of exposure at room temperature. Results were compared for the adequacy of digestion and intensity of staining for FITC-IgG.

Selection of cases

IF-P was performed prospectively in cases where there was inadequate/non representative fresh frozen tissue, in referral blocks where fresh frozen tissue was not available and in cases where the renal pathologists wanted to confirm the findings of routine IF-F. The FITC labeled antibodies to be applied were dictated by light microscopic differential diagnoses in the case and included both full panel (IgA, IgG, IgM, C3, C1q, kappa and lambda) as well as limited panels.

Interpretation of immunofluorescence

In cases where there was optimal digestion and adequate material the IF-P results were evaluated by 2 renal pathologists (LS and GS) and semiquantitatively graded on a 0-3+ scale. In cases where IF-F was available for comparison, these were graded independently in a blinded manner and compared to the grading of IF-P results. All immunofluorescence images were digitally captured and archived.

RESULTS

Enzyme digestion with proteinase K was standardized and the protocol followed is described in Table 1. Standardization was performed at room temperature and slight variations in enzyme exposure depending on ambient temperature (ranging from 15 to 20 min) gave optimal digestion results. This obviated the need for maintaining slides at 37 °C in a water bath.

In the 5-year study period between March 2011 and May 2015, 3171 biopsies (both native and transplant) were received. IF-P was performed on a total of 246 cases (7.7%). The results could not be interpreted in 32 cases (13%) due to technical issues of under digestion (18 cases) and floating of tissue/inadequate tissue (14 cases).

Therefore in 214 cases with adequate tissue, optimal digestion was achieved. Optimal digestion was determined on each individual slide by observing the

Table 1 Protocol for immunofluorescence on paraffin embedded renal biopsies

<p>Cut formalin fixed paraffin embedded tissue at 3-4 μ thickness on poly-L-Lysine coated slides</p> <p>Deparaffinize and rehydrate tissue sections</p> <p>Immerse in Tris EDTA pH 9 for 30 min at room temperature</p> <p>Perform enzymatic digestion with proteinase K 1.25 mg/mL (Sigma Aldrich, United States) at room temperature for 15 min¹</p> <p>Stop digestion by immersing in Tris EDTA at 4 °C</p> <p>Leave in Tris EDTA for 40 min at 4 °C</p> <p>Rinse in PBS for 10 min</p> <p>Apply FITC conjugated polyclonal rabbit antibodies directed against IgG (dilution 1:50), IgM (1:60), IgA (1:60), C3 (1:30), C1q (1:30), kappa (1:25), and lambda (1:40) (BIOSSB, Santa Barbara, CA, United States). Incubate for 2 h in a moist chamber in the dark</p> <p>Rinse with PBS</p> <p>Mount in glycerine</p> <p>Examine slides under a dark field immunofluorescence microscope</p>
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¹Varied based on room temperature. PBS: Phosphate buffered saline; FITC: Fluorescein isothiocyanate.

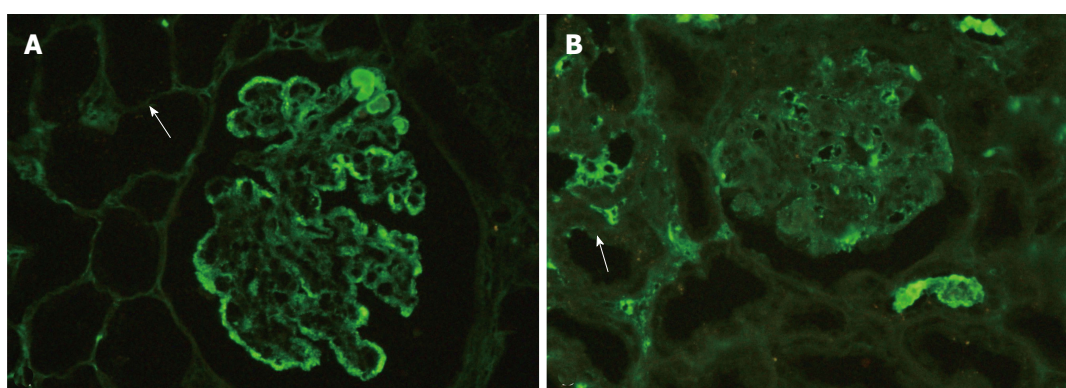


Figure 1 Examples of technically adequate and inadequate digestion. A: Immunofluorescence staining on a paraffin embedded tissue section in a case of diffuse proliferative lupus nephritis after enzymatic digestion with proteinase K. Note the adequate digestion evidenced by disappearance of tubular epithelial cells (arrow) (FITC IgG, \times 200); B: Immunofluorescence staining in a case with inadequate digestion with visible tubular epithelial cells (arrow). Note the antibody sticking to the surface of the capillary wall (FITC IgG, \times 200). FITC: Fluorescein isothiocyanate.

tubules. From experience, the disappearance of tubular epithelial cell outline with only visible tubular basement membranes correlated with optimal digestion and detection of immune complexes in the tissue (Figure 1A). In under-digested glomeruli, a non-specific staining pattern was observed (Figure 1B) with the antibody appearing to stick to the surface of the capillary walls in a blotchy manner rather than labeling immune complexes/complement with granularity. This staining pattern was recognized as false positive.

The major utility of this technique was in classifying glomerular diseases, with limited utility in tubulointerstitial diseases. Table 2 demonstrates the range of renal pathologies that were diagnosed.

In membranoproliferative glomerulonephritis (MPGN, 32 cases), 30 cases could be adequately diagnosed and sub-classified based on the results of IF-P into immune complex mediated (18 cases) and complement mediated (12 cases) MPGN. In two cases with intramembranous dense transformation of the glomerular basement membrane on electron microscopy (dense deposit disease) no significant C3 deposition was noted on IF-P. One of these cases had a comparative IF-F with the diagnostic C3 dominant pattern and intensity 2+ (0-3+ scale) (Figure 2). One case of IC-MPGN showed 1+ IgG, kappa

and lambda with 2+ C3 deposition, but characteristic MPGN type 1 pattern on ultrastructure examination; no comparative IF-F was available. The rest of the cases of MPGN showed at least 2+ intensity of diagnostic immunoglobulin/complement.

Diffuse proliferative glomerulonephritis (12 cases) were diagnosed as post infectious glomerulonephritis (PIGN) in 10 cases based on classical lumpy bumpy deposits of C3 and IgG. In one case with diffuse proliferative exudative pattern of injury and prior episode of febrile illness, a limited IF-P panel of IgA, IgG and C3 was applied to differentiate a PIGN from a proliferative IgA nephropathy. The intensity of IgG and C3 was only 1+, while IgA was negative. The IF-P results were deemed noncontributory in this case. Tissue for electron microscopy was not available.

Within the lupus nephritides (LN, 25 cases) localization of the deposits as mesangial and/or capillary wall aided in accurate classification of the glomerulonephritis (Table 2, Figure 3). The lack of deposits was also significant, as demonstrated by two cases of systemic lupus erythematosus (SLE) presenting with proteinuria and nonspecific light microscopic findings. Further electron microscopic examination confirmed a lupus podocytopathy. In one case of class II LN significant

Table 2 Renal pathologies diagnosed by immunofluorescence on paraffin embedded biopsies

Diagnosis	Total number of cases	Number of cases with non diagnostic IF-P (%)	Remarks
MPGN	32	2 ¹ (6.2%)	Classification into immune complex mediated MPGN (18 cases) and complement mediated MPGN (12 cases) was possible ¹ In two cases C3 was not demonstrated and electron microscopy showed features of dense deposit disease
Membranous nephropathy	11	2 ¹ (22.2%)	In one case staining intensity of IgG was only 1+, however staining pattern was classical ¹ In 2 cases significant fine granular immunofluorescence was not noted
Lupus nephritis	25	1 ¹ (4%)	Classification into Class II (2 cases), Class III/IV (15 cases) and Class V (5 cases) was possible Two cases of lupus podocytopathy were diagnosed ¹ In one case of lupus nephritis only IgM was demonstrated significantly, though electron dense deposits were noted on electron microscopy
Diffuse proliferative glomerulonephritis - post infectious glomerulonephritis	12	1 ¹ (8.3%)	¹ In one case only 1+ IgG and trace C3 deposition noted No tissue for electron microscopy was available
Pauciimmunecrescentic glomerulonephritis	7	-	-
IgAN	39	-	In 64 cases (minimal change morphology, mesangial proliferation or FSGS), IgAN was excluded by IF-P In one case of diabetic nephropathy IF-P was used to exclude secondary IgAN
C1q nephropathy	2	-	-
Light chain deposition disease	1	-	Tubular basement membrane and vascular deposits were also noted in addition to the glomerular deposits
Amyloidosis	4	-	2 cases of AL amyloid (demonstrating light chain restriction) and 2 cases of AA amyloid
CGN	8	3 (37.5%)	The immune complexes could not be demonstrated in 3 cases of chronic glomerulonephritis, one of these was a case of biopsy proven MPGN and the other was a case of IgAN. In one case of CGN no immune complexes were seen, however no previous renal biopsy record was available
Cast nephropathy	2	-	One case also demonstrated light chain restriction
Tubulointerstitial nephritis	9	-	Associated immune complex mediated glomerular disease was excluded

¹Non diagnostic cases and details of their immunofluorescence pattern. MPGN: Membranoproliferative glomerulonephritis; CGN: Chronic glomerulonephritis; IgAN: IgA nephropathy; FSGS: Focal segmental glomerulosclerosis.

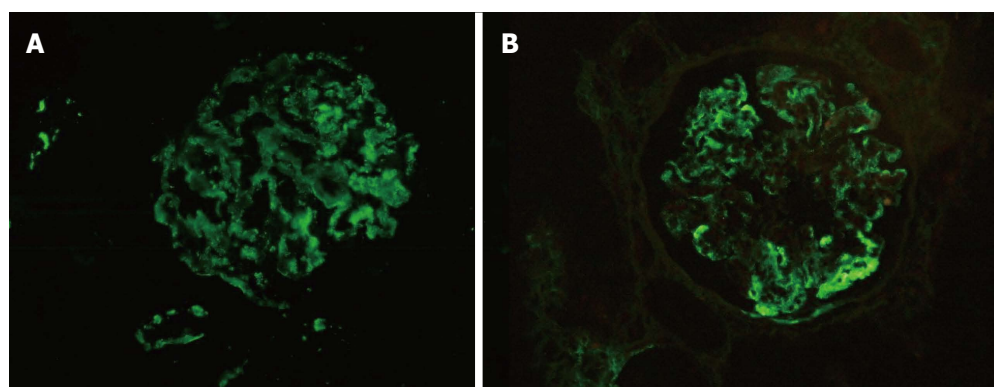


Figure 2 Comparable staining pattern on immunofluorescence on frozen and paraffin embedded tissue. A case of dense deposit disease showing bright C3c deposition 3+ (0-3+ scale) on IF-F (A, FITC C3c, × 200). Note the comparative coarse granular capillary wall staining of C3c (3+) on paraffin embedded tissue section after enzymatic retrieval (B, FITC C3c, × 200). FITC: Fluorescein isothiocyanate; IF-F: Immunofluorescence on fresh frozen tissue.

full house positivity could not be demonstrated. Only IgM showed 2+ mesangial staining and the rest of the immunoglobulins and complements were focal. The EM of this case however revealed numerous predominantly mesangial electron dense deposits along with few subepithelial and subendothelial deposits.

Of 11 cases of membranous nephropathy (MN, 11 cases) diagnostic immunofluorescence with IgG was noted in 8 cases. One case showed weak (1+) staining with characteristic fine granularity and two cases were negative for IgG.

To make the diagnosis of IgA nephropathy (39 cases,

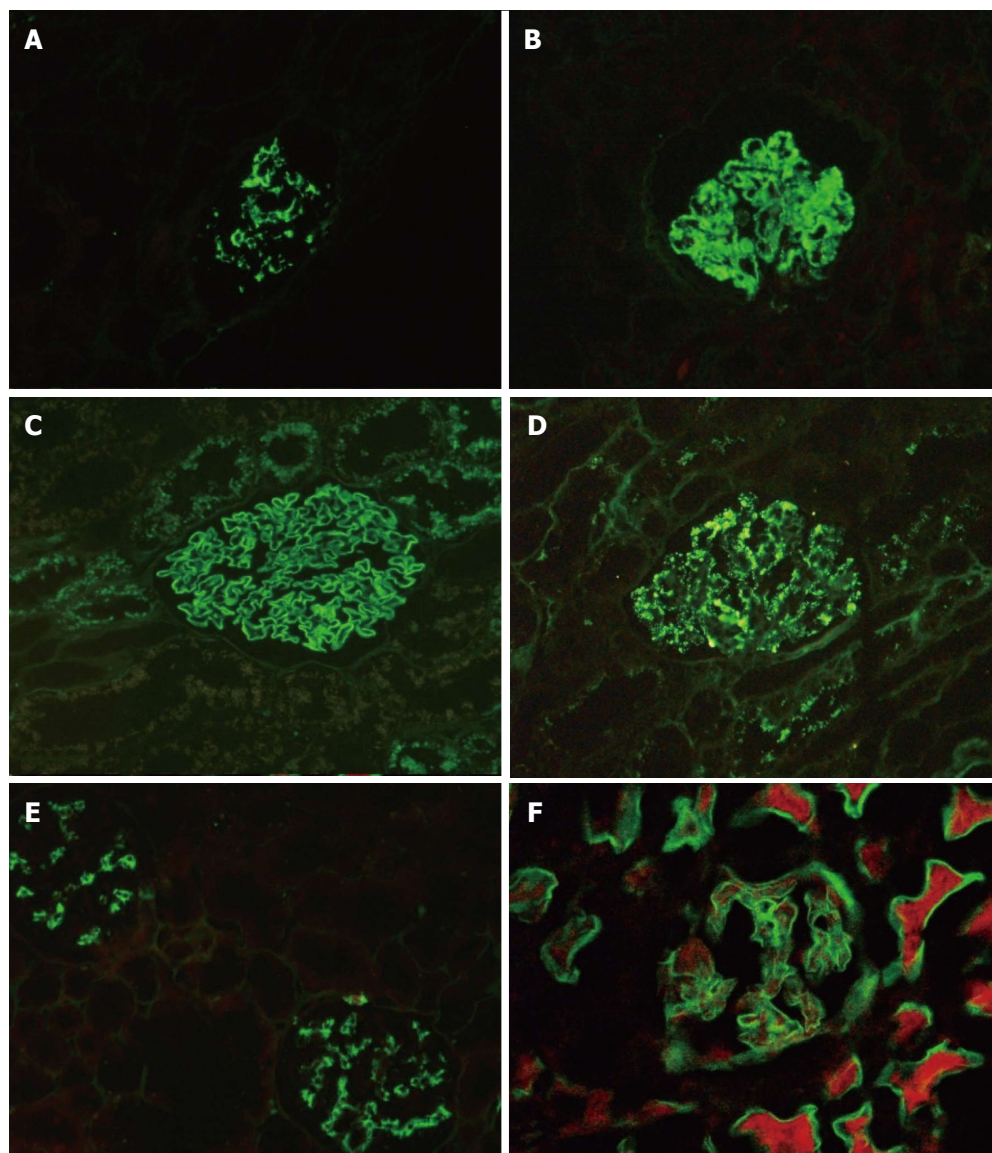


Figure 3 Glomerulonephritis diagnosed on immunofluorescence on paraffin. A: IgA nephropathy: There is predominantly mesangial deposition of IgA (FITC IgA $\times 100$); B: Class IV or diffuse lupus nephritis: Immunofluorescence reveals coarsely granular deposition of immunoglobulins in both mesangium and in the peripheral capillary wall (FITC IgG $\times 200$); C: Membranous nephropathy: Immunofluorescence reveals fine granular capillary wall deposition of IgG (C, FITC IgG $\times 200$); D: Post infectious glomerulonephritis: Garland pattern with elongated peripheral loop deposits is depicted, along with occasional mesangial deposits (D, FITC C3c, $\times 200$); E: C1q nephropathy with mesangial deposition of C1q (FITC C1q $\times 100$); F: Diabetic nephropathy with linear accentuation of glomerular capillary wall and tubular basement membrane (FITC IgG $\times 200$). FITC: Fluorescein isothiocyanate.

16%) or to exclude it in cases with minimal change morphology, mesangial proliferation or focal segmental glomerulosclerosis (64 cases, 26.3%) constituted the bulk of indication for IF-P in our routine practice. In two cases with isolated hematuria, suspected IgA nephropathy and nonspecific light microscopy, IF-P was negative for immunoglobulins which prompted ultrastructural examination of the cases. Classical glomerular basement membrane changes of collagenopathy consistent with Alport syndrome and thin basement membrane disease were identified. IF-P was also performed in patients of diabetic nephropathy with hematuria to exclude secondary IgAN.

In this series there were 8 cases which were diagnosed as chronic glomerulonephritis (CGN), the under-

lying etiology could be established in 5 cases (IgAN = 3, IC-MPGN = 1 and C-MPGN = 1). The immune complexes could not be demonstrated in 3 cases of chronic glomerulonephritis, one of which was a case of biopsy proven MPGN and the other was a case of IgAN. In one case no immune complexes were seen, however no previous renal biopsy record was available.

In one case of post transplant recurrence of nodular glomerulosclerosis of undetermined cause, IF-P resulted in confirming the diagnosis of light chain deposition disease (LCDD) with kappa restriction^[3]. Deposits were identified in the glomerular nodules, tubular basement membranes, arterioles and arteries (Figure 4A and B). Primary amyloidosis was identified in 2 cases demonstrating light chain restriction (Figure 4C and D). Light chains were

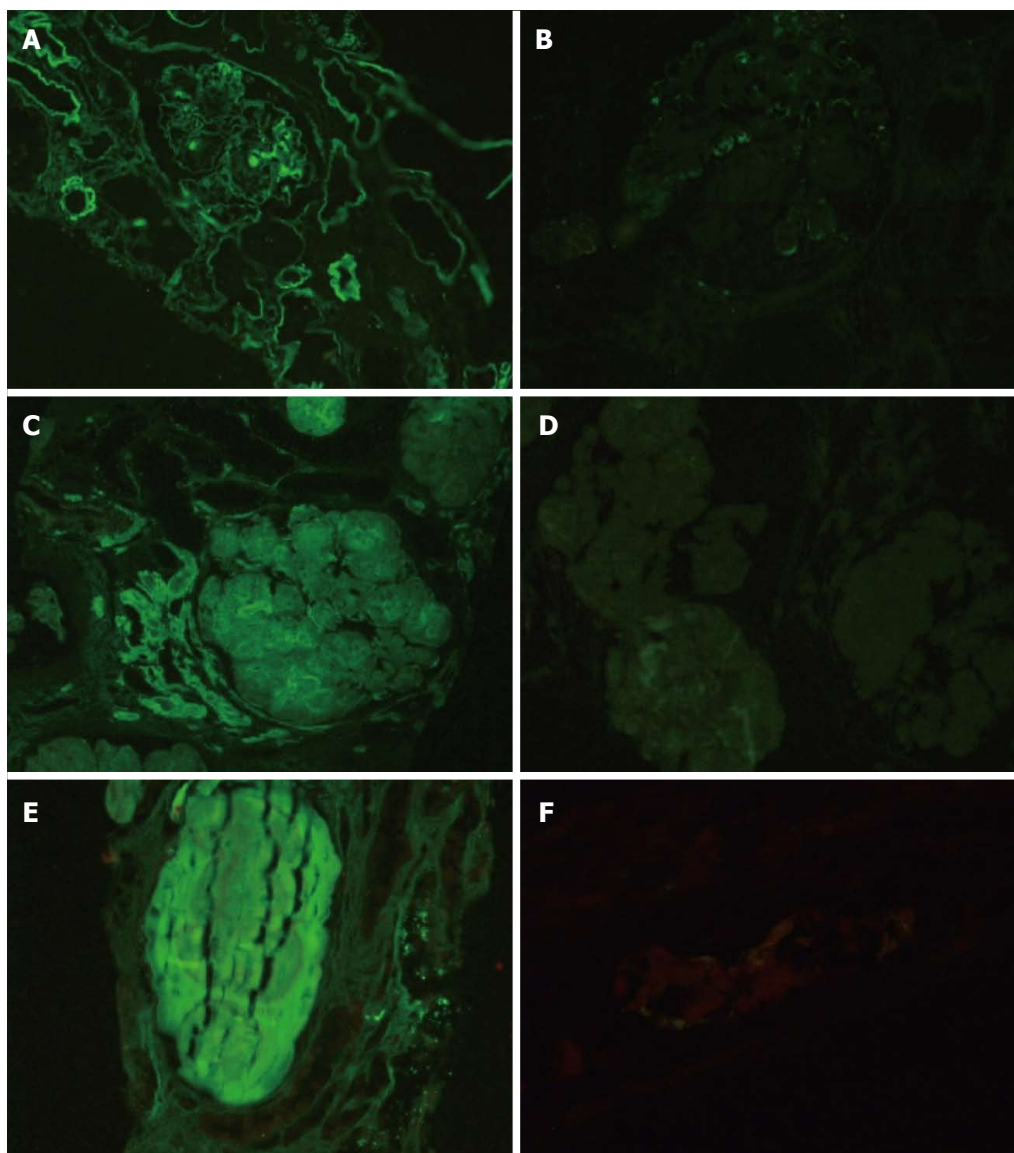


Figure 4 Immunofluorescence on paraffin to demonstrate monoclonal deposits. A case of light chain deposition disease with kappa light chain restriction. There is nodular mesangial, capillary wall and tubular basement membrane deposition of kappa light chain (A, FITC kappa, $\times 100$) while no deposition of lambda is noted (B, FITC lambda, $\times 200$); C: A case of primary amyloidosis with lambda light chain restriction. The lambda deposition is noted in the mesangium (FITC lambda $\times 200$); D: There is no deposition of kappa (D, FITC kappa $\times 200$); E: A case of cast nephropathy with kappa light chain restriction. Note the brightly positive casts for kappa (FITC kappa $\times 200$) with no traces of lambda (F, FITC lambda $\times 200$). FITC: Fluorescein isothiocyanate.

also identified in tubular casts, confirming the diagnosis of cast nephropathy in two cases. One of these cases demonstrated light chain restriction (Figure 4E and F). Other than suspected cast nephropathy, IF-P was performed in cases of primary tubulointerstitial disease with significant proteinuria or hematuria to exclude concomitant glomerular disease.

Comparison between immunofluorescence on frozen and paraffin embedded tissue

Comparative IF-F and IF-P was available in 37 cases. Thirty-five of these cases (93.8%) had either equal intensity or a minor difference in intensity of staining (1+) for the diagnostic immunoglobulin/complement. Significant difference was observed in just 2 cases; a

case of C-MPGN and a case of MN (Table 3).

Technically assessment of immunofluorescence on the paraffin embedded tissue was found to be less challenging than IF-F with clearly observed morphology and ease of comparison with light microscopic findings.

DISCUSSION

IF studies are integral to diagnostic renal pathology and renal pathologists are often left frustrated by a lack of representative tissue in material sent for routine IF. The technique of IF-F is well established however requires a separate representative core of kidney tissue, a cryostat and technical expertise for satisfactory results. Descriptions of enzyme treatment of formalin fixed

Table 3 Comparison of immunofluorescence intensity on fresh frozen and paraffin embedded renal biopsies

Disease	Number of cases with no difference in intensity of diagnostic immunoglobulin/complement (%) IF-F = IF-P	Number of cases with difference in intensity of diagnostic immunoglobulin/complement (%) IF-F > IF-P		Total number of cases	
		Difference of 1 +	Difference of 2 +		
					IgA nephropathy
C-MPGN	1 (25%)	2 (50%)	-	1 (25%)	4
IC-MPGN	4 (100%)	-	-	-	4
Lupus nephritis	3 (50%)	3 (50%)	-	-	6
C1q nephropathy	2 (100%)	-	-	-	2
Membranous nephropathy	3 (43%)	3 (43%)	-	1 (14%)	7
Post infectious glomerulonephritis	1 (100%)	-	-	-	1

IF-F: Immunofluorescence on fresh frozen tissue; IF-P: Immunofluorescence on paraffin embedded tissue; C-MPGN: Complement mediated membranoproliferative glomerulonephritis; IC-MPGN: Immune complex mediated membranoproliferative glomerulonephritis.

paraffin embedded (FFPE) tissue followed by IF studies (IF-P) can be found in literature from as early as 1976, however the technique has still not found a place in most renal pathology laboratories^[2].

The use of a cross linking fixative like formaldehyde leads to masking of antigens. In addition calcium and other divalent ions form complexes with proteins during fixation and these complexes can block the antigenic determinants^[1]. It is to unmask these determinants that enzyme treatment of FFPE tissue is necessary before applying antibodies. In our laboratory the technique of IF-P was standardized using proteinase K and it was applied prospectively as a "salvage" technique with good results.

Proteinase K is an enzyme that exhibits broad substrate specificity. It is isolated from a fungus, *Engyodontium album* (formerly *Tritirachium album*) and is able to digest keratin hence the name proteinase "K"^[4]. Different proteolytic enzymes including pronase, trypsin and pepsin have been tried in various studies as demonstrated in Table 4^[5-15].

As evident from Table 4 multiple studies comparing IF-P and IF-F have clearly established that IF-P is a feasible and valuable "salvage" technique. Comparable staining intensities have been demonstrated for immunoglobulins, with albeit lower sensitivity for detection of complement.

In an early study by Fogazzi *et al.*^[8] paraffin embedded sections were treated with pronase (0.75 g/L for 60 min) and the fluorescence intensity and location was compared with frozen sections in cases of IgAN ($n = 10$), membranous nephropathy ($n = 8$) and proliferative lupus nephritis ($n = 10$). The diagnostic immunoglobulins were detected with equal or increased intensity in 100% cases with a slightly reduced immunoreactivity for C3 in enzyme treated tissue. Structural details were better assessed in terms of location and morphology of deposits. On retrospective digestion of 1 and 2 year old blocks identical staining patterns were obtained in approximately 86% of cases.

Using a similar protocol as Fogazzi *et al.*^[8], Nasr *et al.*^[10] compared IF-F and IF-P in 71 renal biopsies including a spectrum of renal diseases. In glomerular

diseases diagnostic findings were obtained in 100% of cases of lupus nephritis, acute post-infectious glomerulonephritis, cryoglobulinemic glomerulonephritis, fibrillary glomerulonephritis, primary amyloidosis, 88% of cases of IgAN, 80% cases of LCDD, 60% of cases of MPGN type 1, 50% cases of idiopathic MN and 20% of cases of anti-glomerular basement membrane (anti-GBM) disease. In all disease categories studied IF-P was less sensitive than IF-F for the detection of C3 similar to Fogazzi *et al.*^[8]. In addition they found reduced sensitivity for the detection of IgG in cases of MN (50%) and anti-GBM (20%) disease. They also demonstrated utility of the technique in tubulointerstitial diseases such as myeloma cast nephropathy and light chain proximal tubulopathy and found IF-P satisfactory in demonstrating light chain restriction.

More recently Messias *et al.*^[15] studied paraffin immunofluorescence in 304 native renal biopsies. The false positive staining on the surface and within capillary lumina attributed to sera adsorption secondary to fixation by the authors was also recognized in our cases and was more pronounced in under-digested tissue. They described a novel utility of the technique in evaluating masked paraprotein and immune complex deposits. The light chain crystals in light chain proximal tubulopathy were only demonstrated after enzyme digestion. Out of 61 cases where IF-P was performed to unmask immunoglobulins, it was helpful in 20 cases which included 9 cases of membranous like glomerulopathy with masked IgG-kappa deposits (MG MIDK) a novel entity first described by Larsen *et al.*^[16], 4 cases of MPGN with light chain restriction and 7 cases of MPGN with mixed essential cryoglobulinemia, which would have been misdiagnosed as C3 glomerulopathy. They recommended that all cases of C3 glomerulopathy based on routine immunofluorescence should be subjected to paraffin immunofluorescence to reach the correct diagnosis and avoid unnecessary investigations into complement abnormalities. In addition any case where the routine immunofluorescence findings do not match the ultrastructural findings should undergo paraffin immunofluorescence. However the authors reiterated, and we concur that IF on paraffin embedded

Table 4 Studies using the technique of immunofluorescence on enzyme digested paraffin embedded tissue in literature

Ref.	Year	Enzyme used	Cases (n)	IF panel applied	Significant results
[2]	1976	Trypsin for 120 min	NA	Immunoglobulins and complement	Feasible to demonstrate immunoglobulins but not complement Reduced background immunofluorescence
[5]	1979	Trypsin	52 renal biopsies	IgG, IgA, IgM, C3, Fibrinogen	Accurate detection of immunoglobulins (90%) and complement (75%) in comparison with IF on frozen
[6]	1980	Trypsin	21 (LN, MN, IgAN)	IgG, IgM, IgA	IF on trypsin-digested tissue was as sensitive as IF-F for immunoglobulins but less sensitive for complement
[7]	1980	Pepsin (0.4%) and trypsin	Experimental mice model of anti GBM disease	IgG	Pepsin +/- trypsin digestion better than trypsin alone Enzyme digested tissue showed trivial decrease in sensitivity but good preservation in comparison with IF on frozen
[8]	1989	Pronase (0.75 g/L for 60 min at 37 °C)	IgAN (10), MN (8), Proliferative LN (10)	IgG, IgA, IgM, C3, C1q	Correct diagnosis possible in all cases Better structural details and less fading of IF Lower intensity staining for C3 Retrospectively performed digestion on 1 and 2 yr old blocks, satisfactory in 86% cases
[9]	2005	Microwave treatment (10 min) followed by Protease VII (0.05% for 30/60 min) Trypsin (0.25% for 120 min)	IgAN (7), LN (7), MN (7), MPGN (3)	IgG, IgA, IgM, C3	Microwave treatment followed by protease digestion better than trypsin digestion Diagnostic immunoglobulin found in more than 80% cases
[10]	2006	Pronase (0.75 g/L for 60 min at 37 °C)	MN (8), MPGN (5), LN (5), PIGN (5), IgAN (8), Cryo GN (5), Fibrillary GN (5), Anti GBM (5), Cast nephropathy (5), Amyloid (5), LCDD (5), LCFS (10)	IgG, IgA, IgM, C3, C1q, kappa and lambda	Diagnostic utility in 83% cases Useful in dysproteinemia related renal disease particularly LCFS Less sensitive for staining with C3 in MPGN type I, Cryo GN, PIGN Less sensitive for IgG in MGN and anti-GBM disease
[11]	2007	Proteinase XXIV	LN (5), antiGBM (5), MN (9)	NA	IF-P on proteinase XXIV is more sensitive than IF-P with pronase In LN, better intensity staining for C1q and IgG In anti GBM, 80% sensitivity for detection of IgG In MGN, 55% sensitivity for detection of IgG
[12]	2009	Microwave treatment and/or Proteinase K - (30 or 60 min)	IgAN (24), MN (22), LN (24)	IgG, IgA, IgM, C3	Rate of agreement between immunofluorescence on paraffin sections and immunofluorescence on frozen sections with respect to the presence of IgA was 56.5%, IgM - 44.4%, IgG - 73.9%, and C3 - 51.5% IF-P may be used as a salvage technique when frozen tissue is not available
[13]	2011*	Trypsin (30 min), Pepsin	IgAN (20), MN (25)	IgA, IgG, HBsAg, HbcAg	Trypsin digestion better than pepsin digestion IF-P slightly weaker signal than IF-F
[14]	2012	Heat - Tris/Citrate buffer Pronase RTU (60 min at 37 °C)	LN (15), MN (11), IgMN (10), MPGN (2), IgAN (2)	IgG, IgA, IgM, C3, C1q	Heat based retrieval using Tris buffer showed superior results Pronase digestion shows less sensitivity for detection of immunoglobulins and complement
[15]	2015	Proteinase K for 20 min	304 cases (207 cases as salvage and 97 cases for antigen unmasking)	IgG, IgA, IgM, C3, C4, C1q, fibrinogen, kappa and lambda	Not only a good salvage technique but prevents misdiagnosis due to masked immune complex or light chain deposition

LN: Lupus nephritis; MN: Membranous nephropathy; IgAN: IgA nephropathy; IgMN: IgM nephropathy; MPGN: Membranoproliferative glomerulonephritis; anti GBM: Anti glomerular basement membrane nephritis; PIGN: Post infectious glomerulonephritis; Cryo GN: Cryoglobulinemic glomerulonephritis; LCDD: Light chain deposition disease; LCFS: Light chain fanconi syndrome; RTU: Ready to use; HBsAg: Hepatitis B surface antigen; HbcAg: Hepatitis B core antigen; IF: Immunofluorescence.

tissue cannot supplant routine IF-F in renal biopsy interpretation.

In the present series we found comparable results for staining with IF-F and IF-P. As described in other studies in a few cases expected immunofluorescence results were not obtained by IF-P, including two cases of membranous nephropathy, two cases of dense deposit disease, one case of lupus nephritis, one case

of suspected PIGN and three cases of chronic glomerulonephritis; even in the presence of optimal enzyme digestion. Most of these cases (except PIGN and CGN) had electron microscopic confirmation of presence of electron dense deposits, thus they were truly false negative results. We opine that this variability may be a result of differences in time of exposure of the renal biopsy to formalin, making the unmasking of antigenic

determinants more difficult. This of course becomes a limitation of IF-P in a “salvage” scenario, as a negative result in the presence of optimal digestion would always be questionable; however a positive result will always aid in the diagnosis^[3,17].

Nonetheless in the majority of cases undergoing routine fixation and processing, IF-P was successful in providing immunofluorescence results which added to the final diagnosis. Based on our results, we also now offer this technique for skin biopsies and for amyloid characterization in extra renal sites.

Based on the experience in our laboratory, we conclude that immunofluorescence on formalin fixed paraffin embedded tissue is a useful “salvage” technique in case of non-availability of representative fresh frozen tissue; however it is not without pitfalls. Technically assessment of enzyme digestion on each slide is mandatory for accurate interpretation of staining. Antibody staining of under digested tissue can result in both false positive as well as false negative results. Even with optimal digestion expected immunofluorescence results are sometimes not obtained and there is a yet unexplained reduced sensitivity for complement as demonstrated in multiple studies; all of which may result in a misdiagnosis. The extra slices of the renal biopsy taken for IF-P from the paraffin block apart from the routine stains result in insufficient tissue remaining in the block for any further staining or review. Within these limitations, we have demonstrated a significant diagnostic utility of this technique particularly in glomerular diseases and continue to offer it as a “salvage” option.

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COMMENTS

Background

Immunofluorescence (IF) is an indispensable technique for rendering an accurate diagnosis in renal pathology. Diseases such as IgA nephropathy, C1q nephropathy and C3 glomerulopathy cannot be diagnosed without IF. Direct IF on fresh frozen tissue (IF-F) is the most widely used IF technique.

Research frontiers

IF on paraffin embedded renal biopsies after enzyme treatment has not found widespread use in renal pathology laboratories. This leads to incomplete diagnosis and suboptimal patient management.

Innovations and breakthroughs

To overcome the hurdles above, a method of enzymatic digestion of formalin fixed paraffin embedded tissue was standardized and introduced in the authors' laboratory in 2011.

Applications

The authors discussed their experience with this technique in day-to-day diagnostic renal pathology, its utility in reaching final diagnoses and comparing it with usual IF-F where available. Technical and interpretation issues faced are

described in detail, and may be helpful to any laboratory planning to introduce this technique.

Peer-review

It is an interesting paper that could be very useful for pathologists or nephrologists involved in renal pathology.

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