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To cite this article: Alex Laslowski (2016): A modified silver methenamine Masson trichrome stain using methyl green for staining of renal biopsies, Journal of Histotechnology, DOI: 10.1179/2046023615Y.0000000012

To link to this article: http://dx.doi.org/10.1179/2046023615Y.0000000012

Published online: 16 Mar 2016.
Technical Note

A modified silver methenamine Masson trichrome stain using methyl green for staining of renal biopsies

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Renal pathology uses a battery of stains to allow proper assessment of all renal components. One of the most useful of these stains is the silver methenamine with a Masson Trichrome counterstain (SMMT). The SMMT stain uses thin 1-um sections to identify immune complexes deposits on the basement membranes and in the mesangium of the glomerulus and can be an excellent and inexpensive method. Problems can, however, occur when the stain is not performed by a technician or the stain needs to be performed in large numbers. A variation to the SMMT method has proved to provide a better, more robust and reproducible counterstain. The new method substitutes Light Green SF, from the traditional Masson Trichrome stain, for a much smaller dye molecule size Methyl Green allowing for better penetration and a stronger bonding to the tissue components.

Keywords: Light green SF, Masson trichrome, Methyl green, Silver methenamine

Introduction

The pathology department at Monash Medical Centre (Melbourne, VIC, Australia) has routinely used the SMMT stain1 for all manual staining of renal biopsies since the early 1990s. The SMMT stain provides excellent information for the pathologist including information on the glomerular basement membrane (GBM) such as thickening, duplication, and membranous spikes all of which are demonstrated by the silver component of the SMMT stain. The addition of a Masson Trichrome counterstain provides additional information such as the presence of protein deposits on basement membranes and the presence of fibrin. The current issue when performing a SMMT stain it is very difficult to control the staining intensity of the Light Green SF in the various tissue components. This is due to a major problem is the stain retention of the dye Light Green SF in the tissue sections. Experience with the stain in our laboratory has shown that the stain needs to be performed by a skilled technician to achieve a satisfactory result.

It was decided to find an alternative stain, which would provide more consistent and reliable results, but still provide a similar histological image for diagnostic purposes. The commonly accepted theory for the Masson Trichrome stain of dye molecular size was applied to finding a suitable stain, but it was the obverse which proved to be correct in our investigations. We tested numerous modifications to the Masson Trichrome using the traditional dyes of Light Green SF and Aniline Blue with varying concentrations and solvents with little success. The issue was that the Light Green SF did not have the same affinity for the tissue structures after the section had been stained with silver methenamine solution. The Light Green SF would simply wash out of the section in water and/or alcohols leading to problems with dehydration resulting in uneven staining, complete loss of stain, or over staining of the tissue sections due to rapid dehydration techniques.

Staining with silver methenamine Masson trichrome

Methenamine silver is commonly used in renal pathology to demonstrate the fine detail of glomerularopathies, this has been common place since Jones2 first described the advantages of such a technique. The method utilizes periodic acid to oxidize the basement membranes, which is
then subjected to a warm methenamine silver solution, which produces a silver deposition on the carbohydrates on collagen type IV fibers, which make up basement membranes of the GBM. The methenamine silver stain has since undertaken a number of variations in terms of the counterstain used. Jones² originally suggested the use of a Hematoxylin and Eosin counterstain, over the years an alternative counterstain of Light Green SF (CI 42095) has been used in preference by many institutions. The pathological information which the Methenamine silver stain is intended to provide is demonstrated membranous duplication, spikes, or thickening, in addition to vacuolization of the GBM when deposits are present. Early in the 1990s, in Melbourne, Australia, a method¹ was developed, which used a Masson Trichrome counterstain, this was considered by many to be an improvement to a Hematoxylin and Eosin (H&E) or Light Green SF counterstain in that it was able to demonstrate both fibrin and highlight the presence of glomerular protein deposits. The ability to demonstrate these additional features provided information in a paraffin section, which otherwise would have required a more detailed examination by electron microscopy.³ The stain was not intended to replace electron microscopy but to increase the sensitivity by light microscope examination.

**Connective tissue stains**

The term “Trichrome” is used for a number of techniques, which selectively demonstrate muscle, collagen fibers, fibrin, and erythrocytes. The staining mechanism of the MT stain is not entirely known, but the staining theory is thought to be related to tissue permeability and molecular size of the dye molecules.⁴ As tissue undergoes fixation tissue structures such as muscle and collagen are affected differently resulting in altered permeability; the permeability of the tissue structures are then exploited during the Masson Trichrome (MT) staining process. The basis of the theory is that stains are applied to the tissue according to molecular weight from the smallest dye molecule Biebrich Scarlet (Mwt 556)⁵ and finishing with the larger dye molecule Light Green SF (Mwt 815),⁶ and that the larger dye will displace the smaller dye molecules in tissue components which are large enough for that particular molecule to penetrate. The addition of Biebrich Scarlet to Acid Fuchsin in the MT stain helps to differentiate between cytoplasm/muscle and RBC’s.

**Problems with performing the silver methenamine Masson trichrome**

The problems, which were experienced in our laboratory when performing the SMMT stain was that the Light Green SF would readily be removed during dehydration and clearing of the stained sections. This is a common problem, which is also experienced in other laboratories so to compensate the tissue sections need to be rapidly dehydrated with only 2–3 dips in absolute alcohol.¹ Due to the loss of the Light Green SF, the stain needed to be performed by a skilled and adequately trained laboratory staff member. Even in the hands of a skilled operator variations in staining could range from the minor to extreme; the main problems included either complete loss of the Light Green SF, sections completely over stained with no differentiation for tissue structures with the Light Green SF, whilst the variations in between were deemed satisfactory for diagnostic evaluation. The practice of rehydrating and trying corrective action to improve the staining qualities were often not very successful. The reason for the loss of the Light Green component is unknown, but is most likely due to the binding sites of the proteins usually stained with Light Green SF are masked or altered during the silver impregnation stage hindering the ability of the Light Green SF to adequately bind.

**Investigations to correct the silver methenamine Masson trichrome**

Multiple variables of the Light Green SF were tested including altering the concentration, pH, and staining times. These did not produce similar staining characteristics as are traditionally seen in a MT stain. Renal sections were seen to be either completely over stained or had no Light Green SF in the section. Dehydrating through alternative solutions such as acetone, isopropynol, or air drying, which were aimed at retaining the Light Green SF in the tissue sections proved to be unsatisfactory or reproducible. Alternate Trichrome stains were also investigated such as Van Gieson, Martius Scarlet Blue, and Gomori (Aldehyde Fuchsins Gomori), but all failed to stain the renal tissue adequately or demonstrate the same tissue components as seen in the MT.

Aniline Blue was considered as an alternative counterstain to the Light Green SF; Aniline blue is quite often substituted for Light Green SF when performing a Masson Trichrome stain. Staining with Aniline Blue proved to be more consistent with regards to stain retention and staining of relevant cellular components, but due to the intensity of the blue stain pathologists viewing the slides felt that information was obscured and hard to interpret.

A closer look at Aniline blue as a dye to see why this was being retained by the tissue structures and whether there might be a similar dye with a green chromophobe revealed a clue. Aniline blue is
Known by a couple of names including China Blue and Soluble blue but does not possess a C.I number as it is actually a mixture of Methyl blue C.I 42780 and water. This led us to try Methyl Green, which is most commonly used in the demonstration of RNA using the staining procedure Methyl Green Pyronin; it is also suitable for the metachromatic demonstration of amyloid. The molecular size and structure of Methyl Green are vastly different from that of Methyl Blue and Light Green SF and is actually smaller in size than the Biebrich Scarlet.

According to the theory of Masson Trichrome staining, Methyl Green should have displaced the Biebrich Scarlet completely due to its smaller molecular size, but in practice this was not seen. What was seen is that after staining with the silver methenamine solution the Biebrich Scarlet (Fig. 1) behaved in a normal manner staining all tissue components, and was able to disassociate itself from the tissue when treated with phosphotungstic acid, however the Light Green SF (Fig. 2) was not able to enter the same tissue elements specifically the collagen. My theory was that due to its molecular size the Light Green SF could not get close enough to the tissue components it would normally stain to form strong covalent bonds and so instead was at best forming weak electrostatic or dipole-dipole bonds which were easily broken when challenged by water or alcohol. The introduction of Methyl Green (Fig. 3) being a smaller molecule sized dye allowed the molecule to freely enter the tissue forming much stronger bonds and which provided resistance to removal by water and alcohol. This altered staining behavior to the traditional MT would support the theory that the sections have altered tissue structures after treatment with the silver methenamine solution.

**Materials and Methods**

A scientist attends the renal biopsy to assess for tissue adequacy and to divide for the testing into the appropriate fixative for light microscopy, immunofluorescence and electron microscopy. The tissue for light microscopy is then processed on a 2 hour cycle on a Leica Peloris tissue processor and the biopsy is embedded in paraffin wax. Serial sections are cut at 1 μm on a rotary microtome (Leica RM2245) and predetermined slides are collected for H&E, SMMT, PAS, and an OrceinMT stain. Silver methenamine with a Masson trichrome counterstain slides are collected on coated slides (Matsunami) and are dried in an oven at 65°C for 15 minutes. The initial silver methenamine component of the stain is performed on a Ventana Benchmark Special Stain module using a Ventana “Jones Staining H&E Staining Kit” (REF 860-019); deparaffinization time and temperature is set for 8 minutes at 70°C, the silver impregnation step is set for 16 minutes at 58°C, Hematoxylin is set for 8 minutes, and the Eosin is not selected for staining. In our laboratory, we opt to remove both the toner and Eosin vials from the instrument once the run has started so that the sections are not treated with these solutions, this allows the sections to retain the pale tan and intense black color differences seen between collagen type III and collagen type IV fibers when staining with silver methenamine it also ensures that the sections are not stained with eosin so that we can perform the MT counterstain. The silver staining component of this stain can also be substituted for a manual Jones silver methenamine in your laboratory.

At the completion of the staining run, the slides are removed and manually counterstained with the modified MT stain. The MT component of the stain involves staining with 1% Biebrich Scarlet (Waldeck GmbH 1A-398)-1% Acid Fuchsin (Amber Scientific AF25) in 1% Acetic Acid for 10 minutes, and

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**Figure 1** Biebrich Scarlet (C.I 26905) molecule is medium with a molecular weight 556.

**Figure 2** Light Green SF (C.I 42095) molecule is large with a molecular weight of 815.

**Figure 3** Methyl Green (C.I 42585) molecule is small with a molecular weight of 458.
wash in water and apply 1% Tungstophosphoric (Merck Millipore 100582) acid for 2 minutes, wash in water and apply 0.05% Methyl Green (Sigma Aldrich 32389) in 1% Acetic Acid for 2 minutes, wash well in water dehydrate, clear and coverslip. The results should show of the stain black basement membranes, blue nuclei, RBC stain magenta, fibrin stains bright red, connective tissue stains green, immune complex deposits stains red, and amyloid will stain light green.

Sections should not be toned, when staining on a Ventana Benchmark Special Stain module, there is no option to deselect the toner so it need to be removed after the run has begun so that it is not applied to the sections.

**Results**

Once we had identified the optimal staining conditions as outlined in the methods and materials, there was an immediate improvement in the quality and reproducibility of SMSTM stains performed in our laboratory. The ability to wash the sections post staining and dehydrate at a normal pace meant the elimination of a separate set of dehydration alcohols, which were used for the renal slides which were always heavily contaminated with Light Green SF. The improved staining conditions also meant that all staff could perform the test with similar results making the test less specialized and easily incorporated into the laboratory workflow.

A well stained SMSTM should show strong intensely black staining of the collagen structures of the kidney including the GBM, the Bowman’s capsule and collagenous supporting structures surrounding the tubules. Biebrich Scarlet–Acid Fuchsin stains many tissue components with the Biebrich Scarlet staining the cytoplasm of the tubules and the Acid Fuchsin staining RBC’s, fibrin and glomerular and vascular deposits. The Methyl Green stains the mesangium of the glomerulus, which provides contrast to the mesangial and glomerular deposits, interstitial tissue and the luminal surface of tubules.

Figures 4 and 5 demonstrates the fuchsinophilic nature of the protein deposits located on the subepithelial surface of the GBM and contrasts well against the Methyl Green counterstain seen in the mesangium in a standard light microscopy slide. The information gained from a MT stain on the protein deposits has been correlated, whenever possible, with those of immunofluorescent study. Also demonstrated well in Figs. 4 and 5 are the membranous spikes on the GBM which are stained black by the silver methenamine component of the stain.

Fibrin can also be seen within the lumen of a capillary loop in Fig. 5 staining with acid fuchsin.

Figure 6 shows a renal biopsy which has been stained with SMSTM using the methyl green stain. Note how the thickened vessels, which are Congo Red positive, demonstrate a metachromatic color shift to the surrounding structures which are also stained with methyl green. It is not suggested that the SMSTM be substituted for the Congo Red, but can be helpful in identifying some cases.

Figure 7 shows a case of lupus nephritis which shows GBM reduplication stained black and segmental fuchsinophilic subendothelial deposits against the methyl green stained mesangium. Due to the large amount of proteinaceous material present the stain appears a brick red color when viewed under a light microscope. The basement membrane can also be seen to split and outline the deposits.
Figure 8 is a section of normal kidney included to show the general overall appearance of how the SMMT stain should appear. The GBM should be intensely black against a light green mesangium whilst the tubules should demonstrate a two tone staining pattern where the cell body is stained red with Biebrich Scarlet and the luminal brush border stains with methyl green.

Figures 9 and 10 are parallel sections located on the same slide using the Light Green SF as a counterstain showing the dichotomy of staining regularly seen.

The staining method in our department was changed to this new improved SMMT stain and has been in use now for over 12 months. Renal biopsies from both transplant and native kidneys are stained using this protocol with excellent results.
Conclusion
The SMMT stain provides the pathologist with a wealth of information allowing them to assess multiple facets of the tissue pathology on a single slide. The substitution of Methyl Green for Light Green SF in the staining protocol offers a protocol, which is more robust, reproducible and demonstrates the same tissue pathologies as those seen in the previous version using Light Green SF. An added bonus of the Methyl Green staining is the demonstration of amyloid in tissue sections, which still appears green but with a metachromatic color shift to the other tissue components.

The substitution of Methyl Green for Light Green SF in the SMMT stain has produced a stain, which better reflects the staining characteristics seen in a standard MT stain. It has also produced a more reproducible stain which is easier to perform. The investigations, which lead to this paper showed that the SMMT does not necessarily hold true to the commonly accepted theory for the MT stain of tissue permeability possibly due to the sections being initially stained with silver methenamine.

Acknowledgments
I would like to thank all the staff in the Anatomical Pathology department at Monash Medical Centre.

Disclaimer Statements
Contributors Author is the sole contributor of the article.

Funding No funding was received for this article.

Conflicts of interest Part of the staining is performed on a Ventana Benchmark Special Stains instrument. I have been an advisor to Ventana for their immunohistochemical automation in the past and I am currently a member of their patient safety advisory board

Ethics approval As this was part of a laboratory method improvement ethics was not required.

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