Recovering histological sections for ultrastructural diagnosis of glomerular diseases through the pop-off technique

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DECLARATIONS

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ABSTRACT

Introduction: Electron microscopy (EM) represents an indispensable technique for the diagnosis of kidney glomerular diseases. When dedicated tissue is not available, histological and cryostat sections can be reprocessed for EM using the pop-off technique. Here the practical value of this technique is analysed with emphasis on its accuracy in measuring basement membrane thickness and detecting immune deposits.

Methods: Ninety-four histological sections of kidney tissues fixed in Serra's solution, stained with H&E, PAS, Masson's Trichrome; for EM analysis, the sections were recovered from either treated or untreated microscope slides through the pop-off technique. Some sections were recovered from cryosections allocated for immunofluorescence.

Results: The ultrastructural details were sufficiently maintained on tissues fixed with Serra's solution despite being considered disadvantageous for EM. The type of microscope slides and the time of biopsy storage did not affect the quality of sections recovery. The histological stains had only moderate effects on the electron-density of the glomerular basement membrane (GBM). The pop-off technique reduced the GBM thickness when compared to the conventional EM processing but preserved the electron density of immune deposits.

Conclusions: The application of the pop-off method to renal biopsy is a useful recovery method that produces limited but satisfactory results when there is no suitable material for EM. The ultrastructural morphology was retained even from tissues fixed with Serra's solution and deposits maintained the expected electron density; however, we have observed an overall thickness reduction of the GBM that could have a potential impact on thin membrane disease diagnosis.

Keywords: kidney biopsy, glomerular disease, electron microscopy, pop-off technique

INTRODUCTION

The diagnosis of kidney glomerular diseases requires a synergism between nephrologists and pathologists, and abilities for interpreting results from histological stains, immunofluorescence and electron microscopy analysis [1; S1-S2].

The transmission electron microscopy (TEM) represents the gold standard technique to identify specific ultrastructural features of glomerular damage [2; S3-S5] such as deposits [3, S6], modification of the glomerular basement membrane (GBM) [4], endothelial cell injury responsible for different disease [5] and fusion and detachment of foot processes of the podocytes [6; S7]; targets of ultrastructural examination as listed in Table 1.

However, TEM remains a minor activity not always routinely performed as it requires highly trained personnel. Further, when available other reasons can determine the absence of the ultrastructural report, e.g., biopsy core composed of fibrous and adipose tissue exclusively, medulla sampling, the loss of the area of interest.

When one of the above reported conditions occur, alternative approaches are proposed to recover formalin-fixed biopsy fragments from paraffin blocks [7; S8]; however, its applicability is limited to the diagnosis of thin GBM disease [7].

Alternatively, tissue reprocessing for electron microscopy can be obtained from histological sections through the pop-off technique [8; S9]. This recovery approach is rapid and easy to apply for re-embedding in resin, stained paraffin [8; S9-S11] or cryostat sections [9]; one major advantage of this method is the possibility to observe the same area of interest at light and electron microscopy.

In this study, we applied the pop-off method to rapidly recover samples for TEM from paraffin or frozen sections used in light microscopy evaluating the influence on the ultrastructure of the type of microscope slides, the time of tissues, the routine histological stains; different retrieval technical procedures have been also considered. We anticipated the pop-off application in kidney glomerular disease as a suitable tool to quickly provide clinically useful ultrastructural information regarding the presence of electron dense deposits when the dedicated renal tissue is not available for electron microscopy.

METHODS

A retrospective study on ninety-four sections of archival human renal tissues processed from 2006 to 2018, approved by Local Ethical Committee University-Hospital S. Orsola-Malpighi, Bologna (protocol number RecoverEMO; 185/2020/Sper/AOUBo) and carried out in accordance with the Declaration of Helsinki.

Histology

Renal cortical tissues were fixed in Serra's solution and paraffin embedded for histological evaluation. Three-µm thick sections were recovered on untreated (Diamond Microscope Slides) and treated (TOMO® IHC Adhesive Glass Slide) glass slides and stained with hematoxylin and eosin (H&E), Periodic Acid Schiff (PAS), Masson's trichrome (TRICMAS). Additional samples were obtained from routine kidney immunofluorescence; 5-µm thick, frozen, H&E stained sections were recovered.

Transmission Electron Microscopy

Fresh: The renal tissue was fixed in cacodylate buffered 2.5% glutaraldehyde followed by routine TEM processing. Counterstained thin sections were observed in a Philips CM100 (FEI Company, ThermoFisher, Waltham, MA, USA) Transmission Electron Microscope.

Paraffin: Small renal tissue fragments recovered from paraffin block, have been de-waxed in xylene, rehydrated in ethanol and processed as above.

Pop-off: Histological sections stained with H&E or PAS or TRICMAS and H&E frozen sections were retrieved through the pop-off techniques applying a previously described protocol [8]. Details of the protocols are reported in supplementary materials – Part 1; a scheme of the procedure is shown in figure 1.

Measurements of the glomerular basement membrane

For each sample, five random digital images of GBMs of capillary loops were recorded at 13500x using an Olympus digital camera. For each image, twelve random measurements were produced on the GBM thickness. The measurements were performed on linear portions of the GBM exclusively. Values were expressed as mean ± standard deviation.

Statistical analysis

GraphPad Prism software was used to perform statistical analysis and graphs. Nonparametric Mann-Whitney test and one-way ANOVA test followed by Bonferroni's multiple comparison test were applied for determining statistical differences between dissimilar glass and recovery methods respectively. P value < 0.05 was considered to be statistically significant.

RESULTS

The ultrastructural recovery was performed on 94 histological sections as detailed in supplementary materials – Part 2. Indications, advantages and quality of ultrastructural preservation of each approach used to process the renal biopsy are summarized in Table 2.

The influence of routine staining on the ultrastructural analysis of sections recovered from histological sections

The pop-off recovery procedure performed on H&E, PAS and TRICMAS histological sections gave superimposable results. The GBMs were similar in thickness without evident structural alterations even though they showed different electron densities. In H&E, the GBM appeared more electron-lucent whereas the membranes seen in PAS and TRICMAS had enhanced densities (Fig. 2).

The influence of different recovery methods on glomerular basement membrane thickness and electron dense deposits

In order to evaluate the influence of different methods on the GBM thickness as well as on the presence and localization of electron dense deposits, comparative technique analyses were performed on the same renal biopsy from patients with normal (Fig. 3a), thin (Fig. 3b) and thick (Fig. 3c) membranes. GBMs were well-preserved in each condition. The morphometric analysis revealed that the thicknesses of normal GBM was significantly reduced after paraffin block recovery (216 ± 30 nm), paraffin section pop-off (214 ± 43 nm) and frozen section recovery (196 ± 51 nm) when compared to conventional tissue processing (fresh tissue, 302 ± 58 nm). In the renal biopsy with thin membranes, a shorter range of values variability was seen (fresh, 224 ± 31 nm; paraffin block recovery, 222 ± 30 nm; pop-off on paraffin, 201 ± 28 nm; frozen section recovery, 193 ± 38 nm). As to the biopsy with thick GBM, the analysis revealed a progressive reduction of the thicknesses in all recovery methods (paraffin, 633 ± 62 nm; pop-off on paraffin, 592 ± 133 nm; frozen, 420 ± 84 nm) in contrast to fresh (723 ± 215 nm) renal tissue (Fig. 3d). In the same samples with thin GBM, the presence of electron dense IgA deposits was observed in the mesangium regardless of the type of recovery procedure employed (Figs. 3e-h).

DISCUSSION

Routine histological sections of kidney biopsies fixed in Serra's solution were successfully retrieved for TEM examination. The success of the pop-off technique is independent of the characteristics (untreated or treated) of glass slides, the storage time of biopsy, the histological stain performed. In addition to the recovery from the paraffin block [7; S8], also sections can be retrieved through pop-off technique [8; S9-S11]; as reported in table 2, the pop-off technique can be included among salvage methods used in TEM to reprocess dewaxed sections when the dedicated renal tissue is not available. Advantages of this technique are fast executions and precise correlations with areas of interest identified in light microscopy; disadvantages are inferior fine morphology when compared to that provided by samples specifically allocated for TEM.

Although routine histology is performed on formalin-fixed tissues, in renal pathology, Serra's solution, an acid fixative composed by formalin, ethanol and glacial acetic acid, can be preferred in some institutions [S12]. This formulation allows to bring out more cytological details avoiding tissue shrinkage while giving high contrast in the histochemical staining used for diagnostic routine [10]. Although the retrieval for TEM is not recommended [10], our results have showed an acceptable morphology for most targeted cellular and extracellular glomerular compartments.

The type of histological staining had no impact on the morphology of recovered renal tissue. In previous papers, the pop-off method was mostly applied on H&E-stained sections [S9-S11]. Here, the pop-off method was performed on sections routinely stained with H&E, PAS and TRICMAS. We did not see any particular ultrastructural changes in the glomeruli. However, in H&E the GBMs were more electron-lucent than those recovered from PAS and TRICMAS. This effect could be related to PAS specificity for basement membranes and TRICMAS for collagens.

Frozen sections can be used as additional resources to recover samples for TEM. Although artefacts are introduced by freezing procedure, a previous study has showed a preservation of mouse renal glomerulus without freezing damage and related artefacts [9]. Here, renal tissue frozen sections recovered with pop-off showed freezing artefacts which had no impact on the target areas of diagnostic investigation (basement membrane morphology and, localization and density of deposits).

We further focused on the thickness of the GBMs. Changes in the thickness of the GBM have been related to individual biological variability, measurement methods, resin embedding [S13]. Here, biopsies with GBM of normal, reduced and increased thickness were used to execute a morphometric analysis on the same sample which was processed using different methodologies. The comparison showed significant changes in the GBM thickness. Greater reductions were found in the biopsies with thickened GBM probably due to the presence of sensitive membrane components to the reagents in contrast to biopsy with thin GBM whose thickness was almost similar in all recovery approaches probably due to compaction of structural components. As previously mentioned [7] in normal tissue recovered from formalin-fixed paraffin-embedded samples, the recovery procedures reduce the GBM thickness (fresh, 302 ± 58 nm; paraffin block, 216 ± 30 nm; pop-off, 214 ± 43 nm); we also found that the thickness of GBM recovered from a case of thin membrane disease was influenced by the recovery procedures; in this case, the average thickness of GBMs ranged from 224 ± 31 nm seen in fresh samples to 201 ± 28 nm in paraffin sections recovered through pop-off method. Therefore, any ultrastructural diagnosis of thin membrane disease should be made with caution on recovered tissue. On the contrary, in the case of metabolic diseases, such as diabetes, where membranes are thickened, recovery produces useful results as we previously described [11]. Indeed, GBM recovered from paraffin blocks and paraffin sections through pop-off in a diabetic patient remained thicker than the expected values of normality (fresh, 723 ± 215 nm; recovery from paraffin block, 633 ± 62 nm; pop-off, 592 ± 133 nm). In all the recovery procedures, dense deposits were identifiable, and this can be particularly useful when a biopsy from a diabetic patient is not allocated for TEM; in fact, approximately 33% of biopsies with diabetic nephropathy have superimposed glomerular diseases, including

membranous and IgA nephropathy with dominant IgA deposits [12]. Further, the overall deposit morphology is also maintained; this finding expands the application of the recovery technique to those cases in which the identification of structured deposits is mandatory for diagnosis.

A limitation of this study is the quality of ultrastructural morphology that it is not comparable to that of biopsies primarily fixed in glutaraldehyde. Also, sampling is limited to single examples of thin membrane disease, IgA nephropathy, normal tissue with minimal changes, diabetic nephropathy and as such the results cannot be generalized.

CONCLUSIONS

The pop-off technique is a valuable tool for ultrastructural examination of recovered histological sections. This method allows fast correlative light and ultrastructural analyses and can be employed to retrieve tissue sections collected on routine slides glass, stained with common histological dyes and already archived without affecting the ultrastructural morphology. The pop-off technique can be applied on-demand to renal biopsy to rapidly provide ultrastructural diagnosis. Although the fine morphology quality is not comparable to standard processing, the results are generally satisfactory and useful for detecting changes in GBM thickness and electron dense deposits as occurring in metabolic diseases.

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Table 1: Ultrastructural parameters useful for diagnosing glomerular diseases.

Ultrastructural parameters	**Diseases**
GBM thickness and texture changes (thickness changes, basket-weave appearance, wrinkling, multi-layering)	• Diabetes • Thin membrane disease • Alport disease • TMA • Transplant glomerulopathy
Electron dense deposits localization: subepithelial, subendothelial, intramembranous, paramesangial, mesangial	• Different diseases
Electron dense deposits morphology: dense granular, structured and fibrillary	• Different diseases
Foot processes effacement	• Minimal change disease • FSGS (primary and secondary forms)
Cytoplasmic inclusions	• Storage metabolic and genetic diseases, e.g., Fabry disease, mitochondriopathies • Ig crystal storing diseases

GBM, glomerular basement membranes; TMA, thrombotic microangiopathy; FSGS, focal segmental glomerulosclerosis; Ig, immunoglobulin

Table 2: Indication, advantages and quality of preservation in different renal biopsy processing methods.

	Fresh biopsy	**Paraffin-block recovery**	**Pop-off recovery on histological section**	**Frozen biopsy**
Indication	Routine	- No EM biopsy available - No glomeruli in the dedicated EM biopsy	- Paraffin-block saving for additional histological analysis, second opinions, legal purposes	- No EM biopsy available - No glomeruli at histology
Advantages	Best ultrastructural detail	- Histological block is always present	- Urgent reply - Small biopsy core - Minimal residual material - Recovery of a specific area of interest i.e. viral inclusions, Ig crystals, etc	- Last chance of avoiding a second biopsy
Quality of ultrastructural preservation	+++ Excellent	++ Formalin fixation preferable to Serra liquid fixation	++ Ultrastructural morphology comparable to the paraffin block approach	+ Poor ultrastructural morphology when compared to the other savage approaches

EM, electron microscopy

Legend of the Figures

Fig. 1: Pop-off technique on histological renal sections for electron microscopy examination. (a) A stained section of renal tissue was employed to identify the areas of interest at light microscopy. (**b**) A gelatine capsule filled with pure Araldite resin was placed on the previously selected areas. (**c**) After resin polymerization, the capsule was removed from the slide using a hot plate obtaining (**d**) a resin block with the overlapped section ready for thin sectioning and (**e**) grid collection. Representative ultrastructure of glomerular capillaries at (**f**) low and (**g**) high magnification. (**f**) scale bar: 5 μm; (**g**) scale bar: 1 μm.

Fig. 2: Influence of histological staining on ultrastructural morphology of recovered sections. Representative images of histological sections of renal glomeruli stained with H&E (Hematoxylin and Eosin), PAS (Periodic Acid Schiff), TRICMAS (Masson's trichrome) and their respective electron microscopy micrographs. No morphological differences were seen; however, the glomerular basement membrane was electron-lucent in H&E-stained samples and darker in the PAS and TRICMAS ones. H&E, PAS and TRICMAS: scale bar: 20 μm. Ultrastructural images: scale bars: 2 μm.

Fig. 3: Influence of recovery methods on glomerular basement membrane thickness and on electron dense deposits. Representative images of transmission electron microscopy performed on renal biopsies with normal (**a, top row**), thin (**b, middle row**) and thick (**c, bottom row**) glomerular basement membranes (GBM) recovered from fresh, paraffin embedded, pop-off on histological paraffin and frozen sections. (**d**) Morphometric analysis performed on biopsies with normal, thin and thick GBM. The measurement of the GBM thicknesses on renal tissue with normal GBM reveals a significant decrease values in tissue derived from paraffin block and after the recovered with the pop-off technique of paraffin and frozen sections when compared to fresh biopsy; in the biopsy with thin GBM, the thicknesses were similar in fresh and deparaffinized samples while they were statistically reduced in both pop-off recoveries in comparison to fresh and paraffin samples; in the biopsy with thick GMB, the thickness was reduced in all recovery methods with statistical difference when compared to fresh biopsy as well as to paraffin and Pop-off paraffin sections . Values are expressed as mean ± SD. Normal thickness of GBM: *** $P < 0.05$ versus fresh sample. Thin thickness of GBM: °° and °°° $P < 0.05$ versus fresh; ## and ### $P < 0.05$ versus paraffin recovery. Thick thickness of GBM: §§ and §§§ $P < 0.05$ versus fresh; +++ $P < 0.05$ versus paraffin and ççç $P < 0.05$ versus Pop-off paraffin sections. Scale bar of all images: 1μm. Representative ultrastructural images of electron dense deposits localized in the mesangium of (**e**) fresh renal biopsy (white *), (**f**) paraffin embedded biopsy (white head-arrows), (**g**) pop-off on histological paraffin (white arrows) and (**h**) on frozen (white °) sections. (**e-h**): scale bars: 1μm.