

When one plus one equals more than two - a novel stain for renal biopsies is a combination of two classical stains

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Summary. Histologic evaluation of renal biopsies includes multiple ancillary stains, including Periodic acid-Schiff's (PAS) and Masson's trichrome (Trichrome). Herein we report an innovative double-stain, derived from two standard stains (PAS and Trichrome). This novel stain not only has advantages of both ancestor stains, but became more distinguishable and colorful, when basement membranes stain dark-violet, whereas the interstitial collagen remains blue. This allows the pathologist immediate estimation of the amount of collagen, tubular atrophy and the degree of interstitial fibrosis in one section. Using computer-based analysis, we confirmed that our innovative double-stain highlights interstitial collagen better than Trichrome stain alone. We strongly recommend renal pathologists to try this innovative stain in their practice.

Key words: Kidney biopsy, Interstitial fibrosis, Special stains

Introduction

Histologic evaluation of renal biopsies, in addition to the routine hematoxylin-eosin (H&E) stain, involves multiple ancillary stains, including Periodic acid-Schiff's (PAS) and Masson's trichrome (Trichrome) stains (Walker, 2009). These ancillary stains have been used in the renal pathology practice for many years and each of them yields additional information, important for the

final pathologic diagnosis. PAS stain is used to highlight basement membranes, including the glomerular basement membrane (GBM) and tubular basement membrane (TBM) in dark-red to purple (McManus, 1948). Trichrome stain stains all collagens, including those that compose basement membranes, blue (Cohen, 1976). Therefore, determining the degree of interstitial fibrosis based on Trichrome stain often results in overestimation. The degree of interstitial fibrosis correlates well with renal function (Bohle et al., 1987, 1990; Eknayan et al., 1990). Quantitation of interstitial fibrosis is very important in assessing the efficacy of drugs in clinical trials, as well as progression of chronic kidney diseases in native and transplant kidney biopsies (Suzuki, 1994). Alternative methods to Trichrome stain, such as morphometric analysis with special stains (Sirius red or immunohistochemical stains for type III collagen) are burdensome and require additional processing. Recently, an attempt to perform morphometric analysis of renal biopsies with a subtraction morphometry technique was reported (Farris et al., 2009). The authors used computer-based methods to subtract PAS-positive areas from Trichrome stained slides. This methodology involves staining of consecutive sections, requires additional equipment to digitize images, special software to align different slides and is not precisely accurate, since stains are performed on consecutive, but still separate sections.

Herein we describe a novel method for simultaneous double-stain of renal biopsies with PAS and Trichrome stains. This innovative double-stain has advantages of both PAS and Trichrome stains and significantly increases the amount of useful information, which could be obtained from one slide. We have been using this

novel stain in our practice for several months and we believe that this combination of two classic stains is superior to any of the single stains.

Material and methods

We found that the order of applied stains is very important. Paraffin-embedded 3 micron renal biopsy sections were used. The double-stain was possible only if PAS stain was performed first followed by Trichrome stain. Opposite order resulted in bleaching of Trichrome stain. We use Dako Artisan™ Link Special Staining System (Dako North America, Inc., Carpinteria, CA USA). The staining protocols are provided by the manufacturer and briefly they described below:

PAS stain: (slides are rinsed in water after each step): 1) 0.4% Alpha amylase solution (37°C, 10 minutes). 2) 0.5% periodic acid (10 minutes). 3) Schiff's reagent (37°C, 1 hour). 4) 0.5% potassium bisulfite (5 minutes). 5) hematoxylin (Richard Allan, 1 minute). 6) clarifier (Richard Allan, 1 minute). 7) 0.5% ammonia water (until bright blue).

Trichrome stain is performed on the same slides (slides are rinsed in water after each step): 1) Bouin's solution (picric acid, saturated aqueous solution 73%, formaldehyde 23%, glacial acetic acid 4%) (56°C, 1 hour). 2) Weigert's hematoxylin (10 minutes). 3) Biebrich's scarlet-acid fuchsin solution (15 minutes). 4) phosphomolybdic-phosphotungstic acid (2.5% each) solution (12 minutes). 5) aniline blue solution (anilin blue 2.5%, glacial acetic acid 2%) (15 minutes). 6) 1% acetic acid solution for 3 minutes. 7) dehydration with 95% and absolute alcohol, clearing with xylene and mounting.

In the double-stained sections the basement membranes and the interstitial collagen have different colors: the basement membrane turns dark violet, while interstitial fibrosis remains blue.

In order to prove that the novel double-stain better reflects the degree of interstitial fibrosis, we applied a computer-based quantitating analysis, as we described earlier (Brodsky et al., 2007). Briefly, consecutive sections from 10 randomly selected renal biopsies from patients with diabetic nephropathy were stained with PAS, Trichrome or double PAS/Trichrome stain. At least four randomly selected areas of the renal cortex were photographed from each biopsy specimen using 10x or 20x objectives. It is important to note, that the areas of interest were photographed from the same areas on consecutive sections for different stains, which allowed evaluation of the same areas of the renal cortex stained with different stains. Areas stained either with PAS or Trichrome were measured in each photograph as we described earlier (Brodsky et al., 2007). Glomeruli, vessels and areas with interstitial inflammation were not included into analysis. All observations were completed by two independent observers, who were blinded to the origin of the data.

Results

After a careful validation process, we replaced Trichrome stain with the double-stain in our routine practice. After switching to this novel method, the quality and amount of useful information obtained from each biopsy specimen substantially increased. First, each section stained with this double-stain became more distinguishable and colorful, as the result of the combination of two individual stains (Fig. 1). Second, in the new stain, basement membranes stain dark-violet, as a result of the combination of blue (Trichrome) and purple (PAS). The interstitial collagen remains blue, because it is only faintly PAS positive. This allows a pathologist immediate estimation of the amount of collagen and the degree of fibrosis in one section, without spending time for evaluation of two different stains. This is especially important when non-consecutive sections are stained. Third, certain deposits (e.g. amyloid) became more distinguishable in the double-stained sections (Fig. 1).

Using this innovative double-stain, we analyzed the degree of interstitial fibrosis and tubular atrophy in randomly selected biopsies obtained from patients with diabetic nephropathy. Using the computer-based quantitating analysis, we found that the double PAS/Trichrome stain better highlights the degree of interstitial fibrosis, as compared to Trichrome stain (Fig. 2). Thus, atrophic TBM were stained by Trichrome stain blue (Fig. 2A,B), whereas in the double PAS/Trichrome stain TBM become dark-violet. Only areas of true interstitial fibrosis were stained blue by the double PAS/Trichrome stain (Fig. 2E,F). Indeed, in Trichrome stain alone, $27.7 \pm 1.02\%$ of the tubular interstitium was stained blue, whereas in double-stained biopsies only $15.8 \pm 0.86\%$ of the tubular interstitium was stained blue. The PAS positive atrophic TBM accounted for $19.4 \pm 0.94\%$ of the tubulointerstitial area (Fig. 2C,D).

Discussion

The field of renal pathology is relatively young. The evaluation of renal biopsies is now quite standard among different institutions and includes not only routine H&E stain, but methenamine silver, PAS and Trichrome stains. These special stains reveal additional information about different renal compartments and they have been used in practice for many years. Herein we report our innovative stain, derived from two standard stains (PAS and Trichrome), which combines all the advantages of both ancestor stains. Thus, instead of pink (PAS) or blue-red-orange (Trichrome) appearance, the combined stain has many colors, which is almost comparable with switching from black-and-white TV to a color TV. The new stain is not truly better than the conventional stains from the diagnostic point of view (e.g. Congo red for amyloid), but it brings out the lesions as nicely as the other stains with somewhat different color combinations.

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Using our innovative stain, we confirmed that Trichrome stain alone may mislead observers while evaluating interstitial fibrosis, because Trichrome stains not only the areas of true interstitial fibrosis blue, but atrophic TBM as well. Indeed, when we analyzed randomly selected renal biopsies obtained from patients with diabetic nephropathy, we identified significantly less blue-stained areas using our double-stain, as compared to Trichrome stain alone. The differences were due to thickened atrophic TBM, as they were highlighted in dark violet color by our double-stain. The sum of blue areas in the double-stained biopsies and PAS-positive areas was close to but more than the blue-stained areas in Trichrome stain alone ($15.8\pm 0.86\%$, $19.4\pm 0.94\%$ and $27.7\pm 1.02\%$, correspondingly), suggesting that not only

tubular basement membranes, but also other PAS-positive areas in the interstitium contribute to blue areas in the Trichrome stain. Importantly, we stained consecutive sections in order to minimize the error of different degree of staining at different levels of tissue.

Recently, a novel subtraction morphometry technique for assessing fibrosis in renal biopsies has been reported (Farris et al., 2009). The interstitial fibrosis was calculated as (Trichrome blue area-PAS pink area)/total area. This technique requires preparation of consecutive sections, separate staining of the tissue, scanning of the slides and computer-based analysis, which is not achievable in many renal pathology laboratories. Our innovative double-stain simple technique results in the same separation of Trichrome-

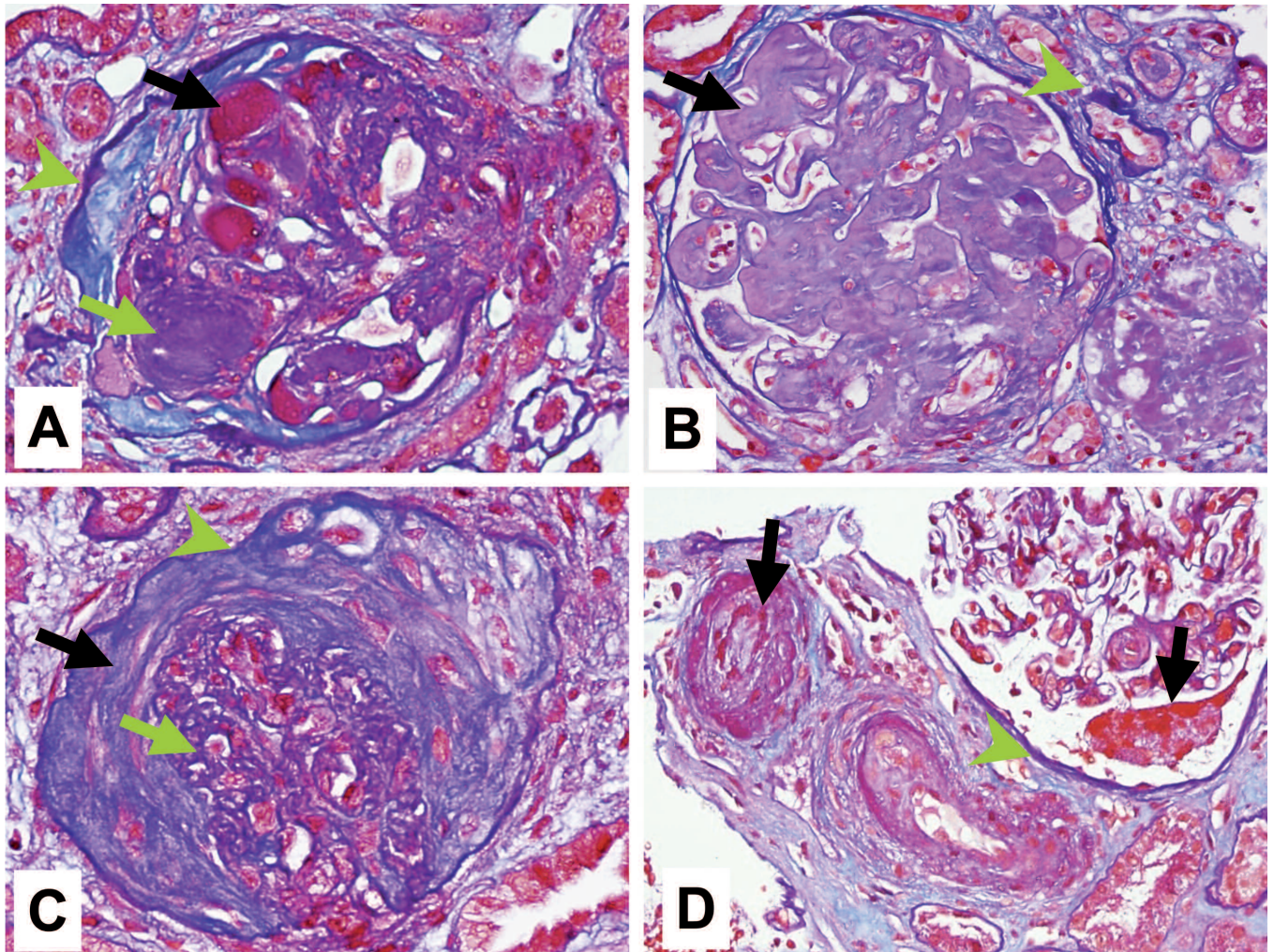


Fig. 1. Appearance of common kidney lesions in the PAS/Trichrome double stain. The Bowman's capsular and tubular basement membranes stain violet (**A-D**, green arrowheads). **A.** Nodular diabetic glomerulosclerosis. Nodular mesangial expansion stains violet (green arrow), whereas hyaline deposits stain dark-red (black arrow). **B.** Amyloid deposits stain light lavender blue (black arrow). **C.** Obsolescent glomerulus. Collagen deposits in the Bowman's space stain blue (black arrow), whereas the retracted glomerular capillary basement membranes stain violet (green arrow). **D.** Thrombotic microangiopathy. The PAS/Trichrome stain highlights fibrin deposits in arterioles and glomeruli (black arrow) in red. x 400

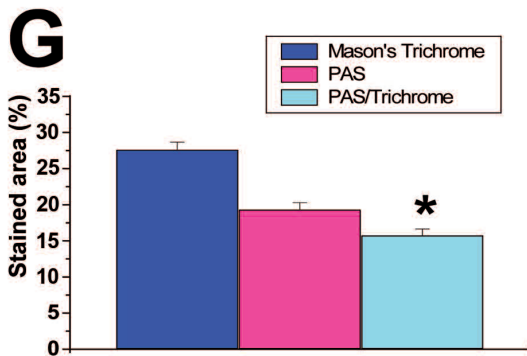
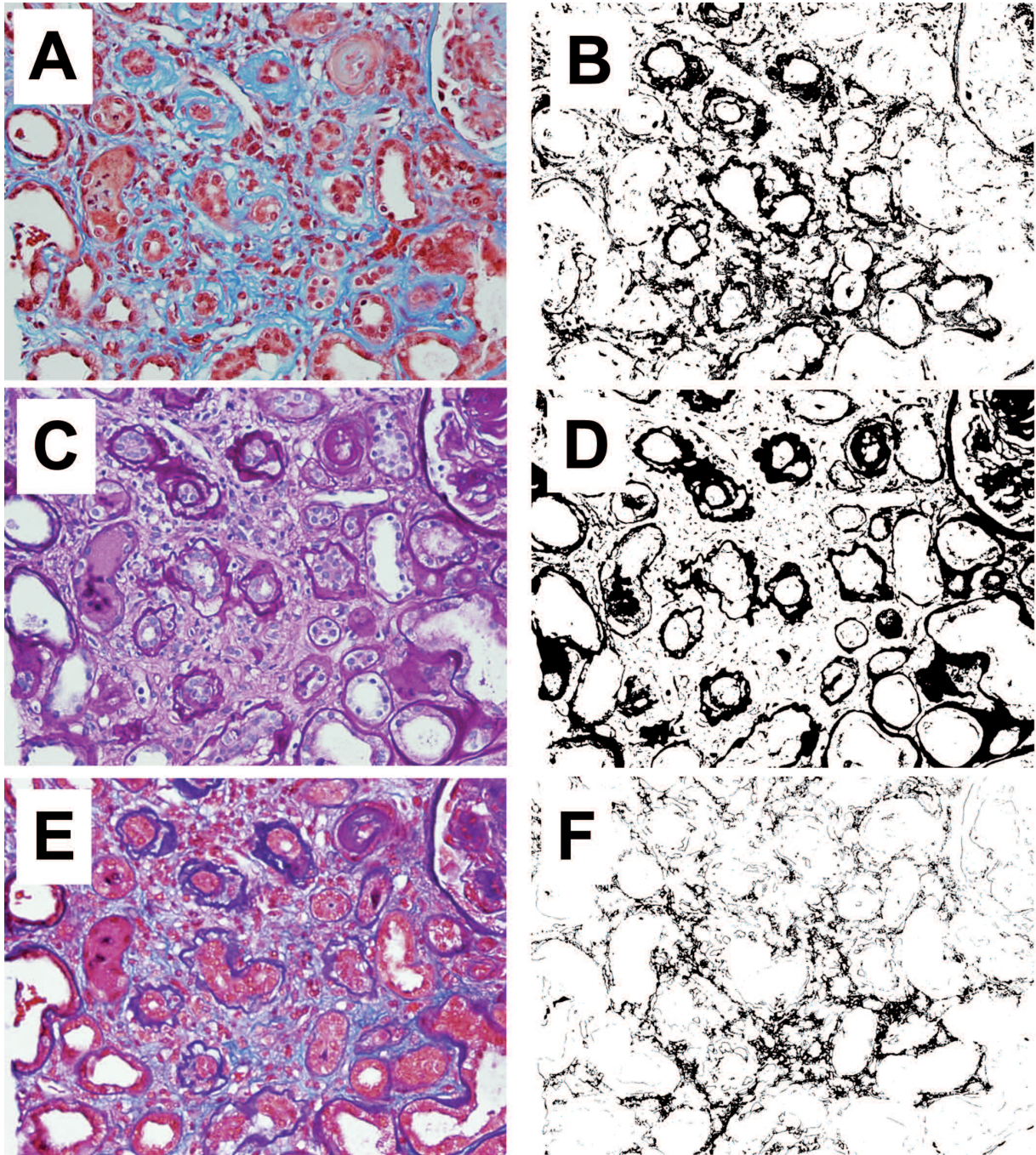


Fig. 2. The degree of interstitial fibrosis is highlighted better by the PAS/Trichrome double stain than with Masson's Trichrome stain alone. Consecutive sections were stained with Masson's Trichrome (A), PAS (C) and PAS/Trichrome double (E) stains. The areas stained blue by Masson's Trichrome (B and F) or purple by PAS (D) were extracted and the percentage of stained areas was calculated in the resulting images (G). *: $p < 0.05$ as compared to Masson's Trichrome stain alone.

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and PAS-stained tissue, without sophisticated and complicated methods.

This novel stain has been used in our routine practice for a several months. We did not lose any of the important features of Trichrome stain alone, but we gained the ability to easily evaluate the degree of interstitial fibrosis and tubular atrophy in renal biopsies. We strongly recommend renal pathologists to try this innovative stain.

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